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Inducible Nitric Oxide Synthase Is Not Required for Long-Term Vaccine-Based Immunity Against *Toxoplasma gondii*¹

Intizal A. Khan, Tadashi Matsuura, and Lloyd H. Kasper

Induction of reactive nitrogen intermediates by IFN-γ is presumed an important mechanism of host resistance against acute and chronic infection with *Toxoplasma gondii*. Although nitric oxide (NO) has been shown to be important in the control of parasite replication in vivo, the role of this molecule in vaccine-based immunity against *T. gondii* is unknown. Mice with a targeted disruption of inducible NO synthase (iNOS) were immunized with an avirulent temperature-sensitive strain of this parasite (ts-4). Both the parental C57BL/6 and the iNOS⁻/⁻ mice survived infection with the ts-4 mutant. Oral challenge of the vaccinated mice with a lethal dose of cysts containing bradyzoites resulted in reduced parasite burden and increased survival compared with nonvaccinated control mice. Host immunity in the iNOS⁻/⁻ mice, similar to that observed in the parental strain, appeared dependent upon both IFN-γ and CD8⁺ T cells. These findings suggest that although vaccine-based long-term immunity against *T. gondii* is dependent upon the induction of IFN-γ, it does not rely upon the anti-microbial effect of NO. *The Journal of Immunology*, 1998, 161: 2994–3000.

Cell-mediated immunity is essential for host resistance to infection with the obligate intracellular protozoan *Toxoplasma gondii* (1). T cell immunity is dependent upon the induction of both an effective IFN-γ and CD8⁺ T cell response (2, 3). IFN-γ plays a pivotal role in protection against acute infection and recrudescence in the chronically infected murine host. Mice treated with an Ab that blocks the activity of IFN-γ do not survive infection with an avirulent parasite strain (4). During chronic infection, blocking of IFN-γ results in the reactivation of disease and the development of toxoplasmic encephalitis (5). The mechanism by which IFN-γ protects against this opportunistic pathogen is most likely multifaceted. IFN-γ mediates the activation of macrophages. There are at least two recognized mechanisms by which activated macrophages exert a parasiticidal effect against *T. gondii*. IFN-γ can induce macrophage-mediated oxidative killing of the parasite by an increase in the production of reactive oxygen metabolites (6–8). Alternatively, the synergistic action of IFN-γ, together with a second effector, will stimulate the production of nitric oxide (NO) (9, 10). The inhibitory activity of NO against both tumor cells and microbial pathogens, including *T. gondii*, is well documented (11, 12).

We have recently reported on the role of NO during acute *T. gondii* infection in mice with a targeted disruption of the gene for inducible NO synthase (iNOS) (13). In normal circumstances, this gene is expressed when the macrophage is stimulated with both IFN-γ and a costimulatory molecule such as TNF (14). This co-stimulation activates a cascade of cellular events culminating in the production of NO. Mice with a deletion of the iNOS gene fail to produce detectable levels of NO following acute oral infection with *T. gondii* tissue cysts containing bradyzoites. The iNOS-deficient mice exhibited greater resistance to acute parasite infection and lived significantly longer than the parental control mice in spite of increased parasite burden. These findings suggested to us that NO plays a significant role in reducing parasite burden during acute infection. The involvement of NO in acquired immunity to chronic infection has been suggested. Studies in our laboratory and by Scharton-Kersten et al. (15) indicate that iNOS-deficient mice eventually die, with evidence of high parasite burden in their tissue. These observations suggested that NO may play a significant role in vaccine-based immunity to this pathogen.

