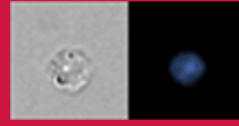


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## Identification of an IFN- $\gamma$ -Producing Neutrophil Early in the Response to *Listeria monocytogenes*

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Jiyi Yin and Thomas A. Ferguson

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# Identification of an IFN- $\gamma$ -Producing Neutrophil Early in the Response to *Listeria monocytogenes*<sup>1</sup>

Jiyi Yin and Thomas A. Ferguson<sup>2</sup>

IFN- $\gamma$  plays a critical role during the immune response to infection with *Listeria monocytogenes*. Early in the innate response NK cells are thought to be a primary source of IFN- $\gamma$ ; however, protection can be mediated by the presence of significant numbers of primed IFN- $\gamma$ -secreting CD8<sup>+</sup> T cells. In this report, we examined the early response to *Listeria* and found that 18 h after infection spleens contain CD11b<sup>+</sup>, Gr-1<sup>high</sup>, or Ly6G<sup>+</sup> cells that produce significant IFN- $\gamma$ . Morphological analysis of sorted Gr-1<sup>high</sup>IFN- $\gamma$ <sup>+</sup> and Gr-1<sup>low</sup>IFN- $\gamma$ <sup>+</sup> or Ly6G<sup>+</sup>IFN- $\gamma$ <sup>+</sup> cells confirmed that these cells were neutrophils. The importance of IFN- $\gamma$  production by these cells was further tested using adoptive transfer studies. Transfer of purified neutrophils from *Ifng*<sup>+/+</sup> mice led to increased bacterial clearance in *Ifng*<sup>-/-</sup> mice. Transfer of *Ifng*<sup>-/-</sup> neutrophils provided no such protection. We conclude that neutrophils are an early source of IFN- $\gamma$  during *Listeria* infection and are important in providing immune protection. *The Journal of Immunology*, 2009, 182: 7069–7073.

**L** *isteria monocytogenes* (LM)<sup>3</sup> is an opportunistic pathogen producing severe infections in immunocompromised individuals. LM is a facultative intracellular parasite that infects macrophages and nonphagocytic cells such as hepatocytes. The innate immune response to LM involves recruitment and activation of phagocytic cells such as neutrophils, macrophages, as well as NK cells (1). Treatment of mice with Abs to deplete neutrophils or NK cells increases LM infection (2, 3), and a number of key cytokines such as TNF- $\alpha$  (4), IL-1 (5), IL-12 (6), and IFN- $\gamma$  (2, 7, 8) are known to be important. LM infection has also been used as a model to study acquired CD8<sup>+</sup> T cell immunity (9); however, the early innate response is clearly important to the development of any long-lasting acquired immunity (1). T cells are apparently more important for defense against secondary infection and, in fact, lymphocyte-deficient SCID mice show heightened resistance to LM infection. This is attributed to the immunosuppressive effects of apoptotic lymphocytes that elicit an immunosuppressive IL-10 response (10).

It has long been established that IFN- $\gamma$  is an important cytokine for clearing infections by LM (2, 7, 8). During the first few days of infection, IFN- $\gamma$  production by NK cells is thought to be critical to host resistance (1, 2, 7). Additionally, memory CD8<sup>+</sup> T cells responding to IL-12 and IL-18 can mediate resistance in an Ag nonspecific manner (11, 12). Interestingly, mice deficient in IFN- $\gamma$

can still establish an Ag-specific CD8<sup>+</sup> T cell response to the bacterium if they were first vaccinated using a nonlethal strain of LM (9, 13). Consequently, it is now thought that IFN- $\gamma$  plays a crucial role in establishing the innate response but not necessarily acquired T cell immunity.

In this paper we examine the early response (<24 h) to LM and identify for the first time a population of neutrophils that produce IFN- $\gamma$  in response to infection. These cells were critical to resistance to LM, as they could provide protection to *Ifng*<sup>-/-</sup> mice if adoptively transferred at the time of infection. Thus, neutrophil-derived IFN- $\gamma$  production provides a critical component in anti-*Listeria* immunity.

