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NK Dendritic Cells Are Innate Immune Responders to Listeria monocytogenes Infection

George Plitas, Umer I. Chaudhry, T. Peter Kingham, Jesse R. Raab, and Ronald P. DeMatteo

NK dendritic cells (NKDC) are recently described immunologic cells that possess both lytic and Ag-presenting function and produce prolific quantities of IFN-γ. The role of NKDC in innate immunity to bacterial infection is unknown. Because IFN-γ is important in the immune response to Listeria monocytogenes (LM), we hypothesized that NKDC play a critical role during LM infection in mice. We found that LM increased the frequency and activation state of NKDC in vivo. Using an in vivo intracellular cytokine analysis, we demonstrated that NKDC are a major source of early IFN-γ during infection with LM. Adoptive transfer of wild-type NKDC into IFN-γ-deficient recipients that were subsequently infected with LM decreased bacterial burden in the liver and spleen and prolonged survival. In contrast, NK cells were depleted early during LM infection, produced less IFN-γ, and conferred less protection upon adoptive transfer into IFN-γ-deficient mice. In vitro, LM induction of IFN-γ secretion by NKDC depended on TLR9, in addition to IL-18 and IL-12. Our study establishes NKDC as innate immune responders to bacterial infection by virtue of their ability to secrete IFN-γ. The Journal of Immunology, 2007, 178: 4411–4416.

Listeria monocytogenes (LM) is a Gram-positive, facultative, intracellular bacterium that is the causative agent in a spectrum of human disease ranging from gastroenteritis to invasive infection. Neonates, the elderly, and immunocompromised patients are particularly at risk of developing meningitis and sepsis when exposed to LM (1). Clearance of LM infection is mediated through a Th1 immune response (2). IFN-γ is a crucial contributor to the Th1 response by activating macrophages, increasing Ag presentation via the MHC class I and II pathways, and inhibiting the expansion of Th2 cells (3, 4). Mice deficient in IFN-γ or its receptor are particularly susceptible to even minute doses of LM (5, 6). Genetic defects in the human IFN-γ receptor system have been described in patients with vaccine-associated bacilli Calmette-Guérin or nontuberculous mycobacteria infections, demonstrating the importance of IFN-γ-mediated immunity in human host defense against intracellular pathogens (7–9). The presence of autoantibodies to IFN-γ in humans results in similar susceptibilities as the receptor defect (10, 11). Detection of pathogen-associated molecular patterns by TLR of the host’s innate immune system initiates the inflammatory response to microbial pathogens (12, 13). TLR2 and TLR5 have been reported to recognize LM (14, 15). Mice deficient in MyD88, an intracellular adaptor protein that mediates most TLR signaling, have a blunted IFN-γ response and are highly susceptible to LM infection (16, 17).

NK dendritic cells (NKDC; NK1.1−CD11c+CD3−) are multifunctional cells with attributes of both NK cells and dendritic cells (DC). NKDC can lyse classical NK cell targets, capture and process Ag, and stimulate naïve T cells (18). Although the human counterpart of murine NKDC has not yet been identified, human NK cells after activation have been shown to up-regulate the expression of DC maturation markers and acquire the capacity to induce proliferation of CD4+ and CD8+ T cells (19). We have previously shown that the TLR9 ligand CpG stimulates NKDC to secrete IFN-γ in vitro (20). In vivo, CpG administration expands NKDC, suggesting that NKDC may have an important role in the innate immune response to infectious organisms. Because of their pleiotropic functions, including their ability to produce IFN-γ, we postulated that NKDC play an important role in the immune response to LM infection.

In this study, we investigated the role of NKDC in the innate immune response to LM infection. We have found that NKDC are a major source of early IFN-γ during infection with LM. Using an in vivo intracellular cytokine (ICC) detection system, we have defined the temporal expression of IFN-γ by NKDC, NK cells, and T cells. Production of IFN-γ by NKDC, but not NK cells, depended on TLR9 ligation. Wild-type (WT) NKDC were able to protect normally susceptible IFN-γ−/− mice by enhancing clearance of LM and prolonging survival. These data establish a new member in the cellular arsenal of the innate immune response to an intracellular bacterial pathogen.

