A Novel Role for Neutrophils As Critical Activators of NK Cells

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A Novel Role for Neutrophils As Critical Activators of NK Cells

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Neutrophils are essential players in innate immune responses to bacterial infection. Despite the striking resistance of Legionella pneumophila (Lpn) to bactericidal neutrophil function, neutrophil granulocytes are important effectors in the resolution of legionellosis. Indeed, mice depleted of neutrophils were unable to clear Lpn due to a lack of the critical cytokine IFN-γ, which is produced by NK cells. We demonstrate that this can be ascribed to a previously unappreciated role of neutrophils as major NK cell activators. In response to Lpn infection, neutrophils activate caspase-1 and produce mature IL-18, which is indispensable for the activation of NK cells. Furthermore, we show that the IL-12p70 response in Lpn-infected neutropenic mice is also severely reduced and that the Lpn-induced IFN-γ production by NK cells is strictly dependent on IL-12. However, since dendritic cells, and not neutrophils, are the source of Lpn-induced IL-12, its paucity is a consequence of the absence of IFN-γ produced by NK cells rather than the absence of neutrophils per se. Therefore, neutrophil-derived IL-12, in combination with dendritic cell-produced IL-12, triggers IFN-γ synthesis in NK cells in Lpn-infected mice. We propose a novel central role for neutrophils as essential IL-12 producers and hence NK cell “helpers” in bacterial infection.

Neutrophils, also called polymorphonuclear leukocytes, are critical effector cells in the innate immune defense against bacterial and fungal infection. Their pivotal role is highlighted by recurrent bacterial and fungal infections in individuals suffering from neutrophil function disorders or neutropenia (1). To eliminate microbial intruders, neutrophils use several oxidases and proteases, as well as other intra- and extracellular microbicidal mechanisms (2). However, a number of pathogens have evolved strategies to evade neutrophil microbicidal activity (3). Of particular interest is the resistance of Legionella pneumophila (Lpn) and other members of the genus Legionella to neutrophil killing (4, 5). Despite the striking ineffectiveness of the classical neutrophil effector functions against Legionella spp., it is clear that neutrophils are vital for the rapid elimination of Lpn from the infected host, as neutropenia is an important risk factor particularly for nosocomial legionellosis (6–8). Moreover, experimental ablation of neutrophils increases the susceptibility to infection with Lpn and exacerbates disease (9, 10). Taken together, these findings imply a role for neutrophils in the control of Lpn infection that does not involve direct bactericidal activity. Lpn is a Gram-negative facultative intracellular bacterium best known as the opportunistic human pathogen causing Legionnaires’ disease. In the mammalian host, Lpn is phagocytosed by macrophages, dendritic cells (DCs), and neutrophils but replicates almost exclusively in macrophages (11, 12). To divert the phagosome from the degradative lysosomal pathway and to establish a replication-permissive vacuole, Lpn injects effector proteins into the cytosol of host cells by means of the bacterial Icm/Dot type IV secretion system (13). Lpn infection in humans and rodents induces a potent innate immune response, manifested most prominently by an early influx of neutrophils into the affected organs and the production of inflammatory cytokines (10, 14). The most crucial effector cytokine for the control of Lpn is IFN-γ, the bulk of which is derived from NK cells (12). Central to the anti-Lpn effect of IFN-γ is its property to activate macrophages and monocytes, thereby inducing a nonpermissive state for intracellular Lpn replication (15).

In the present study, we use experimental infection of mice with Lpn as a model to investigate indirect bactericidal mechanisms by which neutrophils contribute to the control and elimination of bacteria. Surprisingly, neutrophil depletion before infection with Lpn completely abrogated IFN-γ production by NK cells. One cofactor that can facilitate the synthesis of IFN-γ by NK cells is IL-12 (16). Indeed, we found that the level of bioactive IL-12 was strikingly reduced in response to Lpn infection in the absence of neutrophils. However, the defective IL-12 production is not a direct effect of neutropenia as conventional DCs, and not neutrophils, are the primary source of IL-12. The crippling of the IL-12 response is rather due to the disturbed reciprocal activation of DCs for bioactive IL-12p70 secretion in the absence of NK cell-produced IFN-γ (17, 18).

