TLR-independent neutrophil-derived IFN-γ is important for host resistance to intracellular pathogens

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IFN-γ is a major cytokine that is critical for host resistance to a broad range of intracellular pathogens. Production of IFN-γ by natural killer and T cells is initiated by the recognition of pathogens by Toll-like receptors (TLRs). In an experimental model of toxoplasmosis, we have identified the presence of a nonlymphoid source of IFN-γ that was particularly evident in the absence of TLR-mediated recognition of *Toxoplasma gondii*. Genetically altered mice lacking all lymphoid cells due to deficiencies in Recombination Activating Gene 2 and IL-2Rγ$_c$ genes also produced IFN-γ in response to the protozoan parasite. Flow-cytometry and morphological examinations of non-NK/non-T IFN-γ$^+$ cells identified neutrophils as the cell type capable of producing IFN-γ. Selective elimination of neutrophils in TLR11$^{-/-}$ mice infected with the parasite resulted in acute susceptibility similar to that observed in IFN-γ–deficient mice. Similarly, *Salmonella typhimurium* infection of TLR-deficient mice induces the appearance of IFN-γ$^+$ neutrophils. Thus, neutrophils are a crucial source for IFN-γ that is required for TLR-independent host protection against intracellular pathogens.

innate immunity | host defense

IFN-γ is a cytokine that is critical for coordinating protective immunity against infection with intracellular parasites and bacteria. It is produced by natural killer (NK) and NKT cells (1) during the innate immune response and by CD4 and CD8 T cells (2, 3) during the adaptive immune response to a variety of pathogens. IFN-γ mediates its protective effects by triggering activation of lysosomal activity, inducing nitric oxide production and expression of effector genes such as immunity-related GTPases, and modulating metabolic activity of antigen-presenting cells, including dendritic cells and macrophages (4–6). Each of these mechanisms is involved in efficient elimination of pathogens but are too dangerous and metabolically costly to the host to be constitutively active. The importance of IFN-γ is illustrated by studies of pathogens in IFN-γ$^{-/-}$ mice; these mice are highly susceptible to a large number of pathogens, including *Toxoplasma gondii* (7, 8), which is responsible for severe brain damage in humans and experimental animals (9). Similarly, lack of IFN-γ results in uncontrolled bacterial spread during infection with *Salmonella typhimurium* (10). Surprisingly, a lack of NK or T cells failed to recapitulate the phenotype of IFN-γ deficiency in several infectious disease models, suggesting a possibility that there are other cellular sources of this cytokine (11–14).

T. gondii infection in mice has established that the TLR adaptor protein myeloid-differentiation factor 88 (MyD88) is required for the induction of IFN-γ production by NK and T cells (15–17). Lack of MyD88 or IFN-γ results in acute susceptibility to *T. gondii* due to uncontrolled replication of the parasite (7, 15, 17). Toll-like receptor (TLR) 11, a major sensor for *T. gondii* profilin, is responsible for initiation of MyD88-dependent immunity to the pathogen (18). However, in contrast to MyD88$^{-/-}$ or IFN-γ$^{-/-}$ mice, TLR11$^{-/-}$ mice lack acute susceptibility to the parasite (18). Furthermore, TLR11 is a nonfunctional pseudogene in humans (19), yet immunocompetent individuals are resistant to the pathogen. Taken together, these data provoked a question about IFN-γ–dependent immunity to the parasite in the absence of innate TLR11-dependent sensing of *T. gondii*.

In this report, we show that TLR11-independent immunity to *T. gondii* still requires the production of IFN-γ. Depletion of NK, CD4, and CD8 T lymphocytes did not significantly affect the levels of IFN-γ observed in *T. gondii*-infected TLR11$^{-/-}$ mice. Further investigations revealed a large population of IFN-γ$^+$ cells that did not share the cell surface markers of NK, T, or other lymphoid cells. Instead, the identified IFN-γ$^+$ cells expressed lymphocyte antigen 6 complex (Ly-6G) and neutrophil 7/4 (Neu7/4), which are prototypic markers for neutrophils. Additional analysis established that these IFN-γ$^+$ cells were neutrophils. Importantly, depletion of neutrophils in TLR11$^{-/-}$ mice resulted in rapid susceptibility during *T. gondii* infection that was similar to that observed in IFN-γ–deficient mice. A role for IFN-γ production by neutrophils was also observed during infection with *S. typhimurium*. Cumulatively, our experiments demonstrate that neutrophils produce IFN-γ in response to lethal intracellular pathogens and that neutrophil IFN-γ is required for host resistance to *T. gondii*.

