Cutting Edge: Developmental Regulation of IFN-γ Production by Mouse Neutrophil Precursor Cells

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Neutrophils are an emerging cellular source of IFN-γ, a key cytokine that mediates host defense to intracellular pathogens. Production of IFN-γ by neutrophils, in contrast to lymphoid cells, is TLR- and IL-12–independent and the events associated with IFN-γ production by neutrophils are not understood. In this study, we show that mouse neutrophils express IFN-γ during their lineage development in the bone marrow niche at the promyelocyte stage independently of microbes. IFN-γ accumulates in primary neutrophilic granules and is released upon induction of degranulation. The developmental mechanism of IFN-γ production in neutrophils arms the innate immune cells prior to infection and assures the potential for rapid release of IFN-γ upon neutrophil activation, the first step during responses to many microbial infections. The Journal of Immunology, 2015, 195: 000–000.

Induction of immune responses to microbial pathogens is generally divided into distinct stages, in which the recognition of microbial molecules mediated by innate immune receptors results in maturation of dendritic cells and macrophages, followed by secretion of activating cytokines that regulate the effector phase of host defense (1, 2). This model is particularly well studied in the context of TLR-mediated immunity, in which DC-specific TLR activation results in production of IL-12 that subsequently regulates IFN-γ production by NK and T cells (3). In vivo experiments with the protozoan parasite Toxoplasma gondii formally tested this model and revealed that whereas NK and CD4+ T cell IFN-γ production was regulated by TLR11-dependent activation of MyD88 (4–8), there is an unforeseen component of IFN-γ–dependent host response mediated by neutrophils (9). In contrast to NK cells (10, 11), T cells (5, 12, 13), and innate lymphoid cells (14), the ability of neutrophils to produce IFN-γ does not depend on IL-12– or TLR-dependent parasite recognition (9), and therefore how neutrophils produce IFN-γ is incompletely understood. This knowledge is crucial, because although neutrophils have long been viewed as effector cells that mediate their protective effects via the release of lytic granules, recent evidence suggests that neutrophils secrete cytokines that have pleiotropic effects on host defense to microbial infections (15, 16).

In this study, we investigated how neutrophils produce IFN-γ. Our data revealed that neutrophil IFN-γ production is regulated during hematopoietic development of neutrophil precursor cells and that IFN-γ accumulates in primary granules produced in the promyelocyte stage of neutrophil development. The accumulation of IFN-γ in primary granules is independent of microbial infection or the endogenous microbiota. Neutrophil IFN-γ effector mechanisms are achieved by activation-induced degranulation. This represents a broad innate immune mechanism of IFN-γ–mediated host defense required for host protection from intracellular pathogens.

**Materials and Methods**

**Mice**

C57BL/6, germ-free C57BL/6, and TLR11−/− mice have been previously described in experimental toxoplasmosis (5, 6, 17). All experiments were performed with protocols approved by the Institutional Animal Care and Use Committees at the University of Texas Southwestern Medical Center.

**Ex vivo analysis of IFN-γ production by neutrophils**

C57BL/6 and TLR11−/− mice were infected i.p. with 20 cysts of T. gondii (ME49 strain) as previously described (6, 9). Cells were not given any stimulation or Golgi inhibitors unless otherwise indicated. For indicated experiments, peritoneal cells were incubated for 5 h at 37˚C in media with or without GolgiPlug (1 μg/ml). To block protein translation, peritoneal cells were incubated in media with GolgiPlug and increasing concentrations of cycloheximide, with or without PMA (50 ng/ml) and ionomycin (750 ng/ml). To induce neutrophil degranulation, cells were activated with 10 nM fMLP, 100 ng/ml LPS, 50 ng/ml PMA, and/or 750 ng/ml ionomycin, incubated for 1 h at 37˚C, supernatants were collected for IFN-γ ELISA, and cells were stained for flow cytometry.

