Effector Cells of Both Nonhemopoietic and Hemopoietic Origin Are Required for Interferon (IFN)-γ– and Tumor Necrosis Factor (TNF)-α–dependent Host Resistance to the Intracellular Pathogen, *Toxoplasma gondii*

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Summary

Although interferon (IFN)-γ–activated, mononuclear phagocytes are considered to be the major effectors of resistance to intracellular pathogens, it is unclear how they control the growth of microorganisms that reside in nonhemopoietic cells. Pathogens within such cells may be killed by metabolites secreted by activated macrophages or, alternatively, directly controlled by cytokine-induced microbicidal mechanisms triggered within infected nonphagocytic cells. To distinguish between these two basic mechanisms of cell-mediated immunity, reciprocal bone marrow chimeras were constructed between wild-type and IFN-γ receptor–deficient mice and their survival assessed following infection with *Toxoplasma gondii*, a protozoan parasite that invades both hemopoietic and nonhemopoietic cell lineages. Resistance to acute and persistent infection was displayed only by animals in which IFN-γ receptors were expressed in both cellular compartments. Parallel chimera experiments performed with tumor necrosis factor (TNF) receptor–deficient mice also indicated a codependence on hemopoietic and nonhemopoietic lineages for optimal control of the parasite. In contrast, in mice chimeric for inducible nitric oxide synthase (iNOS), an enzyme associated with IFN-γ–induced macrophage microbicidal activity, expression by cells of hemopoietic origin was sufficient for host resistance. Together, these findings suggest that, in concert with bone marrow–derived effectors, nonhemopoietic cells can directly mediate, in the absence of endogenous iNOS, IFN-γ– and TNF-α–dependent host resistance to intracellular infection.

Key words: toxoplasma • interferon γ • tumor necrosis factor α • inducible nitric oxide synthase • cell-mediated immunity

Cell-mediated immune responses are critical for immune defense against a variety of intracellular infections as well as tumors. During the induction of cell-mediated immunity (CMI),[1] T lymphocytes expressing IFN-γ and TNF-α are generated within secondary lymphoid organs in an IL-12–dependent manner (1–3). Their extravasation into sites of infection results in the recruitment of macrophages, which become activated by these T cell–derived lymphokines. Nevertheless, the mechanisms by which the multicellular interactions involved in CMI lead to control of intracellular pathogens and protection of the invaded tissue have only been partially elucidated.

Most of our understanding of CMI is based on studies of microbial agents such as *Listeria*, *Mycobacteria*, and *Leishmania*, which have an absolute or near absolute tropism for macrophages (4–6). Nonetheless, many intracellular pathogens infect not only cells of the mononuclear phagocyte lineage but also those of epithelial, endothelial, mesodermal, and neuronal derivation. The list of organisms belonging to this category include *Salmonella*, *Shigella*, *Chlamydia*, *Rickettsiae*, *Trypanosoma*, and *Toxoplasma*. Although the growth of such intracellular pathogens is generally thought to be controlled by toxic reactive nitrogen or reactive oxygen intermediates, it is unclear in many cases whether the latter effector molecules can be generated and/or function in the nonhemopoietic cell types that they invade. For example, although NO is potentially produced by a wide variety of host cells, its documented antimicrobial functions have been largely restricted to monocytes/macrophages (7). Thus, the question arises as to how T cell–derived lymphokines can act to restrict the growth of the above pathogens within cells of nonhemopoi-

[1]*Abbreviations used in this paper:* BM, bone marrow; CMI, cell-mediated immunity; CNS, central nervous system; IDO, indoleamine dioxygenase; iNOS, inducible nitric oxide synthase; KO, knockout; STAg, soluble tachyzoite antigen; WT, wild-type.

etic origin. Although many possible scenarios have been proposed, these can be distilled into two conceptually distinct "cis" and "trans" models (see Fig. 1 for schematization) that describe how intracellular infections might be controlled within nonmacrophage (i.e., nonhemopoietic) cells.

The trans model argues that the main site for lymphokine-induced activation is at the level of a professional effector cell, such as the macrophage (8, 9). Upon activation, the macrophage releases toxic metabolites, most notably NO generated enzymatically by type 2 nitric oxide synthase (iNOS), which diffuses to neighboring somatic cells, thereby restricting the growth of the intracellular pathogen. In direct contrast, the cis model proposes that lymphokine receptor ligation on nonprofessional effector cells leads to metabolic changes resulting in inhibition of microbial growth within the same cells (10–14). One prediction of the cis model is that receptors for IFN-γ and TNF-α need to be expressed and functional on somatic (in addition to bone marrow [BM]-derived) cellular compartments for complete resistance. In contrast, the trans model requires that these receptors be expressed only on hemopoietic effector cells and postulates that infected nonhemopoietic cells play no active role in host resistance.

