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IL-15 Prolongs the Duration of CD8⁺ T Cell-Mediated Immunity in Mice Infected with a Vaccine Strain of *Toxoplasma gondii*¹

Imtiaz A. Khan² and Lori Casciotti

Immunization of mice with a vaccine (ts-4) strain of *Toxoplasma gondii* is known to induce complete protection against subsequent lethal infection. Ts-4-mediated protection has been reported to be primarily dependent on IFN-γ-producing CD8⁺ T cells. However, duration of CD8⁺ T cell-mediated immunity in the ts-4-vaccinated animals is not known. In the present study, the kinetics of the CD8⁺ T cell response in mice immunized with the ts-4 strain of *T. gondii* was evaluated. Optimal CD8⁺ T cell immunity persisted at least 6 mo after vaccination, and mice at this time point continued to overcome lethal challenge with a more virulent strain. However, at 9 mo postimmunization, CD8⁺ T cell immunity was severely diminished and the mice succumbed to *Toxoplasma* challenge. Pretreatment of animals, vaccinated 9 mo earlier, with rIL-15 prevented the mortality induced by *Toxoplasma* challenge. The protective effect of IL-15 treatment was due to a rise in the frequency of Ag-specific CD8⁺ T cells. CD8⁺ T cells from IL-15-administered animals showed increased proliferation and IFN-γ production in response to antigenic restimulation. These findings suggest that rIL-15 can reverse the decline in the long-term CD8⁺ T cell immune response in mice immunized with vaccine strain of *T. gondii*. *The Journal of Immunology*, 1999, 163: 4503–4509.

Cell-mediated immunity is essential for protection against the obligate intracellular protozoan *Toxoplasma gondii* (1). Host immune response against the parasite can be divided into an innate acute response and an Ag-specific cell-mediated immune response. During the acute response, NK cells are an important element of host defense (2, 3). However, development of parasite-specific T cell responses becomes essential for long-term protection (4). Among the T cell population, CD8⁺ T cells are considered to be the major effector cells responsible for protection against *T. gondii*, with CD4⁺ T cells playing a synergistic role (5–7). Immune CD8⁺ T cells from both infected mice or humans secrete IFN-γ and exhibit in vitro cytotoxicity toward infected cells (8–12). Both of these antimicrobial functions are important for the protective response against *T. gondii* (13, 14). Neutralization of either IFN-γ or CD8⁺ T cells reverses the host-protective immunity (6, 15).

Although natural challenge with *T. gondii* results in the chronic infection, little is known about long-term immunity against the parasite or how long it lasts with progression of time. The depletion of both CD4⁺ and CD8⁺ T cells results in complete reactivation of *T. gondii* infection in the chronically infected animals (16). The treatment of these mice with anti-CD8 Ab resulted in almost 50% mortality in these animals, while depletion of CD4⁺ T cells alone had no effect. Studies by Brown and McLeod suggest that cyst formation during *T. gondii* infection is regulated by CD8⁺ T cells (17). Loss of CD8⁺ T cells in the infected animals can result in the formation of large number of cysts in an otherwise resistant strain of mouse.

In the present study, we demonstrate that CD8⁺ T cell immunity plays a more important role in long-term immunity against *T. gondii* infection. CD8⁺ T cells from mice infected with the vaccine strain of *T. gondii* show increased parasite-specific responses (Ag-specific proliferation and IFN-γ production) as compared with CD4⁺ T cells. However, over the period of time, the CD8⁺ T cell memory response shows a decline and the mice become susceptible to lethal *T. gondii* challenge. This down-regulation of CD8⁺ T cell function is prevented when IL-15 is administered exogenously to these animals.

**Materials and Methods**

**Mice and parasites**

Six- to eight-week-old female C57BL/6 mice obtained from The Jackson Laboratory (Bar Harbor, ME) were used for immunization. The mice were vaccinated with the temperature-sensitive mutant strain of *T. gondii*, ts-4 (kindly provided by Dr. Elmer Pfefferkorn, Dartmouth Medical School, Lebanon, NH). For challenge infection, the PLK strain of *T. gondii* (clonally derived from Me49) was used.