## Materials and Methods

### Mice

C57BL/6 mice were purchased from the National Cancer Institute (Frederick, MD). B6.129S7-*Ifng*<sup>tm1ts/J</sup> (*Ifng*<sup>-/-</sup>) and control (C57BL/6J, *Ifng*<sup>+/+</sup>) mice were obtained from The Jackson Laboratory. All mice were maintained in a specific pathogen-free facility at Washington University School of Medicine. All animal procedures were performed according to National Institutes of Health guidelines and were approved by the Washington University Institutional Animal Care and Use Committee. Groups consisted of five mice and experiments were repeated at least two times.

### Bacterial infection of mice

ActA<sup>-</sup> *L. monocytogenes* that express OVA was a gift from Dr. Thomas S. Griffith (University of Iowa, Iowa City, IA). This is an attenuated version of *L. monocytogenes* created by introducing an in-frame deletion in the *actA* gene (LM-OVA) (14). While we used this strain throughout, parallel studies with the wild-type strain of LM gave identical results. C57BL/6, *Ifng*<sup>-/-</sup> and control littermates were given 1 × 10<sup>6</sup> CFU LM-OVA via i.v. injection. Bacteria were grown and quantified as previously described (9).

### Antibodies

The following Abs were obtained from BD Bioscience and used for surface marker analysis or intracellular cytokines: anti-mouse CD4 (clone GK1.5), anti-mouse CD8 (clone 53-6.7), anti-mouse CD11b (clone M1/70), anti-mouse CD11c (clone N418), and PE anti-mouse Ly-6G (clone IA8), anti-mouse IFN- $\gamma$  (clone XMG1.2). Anti-mouse NK1.1 (clone PK136) and anti-mouse Gr-1 (clone RB6-8C5) used for surface staining were obtained from BioLegend. The 7/4 rat anti-mouse neutrophil Ab was obtained from Serotec.

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<sup>3</sup> Abbreviation used in this paper: LM, *Listeria monocytogenes*.

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### Ex vivo assay and flow cytometry

Mouse spleens were harvested at various time points postinfection. Single-cell suspensions were prepared and the RBCs were lysed with ACK lysing buffer. Splenocytes ( $5 \times 10^6$ ) were plated in RPMI 1640 medium (Invitrogen) supplemented with 10% FCS, 2 mM L-glutamine, 50  $\mu$ M 2-ME, 100 U/ml penicillin, and 100  $\mu$ g/ml streptomycin in 24-well plates (2 ml/well) in the presence of GolgiPlug (2  $\mu$ l/well; BD Biosciences). These plates were cultured at 37°C in 5% CO<sub>2</sub> for 4 h. Spleen cells were stained for 30 min at 4°C with Abs to various surface markers. After two washes in PBS, intracellular IFN- $\gamma$  staining was performed using the Cytofix/Cytoperm kit (BD Biosciences) according to the manufacturer's instructions. Fluorescent intensities were measured using a Cytomics FC 500 and analysis was performed using CXP software (Beckman Coulter). Neutrophils were identified by Wright-Giemsa stain (Sigma-Aldrich) of sorted Gr-1<sup>high</sup>IFN- $\gamma$ <sup>+</sup> and Gr-1<sup>low</sup>IFN- $\gamma$ <sup>+</sup> or Ly-6G<sup>+</sup>IFN- $\gamma$ <sup>+</sup> cells. Cells were sorted on a FACS Vantage SE cell sorter (BD Biosciences).

### Isolation of splenic neutrophils and CD8<sup>+</sup> T cells

Mouse spleens were harvested and single-cell suspensions were prepared. Neutrophils were isolated using a customized negative selection kit (StemCell Technologies) following the manufacturer's instructions. The cocktail contained Ab to CD5, CD4, CD45R, Ter119, F4/80, and CD19. Purity was confirmed by flow cytometry with Gr-1 and Ly-6G Ab staining as well as Wright-Giemsa staining. Purity was 90–95%. Neutrophils ( $1.5 \times 10^6$ ) were recovered from the spleens of single donor mice. CD8<sup>+</sup> T cells were isolated using standard CD8<sup>+</sup> T cell enrichment kit (StemCell Technologies) and purity was >95% as determined by flow cytometry.