We have designed an experimental approach for distinguishing between these two models of antimicrobial effector function that involves infection of BM chimeric mice with the intracellular pathogen *Toxoplasma gondii*. This apicomplexan protozoan parasite invades a wide variety of cells, including epithelial, mesodermal, and neuronal as well as hemopoietic cells (15). Immunity to *T. gondii* is strictly dependent on IFN-γ production by $CD8^+$ T lymphocytes as well as NK and $CD4^+$ T cells (15–17). Although the requirement for CD8 lymphocytes is not completely understood, one possibility is that class I–restricted recognition of infected nonhemopoietic cells leads to the inhibition of parasite growth through localized IFN-γ secretion (18, 19). In addition to IFN-γ, TNF-α has also been shown to play a major role in host resistance to *T. gondii*. However, whereas IFN-γ is necessary for both acute and chronic resistance to parasite infection (20–22), the requirement for TNF-α manifests primarily in the chronic phase, when the parasites reside in the central nervous system (CNS; 21, 23, 24). Similarly, although iNOS-dependent production of NO is also critical for control of the parasite, its role becomes apparent only during the chronic phase of *T. gondii* infection (25, 26).

In this study, we have used BM chimeric mice to evaluate the requirement for IFN-γR, TNFR, and iNOS expression on hemopoietic versus nonhemopoietic cells in host resistance to acute and chronic *T. gondii* infection. Our findings suggest that in both stages of infection, nonhemopoietic cells function as iNOS-independent effectors of IFN-γ/TNF-α–dependent immunity. These observations directly support the cis model of CMI outlined above.

Materials and Methods

Mice. IFN-γR knockout (KO; 27) and IFN-γR wild-type (WT) mice, both on a 129/Sv background, were bred as homozygotes from breeders initially provided by Maria Wysocka (Wistar Institute, Philadelphia, PA). TNFR p55/p75–deficient mice (28, 29) on a mixed 129/Sv × C57BL/6 background were bred in our facility using homozygous breeding pairs provided by Mark Moore (Genentech, Inc., South San Francisco, CA). iNOS-deficient mice (30) on a second generation C57BL/6 backcross were bred through a National Institutes of Allergy and Infectious Diseases contract with Taconic Farms, Inc. C57BL/6 and C57BL/6 × 129/SvEv F1 hybrids obtained from the The Jackson Laboratory were used as WT controls for the iNOS- and TNFR-deficient mice, respectively. Donor (at least 8 wk old) and recipient animals were sex matched.

Bone Marrow Transplantation. Recipient mice were given lethal total body irradiation (950–1,000 rads) and reconstituted intravenously with 10–20 million bone marrow cells within 24 h. Marrow cell suspensions were prepared from donor tibial and femoral bones by flushing with RPMI 1640 (GIBCO BRL) supplemented with antibiotics (penicillin [100 U/ml] and streptomycin [100 U/ml]) using a 25-gauge needle syringe. Irradiated and reconstituted mice were given Bactrim (sulfamethoxazole [150 mg/ml] and *N*-trimethoprim [30 mg/ml]; Teva Pharmaceuticals) in their drinking water for 5 wk. Thereafter, they were switched to sterile drinking water, thus ensuring that the antibiotic treatment would not affect the ensuing experimental infection with *T. gondii*. Unless otherwise stated, mice were used for experimental infection or for analysis of chimerism 8–9 wk after BM cell transfer. For each infection experiment, groups of nonirradiated WT and KO animals were included as positive and negative controls for host resistance, respectively. In every instance except one, sham chimeric KO→KO mice exhibited mortality rates identical to those of nonirradiated KO mice, whereas WT→WT mice behaved like nonirradiated WT mice. However, sham chimeric mice that were used as controls for the studies involving iNOS-deficient mice reproducibly (in three independent experiments) succumbed ~30 d after infection, much earlier than unmanipulated C57BL/6 mice, which survived for at least 60 d. The reason for the earlier mortality of the C57BL/6 sham chimeras is unclear. These mice did not succumb unless infected with *T. gondii* and showed full reconstitution of splenic lymphoid and myeloid cell populations as determined by flow cytometric analysis (data not shown).

Analysis of Chimerism. The extent of hemopoietic cell replacement by donor phenotype cells upon reconstitution was analyzed 8 wk after transfer of BM cells using mice chimeric for iNOS gene deficiency. Spleen cells and d5 thioglycollate–elicited peritoneal cells were harvested from each of three mice per group. Cells were plated in 96-well plates at a concentration of 2×10^6 cells/ml and stimulated with 100 U/ml of IFN-γ and 10 μg/ml of soluble tachyzoite antigen (STAg). The culture medium consisted of RPMI 1640 (GIBCO BRL) supplemented with 10% FBS (Hyclone), penicillin (100 U/ml) and streptomycin (100 U/ml), glutamine (GIBCO BRL), and 2-ME (5 μM; Sigma Chemical Co.). Nitrite production after 24 h was evaluated by the Griess reaction as previously detailed (24).

Assessment of Cytokine Production in IFN-γR KO Mice. IFN-γR KO and WT mice were immunized with 10^6 lethally irradiated (15 Krads) tachyzoites of *T. gondii* (RH strain). 14 d later, IFN-γ and IL-12 p40 production by spleen cells from vaccinated and naive mice was assessed. Spleen cell cultures (3×10^6/ml) were stimulated with either ConA (5 μg/ml) or STAg (10 μg/ml). Supernatants were harvested 24 h later for IL-12 p40 determination and 48 h later for IFN-γ measurement. Previously described ELISA protocols were used to measure IL-12 p40 and IFN-γ levels in culture supernatants (22).