Results

IFN-γ Protects TLR11$^{-/-}$ Mice from *T. gondii* Infection in the Absence of NK and T Cells. To investigate the mechanisms of TLR11-independent immunity to *T. gondii*, we examined the relative contribution of NK and T cells in IFN-γ–mediated resistance to the pathogen. We focused on this cytokine because treatment of TLR11$^{-/-}$ mice with IFN-γ–blocking antibody resulted in acute susceptibility to the infection that was similar to that observed in IFN-γ$^{-/-}$ mice (Fig. 1*A*). Therefore, effector mechanisms responsible for host protection to *T. gondii* in the absence of TLR11 depend on IFN-γ. TLR-mediated recognition of pathogens, including *T. gondii*, regulates IFN-γ production from both NK and T cells (15). To identify the cells contributing to IFN-γ–dependent protection in the absence of TLR11, the major innate immune sensor for *T. gondii* in mice (18), we depleted NK cells, CD4, or CD8 T cells and compared the pathogen burden and IFN-γ$^{-/-}$ levels during infection. No difference in serum levels of IFN-γ or pathogen burden were observed in lymphocyte-depleted mice compared with control animals (Fig. 1*B*). Furthermore, depletion of NK cells, thought to be the early source of IFN-γ during *T. gondii* infection, did not alter the survival of TLR11$^{-/-}$ mice (Fig. S1*A*). This observation suggests that IFN-γ produced by NK or T cells alone is insufficient for early host resistance to the parasite. Surprisingly, even when TLR11$^{-/-}$ mice were simultaneously depleted of NK and T cells, they showed a significant increase in survival compared with mice treated with IFN-γ–blocking antibody (Fig. 1*A*). Analysis of NK and T cells depletion ruled out a possibility that enhanced survival of mice treated with anti-NK1.1, anti-CD4, and anti-CD8 was due to residual production of IFN-γ by those cells (Fig. S1*B*). Consistent with the increased survival of NK and T-cell–depleted TLR11$^{-/-}$ mice, these animals had a reduced pathogen compared with mice treated with an IFN-γ–blocking antibody

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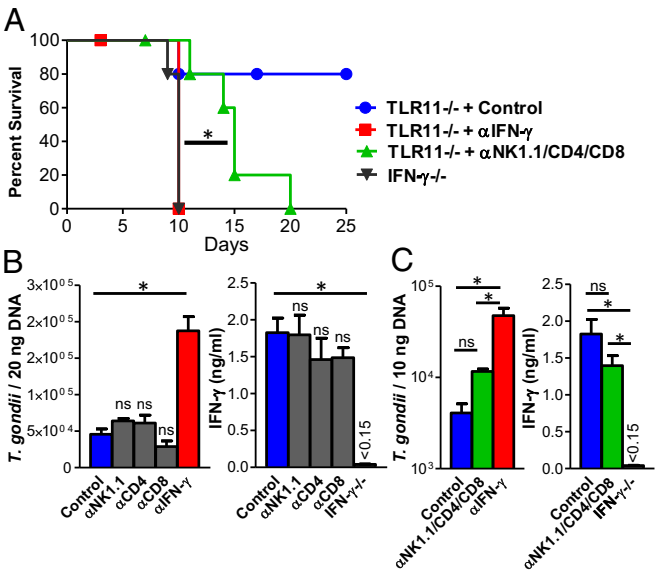

Fig. 1. Survival of TLR11-deficient mice during acute toxoplasmosis depends on IFN-γ but not NK or T cells. TLR11$^{-/-}$ mice were infected intraperitoneally (i.p.) with 20 cysts of the ME49 strain of *T. gondii* and were additionally treated with IFN-γ blocking antibody (red), αNK1.1, αCD4 and αCD8 antibodies to simultaneously deplete NK and T cells (green), or vehicle control (blue) (*A*). The survival of the TLR11$^{-/-}$ mice was compared with similarly infected IFN-γ$^{-/-}$ mice (black). $P \leq 0.009$. (*B*) Infected TLR11$^{-/-}$ mice were treated with IFN-γ–blocking antibody (red), or αNK1.1, αCD4, or αCD8 antibodies to individually deplete NK and CD4 or CD8 T cells, respectively, or vehicle control (blue). *T. gondii* load and IFN-γ production in the peritoneal cavity of infected mice were analyzed 5 d after infection by quantitative RT-PCR and ELISA, respectively. (*C*) *T. gondii* load and IFN-γ production in the peritoneal cavity of infected mice were analyzed on day 5 after infection by quantitative RT-PCR and ELISA, respectively. The data shown are one of three experiments, each involving five mice per group, "ns" is not significant, and error bars shown are the means ± SEM.

(Fig. 1*C*). Moreover, levels of IFN-γ in the peritoneal cavities of NK and T-cell–depleted mice were only slightly reduced compared with those of control mice (Fig. 1*C*). Taken together, these data suggest that there is a source of IFN-γ that is not affected by the depletion of NK and T cells in *T. gondii*–infected TLR11$^{-/-}$ mice. Furthermore, this IFN-γ controls pathogen burden and is essential for enhanced survival of TLR11$^{-/-}$ mice infected with the parasite.

IFN-γ Production Is Not Limited to NK and T cells. To identify cells capable of producing IFN-γ besides NK and T cells, we used flow cytometry to analyze viable cells expressing IFN-γ. A distinct population of IFN-γ$^+$ cells was observed in the peritoneal cavity 5 d after infection in WT and TLR11$^{-/-}$ mice (Fig. 2*A*). Strikingly, substantial numbers of IFN-γ$^+$ cells were negative for NK1.1 and CD3ε, the prototypic surface markers expressed by all NK and T cells, respectively. Additional analysis revealed that lack of TLR11-mediated recognition of the parasite resulted in a dramatic increase in frequency and total number of IFN-γ$^+$ cells (Fig. 2 *A* and *B*). This increase was due to a large expansion of the IFN-γ$^+$ CD3ε$^-$ NK1.1$^-$ cell population (Fig. 2*B*). In addition, combined deficiencies in TLR11 and TLR2, a receptor involved in recognition of GPI anchors expressed on the cell surface of *T. gondii* (9), did not abrogate expansion of the IFN-γ$^+$ CD3ε$^-$ NK1.1$^-$ cells. Furthermore, abrogation of all intracellular TLRs including TLR11, TLR12, TLR7, and TLR9 by using triple D (3d) mice—deficient in unc-93 homolog B1 (UNC93B1) protein (20, 21)—resulted in IFN-γ production dominated by CD3ε$^-$ NK1.1$^-$ cells (Fig. S2). Thus, all TLRs known to be involved in *T. gondii* recognition (18, 22, 23) were not involved in induction of IFN-γ$^+$ CD3ε$^-$ NK1.1$^-$ cells. These IFN-γ$^+$ cells were also observed in WT mice, although at reduced frequencies (Fig. 2 *A* and *B*), because both NK and T cells contribute to IFN-γ production in response to TLR11 activation by *T. gondii* (Fig. 2*B* and Fig. S2). The appearance of IFN-γ$^+$ cells lacking NK1.1, CD4, or CD8 is in agreement with the survival data of lymphocyte-depleted mice (Fig. 1*A*), emphasizing the physiological importance of these cells in host defense against this parasite.