### Real-time PCR

Total cellular RNA from neutrophils and CD8<sup>+</sup> T cells was isolated using an RNeasy Mini kit (Qiagen) according to the manufacturer's instructions. First-strand synthesis was done using high-capacity cDNA archive kit (Applied Biosystems). PCR was done with 12.5  $\mu$ l of TaqMan universal master mix, 1.25  $\mu$ l of 20 $\times$  target or endogenous control primers and probe mix, and cDNA at a 25- $\mu$ l reaction volume. cDNA samples were used at the equivalent of 50 ng of total RNA per reaction. PCR thermal cycling condition was 50°C for 2 min, 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Samples were run on an ABI 7500 real-time PCR system (Applied Biosystems). The data were processed automatically using system's software based on  $\Delta\Delta C_T$  method. The primers used were target gene IFN- $\gamma$  (assay ID Mm00801778\_m1) and endogenous control mouse GAPDH (part no. 4352339E) from Applied Biosystems.

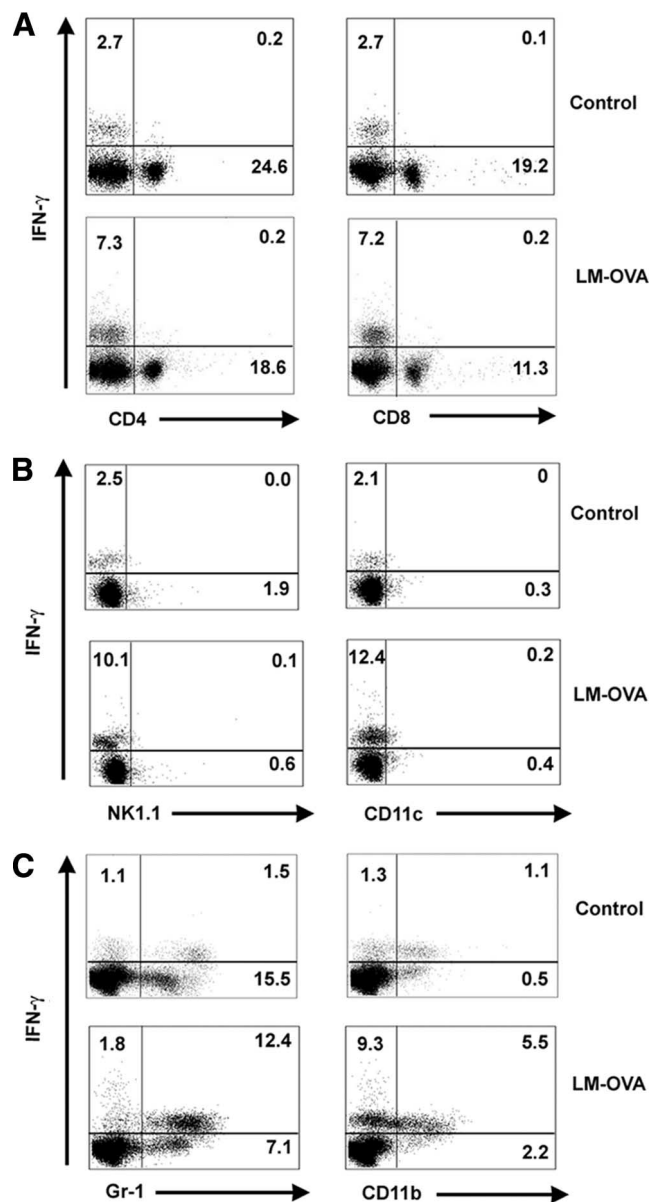
### Neutrophil adoptive transfer and calculation of bacterial loads in liver and spleen

Purified neutrophils ( $3 \times 10^6$ ) were injected i.v. via right retroorbital plexus and  $1 \times 10^6$  CFU of *actA*-LM-OVA were injected i.v. via the left retroorbital plexus. Both injections were done on the same day. The numbers of LM-OVA in the spleens and livers of infected mice were determined on day 3. Spleens and livers were homogenized in PBS by a TissueMiser (Fisher Scientific), the mixtures were serially diluted, and 100  $\mu$ l was spread on a brain heart infusion agar plate containing streptomycin (50  $\mu$ g/ml; Sigma-Aldrich). Plates were incubated for 20 h at 37°C and the colonies were counted.