Materials and Methods

Animals and infections

Adult 6- to 8-wk-old male C57BL/6 (B6) mice were purchased from Taconic Farms and IFN-γ−/− mice on B6 background were purchased from The Jackson Laboratory. Mice were immunized i.v. with 1–5 × 10^7 of the WT LM strain 10403S (provided by Dr. Eric Pamer, Sloan-Kettering Institute, New York, NY). IFN-γ depletion was performed with an anti-IFN-γ mAb (XMG1.2; mAb Core Facility, Sloan-Kettering Institute, New York, NY) or isotype control. The mice received 1 mg of αIFN-γ or isotype matched control Ab i.p. 6 h before transfer of NKDC and everyday thereafter (21). Bacterial CFUs were determined by plating serial dilutions of whole organ lysates on brain-heart infusion agar plates. Mice were maintained in the pathogen-free animal housing facility at Memorial Sloan-Kettering Cancer Center, and all procedures were approved by the Institutional Animal Care and Use Committee.


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3 Abbreviations used in this paper: LM, Listeria monocytogenes; NKDC, NK dendritic cells; DC, dendritic cells; WT, wild type; hkLM, heat killed LM; ICC, intracellular cytokine; tCpG, inhibitory CpG.

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Cell isolation and flow cytometry

Splenocytes were enriched based on CD11c and NK1.1 expression with immunomagnetic beads per the manufacturer’s protocol (Miltenyi Biotec) and stained with fluorescently conjugated Abs to NK1.1, CD3 and CD11c (BD Pharmingen) for further separation into NKDC, NK cells, DC, and T cells using a MoFlo cell sorter (DakoCytomation). Sorted cell populations were consistently >98% pure for the desired set of surface markers. FcRs were blocked with the mAb 2.4G2 (Fc III/IIR block; 1 g/10^6 cells; mAb Core Facility, Sloan-Kettering Institute) before all immunomagnetic bead and Ab incubations. Five-color flow cytometry was performed on a FACScan flow cytometer (BD Biosciences) with modifications by Cytek. Approximately 5 x 10^5 cells were labeled with 0.1 g FITC, PE, PerCP, allophycocyanin, allophycocyanin-Cy7, or biotin-conjugated Ab (BD Pharmingen). Cells were stained for MHC II (I-Ab) [AF6-120.1], CD3 [145-2C11], CD11c [HL-3], CD86 [GL-1], NK1.1 [PK136], and IFN-γ [XMG1.2]. Appropriate Ig isotype controls were used for phenotype analysis. Flow cytometry data were analyzed with FlowJo software (TreeStar).

Lytic assay

Lytic assays were performed using sorted splenic NK cells and NKDC. Cells were cocultured with 1 x 10^3 [51Cr] sodium chromate (PerkinElmer) labeled YAC-1 cells (American Type Culture Collection) for 6 h in 96-well V-bottom plates in a total of 200 μl of medium. [51Cr] sodium chromate release was measured with a TopCount NXT microplate scintillation and luminescence counter (PerkinElmer). Spontaneous release (no effectors) and maximum release (2% Triton-X-100; Sigma-Aldrich) were used to determine differences in maturation marker expression. Values of p < 0.05 were deemed significant.

Cytokine analysis

In vitro cytokine production and serum levels were assessed by culturing 2 x 10^5 cells in a 96-well U-bottom tissue culture plate in 200 μl of medium for 72 h. IL-18 (20 ng/ml; BioSource International), IL-12 (20 ng/ml; R&D Systems), iCpG (10 or 50 μg/ml; InvivoGen), or αIL-12 (C11.5; 10 μg/ml; BD Pharmingen) was added to some wells. IFN-γ and IL-12 concentrations were assayed using a cytometric bead array per the manufacturer’s protocol (BD Biosciences). Serum IL-18 was measured with an ELISA kit (Biosource). ICC analysis was performed with in vitro and in vivo inhibition of the Golgi apparatus. For the in vitro technique splenocytes were cultured for 6 h with brefeldin A (BFA) (GolgiPlug; BD Pharmingen) in antibiotic-free medium without restimulation. For the in vivo technique mice were administered 250 μg of BFA (Sigma-Aldrich) i.v. 6 h before sacrifice. For both techniques cells were stained with surface Abs, fixed, permeabilized, and subsequently stained for intracellular IFN-γ with an ICC kit per the manufacturer’s protocol (BD Pharmingen), and analyzed via flow cytometry.