One possible explanation for the early appearance of IFN-γ$^+$ CD3ε$^-$ NK1.1$^-$ cells is that *T. gondii* infection induces the appearance of an unusual NK cell population not expressing NK1.1. Arguing against this possibility, IFN-γ$^+$ CD3ε$^-$ NK1.1$^-$ cells did not express the NK cell markers DX5, NKp46, and NKG2D, suggesting that these were not NK cells (Fig. S3). Additionally, IFN-γ$^+$ CD3ε$^-$ NK1.1$^-$ cells did not express any other lymphoid cell lineage markers that we examined, including B220, CD19, CD4, CD8, Thy1.2, or CD27, which would identify them as B or T cells (Fig. S3).

To formally determine whether IFN-γ$^+$ CD3ε$^-$ NK1.1$^-$ cells were of the lymphoid lineage, we used a genetic approach and examined the appearance of these cells in Recombination Activating Gene 2 (RAG2)/IL-2Rγ$_c^{-/-}$ mice. These doubly deficient mice do not develop T or B cells as a result of RAG2 inactivation, and they also lack NK cells due to deficiency in IL-2 and IL-15 signaling caused by deletion of the IL-2Rγ$_c$ gene (24, 25). The presence of IFN-γ in these mice would indicate that expression of this cytokine is not limited to lymphoid cells, including NK and T cells. *T. gondii* infection of RAG/IL-2Rγ$_c^{-/-}$ mice resulted in the appearance of IFN-γ$^+$ cells and the induction of IFN-γ transcripts at levels comparable to those observed in TLR11$^{-/-}$ mice (Fig. 2*C*). Taken together, these data suggest that NK and T lymphocytes are not the only cell types capable of producing IFN-γ.

Neutrophils Produce IFN-γ in Response to *T. gondii* and *S. typhimurium* Infections. To define the nature of IFN-γ$^+$ CD3ε$^-$ NK1.1$^-$ cells, we next examined other surface markers expressed by these cells. Expression of CD45 on IFN-γ$^+$ CD3ε$^-$ NK1.1$^-$ cells pointed to their hematopoietic origin although they were negative for the common lymphoid cell surface markers CD25, CD27, and CD122 (Fig. S3). These IFN-γ$^+$ non-NK–non-T cells expressed CD11b, an integrin associated with myeloid lineage cells. They also expressed CD44, an adhesion receptor on activated cells migrating into inflammatory sites (Fig. 2*D*). Nevertheless, IFN-γ$^+$ CD3ε$^-$ NK1.1$^-$ cells did not express the myeloid lineage markers CD11c, F4/80, or MHC-II—present on DC and macrophages (Fig. S3). Instead, IFN-γ$^+$ CD3ε$^-$ NK1.1$^-$ cells shared a surface marker present on monocytes and neutrophils, granulocyte receptor-1 (Gr-1) (Fig. 2*D*). Anti–Gr-1 antibody recognizes two related surface receptors, Ly-6C and Ly-6G (26), and the precise characterization of their expression discriminates between monocytes and neutrophils. Our experiments demonstrated that IFN-γ$^+$ CD3ε$^-$ NK1.1$^-$ cells expressed intermediate levels of Ly-6C compared with monocytes and were highly positive for Ly-6G (Fig. 2*D*), a surface marker highly expressed on neutrophils. The presence of the additional neutrophil-specific marker Neu7/4, also known as Ly-6B (27) (Fig. 2*D*), further suggested that IFN-γ$^+$ CD3ε$^-$ NK1.1$^-$ cells were neutrophils.

To formally examine the ability of neutrophils to produce IFN-γ, Ly-6G$^+$ neutrophils were purified from the peritoneal cavity of *T. gondii*–infected TLR11$^{-/-}$ mice. Their ability to produce IFN-γ was next compared with that of NK cells isolated from the same mice (Fig. 3 *A*–*C*). We established that the majority of neutrophils stained positively for IFN-γ, strongly indicating that these cells were capable of producing this cytokine without any in vitro restimulation (Fig. 3*B*). Furthermore, the presence of IFN-γ transcripts excluded a possibility that neutrophils stained positive for IFN-γ as the result of their phagocytic activity and uptake of the other IFN-γ–producing cells, such as NK and T cells (Fig. 3*C*).