## Results

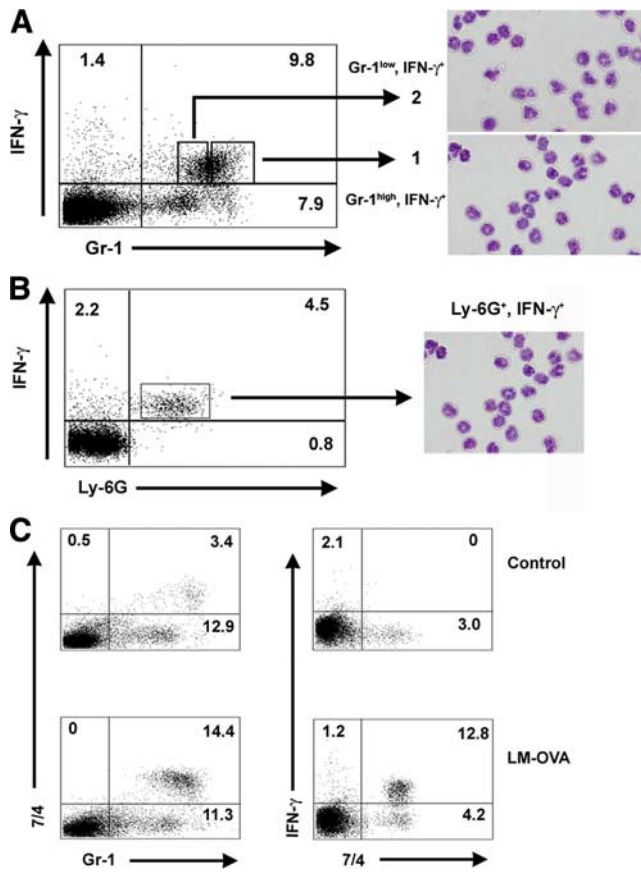
### Identification of a Gr-1<sup>+</sup>CD11b<sup>+</sup> IFN- $\gamma$ -producing neutrophil during LM infection

IFN- $\gamma$  plays a critical role in the host response to LM infection. NK cells and memory CD8<sup>+</sup> T cells can be the source of this cytokine and provide protection against LM infection in adoptive transfer studies (1, 8). Additionally, it is established that neutrophils play a critical role early in the infection, presumably from their phagocytic ability that clears infectious organisms (1). We have reexamined IFN- $\gamma$  production in cell populations of the spleen within the first 24 h following infection. Mice were infected with  $10^6$  CFU of LM-OVA i.v. and 18 h later spleens were removed and cultured in vitro for 4 h with GolgiPlug. Cells were then stained for intracellular IFN- $\gamma$  along with Abs to CD4, CD8, CD11b, CD11c, NK1.1, and Gr-1. Data in Fig. 1A show that very few CD4<sup>+</sup> and CD8<sup>+</sup> T cells producing IFN- $\gamma$  were detected. In



**FIGURE 1.** IFN- $\gamma$ -producing cells during LM infection. C57BL/6 mice were infected i.v. with LM-OVA, control mice received PBS. Mouse spleens were harvested at 18 h postinfection and splenocytes were cultured in 24-well plates in the presence of GolgiPlug for 4 h. Cells were analyzed for cell surface markers and intracellular IFN- $\gamma$ . A, CD4 and CD8 staining. B, NK1.1 and CD11c staining. C, Gr-1 and CD11b staining. Data presented are representative from at least four experiments with a minimum of three mice per group. Numbers in quadrants indicate percentage of cells.

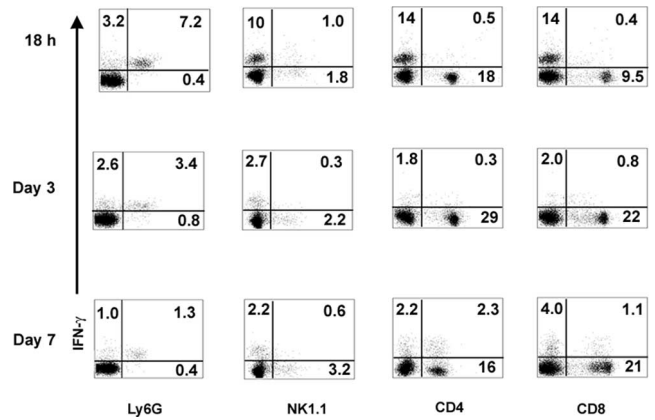
fact, infection did not increase the number of IFN- $\gamma$ -producing T cells at this time point. Infection with LM did, however, increase the number of IFN- $\gamma$ -secreting cells in the non-CD4<sup>+</sup> and non-CD8<sup>+</sup> population. Further analysis of the non-T cell population revealed that the IFN- $\gamma$ -producing cells were not NK cells or dendritic cells (Fig. 1B). Again, the IFN- $\gamma$ -secreting cells were noted only in the non-NK cell and non-dendritic cell population. Interestingly, significant IFN- $\gamma$ -producing cells were found in the Gr-1<sup>+</sup> and the CD11b<sup>+</sup> populations from infected spleens (Fig. 1C). Since these markers identify the neutrophil and monocyte/macrophage fractions of spleen, the cells making IFN- $\gamma$  at 18 h after LM infection likely resided within these populations.