**Vaccination, infection, and Ag preparation**

Vaccination studies were carried with ts-4 strain of *T. gondii*. The ts-4 strain was maintained by a serial passage in a cell line of human fibroblasts (HFF) in Eagle’s MEM (Life Technologies, Gaithersburg, MD) supplemented with 10% newborn calf serum plus antibiotics. The parasites used in the experiments were <50th passage in vitro and were isolated from monolayer by forced extrusion through a 27-gauge needle. Parasites were isolated from host cell debris by separation using a Percoll gradient (1.04 g/ml). Purified parasites essentially free of any fibroblast contamination were washed twice and resuspended in 1× PBS. Mice were injected i.p. with 1 × 10⁵ tachyzoites of ts-4 strain. After a 2-wk period, the animals were boosted for a second time with 5 × 10⁷ parasites of same strain. The immune animals were challenged via i.p. route with 100% lethal dose (5 × 10⁵ tachyzoites) of PLK strain of *T. gondii*. Tachyzoites of PLK strain were cultured, harvested, and purified by the method similar to that utilized for ts-4 strain. Vaccinations of mice were conducted at same time, while the challenge experiments were performed at various time points after second immunization.

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IL-15 PROLONGS CD8<sup>+</sup> T CELL IMMUNITY AGAINST T. gondii

FIGURE 1. Proliferation of T cell subsets following immunization with ts-4 strain of T. gondii. Pooled splenocytes from immune animals (n = 6) were collected between 30 and 90 days p.i. Purified CD<sup>4</sup><sup>+</sup> and CD<sup>8</sup><sup>+</sup> T cells (>95% pure) were isolated from the total spleen cell population and cultured in 96-well plates at the concentration of 2 × 10<sup>5</sup> cells/well. The cultures were stimulated with Con A or TLA in presence of 1 × 10<sup>5</sup> irradiated feeder cells. After 72-h incubation, proliferation was measured by thymidine incorporation. The data are representative of two separate experiments.

Toxoplasma lysate Ag (TLA<sup>1</sup>) was prepared from tachyzoites of the RH strain of T. gondii. The parasites were cultured in human fibroblasts, harvested, and purified, as mentioned above. The purified parasites were pulse sonicated eight times (18,000 Hz) at 10-s intervals at 4°C. The sonicate was centrifuged at 10,000 × g for 15 min to remove nonsoluble Ag, and the protein concentration was determined by a commercial assay (Bio-Rad Laboratories, Cambridge, MA). The Ag was aliquoted and stored at −20°C until further use.

T cell subtype purification, proliferation, and depletion

Following immunization, mice were sacrificed and spleen cell suspensions were prepared following homogenization in PBS. RBC were lysed in a red cell lysis buffer (Sigma, St. Louis, MO). After two to three washes in a HBSS with 5% FBS (HyClone Laboratories, Logan, UT), the CD<sup>4</sup><sup>+</sup> and CD<sup>8</sup><sup>+</sup> T cells were separated by microbeads (Miltenyl Biotech, Auburn, CA). The separation procedure was conducted as recommended by the manufacturer's instructions. The purity of the separated cells was >95%, as determined by FACS analysis. The assay was performed using FITC-labeled L3T4 or Ly-2 Abs (PharMingen, San Diego, CA), respectively, for CD<sup>4</sup><sup>+</sup> and CD<sup>8</sup><sup>+</sup> T cell staining.

Proliferation of CD<sup>4</sup><sup>+</sup> and CD<sup>8</sup><sup>+</sup> T cells was measured by thymidine incorporation assay. Purified cells were suspended in RPMI 1640 (Invitrogen Technologies) with 10% FCS (HyClone Laboratories, Logan, UT), the CD<sup>4</sup><sup>+</sup> and CD<sup>8</sup> T cells were separated by microbeads (Miltenyl Biotech, Auburn, CA). The separation procedure was conducted as recommended by the manufacturer's instructions. The purity of the separated cells was >95%, as determined by FACS analysis. The assay was performed using FITC-labeled L3T4 or Ly-2 Abs (PharMingen, San Diego, CA), respectively, for CD<sup>4</sup><sup>+</sup> and CD<sup>8</sup> T cell staining.