In the same experiments, purified Ly-6G$^+$ cells were morphologically characterized by Giemsa staining. They were observed to have the multilobed nucleus structure characteristic of neutrophils (Fig. 3*D*). Purified neutrophils were stained with anti–IFN-γ and immunofluorescent analysis revealed the presence of discrete puncta of IFN-γ present in their cytoplasm (Fig. 3*E*).

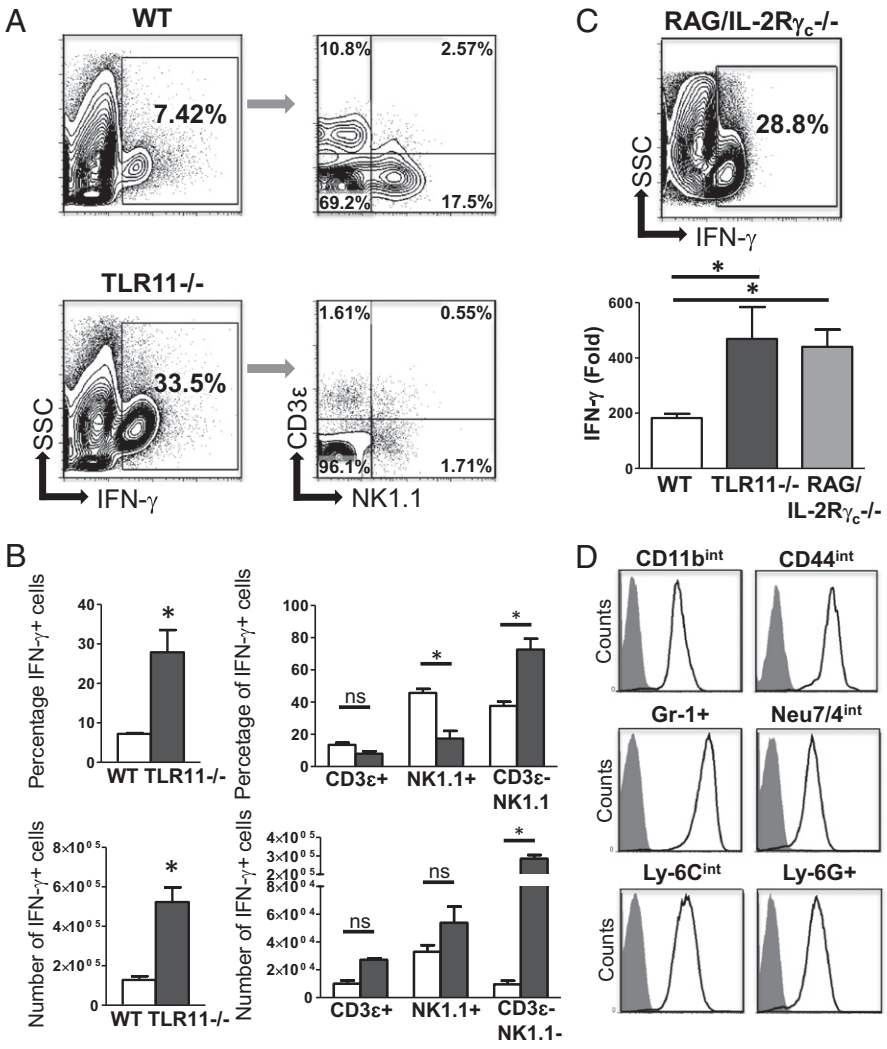

Fig. 2. IFN-γ production by non-NK non-T cells in *T. gondii*-infected mice. (*A*) WT and TLR11$^{-/-}$ mice were infected i.p. with *T. gondii* and IFN-γ$^+$ cells were identified in the peritoneal cavity of infected mice by gating on live IFN-γ$^+$ cells on day 5 after infection. IFN-γ$^+$ cells were further differentiated by plotting NK1.1 vs. CD3ε as markers for NK and T cells, respectively. (*B*) Relative (*Upper*) and absolute quantification (*Lower*) of total IFN-γ$^+$ cells (*Left*) and additionally broken down by cell types (*Right*) in WT (white) and TLR11$^{-/-}$ (black) mice. (*C*) IFN-γ$^+$ cells were identified in the peritoneal cavity of infected RAG/IL-2Rγ$_c$$^{-/-}$ mice by gating on live IFN-γ$^+$ cells (*Upper*) and total IFN-γ expression in infected WT, TLR11$^{-/-}$, and RAG/IL-2Rγ$_c$$^{-/-}$ was measured on day 5 after infection by RT-PCR (*Lower*). $P \leq 0.04$. (*D*) Cell surface markers expressed by IFN-γ$^+$ CD3ε$^-$ NK1.1$^-$ cells isolated from *T. gondii*-infected TLR11$^{-/-}$ mice (open histograms) compared with appropriate isotype controls (filled histograms). The data shown are one of four experiments each involving 3–5 mice per group. Error bars are means ± SEM.

Additionally, TLR11$^{-/-}$ neutrophils in vitro produced IFN-γ in response to incubation with parasite (Fig. S4*A*).

To determine whether IFN-γ production by neutrophils was specific for *T. gondii*, we evaluated neutrophils in response to another pathogen, *S. typhimurium*. In WT mice infected with *S. typhimurium*, neutrophils, NK, and T cells all contributed to IFN-γ production. Importantly, the early IFN-γ responses against *S. typhimurium* were dominated by neutrophils in TLR2/4 doubly deficient mice (Fig. S5), which are deficient in TLR-mediated pathogen recognition of the bacteria. Overall, analyses of IFN-γ responses to *T. gondii* and *S. typhimurium* infections revealed that neutrophils were a major cellular source of IFN-γ, especially in the absence of TLR-mediated pathogen recognition.