Experimental Infection with T. gondii and Listeria monocytogenes. Tachyzoites of the RH strain of *T. gondii* were cultivated in human foreskin fibroblasts maintained in DMEM (GIBCO BRL) supplemented with 1% FCS and antibiotics. The ME49 strain of *T. gondii* was passaged as cysts in C57BL/6 mice. Experimental animals were infected with 20 ME49 cysts by intraperitoneal injection. An additional set of IFN-γR chimeric mice were infected intraperitoneally with 33 *L. monocytogenes* (EGD strain) bacteria, a dose equivalent to 1/100 of LD_{50} for IFNR WT mice. The *L. monocytogenes* inoculum was prepared from a frozen stock. Bacterial counts were confirmed by plating serial dilutions of the stock on Mueller–Hinton agar plates on the day of infection.

Results and Discussion

Functional Assessment of Chimerism in Irradiated and BM-reconstituted Mice. Measurement of the extent of replacement of tissue macrophages by donor-derived cells is required for proper interpretation of the results obtained with BM chimeric mice. We performed this assessment in iNOS BM chimeric mice, in which IFN-γ– and STAg-induced NO production can be used as a readout of macrophage function. 8 wk after reconstitution, the ability of spleen cells to produce NO in response to IFN-γ and parasite antigen was clearly dictated by the genotype of the BM donor (Fig. 2 A). For instance, spleen cells from iNOS KO mice reconstituted with WT marrow exhibited robust NO production. Importantly, there was no residual NO-producing capacity detectable in WT mice reconstituted with iNOS KO BM. Similarly, the capacity of thioglycollate-elicited peritoneal cells to produce NO in response to IFN-γ and STAg stimulation was dictated by the BM donor genotype (Fig. 2 B). Identical results were obtained using chimeric IFN-γR KO mice (data not shown). Thus, in these chimeras, most if not all of the macrophages in a lymphoid organ (spleen) or at a site of cellular recruitment (peritoneal cavity) appear to consist of donor-derived cells.

IFN-γR Expression Is Required on Both Hemopoietic and Somatic Cells for Acute Resistance Against T. gondii but Not L. monocytogenes. Having demonstrated successful and complete reconstitution of macrophages by donor BM-derived cells, we proceeded to construct chimeric mice using WT and IFN-γR KO on the same 129/SvEv genetic background. IFN-γR KO mice fail to respond to IFN-γ and exhibit increased susceptibility to a variety of intracellular pathogens (27, 31). Both the cis and trans models predict that a KO→WT chimera should exhibit susceptibility to infection because the BM-derived elements, including macrophages and neutrophils, will not be able to control infection. More importantly, however, different outcomes are predicted by the two models for the WT BM→KO chimera. The trans model predicts resistance for this chimera, because the WT marrow-derived cells would be armed and protect somatic cells, whereas the cis model predicts susceptibility for this chimera, because the somatic cells would be unable to control infection due to a lack of IFN-γ responsiveness. An assumption made for the KO→WT chimera is that the KO BM-derived cells remain competent for IFN-γ production in the absence of responsiveness to IFN-γ. Taking into account previous reports of IFN-γ amplification of the IL-12 response (32, 33), a concern raised by these observations is that type 1 responses may not develop normally in the absence of IFN-γ responsiveness in APC and T cell populations. To address these concerns, spleen cells from uninfected IFN-γR KO and WT mice were stimulated with tachyzoite extract (STAg), and their capacity to produce IL-12 p40 as well as IFN-γ was measured by ELISA. As shown in Fig. 3, A and B, production of both cytokines in response to STAg stimulation was not compromised by IFN-γR deficiency. To assess possible defects in type 1 cell development, IFN-γR KO mice were immunized with irradiated tachyzoites and their spleen cells restimulated with STAg or mitogen in vitro. Robust, antigen-specific IFN-γ production was observed in cultures from both WT and KO mice (Fig. 3 C). On the basis of these control experiments, we predicted that in WT mice reconstituted with IFN-γ–unresponsive BM cells, IL-12–dependent NK as well as T cell IFN-γ production should not be impaired.

As expected, sham (KO→KO) chimeric mice completely deficient in IFN-γR were acutely susceptible to *T. gondii* infection, whereas WT→WT sham chimeric mice survived infection for at least 60 d (Fig. 4 A). As predicted by both trans and cis models, chimeric WT mice reconstituted with IFN-γR–deficient BM were susceptible to acute *T. gondii* infection. Importantly, however, in WT→KO chimeras, IFN-γR deficiency in the recipient compartment, despite a WT BM donor genotype, resulted in acute mortality. This observation indicates that IFN-γ activation of WT BM-derived cells is not sufficient to confer protection and that nonhemopoietic cells responsive to this lymphokine are required, an interpretation consistent with the cis model.