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To deplete CD<sup>8</sup> T cells, mice received 1 mg of anti-CD8 mAb (clone 2.43; American Type Culture Collection (ATCC), Manassas, VA) for 3 continuous days at the beginning, and every third day thereafter. CD<sup>4</sup> T cell depletion of mice was performed by the administration of rat anti-mouse CD4 (clone GK1.5; ATCC) at a dose of 0.5 mg/day for 3 consecutive days, continuing every third day thereafter. Control animals received an equal amount of rat IgG (Sigma). Treatment was continued until the termination of experiment or death of the animals. The Ab treatment resulted in >95% depletion of the phenotype, as determined by FACS analysis.

IL-15 treatment

Immunex (Seattle, WA) provided rIL-15. Mice were injected with 15 μg of human rIL-15 in 200 μl of PBS on alternate days over a period of 3 wk. Control animals were treated with equal amount of PBS alone.

IFN-γ production by T cell subtype

The IFN-γ production by purified T cell subsets from T. gondii-infected animals was assayed at various time points postimmunization (p.i.). Purified CD<sup>4</sup><sup>+</sup> or CD<sup>8</sup> T cells were stimulated in vitro with TLA in presence of irradiated feeder cells. After 72-h incubation, the cells were harvested and supernatants were collected and assayed for IFN-γ production by commercially available ELISA kit (Endogen, Cambridge, MA).

pCTL frequency analysis

The quantitative analysis of the cytolytic activity of CD<sup>8</sup> T cells was done by determining the precursor CTL (pCTL) frequency of the infected animals by establishing limiting dilution assays (LDAs). Purified CD<sup>8</sup> T cells were cultured by limiting dilution in 96-well round-bottom plates. The cells were grown in RPMI 1640 medium containing appropriate growth factors, including 15 U/ml of rIL-2 (R&D Systems, Minneapolis, MN) irradiated tachyzoites of RH strain and feeder cells. Cells were diluted from 250,000 to 2,500 cells/well. Control wells contained only irradiated parasites and feeder cells. After 1 wk, the cells were harvested and incubated with <sup>51</sup>Cr-labeled parasite-infected and uninfected macrophages. The macrophages were collected and labeled, as described elsewhere (18). Briefly, mouse peritoneal macrophages were obtained by a lavage, 2 days after i.p. inoculation with 1 ml of thioglycolate. The macrophages were washed three times in PBS and dispensed at a concentration of 2 × 10<sup>6</sup> cells/well in 96-well U-bottom tissue culture plates. After overnight incubation, they were radiolabeled with <sup>51</sup>Cr (0.5 μCi/well; Amersham, Arlington Heights, IL) for 8 h to determine DNA synthesis. Pulsed splenocytes were harvested on a glass filter by automated multiple sample harvester and dried, and incorporation of radioactivity was determined by liquid scintillation.

1 Abbreviations used in this paper: TLA, Toxoplasma lysate Ag; LDA, limiting dilution assay; pCTL, precursor CTL; p.i., postimmunization.
cells was greater than $3 \times$ SD above control wells (mean cpm released by the target cells incubated with APCs and irradiated parasites alone). The pCTL frequency was calculated according to a standard formula (19).

### Statistical analysis

Statistical analysis was performed using two sample Student’s $t$ tests (20) for lymphoproliferation studies and log rank test for murine survival data (21).