Neutrophil-Produced IFN-γ Is IL-12–Independent, but Is Regulated by TNF and IL-1β. IFN-γ production from NK and T cells is regulated by TLR11, UNC93B1, and TLR12-dependent induction of interleukin-12 (IL-12) (15, 16, 21, 23). The appearance of IFN-γ$^+$ neutrophils in the absence of TLR11 or UNC93B1 raised the question of whether IL-12 plays a role in triggering a neutrophil-derived IFN-γ response. We therefore analyzed the kinetics of IL-12 and IFN-γ production in WT and TLR11-deficient mice. The experiments revealed a paradoxical result in which *T. gondii*-infected TLR11$^{-/-}$ mice showed a large increase in the number of cells making IFN-γ compared with WT mice (Fig. 4*A*). These data were in contrast to the IL-12 response analyzed by quantification of IL-12p40-YFP–expressing cells (28) that was consistent with published data, demonstrating the dependence on TLR11-mediated parasite recognition (18). Lack of correlation between the appearance of IL-12 and IFN-γ$^+$ cells suggested that in contrast to NK and T cells, neutrophil-derived IFN-γ is not regulated by IL-12. To formally test this hypothesis, TLR11$^{-/-}$ mice were infected with *T. gondii* and IL-12 was blocked during the course of infection by using an anti–IL-12/23p40 antibody. Successful blocking of IL-12 was confirmed by examining serum levels of IL-12 and by the examination of IFN-γ production by NK cells (Fig. S6*A*), which is known to be regulated by IL-12 (16). Quantification of Ly-6G$^+$ CD3ε$^-$ NK1.1$^-$ cells demonstrated that administration of an anti–IL-12 antibody had no effect on the production of IFN-γ by neutrophils (Fig. 4*B*). Additionally, the pathogen load of anti–IL-12 treated mice was intermediate and significantly different from that of control or anti–IFN-γ treated groups (Fig. 4*C*). These levels of pathogen burden are reminiscent of those seen with depletions of both NK and T cells simultaneously (Fig. 1*C*) but are slightly elevated. Because anti–IL-12 treatment has been shown to cause acute susceptibility (13, 15), it may be a fine line between the parasite levels that result in acute versus intermediate susceptibly or, alternatively, that a role of IL-12 exists independent of eliciting IFN-γ from NK and T cells. In complementary experiments, administration of recombinant IL-12 to infected TLR11$^{-/-}$ mice had little effect on the number of IFN-γ$^+$ neutrophils (Fig. S6*B*). Taken together, these results strongly suggest that IFN-γ production by neutrophils does not depend on IL-12.

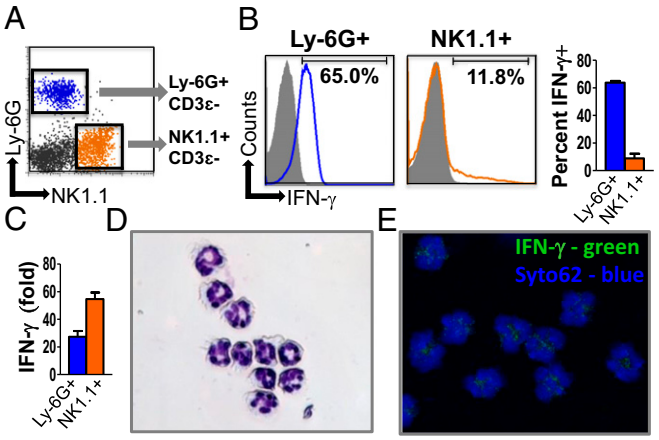

Fig. 3. Ly-6G$^+$ neutrophils produce IFN-γ. TLR11$^{-/-}$ mice were infected i.p. with *T. gondii* and on day 5, cells were sort purified as shown, isolating Ly-6G$^+$ CD3ε$^-$ and NK1.1$^+$ CD3ε$^-$ cells from peritoneal exudate cells (PEC) (*A*). (*B*) Ly-6G$^+$ cells (*Left*) and NK1.1$^+$ cells (*Center*) were defined by histogram expression of IFN-γ (open histograms) compared with its appropriate isotype control (filled histogram) and percent of IFN-γ quantified in a bar graph (*Right*). (*C*) Sorted Ly-6G$^+$ cells contain IFN-γ transcript as quantified by fold change over bulk naïve PEC by quantitative RT-PCR. (*D*) Sort-purified Ly-6G$^+$NK1.1$^-$ CD3ε$^-$ PEC were analyzed morphologically by Giemsa staining. (*E*) Purified Ly-6G$^+$ cells were analyzed for IFN-γ protein by staining with αIFN-γ antibody and analyzed by confocal microscopy. The data shown are one of three independent experiments, and error bars shown are the means ± SEM.