Vaccine-based immunity to *T. gondii* using intact parasites has had variable success. Vaccines made from killed organisms (heat, formalin, etc.) (16) have been unsuccessful in producing effective host immunity. Those studies have shown that partial protection was obtainable, but complete protection against challenge with even an avirulent parasite was not possible. Alternative approaches using either attenuated parasites (1), cross-reactive immunity with the morphologically similar Apicomplexan, *Neospora caninum* (17), as well as the temperature-sensitive mutant (ts-4) (18), have demonstrated resistance against virulent parasite challenge (19). Of these various immunization schemes, most investigation has concentrated on the ts-4 mutant. This parasite has been shown to induce near complete protection against challenge in the rodent model. The parasite has limited survival in the infected murine host and is cleared within 60 days of vaccination (20). Moreover, it has been demonstrated that this parasite can elicit a strong immune response in primates (21). The mechanism by which this mutant stimulates long-term immunity in the host are not well understood. One such mechanism would be to allow for the clearance...
of the parasite by the induction of NO following antigenic reexposure.

The present study demonstrates that vaccination of iNOS-deficient mice with the avirulent ts-4 mutant confers long-term protection against oral challenge with a lethal dose of cysts containing bradyzoites. The level of protection in these deficient mice does not differ from similarly vaccinated parental mice. Vaccination of either mouse strain resulted in decreased parasite burden following challenge. Host immunity appears dependent upon the induction of IFN-γ and CD8+ T cells, but is independent of NO production.

Materials and Methods

Parasites and infection

The temperature-sensitive mutant strain of T. gondii, ts-4 (kindly provided by Dr. Elmer Pfefferkorn, Dartmouth Medical School), was used for immunization of animals. The strain is maintained by continuous passage in human fibroblasts. Mice were challenged with the 76K strain of T. gondii (provided by Dr. Daniel Bout, Tours, France). This strain is maintained by continuous oral passage of cysts as previously described (22). Cysts were isolated from infected tissue, enumerated, and used to orally infect the animals. A challenge dose of 50 cysts was used unless mentioned otherwise.

Mice

A breeding pair of iNOS−/− mice (C57BL/6 × 129) was kindly provided by Dr. Carl Nathan (Cornell University Medical College, Ithaca, NY). The breeding pairs were heterozygotes with the iNOS gene deleted previously described (23). The mice were backcrossed for five generations to wild-type C57BL/6. The absence of the iNOS gene was confirmed by PCR. The breeding pairs of IL-12 p40−/− mice, on a C57BL/6 background, were a gift from Dr. Maurice Gately (Hoffmann-La Roche, Nutley, NJ). These mice were bred under approved conditions in the Animal Research Facility at Dartmouth Medical School. Five- to six-week-old female mice were used in the studies. Age- and sex-matched C57BL/6 mice obtained from The Jackson Laboratory (Bar Harbor, ME) were used for wild-type controls.

Vaccination and challenge

The immunizations of iNOS−/− and wild-type C57BL/6 mice were carried out by injecting the animals i.p. with 1 × 104 ts-4 parasites. After 2 wk, the mice were boosted a second time with 5 × 104 parasites of the same strain. The immunized animals were challenged later with the 76K strain of T. gondii.

In vivo cytokine and T cell depletion

For IFN-γ depletion, mice were treated with 3 mg of rat anti-mouse IFN-γ (XM6G; American Type Culture Collection [ATCC]; Manassas, VA) weekly. CD4+ T cell depletion was performed by administration of affinity-purified rat anti-mouse CD4 Ab (GK1.5) at a dose of 0.5 mg/day for 3 consecutive days followed by every third day thereafter. To deplete CD8+ T cells, mice received 1 mg of anti-CD8 mAb (clone 2.43, ATCC) for 3 days at the beginning and every third day until termination of the experiment. The Ab treatment resulted in >95% depletion of the respective T cell phenotype. The control animals received an equal amount of rat IgG.