**FIGURE 2.** Characterization of IFN- $\gamma$ <sup>+</sup>-producing cells. C57BL/6 mice were infected i.v. with LM-OVA, while control mice received PBS. Mouse spleens were harvested at 18 h, and splenocytes were cultured in 24-well plates in the presence of GolgiPlug for 4 h. Splenocytes were then stained for cell surface markers and intracellular IFN- $\gamma$ . **A**, The cells were sorted based on expression of these markers into Gr-1<sup>high</sup>IFN- $\gamma$ <sup>+</sup> and Gr-1<sup>low</sup>IFN- $\gamma$ <sup>+</sup>. Cells were placed on slides and stained with Wright-Giemsa solution. *Insert*, Representative Gr-1<sup>high</sup>IFN- $\gamma$ <sup>+</sup> cells (population 1) and Gr-1<sup>low</sup>IFN- $\gamma$ <sup>+</sup> cells (population 2). **B**, The cells were sorted based on expression of Ly-6G, and these cells were placed on slides and stained with Wright-Giemsa solution. *Insert*, Representative Ly-6G<sup>+</sup>IFN- $\gamma$ <sup>+</sup> cells. **C**, Spleens were stained with Ab to 7/4 and Gr-1 (*left panels*) or 7/4 and IFN- $\gamma$  (*right panels*). Uninfected control mice (*top panels*) were compared with LM-OVA-infected mice (*bottom panels*). Numbers in quadrants indicate percentage of cells.

#### Characterization of Gr-1<sup>+</sup>IFN- $\gamma$ <sup>+</sup> and Ly-6G<sup>+</sup>IFN- $\gamma$ <sup>+</sup> cells

Further characterization of the IFN- $\gamma$ -producing cells in the spleen was conducted by flow cytometry and morphological analysis. Mice were infected with  $1 \times 10^6$  CFU of LM-OVA i.v., and 18 h later spleens were removed and cultured in vitro for 4 h with GolgiPlug. Cells were then stained for Gr-1 and intracellular IFN- $\gamma$  and sorted based on these markers. Sorted cells were then placed on slides by cytopsin, and morphological analysis was conducted following Wright-Giemsa staining. The flow cytometry plot in Fig. 2A shows that the Gr-1 Ab identified two populations of IFN- $\gamma$ -secreting cells. Population 1 was the Gr-1<sup>high</sup>IFN- $\gamma$ <sup>+</sup> cells, and >98% of these cells had ring-shaped and closed nuclei (as shown in the insert for Fig. 2A), and they were therefore neutrophils. The majority (80%) of the Gr-1<sup>low</sup>IFN- $\gamma$ <sup>+</sup> cells (population 2) also with ring-shaped and closed nuclei; however, cells with open nuclei were also observed (20%), suggesting that the latter cells are of the monocyte/macrophage type (15, 16).



**FIGURE 3.** IFN- $\gamma$  production by spleen cells from LM-infected mice. Mouse spleens were harvested at 18 h, 3 days, and 7 days postinfection and stained for IFN- $\gamma$  along with Ly-6G, NK1.1, CD4, or CD8. For Ly6G, NK1.1, and CD8 staining, splenocytes were cultured and treated as in Fig. 1. For CD4 and IFN- $\gamma$  staining, splenocytes were cultured overnight and restimulated with listeriolysin O 190 peptide (NEKYAQAYPNVS; Bio-Synthesis) in 24-well plates. GolgiPlug was added during the last 4-h incubation. Data presented are representatives of two independent experiments. Numbers in quadrants indicate percentage of cells.