TNF and IL-1β have been implicated in the regulation of IFN-γ (29). Therefore, these cytokines were blocked during *T. gondii* infection, and IFN-γ$^+$ neutrophils were quantified. Administration of anti-TNF significantly decreased the number of IFN-γ$^+$ neutrophils (Fig. 4*D*) without affecting early IFN-γ production by NK or T cells (Fig. S6*C*). Flow cytometry experiments revealed that Ly-6C^{hi} proinflammatory monocytes were the major source of TNF and IL-1β during *T. gondii* infection (Fig. S6*D*). Neutrophils were also capable of TNF and IL-1β production but at lower levels than was seen from monocytes, suggesting neutrophils themselves may have some minor positive feedback in this system (Fig. S6*D*). Blocking responses to IL-1β by using a receptor antagonist revealed a decrease in IFN-γ$^+$ neutrophils (Fig. 4*D*). IL-1R$^{-/-}$ mice provided a more decisive model to examine the role of IL-1β. Comparison of WT and IL-1R$^{-/-}$ mice exposed a significant decrease in IFN-γ$^+$ neutrophils in the absence of IL-1R (Fig. 4*E*). This observation is in agreement with the phenotype of MyD88-deficient mice that fail to induce IFN-γ from neutrophils (Fig. S7). Together these data suggest that both TNF and IL-1β, but not IL-12 regulate IFN-γ$^+$ neutrophils.

Neutrophils Protect Against *T. gondii* in the Absence of TLR11. Neutrophils participate in host response to *T. gondii* but play a limited role in survival of WT mice infected with the parasite (30–32). Instead, IFN-γ from NK and T cells predetermined susceptibility vs. resistance to the parasite (12, 16, 33). The dominance of neutrophils in IFN-γ production observed in *T. gondii*-infected TLR11$^{-/-}$ mice prompted us to investigate their physiological importance. Depletion of neutrophils using the highly specific anti–Ly-6G antibody resulted in early mortality of TLR11$^{-/-}$ mice with kinetics nearly indistinguishable from those observed in IFN-γ$^{-/-}$ mice (Fig. 5*A*). These results were in contrast to neutrophil-sufficient TLR11$^{-/-}$ mice that can control the parasite during the acute phase of the infection (Fig. 1*A*) and demonstrate enhanced susceptibility to *T. gondii* only during the chronic phase of the infection (25). Importantly, the selective depletion of neutrophils in *T. gondii*-infected TLR11-deficient mice did not affect the levels of IFN-γ produced by NK, CD4, or CD8 T cells (Fig. 5*B* and Fig. S8), yet lymphoid cell IFN-γ production was insufficient to control the parasite in the absence of neutrophils (Fig. 5*A*). Treatment of neutrophil-depleted TLR11$^{-/-}$ mice with recombinant IFN-γ (rIFN-γ) enhanced host resistance during the acute stage of the parasitic infection (Fig. 5*A*). Overall, these results revealed that neutrophils are crucial effector cells for IFN-γ–mediated host resistance to the parasite in the absence of TLR11-dependent activation of NK and T cells.

Discussion

It is generally accepted that activation of TLRs on DCs is a crucial event for triggering IL-12–dependent IFN-γ production by NK and T cells. The currently proposed models of TLR-mediated immune responses implies that lack of TLRs, IL-12, or NK and T cells would completely abrogate IFN-γ production and activation of inflammatory cells at the site of infection; hence, infection would be more severe. The significance of IFN-γ is exemplified by studies performed in IFN-γ$^{-/-}$ mice that are extremely susceptible