Statistical analysis

Statistical significance was determined by Student’s t test using Prism 4.0 statistical software. Probability binning analysis (FlowJo software) was used to determine differences in maturation marker expression. Values of p < 0.05 were deemed significant.

Results

NKDC increase in number and are activated in vivo by LM

To determine the role of NKDC during LM infection, we inoculated B6 mice i.v. with a sublethal dose of LM and analyzed
FIGURE 2. NKDC secrete IFN-γ in vivo. At the indicated time points following LM infection, mice were either sacrificed and splenocytes were cultured with BFA without restimulation for 6 h (in vitro plug) or the mice received 250 μg BFA i.v. (in vivo plug) and 6 h later their splenocytes were isolated and analyzed by flow cytometry for intracellular IFN-γ. A. A timeline of IFN-γ expression is plotted based on all IFN-γ+ events. B. Serum from LM-infected B6 mice was analyzed for IFN-γ, IL-18, and IL-12 content. C. Phenotype of IFN-γ+ in vivo ICC events is shown, and expressed as a percentage of total IFN-γ+ cells for NKDC, NK cells, T cells, and DC. D. Flow cytometry histograms depict IFN-γ expression from in vivo ICC analysis. Gates were set based on isotype controls, which are shown as open histograms. E. In brief, 2 × 10⁵ sorted NKDC, NK cells, T cells, and DC from 24-h LM-infected mice were cultured in 200 μl of medium without stimulation for 36 h, and supernatant IFN-γ content was determined. Bars represent the means ± SEM of at least two independent experiments with a total of three to five mice per group. * p < 0.05.

NKDC produce IFN-γ in vivo following Listeria infection

To determine the cellular source of IFN-γ in response to LM infection in vivo, we used a recently described technique of in vivo administration of BFA, a Golgi apparatus inhibitor, followed by ICC analysis (23). We found the greatest number of IFN-γ+ cells (~0.4% of all splenocytes) at 24 h (Fig. 2A), which correlated with the serum peaks of IFN-γ, IL-18, and IL-12 (Fig. 2B). For comparison, we used BFA in vitro on splenocytes isolated at serial time points after in vivo infection. There was a trend toward the IFN-γ+ cells appearing later and less frequently when analyzed by the in vitro technique (Fig. 2A). Subset analysis of our in vivo ICC data revealed that at 12 h, NKDC accounted for the majority of IFN-γ+ cells (Fig. 2C). At 24 h, NKDC, NK cells, and T cells (NK1.1+CD3+) contributed equally, and by 36 h the majority of IFN-γ+ cells were T cells. Meanwhile, DC represented the lowest percentage of IFN-γ+ cells at all time points. Phenotype analysis of the in vitro ICC, in which the cells are removed from their native environment, showed that NK cells, NKDC, and T cells contributed equally to the total number of IFN-γ+ events at all the time points examined (data not shown).

Of the cell types examined, NKDC had the highest percentage of IFN-γ staining following LM infection (Fig. 2D). Although ICC staining provides a means to identify which cells are producing IFN-γ, the relative amounts of IFN-γ being made may not be accurately reflected. To determine the amount of IFN-γ produced per cell type, we sorted NK cells, NKDC, DC, and T cells 24 h after LM infection. Cells were cultured without additional stimulation for 72 h. Consistent with our ICC results, NKDC secreted more IFN-γ on a per cell basis than NK cells, and substantially more than DC or T cells (Fig. 2E).