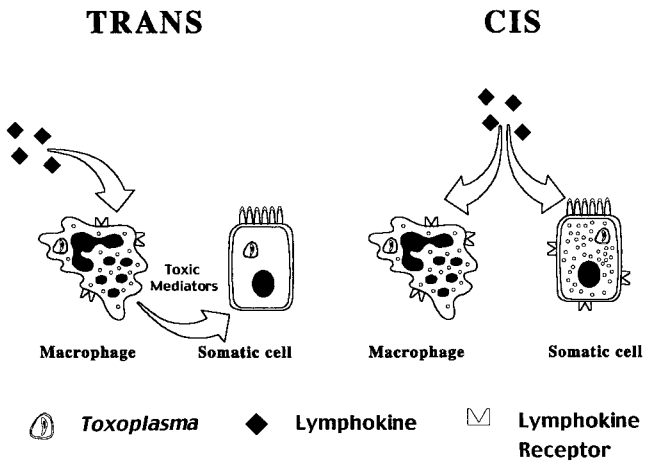

Figure 1. Schematic representation of the trans and cis models of cell-mediated effector function against *T. gondii* within nonhemopoietic cells (i.e., somatic cells). In the trans model, the protective lymphokines (IFN-γ/TNF-α) activate (designated by stippling) the professional effector cell, the macrophage resulting in the synthesis and diffusion of toxic metabolites (e.g., NO) to neighboring somatic cells. These mediators suppress the growth of *T. gondii* within the somatic cell and protect it from lytic infection. In the cis model, the lymphokines directly activate the somatic cell to suppress intracellular *T. gondii* growth.

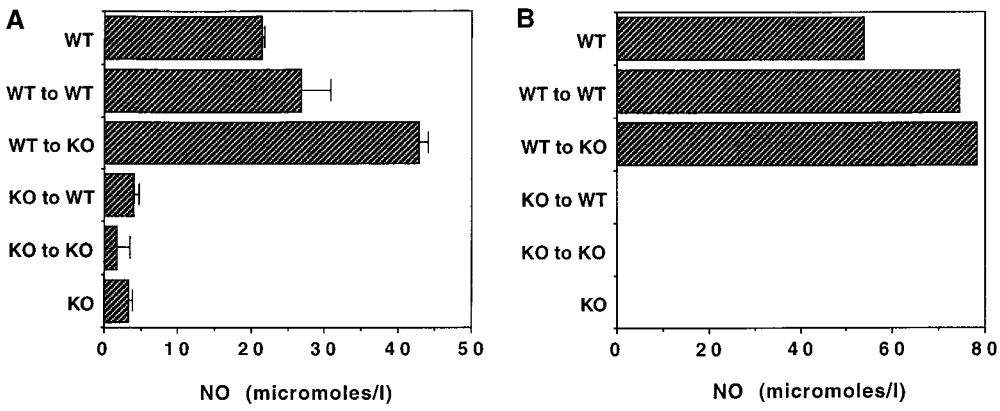

Figure 2. Assessment of hemopoietic reconstitution by donor-type progenitors in BM chimeric mice. Mice chimeric for iNOS gene deficiency were constructed by lethal irradiation and BM reconstitution as described in Materials and Methods. Unmanipulated iNOS KO and WT C57BL/6 mice were included as negative and positive controls, respectively. Spleen cells (A) and day 5 thioglycollate-elicited peritoneal cells (B) were stimulated with IFN-γ (100 U/ml) and STAg (10 μg/ml). Nitrite levels were measured in 24-h culture supernatants. Each bar represents the mean of 2–4 mice per group. All supernatants from unstimulated spleen and peritoneal exudate cells produced <5 μmol/liter of NO_2^-, which also represented the threshold value for a positive signal in this assay.

A possible caveat is that at week eight, WT→KO BM reconstitution may be incomplete in tissue sites other than the spleen and the peritoneum, where chimerism was initially assessed (Fig. 2). The latter explanation is unlikely, however, because even after an additional 2 mo of reconstitution, these animals remained fully susceptible to acute infection (data not shown).

To further confirm that full functional reconstitution was achieved after 8 wk, similarly constructed sets of chimeric mice were infected with the macrophage-trophic intracellular bacterium *L. monocytogenes* and survival of chimeric mice assessed after challenge with a sublethal dose. In the case of this pathogen, IFN-γR expression on the BM but not in the somatic cell compartment was required for resistance (Fig. 4 B). The latter finding confirms that after only 2 mo, WT BM reconstitution of IFN-γR KO recipients is sufficiently complete to confer potential resistance to intracellular infections. The above experiments, by demonstrating a differential requirement for IFN-γR expression on somatic cells for protection against *Toxoplasma* versus *Listeria*, underscore the critical importance of host cellular niches in determining the effector cell types and mechanisms required for control of these pathogens.