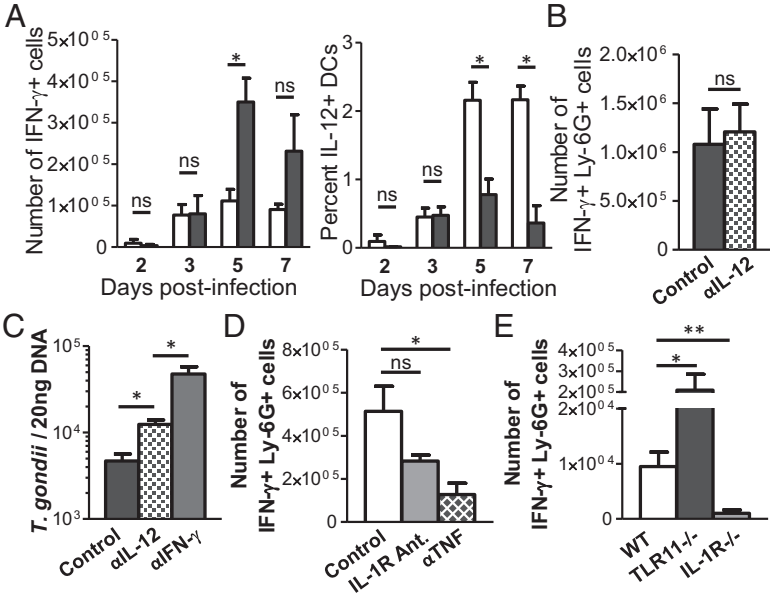

Fig. 4. Neutrophils produce IFN-γ in an IL-12–independent, TNF- and IL-1β–dependent manner. Mice were infected i.p. with 20 cysts of the ME49 strain *T. gondii*. (*A*) PEC were analyzed by flow cytometry on days 2, 3, 5, and 7. Total IFN-γ$^+$ cells were examined by absolute quantification in WT (white) and TLR11$^{-/-}$ mice (black). IL-12 in PEC CD11c$^+$ DCs was examined by relative quantification of IL-12p40 expression from IL-12–reporter mice (Yet40) (white) and Yet40xTLR11$^{-/-}$ mice (black). (*B*) Absolute quantification of IFN-γ$^+$ Ly-6G$^+$ cells day 5 after infection from TLR11$^{-/-}$ mice that received control (black) or αIL-12 antibody (checkered). (*C*) *T. gondii* load in the peritoneal cavity of infected TLR11$^{-/-}$ mice that received control (black), αIL-12 antibody (checkered), or αIFN-γ (gray) were analyzed on day 5 after infection by quantitative RT-PCR. $P \leq 0.004$. (*D*) Absolute quantification of IFN-γ$^+$ Ly-6G$^+$ cells from infected TLR11$^{-/-}$ mice that received control (white) or IL-1R antagonist (light gray) or αTNF antibody (hatched). $P \leq 0.05$. (*E*) WT (white), TLR11$^{-/-}$ (black), and IL-1R$^{-/-}$ mice (light gray) were infected and then analyzed by absolute quantification of IFN-γ$^+$ Ly-6G$^+$ cells day 5 after infection. *$P \leq 0.05$ and **$P \leq 0.002$. The data shown are one of three independent experiments with three to seven mice per group, and error bars shown are the means ± SEM.

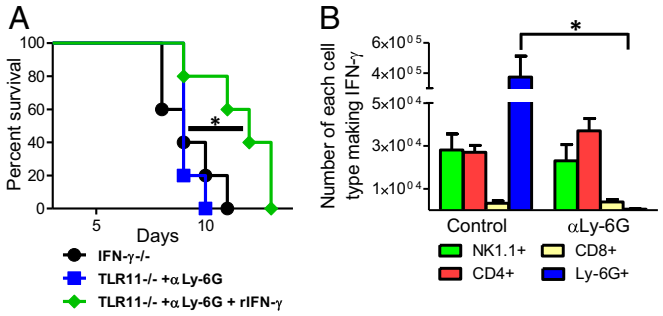

Fig. 5. Neutrophils are protective during *T. gondii* infection. TLR11$^{-/-}$ mice were infected i.p. with *T. gondii* and were additionally treated with αLy-6G antibody (blue) or a combination of αLy-6G antibody and rIFN-γ (green) (*A*). The survival of the TLR11$^{-/-}$ mice was compared with similarly infected IFN-γ$^{-/-}$ mice (black). $P \leq 0.02$. (*B*) Absolute quantification of IFN-γ^{+} NK, T cells, and neutrophils present in the PEC on day 5 after infection during Ly-6G depletion. The data shown are one of two independent experiments with five mice per group. Error bars shown are the means ± SEM.

to a broad range of pathogens, yet this susceptibility is not recapitulated in mice lacking NK or T cells. These results have raised the question of the cellular origin of this cytokine in the initial stages of the innate immune response. In this regard, we observed that neutrophils are the major IFN-γ–producing cells during acute responses to both *T. gondii* and *S. typhimurium*. The presence of IFN-γ–producing cells in *T. gondii*-infected mice lacking T and NK cells due to deficiency in RAG and IL-2Rγ$_c$ has formally proven the existence of non-NK/non-T IFN-γ–producing cells. In WT mice, neutrophils produce IFN-γ; however, IFN-γ^{+} neutrophils were especially evident in the absence of TLR11 or UNC93B1. We also established that IFN-γ production by neutrophils is IL-12 independent but is regulated by TNF and IL-1. These results indicated the existence of an IFN-γ pathway for controlling intracellular pathogens that is independent of TLR recognition as well as NK and T cells. Based on what we know about neutrophils, their large numbers and rapid deployment to the site of infection should provide an important means of very early, robust, and rapid elimination of pathogens.

NK cells are a well characterized source of IFN-γ that restricts *T. gondii* replication during the first week of acute infection (11, 13). This conclusion stems from the extended survival of lymphocyte-deficient mice during parasite infection in comparison with IFN-γ$^{-/-}$ mice and WT mice treated with IFN-γ–blocking antibody (7). In addition to NK cells, CD4 T cells participate in IFN-γ production as early as 5 d after infection. The contribution of CD4 T cells to the initial IFN-γ–mediated control of the parasite is particularly evident in mice lacking the common cytokine receptor γ-chain (γc) gene that leads to the defective development of NK and T cells (34). Nevertheless, selective depletion of either NK cells or T lymphocytes does not diminish the resistance during the acute stages of infection, suggesting the redundancy of these cell types in mediating IFN-γ–dependent host resistance (34). Paradoxically, the combined deficiency in NK and T cells does not abrogate IFN-γ triggered by *T. gondii* infection (13). As in *T. gondii* infection, *Salmonella*-induced IFN-γ expression only partially depends on NK and T cells (35). Taken together, these results suggest that in addition to NK and T cells, there are innate immune cells, likely of myeloid origin, producing IFN-γ in response to microbial infections. Furthermore, neutrophils producing IFN-γ were identified in *Salmonella*-, *Listeria*-, and *Nocardia*-infected mice (36–38), but this possibility is not well accepted and is usually interpreted as a result of incomplete depletion of IFN-γ–producing NK or T cells during analysis of myeloid cells (39, 40). This scenario is particularly applicable to the description of IFN-γ production by DCs and macrophages, because these cells can form tight functional contacts with NK cells (41–43). In addition, the presence of IFN-γ protein in antigen-presenting cells may result from the phagocytic activities of DCs and macrophages rather than cytokine production. Overall, the concept of myeloid-derived IFN-γ is still the subject of debate, in part because of the failure to identify cytokines regulating IFN-γ production in myeloid cells that are distinct from those involved in IFN-γ production by NK and T cells (44). Our experimental infections of RAG2/IL-2Rγ$_c^{-/-}$ mice with *T. gondii* provided definitive evidence for the ability of neutrophils to produce protective IFN-γ. This conclusion was further supported by identification of TNF and IL-1β as selective regulators of IL-12–independent IFN-γ production by neutrophils required for host resistance to *T. gondii* in the absence of TLR11.