Ab to Gr-1 (clone RB6-8C5) reacts with both Ly-6G and Ly-6C and identifies neutrophils; however, this Ab can also react with subsets of monocytes, dendritic cells, macrophages, and lymphocytes (15, 17, 18). Although we did not detect IFN- $\gamma$ -producing cells in these latter populations, we wanted to be sure that we had in fact identified neutrophils as the source of IFN- $\gamma$  during LM infection. Consequently, we further analyzed IFN- $\gamma$ -producing cells with the Ly-6G and 7/4 Abs. Spleens from mice infected as above were stained for Ly-6G and intracellular IFN- $\gamma$  followed by sorting and morphological analysis. The flow cytometry plot in Fig. 2B shows that most cells making IFN- $\gamma$  were found in the Ly-6G<sup>+</sup> population and 95% of these cells had ring-shaped and closed nuclei (Fig. 2B, *insert*). Fig. 2C explores expression of 7/4, an allotypic marker expressed on neutrophils, but absent on macrophages (19, 20). Dual staining with 7/4 and Gr-1 LM shows that infection increased the number of Gr-1<sup>+</sup>7/4<sup>+</sup> cells from 3.4% to 14.4% of total spleen cells. Costaining for 7/4 and IFN- $\gamma$  revealed that these cells were virtually absent in uninfected cells but were increased to 12.8% of total spleen cells 18 h following LM infection.

Based on our data in Figs. 1 and 2 we can calculate the percentage of neutrophils producing IFN- $\gamma$ . In uninfected mice, 8% of the Gr-1<sup>+</sup> cells produce the cytokine; this increases to ~60% 18 h following infection. When cells are gated on the 7/4 marker, IFN- $\gamma$ -producing cells increased from 0% in uninfected mice to >75% 18 h following LM infection. Thus, the vast majority of neutrophils in infected mice produced IFN- $\gamma$  protein.

The highest numbers of IFN- $\gamma$ -producing neutrophils were found at 18 h postinfection (Fig. 3). This number declined by day 3 and the cell counts were near control numbers 7 days following LM infection. This contrasts IFN- $\gamma$ -producing cells in the NK and T cell populations. We never observed >1% NK1.1<sup>+</sup> cells producing IFN- $\gamma$  in the spleen at 18 h following infection (Fig. 3), and in most other cases IFN- $\gamma$ -producing NK1.1<sup>+</sup> cells represented <0.1% of the spleen (see Fig. 1B). T cells producing IFN- $\gamma$  were at low numbers 18 h and 3 days following infection. Higher numbers of CD4<sup>+</sup> T cells were found on day 7, while the number of CD8<sup>+</sup> T cells increased slightly over the course of the experiment (Fig. 3).

Table I. *IFN- $\gamma$  mRNA expression at 18 h following LM-OVA infection<sup>a</sup>*

	CD8 <sup>+</sup> T cells	Gr-1 <sup>+</sup> Neutrophils
Control	1.00 ± 0.19	1.00 ± 0.06
LM-OVA	15.97 ± 0.04*	1115.77 ± 106*

<sup>a</sup> C57BL/6 mice were infected with LM-OVA and 18 h later CD8<sup>+</sup> T cells and neutrophils were isolated using magnetic beads (see *Materials and Methods*). CD8<sup>+</sup> T cells and neutrophils were also isolated from uninfected (Control) mice. Quantitative real-time PCR was performed and values were normalized to an internal GAPDH control. Numbers represent the means of three replicates ± SE and expressed as fold change vs control mice. \*,  $p < 0.01$ .

Further examination of IFN- $\gamma$  production by neutrophils in the spleen of infected mice was carried out by quantitative RT-PCR with magnetic bead-purified cell populations. Splenic neutrophils and CD8<sup>+</sup> T cells were isolated from LM-OVA-infected cells 18 h postinfection, and mRNA levels were evaluated in comparison to uninfected control mice. Table I shows that while CD8<sup>+</sup> T cells produced significant levels of IFN- $\gamma$  mRNA, purified splenic neutrophils made nearly 70-fold more. Additionally, stimulation of isolated Ly-6G<sup>+</sup> cells with LM in vitro resulted in a 53.3-fold

increase in IFN- $\gamma$  mRNA vs unstimulated cells. We conclude from our data in Figs. 1–3 and Table I that the vast majority of splenic IFN- $\gamma$ -producing cells 18 h postinfection were in the neutrophil fraction of spleen.