We have demonstrated in an earlier study that Lpn-induced IFN-γ production in vivo is dependent on MyD88 expression in NK cells but could not assign a critical role for TLRs 2, 4, 5, and 9 or IL-1R in this process (12), although there is some experimental evidence that Lpn can be recognized via TLRs 2 and 9 (19–23). In addition to TLRs and IL-1R, the IL-18R signals via MyD88 (24). The data presented here show that the MyD88-dependency of NK cells to produce IFN-γ in response to Lpn infection can be attributed entirely to their absolute requirement for IL-18. Most importantly, we identify the neutrophils as the source of IL-18 in mice infected with Lpn. We therefore propose a novel role for neutrophils in the control of bacterial infection, where they act as crucial accessory cells for NK cell activation through the secretion of IL-18 rather than by directly exerting classical microbicidal functions.
Materials and Methods
Mice, bacteria, and immunizations
All mice (C57BL/6, B6-Ly5.1, IL-12p40−/−, IL-12p35−/−, MyD88−/−, IL-1R−/−, Caspase-1−/−, IL-18−/−, and IL-18R−/−) were bred at the ETH Zurich or purchased from Janvier Elevage (Le Genest Saint Isle, France), and were kept in individually ventilated cages and used at 6–16 wk of age (sex- and age-matched within experiments; backcrossed over at least nine generations). All animal experiments were in accordance with institutional policies and have been reviewed by the cantonal veterinary office. For generation of chimeric mice B6-Ly5.1 mice were γ-irradiated (950 Rad) and reconstituted with 1 × 10⁶ bone marrow (BM) cells from B6-Ly5.1 mice mixed with an equal number of cells derived from either B6, MyD88−/−, or IL-18R−/− mice. Six to eight weeks after reconstitution, mice were tested for chimerism.

The Lpn strain used in this study was JR32 (wild-type (WT) Philadelphia-1) (25). Lpn was grown for 3 days on charcoal yeast extract agar plates. Mice were infected by i.v. injection of 5 × 10⁵ cfu Lpn suspended in 200 µl prewarmed PBS.

For the depletion of neutrophils, 150 µg of the RB6-8C5 mAb or 75 µg of the NimpR14 mAb were injected i.p. 1 day before immunization.

Antibodies
All Abs and streptavidin used for FACS analysis were purchased from BD Biosciences, with the exception of the mAb recognizing mature IL-18 (clone 93-10C; biotinylated; R&D Systems). The RB6-8C5 and NimpR14 mAbs were kind gifts of Dr. P. Seiler (MPI for Infection Biology, Berlin) and Dr. Nancy Hogg (Cancer Research U.K., London), respectively.

Neutrophil adoptive transfer
Neutrophils from WT, IL-18−/−, or IL-12p40−/− mice immunized with Lpn 4 h previously were isolated using the anti-Ly6G MicroBead Kit (Miltenyi Biotec). Briefly, single splenocyte suspensions were labeled with biotinylated anti-Ly6G mAb for 15 min, followed by anti-biotin MicroBeads for additional 15 min on ice. The suspensions were then washed and labeled cells were positively selected using an AutoMACS (Miltenyi Biotec) to a purity of approx. 95%. A total of 5 × 10⁶ neutrophils were injected i.v. into Lpn-immunized IL-18−/− mice (4 h post infection).

FACS analysis
Sample preparation, flow cytometric data acquisition, and analysis were performed as described previously (12). The total numbers of NK cells (DX5⁺) and neutrophils (CD11b⁺Ly6G⁺mp) were assessed with CaliBRITE beads (BD Biosciences).

Ex vivo measurement of active caspase-1
A total of 1 × 10⁷ nucleated splenocytes from single-cell suspension prepared as described previously (12) were incubated for 1 h in the presence of FAM-YVAD (Alexis) according to the manufacturer’s instructions and then surface stained for FACS analysis.

Cytokine analysis in serum
The concentration of TNF-α, IL-12p70, and IFN-γ in sera was determined using the BD Cytometric Bead Array (BD Biosciences) according to the manufacturer’s instructions. The serum concentration of IL-12 p40 was measured by standard sandwich ELISA using C15.6 for capture and biotinylated C17.8 for detection at the concentrations recommended by the manufacturer (BD Biosciences).