Listeria induces NKDC to produce IFN-γ via IL-12, IL-18 and TLR9 ligation

To identify the mechanism of IFN-γ production by NKDC in response to LM, we cultured NKDC and NK cells with LM and measured supernatant IFN-γ. LM alone did not induce detectable IFN-γ (Fig. 3A), consistent with a previous report of NK cells (24). IL-18 is essential to the induction of IFN-γ during Listeria infection and may have anti-LM effects that extend beyond its ability to stimulate IFN-γ production (25). Although IL-18 alone induced minimal (<1 ng) IFN-γ production by NK cells and NKDC, the
addition of IL-18 to LM induced substantial amounts of IFN-γ from NKDC, and to a lesser extent, NK cells. IL-12 has been shown to synergize with IL-18 to stimulate IFN-γ production by NK cells, and IL-12-deficient mice are highly susceptible to LM and generate reduced IFN-γ levels (26, 27). We found that the addition of IL-12 to NKDC and NK cells exposed to LM did not result in IFN-γ production (data not shown). However, a blocking anti-IL-12 mAb abrogated the effects of IL-18 on both NK cells and NKDC cultured with LM (Fig. 3A). We have previously shown that blocking IL-12 inhibits IFN-γ production by NKDC stimulated with CpG and IL-4 (20). Early studies on NK cells in listeriosis found IL-12 blocking Abs to inhibit IFN-γ secretion induced with heat-killed LM (hkLM) (28). Similarly we found that viable bacteria were not necessary to cause NKDC to produce IFN-γ, because hkLM produced similar results (Fig. 3B).

Previously we have shown that NKDC secrete IFN-γ when stimulated by the TLR9 ligand CpG (20). Although it is known that the common TLR signaling protein MyD88 participates in the ability of the innate immune system to recognize LM, the role of TLR9 is unknown. To determine whether TLR9 is involved in IFN-γ production by NKDC after LM infection, we used an inhibitory CpG (iCpG) sequence to block TLR9 (29). To test the specificity of this inhibitory sequence, iCpG (10 μg/ml) was added to a culture of NKDC activated by CpG (10 μg/ml) plus IL-18 (20 ng/ml) for 72 h. Treatment with the inhibitor led to a decrease in supernatant IFN-γ content from 78 ± 6 to 2 ± 0.3 ng/ml (p < 0.05). The addition of iCpG diminished LM-induced IFN-γ production by NKDC in a dose-dependent manner. In contrast, NK cell IFN-γ secretion was not altered by blocking TLR9 (Fig. 3C).

**FIGURE 3.** IL-18, IL-12, and TLR9 ligation regulate NKDC IFN-γ production. A, Sorted NK cells and NKDC were cultured with LM at a multiplicity of infection (MOI) of one for 72 h in antibiotic-free medium alone, with IL-18, or IL-18 and a blocking Ab to IL-12. IFN-γ content was measured. B, The above experiment was repeated with hkLM at the same MOI.Heat inactivation of LM was accomplished by incubating at 60°C for 2 h and confirmed by plating on BHI agar. C, The role of TLR9 on IFN-γ production was investigated by the addition of an iCpG sequence (10 μg/ml or 50 μg/ml; the total amount per well is shown) to NK cells and NKDC cultured with LM and IL-18. Mean and SEM are based on triplicate wells as shown; at least two independent experiments were performed with similar results. * p < 0.05.

**Discussion**

We have identified NKDC as a new participant in the innate response to bacterial infection. LM induces the expansion and activation of NKDC in vivo. Their activation is marked by an increase in the expression of maturation markers and lytic capability, but it is the production of IFN-γ that appears to have the most significant physiological relevance. The absence of IFN-γ or its receptor in murine models and humans significantly increases the susceptibility of the host to bacterial infections, validating the importance of this cytokine in immunity against microbial pathogens. The source of IFN-γ in the innate phase of LM infection has been controversial, with reports of multiple cell types, including NK cells and T cells as being major contributors (31, 32). The controversy probably exists because IFN-γ synthesis following infection is likely sporadic, varying by the anatomic distribution of the bacteria and the fluctuations of other cytokines. It is probable then that there are multiple cell types that can secrete IFN-γ but our ability to detect them is constrained by the artificial environment created by ex vivo processing. In this study, we used injection of BFA directly into infected animals, as first described by Liu et al. (23), leading to the production of IFN-γ by NKDC and NK cells exposed to LM did not result in IFN-γ. To determine whether TLR9 is involved in IFN-γ production by NKDC after LM infection, we used an inhibitory CpG (iCpG) sequence to block TLR9 (29). To test the specificity of this inhibitory sequence, iCpG (10 μg/ml) was added to a culture of NKDC activated by CpG (10 μg/ml) plus IL-18 (20 ng/ml) for 72 h. Treatment with the inhibitor led to a decrease in supernatant IFN-γ content from 78 ± 6 to 2 ± 0.3 ng/ml (p < 0.05). The addition of iCpG diminished LM-induced IFN-γ production by NKDC in a dose-dependent manner. In contrast, NK cell IFN-γ secretion was not altered by blocking TLR9 (Fig. 3C).
phenotyped the IFN-\(\gamma\)-requirement for adaptive immunity (5). Although we have not further investigated the innate immune response to LM. We show that NK cells and T cells are both in fact major producers of IFN-\(\gamma\), but with the ability of the in vivo ICC technique to detect IFN-\(\gamma\)-\(^{+}\) cells earlier and in greater numbers, we identified NKDC as the initial IFN-\(\gamma\)-responders. IFN-\(\gamma\) production by CTLs is considered to be an important component of the adaptive immune response to infectious agents. However, in LM infection IFN-\(\gamma\) is most important during the innate immune response. The ability of IFN-\(\gamma\)-\(^{-}\) mice to develop resistance to WT LM after infection with an attenuated strain demonstrates that IFN-\(\gamma\) is not a requirement for adaptive immunity (5). Although we have not further phenotyped the IFN-\(\gamma\)-\(^{+}\) T cells, memory CD8\(^{+}\) T cells have been shown to participate in the innate immune response to LM (33).