Quantitation of tissue parasite burden

Tissues (liver and brain) were recovered fresh from vaccinated mice at 7 days postchallenge with the 76K strain. Genomic DNA from the organs was prepared as previously described (13). To quantify the number of parasites from the tissue, T. gondii DNA was isolated from a known number of cell culture–derived extracellular tachyzoites. The toxoplasma B1 sequence was used as a probe to quantify tissue parasite load. This gene, previously used for detection of this parasite (24), is a 35-fold repetitive sequence and is found in all parasite strains, including 76K and RH. A 1018-bp sequence was amplified by using primers 5'-GTGTGGTCCGGCTTCGTC and 3'-CGAATCACCAGGAACTGTTATG with a cycle of 1 min denaturation at 93°C, 1 min annealing at 55°C, and 1.5-min extension at 72°C. A corresponding 1-kb band observed on 1% agarose gel was purified by QIA gel extraction kit (Qiagen, Chatsworth, CA) and T-PipATP tagged with a random-primed labeling kit (Boehringer Mannheim, Indianapolis, IN). Slot blot hybridization was conducted according to a standard protocol (25). Five micrograms of genomic DNA from each tissue were denatured with 0.4 N NaOH and 0.2 mM EDTA and then blotted onto a Hybond-N+–positive charged membrane. The membranes were analyzed by autoradiography and scanned by a Silverscanner III flatbed scanner, model G550A (Epson, Torrance, CA). Data analysis was performed using a public domain National Institutes of Health Image program (developed at the National Institutes of Health and available from the Internet at zippy.nimh.nih.gov). To quantitate the number of parasites in the tissue, DNA was isolated from a known number of parasites and run in parallel with the infected tissues. The intensity of the signal in the autoradiograph was measured and a known number of parasites were used as a standard to quantitate the number of parasites/ microgram of tissue DNA.

Cytotoxicity assays

Cell-mediated cytotoxicity was determined by using a microcytotoxicity assay. The quantitative analysis of the cytolytic activity of the T cells was done by determining the precursor CTL (pCTL) frequency of the vaccinated mice by establishing limiting dilution assays (LDA). Splenic CD8+ T cells were isolated by magnetic separation (26). Purified 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 IL-2, irradiated parasites, and feeder cells. The dilution of cells was carried out from 100, 500, 1,000, 5,000, 10,000 to 25,000 cells/well. Control wells contained only irradiated parasites and feeder cells. After 1 wk, the cells were harvested and incubated with 31Cr-labeled parasite-infected and uninfected macrophages. The macrophages were collected and labeled as described elsewhere (26). Briefly, peritoneal macrophages were cultured at a concentration of 2 × 104 cells/well in 96-well U-bottom tissue culture plates. After overnight incubation they were radiolabeled with 31Cr (0.5 μCi/well); New England Nuclear, Boston, MA) for 3 h at 37°C. After several washes in PBS, macrophages were infected with 1 × 106 freshly obtained RH parasites. The next morning, spontaneous lysis caused by overnight parasite infection was measured and all wells exhibiting >250 cpm in the supernatant were excluded from the experiment. Macrophages were washed in PBS and incubated with CD8+ T cell cultures. The amount of radioisotope release was measured following a 4-h incubation. The wells were considered to be positive for lytic activity if total cpm released by effector cells plus target cells was greater than 3 × 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 (27).

IFN-γ assay

The cytokine assay of CD8+ T cell subtype from the immunized animals was performed. Two weeks after the last immunizing dose the CD8+ T cells from the splenocytes were separated by magnetic separation and stimulated in vitro with toxoplasma lysate in presence of irradiated feeder cells. After incubation for 72 h, the cells were harvested and supernatants were collected and assayed for IFN-γ production by commercially available ELISA kit (Genzyme, Cambridge, MA).