Statistics
Statistical analysis was performed with Student’s t test. Quantitative data are expressed as mean ± SEM unless otherwise stated.

Results
Impaired control of Lpn infection in neutrophil-depleted mice
Neutrophils play a vital role in the defense against infection by many clinically relevant pathogens. Accordingly, their dysfunction or absence renders hosts susceptible to life-threatening infection by otherwise relatively harmless microbes. In the case of infection with Lpn, neutropenia was described as a risk factor for developing Legionnaires’ disease (6–9). Neutrophils typically engulf and inactive bacteria by means of proteolytic enzymes, reactive oxygen species, or extracellular traps (2); however, Legionella spp. resist the direct bactericidal functions of neutrophils (4, 5). We therefore reasoned that other functions must account for the role of neutrophils in the control of Lpn infection. To investigate this, we depleted naive B6 mice of neutrophils by injecting carefully titrated doses of depleting Abs (clones RB6-8C5 or NimpR14, recognizing Ly6G/C) 1 day before infection with Lpn. Although mice left untreated almost completely cleared Lpn from the spleen by 36 h post infection, neutrophil ablation resulted in a failure to control the bacteria, leading to a roughly 10-fold increased bacterial burden in the infected tissue (Fig. 1A). Albeit at considerably lower levels than on neutrophils, Ly6G/C is also expressed on other leukocytes such as monocytes and plasmacytoid DCs. Collateral ablation of these cells by injection of the neutrophil-depleting Abs could potentially account for the observed phenotype. However, the inability of neutrophil-depleted mice to eradicate Lpn was not due to a generalized malfunction of the innate immune system. The numbers of plasmacytoid DCs and infiltrating monocytes was unaffected by the Ab treatment and, more importantly, the latter produced normal amounts of TNF-α as measured by intracellular staining 9–14 h after infection (Fig. 1B and data not shown). Furthermore, the serum levels of Lpn-induced TNF-α as well as IL-12p40 were comparable in normal and neutrophil-depleted mice (Fig. 1C). In sharp contrast, IFN-γ was virtually absent in the serum when neutrophils were ablated before Lpn-infection (Fig. 1C). IFN-γ is required to activate macrophages to enable them to target phagocytosed Lpn for lysosomal degradation (15). We have previously shown that early in Lpn infection, IFN-γ is derived primarily from NK cells (12), whereas neutrophils do not produce this cytokine (unpublished observation). We therefore hypothesized that either neutrophil depletion causes a functional impairment of NK cells or, alternatively, the ablation of neutrophils with depleting Abs impairs the recruitment of NK cells or even causes their collateral loss. To distinguish between these possibilities, mice were neutrophil-depleted and infected with Lpn, and 9–14 h later, the IFN-γ production by NK cells was analyzed. Although the Ab treatment did not affect NK cell numbers, it completely abrogated the IFN-γ production by these cells (Fig. 1D and unpublished observation). Of note, the IFN-γ production by cells with low or absent CD49b surface expression (recognized by the DX5 mAb) is also ablated in RB6-8C5- and NimpR14-treated mice (Fig. 1D). Control experiments revealed that a large fraction of these cells, although surface CD49b−/low, stain positive for this marker when permeabilized, suggesting that these cells are NK cells that have internalized CD49b (unpublished observation). Taken together, these results clearly show that the absence of neutrophils is directly linked to the failure in NK cell activation and lack of IFN-γ production.