### Results

**CD8$^+$ T cells from ts-4-immunized animals exhibit an enhanced proliferation in response to recall Ags**

Proliferation in response to antigenic restimulation is an important characteristic of memory T cells (22). A proliferation assay was performed to study the memory response of T cell subtypes during *T. gondii* infection. CD4$^+$ and CD8$^+$ T cells were isolated at days 0, 30, and 90 p.i. Purified CD4$^+$ or CD8$^+$ T cells were cultured in vitro in the presence of Con A or TLA. After 72-h incubation, proliferation was measured by $[^3]$H]thymidine incorporation. As demonstrated in Fig. 1, the proliferative response of CD8$^+$ T cells was significantly greater than CD4$^+$ T cells both at day 30 ($p = 0.03$) and day 90 ($p = 0.005$) p.i. CD8$^+$ T cells from immunized animals continued to exhibit increased stimulation in response to TLA at day 180 p.i. (data not shown). These observations suggest that as compared with CD4$^+$ T cells, CD8$^+$ T cells may be more important in long-term immunity against *T. gondii* infection.

**Duration of IFN-γ production after ts-4 vaccination**

In addition to the proliferation, another characteristic of memory T cells is the release of cytokines upon antigenic exposure (23). IFN-γ is one of the important cytokines produced by both CD4$^+$ and CD8$^+$ T cells during *T. gondii* infection (24, 25). The IFN-γ-producing ability of the CD4$^+$ and CD8$^+$ T cell population during long-term *T. gondii* infection was determined by an in vitro assay. Purified T cell subtypes were cultured in presence of Ag and irradiated feeder cells. After 72-h incubation, the supernatants from the cultures were analyzed for IFN-γ release. At day 30 p.i., although both CD4 and CD8$^+$ T cells secrete IFN-γ, the level of cytokine produced by CD8$^+$ T cells was higher in comparison with CD4$^+$ T cells (Table I). At day 90 p.i., immune CD8$^+$ T cells continued to show a significant release of IFN-γ in response to TLA stimulation. In contrast, CD4$^+$ T cells from immune animals show a minimal IFN-γ production at this time point (Table I).

**Ts-4 immunization results in long-term CTL response**

Ts-4 immunization has been reported to induce an Ag-specific CTL response in the vaccinated animals (9, 10). Splenocytes from the vaccinated animals exhibited cytolytic activity in vitro against parasite-infected target cells. To estimate the frequency of Ag-specific cytolytic spleen cells in the immune population following ts-4 immunization, pCTL assay was performed. As shown in Fig. 2, pCTL frequency of the immune CD8$^+$ T cells after 30 days post-ts-4 immunization is 1/3,150 cells compared with 1/5,771 cells 3 mo p.i. The frequency of pCTLs continues to stay high up to 6 mo p.i. (1/10,564 cells). Differences in these values are considered to be within the range of variability for this assay (26). The high incidence of the pCTL frequency in the ts-4-immunized animals can be compared favorably with that observed during viral infection in which CD8$^+$ CTLs play an important role in host protection (27).

**Protective immunity in the vaccinated animals wanes with time**

Studies from various laboratories have shown that mice immunized with ts-4 parasite can survive a lethal *T. gondii* challenge (28–30). However, the duration of protective immunity in the vaccinated animals has not been studied. Experiments were performed in which the vaccinated mice were challenged with a LD$_{100}$ of the...
PLK strain of *T. gondii* at 3, 6, and 9 mo post-last vaccinating dose. All of the mice challenged at 3 or 6 mo p.i. survived lethal *T. gondii* infection similar to the animals infected 2 wk after vaccination (data not shown). These animals continued to survive until the termination of experiment. Although the mice immunized 9 mo earlier survive significantly longer (*p* = 0.002) than the age-matched nonimmune controls, they ultimately succumbed to *Toxoplasma* infection (Fig. 3).

**IL-15 treatment enhances the longevity of ts-4-mediated protection by up-regulating the long-term CD8⁺ T cell immune response**

The role of IL-15 in augmenting the long-term CD8⁺ T cell immunity against *T. gondii* infection has been previously demonstrated (31, 32). We investigated whether IL-15 treatment could sustain the protective immunity in the ts-4-immunized animals. Mice vaccinated with ts-4 strain 9 mo earlier were administered IL-15 starting 2 wk before *T. gondii* challenge and continued for a total period of 3 wk. IL-15 treatment completely restored the protective immunity of the immune animals, as no mortality was observed in these mice (Fig. 4). On the contrary, as observed earlier, untreated control animals succumbed to *T. gondii* infection.