Results

iNOS−/− mice are resistant to ts-4 infection

iNOS−/− and parental C57BL/6 mice were infected with varying numbers (1 × 103 to 1 × 106) of ts-4 strain tachyzoites. Both the parental and iNOS−/− mice could tolerate the highest parasite challenge without evidence of morbidity (as determined by body weight change, ruffled fur) or mortality. Mice vaccinated with ts-4 remained asymptomatic throughout the entire experiment, which was terminated at day 60 postinfection (data not shown). Since production of NO is an important outcome of IFN-γ production (28). IL-12-deficient mice vaccinated with ts-4 were susceptible to infection compared with both the parental control mice and the iNOS−/− (Fig. 1). All mice in the IFN-γ−/− group had succumbed by day 11 postinfection. Since IL-12 is a primary stimulator of IFN-γ, mice with a disruption of the IL-12 p40 subunit (p40−/−) are deficient in IFN-γ production (28). IL-12-deficient mice were susceptible to infection with ts-4 and succumbed to infection, unlike parental controls or the iNOS−/−, which showed no signs of morbidity or mortality throughout the study. These observations suggested that IFN-γ was an important mediator of protection but not NO.
In addition to IFN-γ, CD8⁺ T cell immunity is essential for host protection against this parasite (2, 29). Host immunity was abrogated in both the iNOS⁻/⁻ and parental control mice when ts-4-infected mice were treated with either anti-IFN-γ or anti-CD8 T cell mAb starting at day 7 postinfection (Fig. 2, A and B). Mice treated with either anti-IFN-γ or anti-CD8 Ab at 1 mo postinfection showed no increase in mortality (data not shown).

Production of IFN-γ by CD8⁺ T cells from ts-4-vaccinated mice was determined. The CD8⁺ T cell subset from the mice immunized with ts-4 strain of parasites was isolated and cultured in the presence of toxoplasma lysate and irradiated feeder cells. The supernatants from the cultures were analyzed for IFN-γ production. CD8⁺ T cells from both the iNOS⁻/⁻ and parental mice secreted equal amounts of IFN-γ in response to antigenic exposure (Table I).

pCTL frequency is similar in iNOS⁻/⁻ and parental mice after ts-4 vaccination

It has been demonstrated that ts-4 vaccination leads to the induction of a CD8⁺ T cell response (19, 30). These CD8⁺ T cells exhibit cytolytic activity in vitro against parasite-infected target cells. To estimate the frequency of Ag-specific cytolytic CD8⁺ T cells in the immune population following ts-4 immunization, a precursor CTL assay was performed. By limiting dilution analysis, it was determined that the precursor frequency of the two vaccinated mouse strains was similar. One month after the final immunization, the pCTL frequency of iNOS⁻/⁻ animals was 1/3000 compared with 1/4100 in the parental control group (Fig. 3). Since this is considered to be within the range of variability for this assay (31), it appears that the magnitude of Ag-specific CD8⁺ T cell response in iNOS⁻/⁻ mice is similar to parental wild-type controls.

iNOS⁻/⁻ mice vaccinated with ts-4 survive lethal parasite challenge

iNOS⁻/⁻ and parental mice were vaccinated with ts-4 and 1 mo later challenged with 50 tissue cysts (76K strain) containing bradyzoites. Vaccination of both iNOS-deficient and parental mice with ts-4 provided complete protection against lethal parasite challenge until the experiment was terminated at day 40 postchallenge (Fig. 4). Nonvaccinated control mice succumbed to infection between days 10 and 12 postchallenge whereas nonvaccinated

<table>
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<th>Mice</th>
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<tr>
<td>Parental infected</td>
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<td>1565 ± 150</td>
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<td>Not detected</td>
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<tr>
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<td>37 ± 8.5</td>
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*CD8⁺ T cells from the immunized knockout and parental wild-type mice were separated 2 wk after the last immunizing dose. A total of 1 × 10⁶ purified cells were cultured in presence of 15 μg/ml of toxoplasma lysate Ag and irradiated feeder cells. After 72-h incubation at 37°C, the supernatants were collected and analyzed for IFN-γ by ELISA.

FIGURE 1. Survival of gene knockout mice from i.p. challenge of tachyzoites of ts4 strain of T. gondii. iNOS⁻/⁻, p40⁻/⁻, IFN-γ⁻/⁻, and parental mice (n = 6/pc) were infected with 1 × 10⁵ tachyzoites and mortality was monitored. The study was performed thrice with similar findings. Data presented are representative of one experiment.

FIGURE 2. Effect of IFN-γ and CD8⁺ T cell depletion on survival of iNOS⁻/⁻ mice. iNOS⁻/⁻ (A) and parental wild-type (B) mice were challenged i.p. with 1 × 10⁵ tachyzoites of the ts-4 strain. Animals were treated with either rat anti-mouse IFN-γ or rat anti-mouse CD8 starting day 7 postchallenge. The control animals were treated with rat IgG. The survival of the mice was monitored up to day 30 postinfection when the experiment was terminated. There were six animals per group and the experiment was performed twice. The data are representative of one experiment.