**Flow cytometry**

For bone marrow (BM) and peritoneal cells, single-cell suspensions were stained in PBS plus 1% FBS and 0.5 mM EDTA. Blood samples were directly stained and erythrocytes lysed with ACK lysing buffer. After extracellular staining, samples were fixed and permeabilized for intracellular staining using the Foxp3 staining buffer kit by eBioscience, per the manufacturer’s instructions. The following Abs were used for staining and were from eBioscience unless otherwise noted.

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Abbreviation used in this article: BM, bone marrow.

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Results

Constitutive production of IFN-γ by neutrophils in the absence of microbial stimulation

T. gondii elicits potent innate and adaptive IFN-γ responses from multiple cell types, including neutrophils, innate lymphoid cells, NK cells, and T cells (19). Although TLR activation and IL-12 production by dendritic cells is required for IFN-γ production by lymphoid cells, how neutrophils mediate TLR- and IL-12-independent IFN-γ production is largely unknown (20). Therefore, we began to address this question by examining the appearance of IFN-γ-producing neutrophils triggered by T. gondii infection.

Neutrophils are present in large numbers in the peritoneal exudate cells isolated from the site of T. gondii infection (Fig. 1A). However, the amount of IFN-γ detected in neutrophils by intracellular staining was not increased by in vitro incubation with GolgiPlug, a protein transport inhibitor that leads to intracellular accumulation of IFN-γ seen in NK and T cells.

Quantitative real-time PCR

RNA from defined immature neutrophils was isolated using the PureLink RNA Mini kit, cDNA synthesis was done using SuperScript III reverse transcriptase. Samples were analyzed on MyiQ real-time PCR system, and data were processed for relative expression to control gene hypoxanthine phosphoribosyltransferase.

Microscopy

Magnetic bead sort-purified Ly-6G+ BM cells were fixed in 4% PFA, blocked in PBS with 2% BSA and 0.2% Triton X-100, and stained with Abs of interest. The following Abs were used for microscopy analysis: anti–IFN-γ Alexa Fluor 488 (XMG1.2; eBioscience), anti–myeloperoxidase (ab9535; Abcam), anti-lactoferrin (ab135710; Abcam), anti-gelatinase (ab38898; Abcam), and donkey anti-rabbit IgG Alexa Fluor 568 (A10042; Life Technologies). Images were acquired with Leica TCS SPE or SP5 laser scanning confocal microscopes. Downstream images were examined with ImageJ, Z stacks underwent three-dimensional blind deconvolution with AutoQuant X software, colocalization analysis (Manders coefficient) (18) was performed, and three-dimensional modeling was done with Imaris.

Statistical analysis

All statistical analysis on bar graphs is done using the standard unpaired t test, and error bars shown are mean ± SD.

FIGURE 1. Neutrophils express IFN-γ independently of pathogens or microbes. (A and B) C57BL/6 mice (n = 5) were infected i.p. with T. gondii or (C and D) left untreated and peritoneal cavity cells, blood, and BM were examined for the presence of Ly6G+IFN-γ neutrophils on day 5 postinfection. Histograms in (B) and (D) show mean fluorescence intensity (MFI) of neutrophil IFN-γ in the peritoneal cavity (blue), blood (red), and BM (black) in infected and naive mice, respectively. Isotype control stain (filled histogram) is shown for BM neutrophils. (E) IFN-γ neutrophils and (F) MFI of neutrophil IFN-γ was quantified from (A) and (B). (G) BM from naive conventional (CV) or germ-free (GF) C57BL/6 mice (n = 3) was examined for the presence of IFN-γ neutrophils. Quantification of data seen in (G), the percentage of total BM that are Ly6G+ neutrophils (H), the percentage of IFN-γ neutrophils (I), and MFI of neutrophil IFN-γ (J) are shown. The data shown are representative of more than three independent experiments, each involving two to four mice per group, and error bars shown are mean ± SD. **p < 0.001.