To determine whether survival of IL-15-treated animals is a result of an augmented CD8⁺ T cell response, Ab depletion studies were performed. Cytokine-treated animals were administered either anti-CD8 or anti-CD4 Ab just before the challenge. Depletion of CD8⁺ T cells in the IL-15-treated mice completely reversed the protective effect of the cytokine (Fig. 5), and these animals died almost at the same time as untreated controls. There was no significant difference in the survival time between these two groups (*p* > 0.01). Treatment with anti-CD4 Ab had no effect on the survival of the IL-15-administered animals and, similar to the mice injected with isotype control, they continued to survive until the termination of the experiment. The animals treated with anti-CD4 Ab showed significantly increased survival as compared with those receiving anti-CD8 Ab (*p* = 0.001).
The role of IL-15 treatment in stimulating long-term CD8+ T cell response was further evaluated by determining the Ag-specific proliferation of this T cell subtype. CD8+ T cells from immune mice were isolated at 9 mo postvaccination. Purified cells were stimulated with TLA, and lymphoproliferative assay was performed. Immune CD8+ T cells from the IL-15-treated mice showed significantly greater proliferation (\(p < 0.001\)) in comparison with the cells isolated from immune animals treated with saline (Fig. 6), suggesting an increase in the frequency of Ag-specific cells in response to cytokine treatment. CD8+ T cells from nonimmune mice treated with IL-15 or saline did not proliferate in response to TLA stimulation. There was no significant difference in the Con A stimulation of CD8+ T cells between different groups of animals used in the study.

**IL-15 administration increases the pCTL frequency and cytokine profile of CD8+ T cell population from vaccinated animals**

In addition to the lymphoproliferative response to recall Ag, CD8+ T cells from the IL-15-treated animals were evaluated for pCTL frequency and IFN-\(\gamma\) production. Frequency of CD8+CTLs at 9-mo p.i. was severely diminished (1/154,000) in comparison with the response observed at earlier time points (Fig. 7). Administration of IL-15 to these animals increased the magnitude of pCTL frequency response by more than 1 log (1/8,271). Attenuation of the memory CD8+ response in the vaccinated mice was also evident by decreased IFN-\(\gamma\) levels in response to antigenic stimulation (Table II). CD8+ T cells from immune mice injected with saline produced significantly less IFN-\(\gamma\) than CD8+ T cells from those treated with IL-15 (\(p < 0.003\)).

**Discussion**

The data presented in these studies demonstrate that long-term memory against *T. gondii* infection in a vaccine model is primarily dependent on CD8+ T cells. Both CD4+ and CD8+ T cells are known to be essential for ts-4-mediated protective response (6, 33). However, CD8+ T cells are considered to be the major effector cells against lethal *Toxoplasma* challenge. Studies by...
Gazzinelli et al. have shown that depletion of the total T cell population abrogates protective immunity against Toxoplasma challenge in ts-4-immunized animals (6). While treatment with anti-CD8 Ab alone resulted in delayed mortality, loss of CD4+ T cells did not alter the protective effect of vaccination. In the present study, we have demonstrated that CD8+ T cells from ts-4-vaccinated animals are able to proliferate and secrete IFN-γ in response to recall Ag for a longer duration than the CD4+ T cells from the same mice. The data obtained from these studies imply that CD8+ T cells may be the mediator of long-term immune response in mice.

The role of memory CD8+ T cells in the long-term protection against reinfection has been reported in several intracellular pathogens (34, 35). For example, mice that have undergone an acute infection with influenza virus, Sendai virus, or lympho choriomeningitis virus exhibit lifelong CD8+ T cell-mediated immunity (34, 36, 37). A recent study has documented long-term CD8+ T cell memory in humans by isolating vaccinia virus-specific CTLs from individuals vaccinated more than 30 yr earlier (38). Similarly, protection against Listeria monocytogenes has been reported to be mediated by CD8+ T cells (39). A CD8+ T cell immune response generated during ts-4 vaccination in mice has been documented by several laboratories (15, 24, 33); however, not much is known about the duration of this response. In the present study, the kinetics of the T cell memory response in the mice immunized with ts-4 strain of parasite was evaluated. Although robust immunity continued through 6 mo after the last immunizing dose, down-regulation of CD8+ T cell immunity was observed at 9 mo p.i. This was determined by a less than optimal response to recall Ag by CD8+ T cells and substantial decrease in pCTL frequency in this population. The loss of memory CD8+ T cells was further confirmed by the susceptibility of the host to Toxoplasma challenge at this time point.