Table 1. IFN-γ production (pg/ml) by CD8⁺ T cells from ts-4 immunized mice

*CD8⁺ T cells from the immunized knockout and parental wild-type mice were separated 2 wk after the last immunizing dose. A total of 1 × 10⁶ purified cells were cultured in presence of 15 μg/ml of toxoplasma lysate Ag and irradiated feeder cells. After 72-h incubation at 37°C, the supernatants were collected and analyzed for IFN-γ by ELISA.
Parasite burden in vaccinated iNOS<sup>−/−</sup> and parental mice is similar

Tissue from both the iNOS<sup>−/−</sup> and parental mice was analyzed by Southern blot to determine whether vaccination had an effect on reducing parasite burden after challenge. The assay was performed at day 7 postchallenge by determining the relative abundance of B1 gene, a genetic marker for T. gondii. Analysis of brain and liver tissues demonstrated a very low number of parasites in the vaccinated parental (Fig. 6A) and iNOS<sup>−/−</sup> (Fig. 6B) mice as compared with nonvaccinated control animals. There was no difference in the relative parasite burden between the tissues of the two mouse strains. In comparison, the nonvaccinated control mice exhibited a 2- to 10-fold increase in parasite burden in the brain and liver of iNOS<sup>−/−</sup> mice as compared with the parental control consistent with earlier observations (13).

Discussion

Our data demonstrate that NO has limited function in vaccine-based immunity against T. gondii. The implication of this observation could perhaps be extended to include an uncertain role for this molecule during recrudescence infection in the chronically infected host, such as those with AIDS. The principal finding in this study demonstrated that mice with a targeted deletion of the gene for iNOS could tolerate vaccination with an avirulent parasite strain and, moreover, were completely protected against subsequent lethal challenge.

The release of NO is mediated by the key Th1-type cytokine, IFN-γ. Secretion of IFN-γ by either T cells or NK cells in response to infection activates macrophages (32, 33). Following a cascade of complex molecular events, including costimulation with TNF, the activated macrophages release NO. This molecule has demonstrated microbicidal and tumoricidal activity, including a static role in response to infection with T. gondii (12). Earlier studies from our laboratory have shown that acute infection with T. gondii results in significantly increased parasite burden in iNOS<sup>−/−</sup> mice as compared with wild-type controls (13). In the present study, we demonstrate that vaccination of iNOS-deficient mice with an attenuated parasite strain is not lethal. Analysis of parasite burden indicates that the ts-4 strain is cleared with equal efficiency in the two strains of mice. This finding may be considered inconsistent with previous reports that NO is essential for the control of persistent parasitemia in the murine model (15). One explanation for this difference could be attributed to the efficient elimination of the parasite by a NO-independent mechanism. Establishment of CD8<sup>+</sup>-mediated long-term immunity appears independent of an early NO response. Furthermore, once primed, the CD8<sup>+</sup> memory T cells do not require NO to carry out their toxoplasmacidal activity.

The protective immunity in both iNOS<sup>−/−</sup> and wild-type mice was dependent on CD8<sup>+</sup> T cells. The role of CD8<sup>+</sup> T cells during the natural infection or experimental immunizations with T. gondii has been described by various laboratories (29, 30). Similarly, the importance of CD8<sup>+</sup> T cells during vaccination with ts-4 parasites has been established (2). In the present study, the depletion of CD8<sup>+</sup> T cells resulted in the abrogation of vaccine-mediated protection in both the parental and iNOS<sup>−/−</sup> mice, resulting in earlier death. This differs from previous observations in which β<sub>2</sub>-microglobulin-deficient mice were effectively vaccinated with ts-4 parasite (34). Since these mice are CD8<sup>+</sup> deficient, a possible explanation for this discrepancy is that β<sub>2</sub>-<sup>−/−</sup> mice can generate unusually high NK cell response (35). This NK response could protect the mice in the absence of CD8<sup>+</sup> T cells, either via the induction of protective cytokines or cytolysis of infected targets.
IFN-γ plays a critical role in protection against *T. gondii* infection (4). Protective immunity against *T. gondii* conferred by immune CD8⁺ T cells is dependent on IFN-γ (3, 36). Mice with targeted disruption of IFN-γ gene are susceptible to vaccination with ts-4 (37). Our studies support the essential role for this cytokine since depletion in either the parental or iNOS−/− mice results in increased susceptibility. Although one of the parasiticidal effects of IFN-γ is macrophage activation and subsequent release of NO, another consideration is enhancement of MHC class I expression and Ag presentation (38), resulting in greater proliferation.