FIGURE 2. Developing neutrophils express IFN-γ at the promyelocyte stage. (A) The gating strategy to identify neutrophil precursor cells excludes other potentially IFN-γ cells by negative selection (CD3e, CD19, NK1.1, and Ter119), with the gating of the neutrophil precursor populations shown in the third box from the left. Neutrophils develop from the granulocyte-monocyte progenitor (GMP) through a series of stages shown in red (myeloblast), orange (promyelocytes), purple (myelocytes), green (metamyelocytes), and blue (band/segmented neutrophil). (B) Neutrophil precursor populations were examined for expression of IFN-γ by flow cytometry. These data are representative of at least three independent experiments each involving two to four mice per group.
examined for neutrophil IFN-γ production. However, confined to the site of experimental infection during the first day after infection, IFN-γ positivity, and cycloheximide treatment had no dose-dependent effects on the levels of IFN-γ detected in neutrophils (Supplemental Fig. 1B). Taken together, these data revealed that neutrophils expressed IFN-γ prior to isolation from T. gondii–infected mice.

To address the question of whether neutrophil IFN-γ production is limited to the site of infection in vivo, wild-type mice were infected with T. gondii and several tissues were examined for neutrophil IFN-γ positivity, including the peritoneal cavity, peripheral blood, and BM. Neutrophils stained positive for IFN-γ at a similar level in all locations examined (Fig. 1A, 1B, 1E). T. gondii parasites are largely confined to the site of experimental infection during the first 5 d, and thus these data suggest that an indirect sensing of infection may be responsible for IFN-γ production by neutrophils. However, T. gondii–infected TLR11−/− mice also have IFN-γ+ neutrophils in all tissues examined (Supplemental Fig. 1C). Additionally, the analysis of neutrophils in naïve mice revealed IFN-γ+ neutrophils in the blood and BM but not in the peritoneal cavity (Fig. 1C–E, Supplemental Fig. 1C). The amount of IFN-γ positivity by neutrophils in the BM and blood was comparable in naïve and T. gondii–infected mice (Fig. 1E, 1F). Lack of IFN-γ+ neutrophils in the peritoneal cavity of naïve mice is not surprising, given that neutrophils are not present in the peritoneal cavity of naïve mice. Nevertheless, thioglycollate was used to elicit neutrophils into the peritoneum of otherwise naïve mice, neutrophils stained uniformly positive for IFN-γ (Supplemental Fig. 1D) when combined, these experiments suggest that neutrophils constitutively produce IFN-γ independent of infection.

One possible explanation for the appearance of IFN-γ–producing neutrophils in naïve mice is that microbial products derived from intestinal microbiota could be stimulating neutrophils in peripheral tissues to produce IFN-γ, because commensal bacteria are involved in the activation of mature neutrophils (21, 22). To examine this possibility we quantified the presence of IFN-γ+ neutrophils in the BM of germ-free C57BL/6 mice. We observed that the colonization status of mice had no effect on the appearance of IFN-γ+ neutrophils (Fig. 1G–J). Thus, IFN-γ production by neutrophils is regulated differently from lymphoid cells and does not require the presence of T. gondii or other microbial stimuli. Instead, T. gondii infection triggers neutrophil recruitment to the site of infection, explaining the selective appearance of IFN-γ+ neutrophils in the peritoneal cavity of infected but not naïve mice (Fig. 1A, 1C).