IFN-γR Expression on Both Hemopoietic and Somatic Cells Is Also Required for Control of Established T. gondii Infection. Resistance to *T. gondii* is exquisitely dependent on IFN-γ, not only during acute infection but also during the chronic phase of infection (20, 21). For instance, administration of neutralizing antibodies to IFN-γ 30 d after inoculation allows uncontrolled cyst reactivation and tachyzoite replication in the brain parenchyma and invariably causes death of the host within 10 d. To further explore the role of nonhemopoietic cells versus BM-derived inflammatory cells as effectors in the chronic phase of the infection using the IFN-γR KO chimeric mice, partial chemotherapy (commencing 3 d after parasite inoculation) was employed to allow host survival through the acute phase and establishment of persistent infection (as evidenced by cyst formation in brain tissue). Once infection was established (at day 20), further drug treatment was terminated and the survival of the chimeric mice monitored.

As shown in Fig. 5, even with oral drug treatment, a majority of sham IFN-γR KO chimeric mice succumbed to infection by week three postinfection. Chimeric mice with IFN-γR deficiency in either the hemopoietic or nonhemopoietic compartments also died within 5–11 d following cessation of chemotherapy. The kinetics of mortality in these two sets of chimeric mice were essentially identical. As expected, WT sham chimeric mice survived the infection even after drug treatment was withheld. Thus, as observed

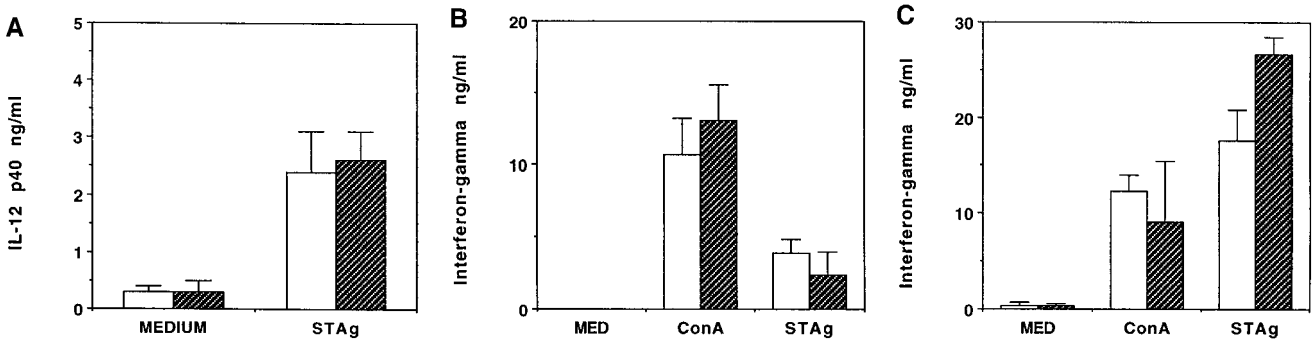

Figure 3. *T. gondii*–induced IL-12 p40 and IFN-γ production in IFN-γR–deficient mice. IFN-γR WT and KO mice (three per group) were either unimmunized (A and B) or immunized with 10^6 lethally irradiated *T. gondii* tachyzoites (C). 2 wk later, spleen cells were harvested and their IL-12 p40 and IFN-γ responses to ConA (5 μg/ml) and STAg (10 μg/ml) assessed. A, IL-12 p40/naive spleen; KO, white bar; WT, hatched bar. B, IFN-γ/naive spleen; naive KO, white bar; naive WT, hatched bar. C, IFN-γ/immune spleen; immune KO, white bar; immune WT, hatched bar.

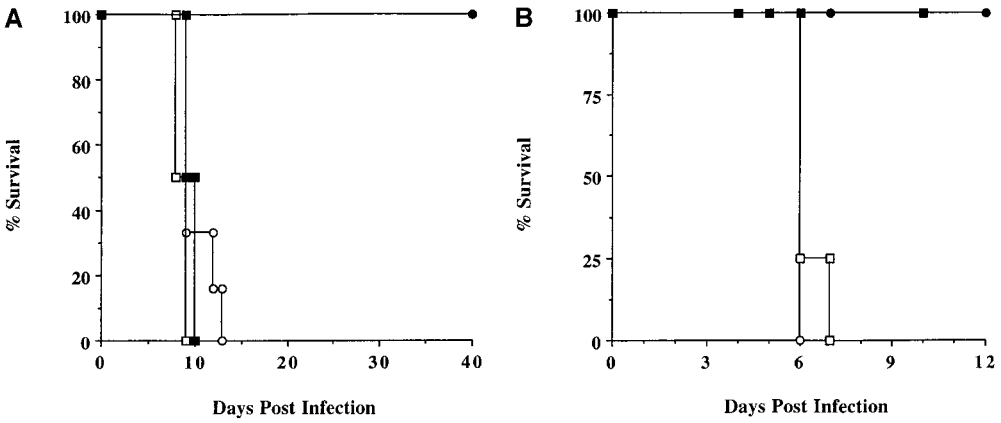

Figure 4. Survival of IFN-γR BM chimeric mice infected with (A) *T. gondii* (ME49 strain; n = 4–7/group) or (B) *L. monocytogenes* (EGD strain; n = 4/group). IFN-γR–WT and –KO mice (both on a 129/Sv background) were used as partners for chimera construction. 2 mo after reconstitution, chimeric and unmanipulated control mice were infected with either (A) 20 *T. gondii* cysts or (B) an LD_{50} dose of *L. monocytogenes*. Data presented are representative of four experiments performed. In each experiment, all IFN-γR KO→KO (○), KO→WT (□), and WT→KO (■) chimeric mice succumbed acutely to *T. gondii* infection, whereas only IFN-γR KO→KO and KO→WT died after *L. monocytogenes* infection. In both infections, IFN-γR KO→KO chimeric mice succumbed acutely, as did control IFN-γR KO mice, whereas IFN-γR WT→WT (●) and control WT mice survived >60 d (not shown).