**FIGURE 5.** The effect of T cell depletion on the protective immunity induced by ts-4 vaccination. Immune iNOS−/− (A) and wild-type mice (B) (n = 6/gp) were treated with mAbs directed against either CD4, CD8, or IFN-γ. The Ab treatment was started 2 days before the challenge with 76K strain of *T. gondii* and continued throughout the course of experiment. Control mice were injected with rat IgG. The survival of the animals was monitored daily until the experiments were terminated. The study was conducted twice and data are representative of one experiment.

**FIGURE 6.** Levels of parasite DNA in the organs of immune parental C57BL/6 (A) and iNOS−/− mice (B). Mice (n = 3/gp) vaccinated with ts-4 strain (IM) or sham immunized (NI) were infected orally with 50 cysts of 76K strain of *T. gondii*. DNA was prepared from the brain and liver of mice 7 days postchallenge. A slot blot analysis was probed with *T. gondii*-specific B1 sequence. The scanned autoradiogram was quantitated by National Institutes of Health Image using DNA from a known number of extracellular tachyzoites as control. Bars represent the average number of parasites detected in each tissue.
of CD8+ T cells. T. gondii infects a wide variety of cell types that express class I Ags (39). Enhanced expression of class I can provide for a stronger interaction between MHC class I expressing cells and Ag-specific CD8+ T cells. Even though CD4+ T cells have been demonstrated to produce equal, if not greater, levels of IFN-γ in vitro, as compared with CD8+ T cells (30), their participation in vaccine-based immunity to this parasite is primarily synergistic.

Further support for an IFN-γ-mediated CD8+ mechanism of protection can be found in the observation that iNOS−/− mice vaccinated with the ts-4 strain could generate a CD8+ memory CD8+ T cells that are cytotoxic for the parasite and per-

ceptibility during infection with LCMV and Listeria monocytogenes (46, 47). Perforin−/− mice infected with T. gondii exhibited significantly accelerated mortality and 3- to 4-fold increase in the cyst number (45). We have further confirmed the importance of IFN-γ in stimulating the production of CTL, using mice deficient in the production of IL-12. These IL-12 knockout mice exhibit a substantially lower precursor CTL frequency response during toxoplasmosis infection compared with the parental control. Of note is the increase in pCTL frequency when these knockout mice are treated with exogenous IFN-γ. Together these data suggest that a very important role for IFN-γ may be the regulation of CD8+ T cell response during microbial infection.

The observations in this report indicate that NO is not essential in the development of vaccine-based immunity to this intracellular parasite. Mice deficient in the production of NO do not display increased susceptibility to vaccination with an attenuated parasite. Moreover, these vaccinated deficient mice are able to control challenge with a more virulent parasite strain. IFN-γ produced by NK cells and CD4+ T cells during the acute stages of infection results in macrophage activation and subsequent killing of the parasite by NO-dependent mechanism. The overproduction of NO may cause a lethal hyperimmune response in some hosts (e.g., C57BL/6 mice) (13). Continued production of IFN-γ enhances the production of memory CD8+ T cells that are cytotoxic for the parasite and perhaps home to the appropriate organ. Upon antigenic restimulation they provide the protective immunity to the host either by cytolytic or noncytolytic pathway as reported in viral systems (48).

Acknowledgments

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