**Developmental regulation of IFN-γ production by neutrophils**

Analysis of BM neutrophils revealed that in addition to IFN-γ+Ly6Ghigh cells, there is an appearance of Ly6G− and Ly6Glow cells that produce IFN-γ in germ-free, naïve, and T. gondii–infected mice (Fig. 1). The presence of IFN-γ+ in the BM suggests a model of developmental regulation for IFN-γ production by neutrophils. To obtain insight into this hypothesis, we investigated the stages of neutrophil development and their association with IFN-γ production. Flow cytometric analysis of neutrophil precursors in BM excluded T, B, and NK cells, followed by the analysis of c-Kit−, CD34−, and Ly-6G−expressing cells (Fig. 2A). The cells were divided into five populations: myeloblasts (red), promyelocytes (orange), myelocytes (purple), metamyelocytes (green), and mature neutrophils (blue) (Fig. 2). To confirm the successful identification of neutrophil precursors, the immature neutrophil populations were sort purified and analyzed for stage-specific proteins by quantitative RT-PCR, including proteinase 3, a primary granule protein expressed mostly in early promyelocytes, lactoferrin (a secondary granule protein expressed mostly in metamyelocytes), and gelatinase (a tertiary granule protein expressed mostly in late band cells) (Supplemental Fig. 2A) (23). Examination of immature neutrophils for IFN-γ positivity by flow cytometry revealed that neutrophils acquire IFN-γ positivity at the promyelocyte stage, exhibiting a nearly equal split of IFN-γ+ and IFN-γ− cells at this stage.
We also examined whether earlier hematopoietic cells were capable of producing IFN-γ. Lineage `-sca-1+c-Kit+ (LSKs), which included the long-term hematopoietic stem cells, short-term hematopoietic stem cells, and multipotent progenitors, did not stain positive for IFN-γ (Supplemental Fig. 2B). Additionally, the immediate precursor to all granulocytes, the GMPs, also did not stain positive for IFN-γ (Supplemental Fig. 2B). These data revealed that IFN-γ expression in the BM is restricted to the neutrophil-specific lineage development (Fig. 2).

**Neutrophil IFN-γ accumulates in primary granules**

Given that promyelocyte stage neutrophils produce IFN-γ (Fig. 2B) and that immature neutrophils begin packaging granules as they transition to the promyelocyte stage (24), we hypothesized that neutrophil IFN-γ could be stored in granules. To examine the localization of IFN-γ, we used immunofluorescence detection of IFN-γ together with known granular proteins. Simultaneous visualization of IFN-γ with myeloperoxidase, lactoferrin, or gelatinase, which localize in primary, secondary, and tertiary granules, respectively, revealed colocalization between IFN-γ and myeloperoxidase (Fig. 3A). Minimal to no colocalization was observed between IFN-γ and lactoferrin or gelatinase (Fig. 3B, 3C). Quantitative colocalization analysis, using Manders coefficient, confirmed that IFN-γ is localized in the same granules that contain myeloperoxidase (Fig. 3). These results formally established that IFN-γ is largely contained within the primary granules of neutrophils.

**Neutrophil degranulation releases IFN-γ**

To further examine the physiological significance of IFN-γ distribution in primary granules, we tested whether induction of neutrophil degranulation would result in IFN-γ release. We first induced neutrophil degranulation with fMLP and LPS and observed that this stimulation resulted in tertiary granule, but little primary granule, degranulation, and minor loss of IFN-γ positivity (Fig. 4A–C). However, the combined treatment of neutrophils with fMLP, LPS, PMA, and ionomycin resulted in primary granule degranulation as seen by the loss of myeloperoxidase staining in the stimulated neutrophils (Fig. 4B). Importantly, primary granule degranulation also resulted in a nearly complete loss of intracellular IFN-γ staining and simultaneous release of IFN-γ into the cell culture supernatant (Fig. 4C, 4D). Taken together, our data revealed a developmental mechanism for IFN-γ expression in immature neutrophils and that the release of IFN-γ is regulated by activation-induced primary granule degranulation.

**Discussion**

In the present study, we have identified developmental regulation of IFN-γ expression in neutrophil precursor cells, which represents a novel mechanism for IFN-γ-mediated host defense. Whereas IFN-γ production by innate lymphoid cells, NK cells, and T cells is largely regulated by IL-12–dependent induction of IFN-γ expression postinfection, neutrophil IFN-γ is produced prior to infection in immature neutrophils at the promyelocyte stage. This mechanism prearms neutrophils, ensuring a rapid response to pathogens.