#### *Adoptive transfer of neutrophils to *Ifng*<sup>-/-</sup> mice increases bacterial clearance*

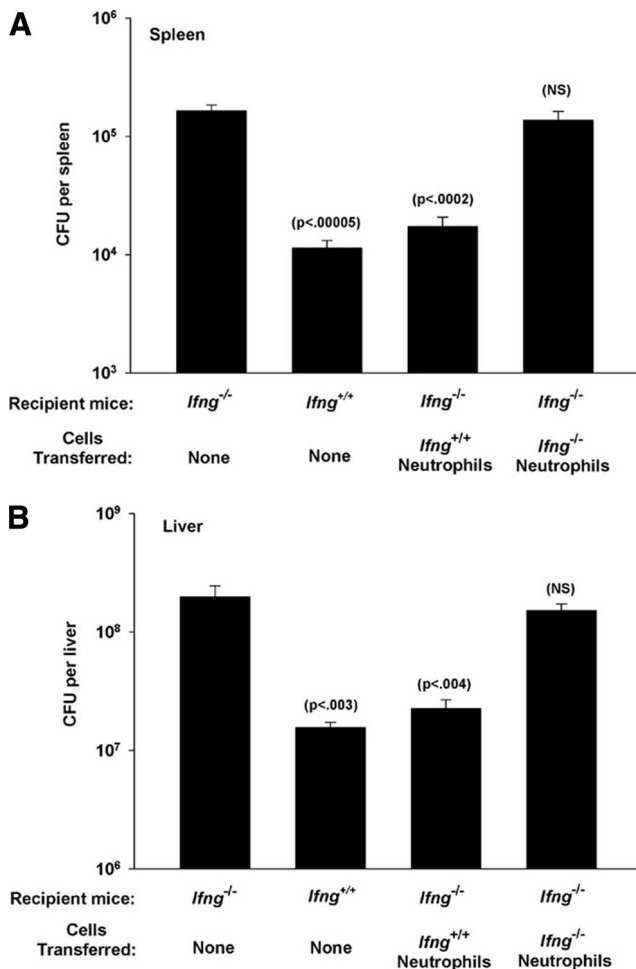
IFN- $\gamma$  production during the early stages of the innate response is critical to resistance to LM, and consequently *Ifng*<sup>-/-</sup> mice are highly susceptible to infection (9, 13). These mice have difficulty clearing the bacterium from infected organs, and this can lead to a lethal infection. To evaluate the importance of IFN- $\gamma$ -producing neutrophils in this model we used an adoptive transfer system with *Ifng*<sup>+/+</sup> (wild-type C57BL/6) and *Ifng*<sup>-/-</sup> mice (also C57BL/6 background). Neutrophils were purified from the spleens of normal (not infected) *Ifng*<sup>+/+</sup> mice at 18 h and transferred to *Ifng*<sup>-/-</sup> mice (recipient mice) who were infected with LM on the same day. Three days later bacterial loads in the spleen and liver were quantified. As shown in Fig. 4, *Ifng*<sup>-/-</sup> mice had very high bacterial loads in the spleen (Fig. 4A) and liver (Fig. 4B) compared with *Ifng*<sup>+/+</sup> mice. In fact, in several experiments at least 50% of infected *Ifng*<sup>-/-</sup> mice succumbed to the infection before day 3 (not shown). In contrast, *Ifng*<sup>-/-</sup> mice that receive *Ifng*<sup>+/+</sup> neutrophils had reduced bacterial loads in both the spleen and liver. Additionally, no mortality in the *Ifng*<sup>-/-</sup> recipients of *Ifng*<sup>+/+</sup> neutrophils was observed (not shown). In sharp contrast, purified neutrophils from *Ifng*<sup>-/-</sup> mice provided no such protection when transferred to *Ifng*<sup>-/-</sup> mice and mortality remained at 50% for these animals (not shown). Thus, neutrophil-derived IFN- $\gamma$  is critical to controlling bacterial colonization of the spleen and liver.

#### Discussion

The immune response to pathogenic microorganisms involves both the innate and acquired immune responses. Innate immunity involves the rapid, Ag nonspecific response of phagocytic cells and serum components, while acquired immunity is delayed, often requiring days or weeks to develop. LM is an intracellular parasite that infects macrophages of immunocompromised hosts. Resistance to LM requires both innate and acquired immunity, with both arms of the response being required for successful clearance of the most virulent strains (1, 7). Key cytokines such as TNF- $\alpha$  (4), IL-1 (5), IL-12 (6), and IFN- $\gamma$  (2, 7, 8) are also critical to the response to LM. IFN- $\gamma$  is very important during the innate phase, being required for clearing the organism, but it plays a lesser role in the establishment of the T cell response to LM, as shown in studies with *Ifng*<sup>-/-</sup> mice (9, 13).