for resistance to acute infection, IFN-γR expression on nonhemopoietic as well as BM-derived cells is essential for host survival in established infection, as assessed in this drug treatment model.

TNFR Are Also Required on Both Compartments for Resistance to T. gondii. Although IFN-γ–mediated signals are clearly important and required on both somatic and hemopoietic cells during the acute and chronic phases of infection, IFN-γ alone does not suffice, at least during the chronic phase. At this stage, TNF-α is also crucially required for host resistance. Thus, administration of anti-TNF Ab (without additional neutralization of IFN-γ) is sufficient to reactivate infection and induce lethal encephalitis in chronically exposed C57BL/6 mice (21). Additionally, *T. gondii* infections

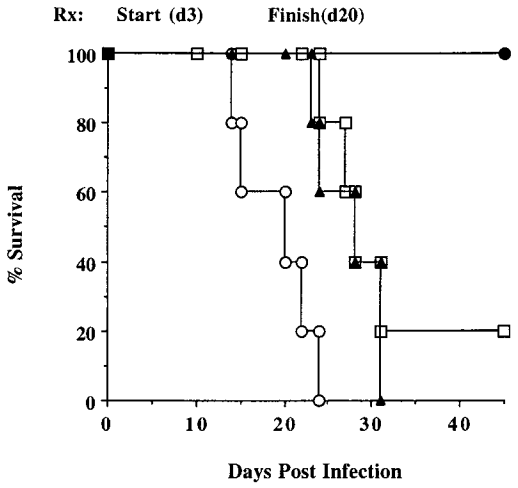

Figure 5. Survival of *T. gondii*–infected and drug-treated IFN-γR BM chimeric mice. IFN-γR WT and KO mice (both on a 129/Sv background) were used as partners for chimera construction. Groups (n = 5/group) of nonirradiated controls and irradiated BM reconstituted mice were infected with 20 *T. gondii* cysts. Bactrim-containing drinking water was administered on day 3 and withdrawn on day 20 postinfection. The data shown are from a single experiment performed. ○, KO→KO; □, KO→WT; ▲, WT→KO; ●, WT→WT.

are lethal in mice lacking either the TNF p55 receptor or both the p55 and p75 receptors (23, 24). In the case of this as opposed to IFN-γR deficiency, lethality occurs only after infection establishes in the CNS (∼3 wk after infection).

Because TNFRs are expressed not only on macrophages and other hemopoietic cells but also on nonhemopoietic cells, including astrocytes and neuronal cells, we evaluated the requirement for TNFR signaling on these cellular compartments for resistance to chronic infection. Reciprocal chimeras were constructed between WT (B6 × 129/J F1) and TNFR p55/p75 KO mice on the same hybrid background. As shown in Fig. 6, TNFR KO mice reconstituted with KO BM cells succumbed to infection within 20 d, as previously reported for unmanipulated TNFR KO mice. WT controls and WT→WT sham chimeric mice survived *T. gondii* infection for at least 60 d. However, TNFR KO BM cells in the context of a WT recipient did not confer the same susceptibility phenotype observed in completely deficient mice. Similarly, WT BM→KO chimeras also exhibited only partial resistance to chronic infection. Thus, both compartments must be receptor deficient or WT to exhibit a completely susceptible or resistant phenotype, respectively. In contrast to these findings, resistance to *Listeria monocytogenes* has been shown to require TNFR expression only on BM-derived cells (34). These divergent requirements for TNFR expression in *Toxoplasma* and *Listeria* systems further highlight the importance of the parasitized cellular niche in determining which effector cell populations are required for host resistance.

iNOS Expression Is Critically Important for the Resistance Phenotype only in Hemopoietic Cells. We have previously proposed that TNFRs expressed on nonhemopoietic, somatic cells such as neurons and astrocytes may be important in activating them to control intracellular tachyzoite replication by an iNOS-independent mechanism (24). This hypothesis was based on the finding that although TNFR (p55/p75) KO mice develop necrotizing encephalitis and die at the same rate as iNOS-deficient mice, they nevertheless display significant iNOS induction in brain tissue. Be-