TLR11 is a major innate immune sensor for *T. gondii* that is responsible for IL-12–dependent activation of NK and T cells. Nevertheless, in contrast to IFN-γ$^{-/-}$ mice, those lacking TLR11 are relatively resistant to the parasite (18). It was recently suggested that TLR12-dependent activation of NK cells in the absence of TLR11 explains the enhanced survival of TLR11$^{-/-}$ mice compared with MyD88$^{-/-}$ or IFN-γ$^{-/-}$ mice (23). Nevertheless, depletion of NK cells in *T. gondii*-infected TLR11$^{-/-}$ mice did not affect the survival of these mice, strongly suggesting that NK cells play little or no significant role in TLR11-independent IFN-γ–dependent host resistance to *T. gondii*. Furthermore, TLR11, as well as TLR12 inactivation, or combined deficiencies in TLR11, TLR12, TLR7, and TLR9 signaling pathways caused by mutation in UNC93B1, abolished IL-12 production in vivo and in vitro, but had a minor effect on the levels of IFN-γ observed in *T. gondii*-infected mice (18, 21). This unexpected disconnect between TLR-mediated pathogen recognition and IFN-γ–dependent host resistance is not unique to *T. gondii*; it has been observed with other intracellular pathogens and is frequently interpreted as a functional redundancy among TLRs in the activation of NK or T cells. This conclusion seems to be supported by the phenotypes of MyD88$^{-/-}$ mice that are highly susceptible to both *T. gondii* and *S. typhimurium* (15, 45). In addition to TLRs, IL-1R–mediated activation of MyD88 is indispensable for host resistance to intracellular pathogens (46) although IL-1R regulation of IFN-γ production is not completely understood, because IL-1R contributes to but is not absolutely essential for the IL-12 and IFN-γ production by DCs and NK cells, respectively (29, 47). IL-1R or caspase-1 deficiency during *T. gondii* infection has no effect on IFN-γ production by T cells (47, 48). IL-12–independent functions of MyD88 are also supported by the inability of recombinant IL-12 to rescue *T. gondii*-infected MyD88$^{-/-}$ mice (16). Although this treatment protects DC-specific MyD88-deficient mice as well as 3d mice with impaired functions in all endosomal TLRs (20), exogenous IL-12 fails to protect complete MyD88-deficient mice from *T. gondii* (16, 21). In this study, we established that IL-1R–mediated MyD88 activation cooperates with TNF to regulate IL-12–independent IFN-γ.

Our results suggest that neutrophils are the early cellular source of IL-12–independent IFN-γ. The proposed model suggests that IL-1β and TNF produced by resident macrophages and circulating proinflammatory monocytes at the site of the infection regulate IFN-γ production by neutrophils. Although neutrophil IFN-γ alone is not sufficient for complete host protection, it significantly reduces the pathogen loads and extends the survival of mice, probably because the response is rapid and robust. The importance of this myeloid-derived IFN-γ is supported by several clinical observations that human neutrophils produce IFN-γ (49, 50). These observations are particularly relevant to human toxoplasmosis, because humans lack functional TLR11 and TLR12 (19), and the host resistance to the parasite is mediated by unknown mechanisms responsible for IFN-γ–mediated parasite killing. Taking into account the dominance of neutrophils among all immune cells, it is highly possible that in addition to direct pathogen elimination, IFN-γ produced by neutrophils may have broad immunoregulatory effects on establishing type I immunity by suppressing IL-4 secretion by CD4 T cells (51) and influencing the isotypes of Ig secreted by B cells during the adaptive immune response (52, 53). Our results have unveiled a unique arm in TLR-independent innate immunity and provide an explanation for IFN-

γ–dependent host resistance to intracellular pathogens in the absence of NK and T cells.

Materials and Methods

Full details of experimental methods are described in *SI Materials and Methods*.

***T. gondii* Infection and Intervention.** Mice were infected i.p. with 20 brain tissue cysts of the avirulent ME49 strain of *T. gondii* as described (18). In some experiments, mice were injected with 500 μg of αIFN-γ, combinations of 200 μg of αCD4, 200 μg of αCD8, and 100 μg of αNK1.1, 200 μg of αIL-12, 500 μg of αTNF, or 4 mg of IL-1R antagonist twice a week. In additional experiments, mice were injected 500 μg of αLy-6G initially and 100 μg every day or recombinant 1-μg IFN-γ every other day for the duration of the experiment.

Flow Cytometry and Quantitative PCR. For flow cytometry, cells were stained with the indicated antibodies and were permeabilized with the Foxp3/Transcription Factor Staining Buffer Set according to the manufacturer's instructions (eBioscience). Data were acquired with the FACSCalibur (BD Bioscience) or the MoFlo cytometer (Dako Cytomation) and were analyzed with FlowJo software (TreeStar).

Statistical Analysis. All data were analyzed with Prism (version 5; GraphPad). These data were considered statistically significant when P values were ≤ 0.05 by a two-tailed t test.

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