LM enters host cells through passive uptake by cells capable of phagocytosis or by inducing phagocytosis in normally nonphagocytic cells (34). After being internalized in a phagosome, the bacterium lyses its membrane bound compartment and replicates in the cell cytoplasm. CpG causes TLR9 to localize to endocytic vesicles from the endoplasmic reticulum and has potent immunostimulatory effects and can increase host resistance to LM (35, 36). TLR9 was considered to be the only sensor of foreign DNA until recently when a TLR-independent pathway was found to lead to type I IFN expression (37). Our finding that TLR9 ligation is necessary for optimal LM-induced IFN-\(\gamma\) production by NKDC is the first report implicating TLR9 in the innate immune response to LM. This is consistent with the known importance of colocalization of TLR9 and pathogen DNA in intracellular compartments during recognition of other intracellular pathogens (38). Recently, Streptococcus pneumoniae and Neisseria meningitidis have been shown to activate TLR9 in human PBMC leading to the induction of NF-\(\kappa\)B and IL-8 (39).

The differential response of NKDC vs NK cells to TLR9 signaling by LM may explain the rapid IFN-\(\gamma\) response by NKDC in vivo. Another possibility is that NKDC have increased access to LM DNA in vivo. The finding that NKDC harbored more LM than NK cells (Fig. 1D) may result in preferential activation and more efficient cytokine production. The preferential activation of cells containing viable bacteria has been shown for DC as well, where LM up-regulates the expression of costimulatory molecules on DC in the presence of live, cytosol-invasive bacteria (40). Expression of CD11c, a receptor for the opsonin C3bi, has been shown to play a role during the internalization of apoptotic cells by DC, and may be important for NKDC as well (41). The uptake of infected apoptotic bodies by DC can be a significant source of Ag for cross-presentation and immunostimulatory CpG sequences (41, 42). The importance of CD11c expression in host defense against bacteria is highlighted in CD11c-deficient mice that have an increased susceptibility to Borrelia burgdorferi and S. pneumoniae (43, 44).

Adaptive transfer of NKDC into IFN-\(\gamma\)-\(^{-}\) mice provides innate immune protection and enhances survival. Using a blocking Ab, we showed that the mechanism of innate protection is mediated by IFN-\(\gamma\). Although this study focused primarily on the ability of NKDC to produce IFN-\(\gamma\), their contribution to immunity against bacterial pathogens likely extends to their other functions as well. Their lytic function may enable NKDC to obtain bacterial Ags for presentation to T cells. Recently a similar multifunctional cell type was shown to be able to present Ag to naive T cells following infection with a recombinant LM, posing NKDC as a potential link between the innate and adaptive immune systems (45).
Our data identify NKDC as the principal initial source of IFN-γ during LM infection. NKDC differ from NK cells and T cells in that they respond to LM by producing IFN-γ earlier and in greater amounts. Their ability to produce IFN-γ results in a decreased bacterial burden and enhanced survival in IFN-γ-deficient mice. We show for the first time that TLR9 is involved in host detection and that they respond to LM by producing IFN-γ.

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Disclosures

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