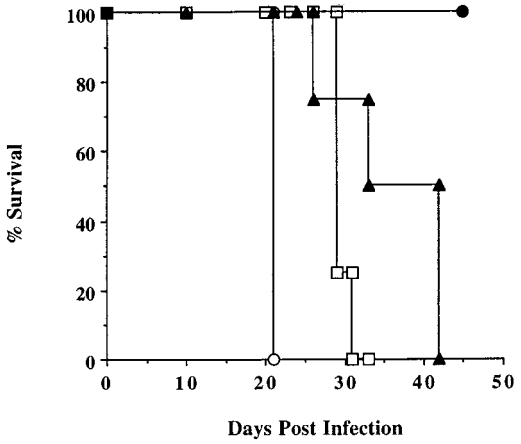

Figure 6. Survival of *T. gondii*–infected TNFR BM chimeric mice. TNFR p55/p75 KO and C57BL/6 × 129 F1 WT hybrids were used as partners for chimera construction. Nonirradiated and 8-wk reconstituted chimeric mice ($n = 4$/group) were infected with 20 *T. gondii* cysts and their survival monitored daily. Nonirradiated TNFR p55/p75 KO mice succumbed at the same time as TNFR KO→KO chimeras, whereas both control WT and WT→WT chimeric mice survived >60 d (data not shown). Data shown are representative of two independent experiments. Mean survival time of TNFR chimeric mice (KO→WT and WT→KO) was significantly different from that of KO→KO mice ($P < 0.01$ in both experiments done) but not different from each other ($P = 0.16$ and 0.06). ○, KO→KO; □, KO→WT; ▲, WT→KO; ●, WT→WT.

cause macrophages from the same TNFR KO mice can exhibit both NO production and microbicidal activity in vitro and ex vivo, these observations suggested that the defect responsible for death of the infected TNFR-deficient animals resides in nonhemopoietic effector cells. The intermediate level of resistance displayed by KO→WT and WT→KO TNFR chimeric mice (Fig. 6) argues instead that control of chronic infection may require TNFR expression on both hemopoietic and nonhemopoietic compartments.

If the extreme susceptibility of the TNFR KO mice is explainable solely by iNOS deficiency, then the resistance patterns in iNOS chimeric mice should be identical to those exhibited by TNFR chimeric animals. As shown in Fig. 7, this is clearly not the case. In contrast to the parallel TNFR-deficient chimera, iNOS→WT BM chimeric mice displayed susceptibility indistinguishable from either iNOS→iNOS (Fig. 7 A) or nonirradiated iNOS KO mice (Fig. 7 B). Furthermore, the WT→KO reciprocal chimera exhibited a survival pattern virtually indistinguishable from the control WT→WT chimera. For reasons that are not clear (see Materials and Methods), these sham chimeric mice succumbed early, at ~30 d, in contrast to nonirradiated WT C57BL/6 mice, which, as previously reported (26), survived greater than 60 d (Fig. 7 B). Nevertheless, the findings clearly indicate that, in the iNOS system, the BM genotype is the main determinant of the resistance phenotype of the chimera. This is in direct contrast to the situation in both IFN-γR and TNFR chimeric animals, in which both hemopoietic and nonhemopoietic cells contribute to host resistance during the chronic phase of infection.

Taken together, these results indicate that the trans mechanism, whereby IFN-γ– and TNF-α–dependent production of toxic metabolites such as NO by mononuclear phagocytes is, by itself, not sufficient to account for immune control of a pathogen that infects both hemopoietic and nonhemopoietic cells. Instead, our findings are more consistent with the cis model of host resistance against intracellular pathogens, which proposes that nonhemopoietic cells need to be directly activated by lymphokines and play an active role as effectors of IFN-γ/TNF-α–dependent CMI to *T. gondii*. The latter requirement may explain why, although not essential for cell survival or tissue homeostasis (24, 27–29), the expression of IFN-γR and TNFR has been retained in all nucleated cell types during the course of evolution (35, 36).

Nevertheless, it remains unclear whether or not the direct activation of nonhemopoietic cells by IFN-γ and TNF-α is sufficient to completely restrict parasite growth within these cells. Although in vitro experiments have clearly doc-