Regulated secretion of IFN-γ from neutrophil granules prevents spontaneous effects of IFN-γ in the absence of microbial infections. Whereas IFN-γ–expressing neutrophils are in blood and BM, the effector mechanisms of neutrophil-derived IFN-γ are restricted to the sites of infection because their IFN-γ secretion requires microbial or inflammatory environment–driven degranulation of primary granules. Granular localization of IFN-γ may explain the confusion in the literature regarding the ability of neutrophils to produce IFN-γ, because both isolation-induced degranulation or insufficient granular protein fixation will result in an inability to detect neutrophil IFN-γ. In addition to the priming effects of IFN-γ on other cell types, IFN-γ can prime neutrophils themselves for increased migration, pathogen clearance, Ag presentation abilities, and cytokine production (25), the complexities of which will need to be revisited in light of neutrophil-derived IFN-γ.

The release of IFN-γ by neutrophils represents a bona fide type I innate immune response that can potentially be elicited by a variety of pathogens and host inflammatory responses sufficient to trigger neutrophil primary granule degranulation. In this regard it is of interest that neutrophil IFN-γ expression is developmentally regulated, which is distinct from their cytokine and stimuli-induced production of IL-22 and IL-17, two additional cytokines that were also, until recently, considered to be lymphoid cell–specific effector molecules (26, 27). Both IL-22 and IL-17 production by neutrophils is restricted to mucosal tissues, and in the case of IL-17, the transcription factor retinoic acid–related orphan receptor γt is a master regulator of IL-17 expression by neutrophils (27) and lymphoid cells (28). The localization of IFN-γ within primary granules of neutrophils provides an activation-dependent regulatory mechanism, because stronger inflammatory signals at the site of infection or inflammation are involved in primary granule degranulation. Overall, in this study we unveiled an arm of IFN-γ–mediated innate immunity that is prepared for immediate effector responses and is regulated by neutrophil recruitment and activation.

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**Disclosures**

The authors have no financial conflicts of interest.

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to default to a Th2 pattern and are host protective in an IL-10


Supplemental Figure 1. Neutrophils express IFN-γ prior to isolation from naïve or T. gondii infected WT and TLR11-/− mice.

C57BL/6 mice were infected i.p. with T. gondii and PECs were harvested day 5 post-infection. (A) PECs were incubated at 37C for 5 hours in media with GolgiPlug (Brefeldin A) or placed in media on ice for 5 hours. IFN-γ expression in neutrophils, T cells, and NK cells was assessed by flow cytometry. (B) PECs were incubated for 5 hours in media with GolgiPlug with or without stimulation (PMA-ionomycin) and with increasing concentrations of Cycloheximide (CHX), a de novo protein synthesis inhibitor. The percentage of IFN-γ-positive T cells or neutrophils were assessed by flow cytometry. (C) TLR11 KO mice were infected i.p. with T. gondii or were kept naïve and examined day 5 post-infection. PEC, blood, and bone marrow were examined for the presence of Ly6G+ neutrophils and IFN-γ positivity as in Figure 1. (D) Thioglycollate elicited Ly6G+ neutrophils were examined for IFN-γ positivity (blue). These data are representative of three independent experiments. * p<0.05, ns - not significant.
Supplemental Figure 2. Neutrophil developmental stage confirmation and earlier hematopoietic cells.

Bone marrow from C57BL/6 mice were harvested and examined for different stages of their development. (A) Neutrophil-specific lineage development was examined as in (Fig. 2A): promyelocytes (orange), myelocytes (purple), metamyelocytes (green), band/segmented neutrophils (blue). Relative expression of granule proteins, Proteinase 3, Lactoferrin, and Gelatinase, characteristic of different neutrophil precursor developmental stages. (B) Bone marrow from WT mice were harvested and examined for different stages of hematopoietic development. The neutrophil-specific lineage develops from the granulocyte-monocyte progenitor (GMP) and prior to that, the common-myeloid progenitor (CMP) and Lineage-negative Sca-1-positive c-kit-positive (LSKs). LSKs and GMPs were examined for IFN-γ expression (black) and isotype control (gray) by flow cytometry. The data shown are representative of at least 3 independent experiments.