IFN-γ production by NK cells upon Lpn infection is IL-12 dependent
A further analysis of the cytokine content in serum from infected neutrophil-depleted mice revealed that the concentration of bioactive IL-12p70 was severely reduced compared with infected control animals (Fig. 1C). Since IL-12 has the well documented ability to activate NK cells (26), the defect in IFN-γ production of NK cells in Lpn-infected neutropenic mice could be explained by the absence of IL-12 in these mice. To test this, we immunized IL-12p40−/− mice with Lpn and analyzed the induction of IFN-γ in NK cells by intracellular staining 9–14 h later. Although roughly two thirds of the NK cells in Lpn-infected WT mice produced IFN-γ, virtually none stained positive in IL-12p40−/− mice (Fig. 2A, left and middle panels). This defect was not due to the absence of IL-23, a heterodimeric cytokine that shares the p40 subunit with IFN-γ.
FIGURE 1. Effect of neutrophil depletion on NK cell activation. Mice were depleted of neutrophils using RB6-8C5 mAb (A, C, and D), NimpR14 (A–D) or left untreated 1 day before infection with Lpn. A. Then, 36 h later the bacterial burden in the spleen of infected mice was measured by plating of homogenates. Results are presented as mean ± SD of three mice per group (*, p = 0.015; **, p = 0.0057). B. Nine to twelve hours after immunization, the production of TNF-α in splenic monocytes was analyzed by flow cytometry. Numbers adjacent to boxes indicate the frequency of TNF-α+ cells among total monocytes (CD11bhighLy6Chigh cells). Dot plots of stainings for TNF-α and Ly6C of CD11bhigh splenocytes falling into a leukocyte scatter gate are shown. C. The concentration of IL-12p40 was determined by ELISA, that of TNF-α, IFN-γ, and IL-12p70 was measured by cytometric bead array in the sera 9–12 h post infection. Results are presented as mean ± SD of three mice per group (*, p = 0.0027; **, p = 0.0054; ***, p = 0.007; ****, p = 0.0058). D. The production of IFN-γ in splenic NK cells was assessed 9–12 h after immunization. Dot plots of stainings for IFN-γ and DX5 on splenocytes falling into a leukocyte scatter gate are shown. Numbers in box gates indicate the percentage of total NK cells (DX5+; ×10⁶) staining positive or negative for IFN-γ, respectively. Numbers in parentheses indicate the extrapolated number NK cell (DX5+, ×10⁶) per spleen. Data representative of two independent experiments (per mAb) with three mice per group are shown.
FIGURE 2. IFN-γ production by NK cells upon Lpn infection is dependent on IL-12 derived from non-neutrophils. A, WT (left panels), IL-12p40−/− (middle panels), and IL-12p35−/− (right panels) mice were immunized with Lpn (lower panels) or injected with PBS. Nine to twelve hours later, production of IFN-γ in splenic NK cells was assessed. Dot plots of stainings for IFN-γ and DX5 on splenocytes falling into a leukocyte scatter gate are shown. Numbers in box gates indicate the percentage of total NK cells (DX5+) staining positive or negative for IFN-γ, respectively. B, The serum concentration of IFN-γ 9–12 h post infection was measured by cytometric bead array (n.d.: not detectable). Results are presented as mean ± SD of three mice per group. C, The CFU load in spleens from animals treated as in A was measured by plating homogenized organs 36 h after immunization. Results are presented as mean ± SD of three mice per group.
subunit with IL-12p70, as the NK cell phenotype in IL-12p35−/− mice infected with Lpn resembled that seen in IL-12p40−/− mice (Fig. 2A, right panels). Moreover, Lpn-induced IFN-γ was absent from the serum of IL-12p40−/− as well as IL-12p35−/− mice (Fig. 2B). This result was surprising, as in vivo neutralization of IL-12 was previously shown to leave IFN-γ production in the Lpn-infected tissue unaffected (27); however, a subsequent study assigns its involvement in TLR signaling. Although an in vivo role for MyD88 has been reported (12), MyD88 is an adaptor molecule whose most prominent role is the recruitment of neutrophils upon Lpn infection and the IL-12p40−/− or IL-12p35−/− mice to produce IFN-γ is accompanied by a more than 10-fold increased bacterial load as compared with control WT animals 36 h after immunization (Fig. 2C). Importantly, the recruitment of neutrophils upon Lpn infection was comparable in WT, IL-12p40−/−, and IL-12p35−/− mice (Fig. 2D). Nevertheless, it is possible that the defective IL-12p70 production in Lpn-infected neutrophil-ablated mice is a direct consequence of the absence of neutrophils (10). On the one hand, the severely impaired IL-12p70 response in these mice could potentially indicate that IL-12 originates from the neutrophils themselves. On the other hand, the comparable level of Lpn-induced IL-12p40 subunit in the serum of control and neutrophil-depleted mice suggests that neutrophils are not the source of IL-12p40 and consequently not of bioactive IL-12p70. Confirming this, flow cytometric measurement of intracellular IL-12 in leukocytes from mice infected with Lpn 9–14 h earlier revealed that the main producers of IL-12 were neither neutrophils nor plasmacytoid DCs (included in Gr-1+ cells) but CD11c[high] DCs and some CD11c[low] cells (Fig. 2E). All of the IL-12+ cells were CD11b+, ruling out plasmacytoid DCs as the main producers of IL-12 (unpublished observation). Thus, the crippling of the IL-12p70 response observed in neutrophil-depleted mice is most likely due to the missing IFN-γ positive feedback signal from NK cells to DCs (17, 18) and not the absence of neutrophils per se. Although pattern recognition by DCs is sufficient to induce robust amounts of the IL-12p40 subunit, they require a feedback signal to generate the bioactive IL-12p70 heterodimer.