Cytokines are known to play a critical role in determining the outcome of T. gondii infection (12, 40–43). One of these cytokines, IL-15, has been reported by us to have a profound effect on the augmentation of CD8+ T cell response against murine T. gondii infection (31). Subsequent studies by other groups have confirmed the role of IL-15 in the stimulation of memory CD8+ T cell response (44–47). Recent studies by Zhang et al. have demonstrated that IL-15 causes a strong and selective stimulation of memory CD8+ T cells in vivo (45). Studies with IL-15Ra−/− mice have shown that in addition to other abnormalities, CD8+ memory T cells are selectively reduced in these mice (46). In the current studies, we observed a reversal in the loss of memory CD8+ T cell response by exogenous IL-15 administration. CD8+ T cells from the IL-15-treated animals exhibited robust pCTL frequency, proliferation, and IFN-γ production. These animals were able to overcome the infection with lethal T. gondii strain. The protective response induced by IL-15 administration was lost when the animals were simultaneously depleted of CD8+ T cells. In contrast, CD8+ T cells from untreated immune animals at this time point failed to show optimal memory CD8+ response and succumbed to Toxoplasma challenge.

Based on our findings, we postulate that immunization with a vaccine strain of T. gondii induces a long-term immunity by induction of a pool of Ag-specific memory CD8+ T cells. These CD8+ T cells are primary effector cells responsible for immune surveillance against T. gondii challenge. Over the passage of time, the pool of memory CD8+ T cells gets diminished and protective immunity against the challenge infection is reduced. One reason for this to happen may be that ts-4 infection is completely cleared 2–3 mo postinfection (29, 48). It is very likely that, unlike viral infections in which memory CD8+ T cells can be maintained in the absence of Ag (49, 50), the presence of antigenic stimulation is essential for long-term CD8+ T cell immunity against T. gondii.

### Table II. IFN-γ production (pg/ml) by CD8+ T cells from immune animals treated with IL-15

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Unstimulated</th>
<th>Stimulated</th>
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<tbody>
<tr>
<td>Saline</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>IL-15</td>
<td>ND</td>
<td>28 ± 4.6</td>
</tr>
<tr>
<td>Immune</td>
<td>64 ± 11.8</td>
<td>316 ± 74</td>
</tr>
<tr>
<td>Immune + IL-15</td>
<td>188 ± 44.0</td>
<td>1085 ± 132</td>
</tr>
</tbody>
</table>

* CD8+ T cells from immune mice (vaccinated nine months earlier) and treated with IL-15 were isolated 1 wk after the completion of cytokine treatment. A total of 1 × 10^6 purified CD8+ T cells were cultured in 24-well plates in the presence of 5 × 10^4 irradiated feeder cells. Cultures were stimulated with 15 µg/ml of TLA. After 72 h incubation, supernatants were collected and stored at −70°C. Supernatants were assayed for IFN-γ by ELISA. The data represents mean ± SD of triplicate cultures. ND, Not detected.
infection. Ongoing studies in our laboratory involving cyst-forming strains of *T. gondii* will help to clarify the importance of antigenic persistence for long-term CD8+ T cell immunity during *Toxoplasma* infection. However, the present findings conclusively demonstrate the role of IL-15 in expanding the memory CD8+ T cell population in *Toxoplasma*-vaccinated animals. The novel feature of our studies is that it is the first report that demonstrates that *Toxoplasma*-specific CD8+ T cell immunity in mice is depleted over time, but can be rescued by IL-15 treatment.

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