In this report we identify for the first time IFN- $\gamma$ -producing neutrophils early in the response to LM. The cell was identified by cell surface staining and morphological analysis of splenic IFN- $\gamma$ -producing cells. The importance of these cells to LM resistance was demonstrated by adoptive transfers to *Ifng*<sup>-/-</sup> mice. These mice succumb to LM infection; however, transfer of neutrophils from *Ifng*<sup>+/+</sup> mice eliminated mortality and increased bacterial clearance.

To our knowledge this is the first demonstration of IFN- $\gamma$ -producing neutrophils in LM infection and one of only a few reports showing that neutrophils can make this cytokine. Myeloid cells (including neutrophils and monocytes/macrophages) are typically viewed as targets of IFN- $\gamma$  rather than producers; however, IFN- $\gamma$ -producing neutrophils have been observed in human tissue and in cultured human peripheral blood cells (21, 22). It was also observed that human neutrophils can contain preformed IFN- $\gamma$  that is



**FIGURE 4.** Adoptive transfer of neutrophils to *Ifng*<sup>-/-</sup> mice. Mouse splenic neutrophils were isolated by negative selection from uninfected *Ifng*<sup>+/+</sup> (C57BL/6) mice at 18 h. *Ifng*<sup>-/-</sup> mice (Recipient mice) received  $3 \times 10^6$  purified neutrophils (Cells transferred) or no cells (None) along with LM-OVA i.v. The number of LM-OVA in the spleens (A) and livers (B) was determined on day 3. Data were derived from two independent experiments (A and B) with five mice per group. Values of  $p$  represent comparisons to infected *Ifng*<sup>-/-</sup> mice by Student's  $t$  test using a 95% confidence interval.

rapidly released in response to granulating agents (23). IFN- $\gamma$ -producing neutrophils have also been shown in the mouse by intracellular staining of the Gr-1<sup>high</sup> population during infections with *Salmonella typhimurium* (24) and *Nocardia asteroides* (25). Our data demonstrated that IFN- $\gamma$  is rapidly made in response to LM infection, coming predominantly from the induction of mRNA transcription. This is because we observe a small percentage of neutrophils containing IFN- $\gamma$  protein in uninfected mice (Fig. 1C, 8% of GR-1<sup>+</sup> cells; Fig. 2C, 0% 7/4<sup>+</sup> cells). This number increases to ~80% following LM infection.

Studies by North and colleagues more than a decade ago established that neutrophils and NK cells were important to protection from LM infection (1–3, 26). More recent reports have confirmed the role of NK cells and revealed a role for IFN- $\gamma$ -producing CD8<sup>+</sup> T cells early in the response to the bacterium (7, 11, 12). Neutrophils are seen as critical to phagocytosis and destruction of the bacterium, while NK cells are the source of IFN- $\gamma$ . Both of these innate response cells can be detected in the first 3 days following infection, and various experiments involving Ab depletion and adoptive transfers were used to define their importance. For example, depletion of NK cells reduced the number of IFN- $\gamma$ -producing cells in the spleen of infected mice (2). Additionally, NK cells producing IFN- $\gamma$  were identified in the spleens of mice 3 days following infection (7). We do not think that our data contradict these findings, as we have observed the presence of IFN- $\gamma$ <sup>+</sup> neutrophils earlier (18 h) in the infection. By day 3 this number had fallen dramatically (see Fig. 3). Additionally, it was reported by Andersson et al. (27) that mice deficient in NK cells mounted a normal innate immune response to LM infection and these mice had increased blood levels of IFN- $\gamma$ . The authors concluded that the response in this case was not NK cell dependent; however, they did not identify the IFN- $\gamma$  source in vivo. We would suggest that these cells may have been neutrophils, although in vitro analysis by these authors suggested that T cells may be an important source. Importantly, these studies were conducted on day 2 following infection while our studies were performed at 18 h. However, we did not observe significant increases in NK1.1 cells at any time point after LM infection, supporting a lesser role for NK cells in this infection. It is clear from our adoptive transfer studies that the rapid neutrophil-derived IFN- $\gamma$  is a critical component in resistance to this infection. Thus, we propose that the neutrophilic response to LM may be more rapid than the NK or T cell response, and it may promote the development of the innate immune response to LM.

## Disclosures

The authors have no financial conflicts of interest.

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