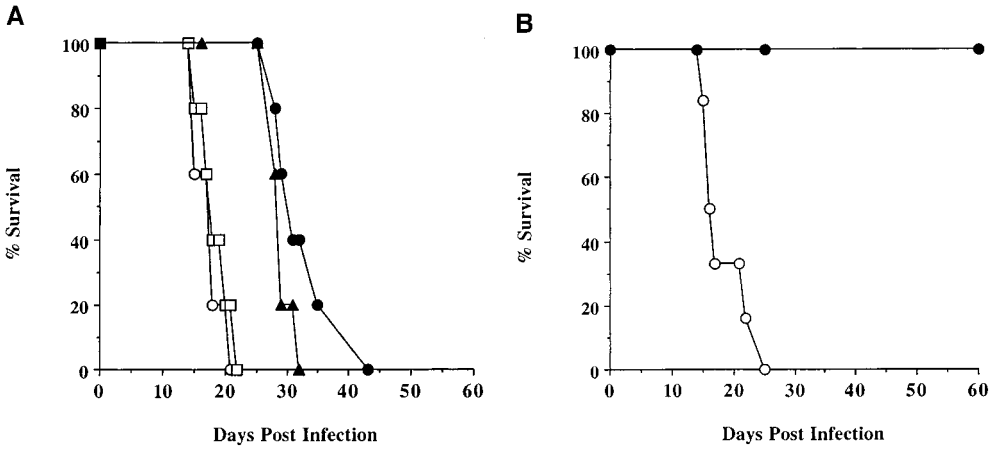

Figure 7. Survival of *T. gondii*–infected iNOS KO BM chimeric mice. C57BL/6 and iNOS-deficient mice were used as WT and KO partners for the construction of the chimeras. BM chimeric (A) and nonirradiated (B) control mice ($n = 5$/group) were infected with 20 *T. gondii* cysts. Results shown are representative of three independent BM chimera experiments. In all three cases, survival time of iNOS KO→WT chimeras was not statistically different from that of either iNOS KO→KO sham control mice ($P > 0.3$) or nonirradiated iNOS KO mice ($P > 0.3$; data not shown). Similarly in all three experiments, mean survival times were not different between WT→iNOS KO and WT→WT chimeras ($P > 0.2$). However, the latter groups of mice survived significantly longer than mice with iNOS-deficient BM ($P < 0.001$). The explanation for the reproducible early death of the WT→WT chimeras with respect to infected, nonirradiated C57BL/6 mice (which, as expected, survived >60 d) is unclear (see above). (A) ○, KO→KO; □, KO→WT; ▲, WT→KO; ●, WT→WT. (B) ○, KO; ●, WT.

umented that nonhemopoietic cells can do so in the absence of macrophages (11), our in vivo results do not exclude potential synergism between trans-acting diffusible metabolites such as NO derived from hemopoietic cells and the cis-acting IFN-γ/TNF-α–dependent mechanism in limiting tachyzoite replication within nonhemopoietic cells.

The identical susceptibility observed in iNOS→iNOS and iNOS→WT chimeric mice underscores the critical importance of NO production by cells derived from the hemopoietic lineage in host resistance. This conclusion agrees with previous reports of the iNOS dependence of the antitoxoplasmic activity of lymphokine-activated macrophages and microglial cells (37, 38). In parallel, the comparable survival curves exhibited by either iNOS-deficient or WT recipients reconstituted with WT BM suggests that iNOS expression in the nonhemopoietic cell compartment plays a limited if not minimal role relative to that in hemopoietic cells. Consistent with this conclusion is the recent observation that lymphokine-induced control of intracellular tachyzoite growth occurs effectively in astrocytes (putative nonhemopoietic effector cells in the brain) derived from iNOS-deficient mice (39). Nevertheless, because of technical problems uniquely observed in the iNOS BM chimera experiments, we cannot definitively conclude that iNOS expression in the nonhemopoietic compartment is absolutely without antimicrobial function. Thus, the premature mortality exhibited by sham chimeric WT mice suggests that lethal irradiation may have damaged a subset of nonhemopoietic cells required for long-term survival after infection and could have, in theory, masked any protective effect of iNOS in the nonhemopoietic compartment. Notwithstanding, it is reasonable to conclude, based on the marked differences in mortality observed between iNOS→WT and WT→iNOS animals, that the IFN-γ– and TNF-α–dependent resistance mechanism(s) operating within nonhemopoietic cells has a major iNOS-independent component.

The nature of the cis-acting effector mechanism(s) responsible for restricting the growth of *T. gondii* within nonhemopoietic cells is presently undefined. A primary candidate mechanism is the depletion of intracellular tryptophan stores by the enzyme indoleamine dioxygenase (IDO; 12). IFN-γ and TNF-α synergistically induce the transcription and activation of this enzyme in many human cell types, including fibroblasts, retinal pigmented epithelium, and neurons as well as macrophages (12, 40–42). Nonetheless, the evidence for the importance and contribution of the IDO mechanism to host resistance in murine cells is more tenuous and controversial than in human cells (43). IFN-γ reportedly fails to induce IDO and toxoplasmastatic activity in mouse fibroblasts (44). Furthermore, in murine macrophages, NO induction by IFN-γ results in cross-inhibition of IDO gene transcription and enzymatic activity (42, 45). The above observations suggest that other as yet unidentified iNOS- and IDO-independent mechanism(s) are responsible for resistance to *T. gondii* infection, a conclusion also reached in a study involving IFN-γ–induced control of the parasite by endothelial cells (46).

The existence of iNOS-independent, IFN-γ-dependent mechanisms of host resistance is not unique to *T. gondii* infection. A similar divergence in the resistance phenotypes of IFN-γ– and iNOS-deficient mice has been described in *Chlamydia* infections (47). In the case of these two pathogens, in vitro transfection experiments have directly implicated the IDO pathway in the control of microbial growth within nonhemopoietic cells (48, 49). An IFN-γ/TNF-α–dependent but iNOS-independent mechanism of CD8 T cell–mediated host resistance has also been described for hepatitis B infection, based on adoptive transfer experiments in a transgenic mouse model (50). Clearly, the identification of these important and potentially novel effector pathways is a highly relevant area for future investigation and represents a major frontier for the field of microbial immunity.

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