Lpn-induced IFN-γ production by NK cells is strictly IL-18 dependent

Although the above experiments demonstrate an important and nonredundant role for IL-12 in the induction of IFN-γ by NK cells in response to Lpn and the subsequent clearance of the pathogen, the results do not explain the involvement of neutrophils in this process, and they cannot account for our previous observation that there is a strict requirement for MyD88 in NK cells for this process (12). MyD88 is an adaptor molecule whose most prominent role is its involvement in TLR signaling. Although an in vivo role for TLRs in the immune response to Lpn has been reported, we could not find evidence that the IFN-γ production by NK cells is directly dependent on Lpn recognition by TLRs (12, 19–21). The function of MyD88 as a downstream signaling adaptor is not restricted to TLRs but also includes the receptors for IL-1 as well as IL-18 (24). IL-18 in particular is an attractive candidate for mediating the observed NK cell response, as it was originally described as a potent LPS-elicited activator of IFN-γ production in NK and T cells (29). Therefore, we immunized IL-18R−/− mice with Lpn, and 9–12 h later the IFN-γ production in NK cells was assessed by intracellular staining. Identically treated MyD88−/− or IL-1R-deficient and WT mice served as controls. Confirming our previous results, the IFN-γ response of mice deficient in IL-1R to Lpn was normal (Fig. 3A) (12). In contrast, NK cells from IL-18R−/− mice (or from IL-18−/− mice, see Fig. 5) infected with Lpn completely failed to produce IFN-γ, resembling the NK cell phenotype seen in MyD88−/− animals (Fig. 3A). Importantly, in contrast to MyD88−/− controls, the defect of IL-18R−/− mice in the innate response to Lpn infection was selective for IFN-γ production (and consequently IL-12p70, as these two cytokines are interdependent), as illustrated by the normal concentration of TNF-α in the serum (Fig. 3B). Furthermore, the recruitment of neutrophils in Lpn-infected IL-18R−/− mice was comparable to control WT mice, while no influx of neutrophils was detectable in MyD88−/− mice upon infection (Fig. 3C). The selective defect in IFN-γ production by NK cells (and consequently the downstream parameters) in Lpn-infected IL-18R-deficient mice indicates that, although the innate recognition per se is not affected, the “inflammatory information” is not relayed to NK cells.

Based on the crucial role of NK-deriven IFN-γ in Lpn clearance, we suspected that the failure of IL-18R-deficient mice to produce this cytokine may result in a higher bacterial burden as compared with WT mice. Indeed, the number of culturable Lpn recovered from the spleen of IL-18R−/− mice was almost 10-fold higher than that of control WT mice 36 h postinfection (Fig. 3D). Corresponding to the cytokine data, IL-1R−/− mice controlled Lpn normally while MyD88−/− animals carried a bacterial burden comparable to IL-18R−/− mice. Interestingly, the administration of a blocking anti-IL-18R Ab had no effect on the bacterial burden in the lung of Lpn-infected A/J mice (28). Differences in the bacterial strain and its administration, the genetic background of the host mice, as well as incomplete blockage of the IL-18R by the Ab treatment could all contribute to these discrepancies.

To clarify that the NK cell defect in IL-18R−/− mice cannot be attributed to the diminished IL-12p70 response, we generated mixed BM-chimeric mice where half of the hematopoietic cells were of IL-18R−/−-Ly5.1− origin and the other half as well as the host animals were WT-Ly5.1+ (test chimeras). Control chimeras were produced where the Ly5.1+ cells were either WT or MyD88−/−. As the Ly5.1+ WT NK cells in the chimeras are able to produce IFN-γ upon Lpn infection, they allow for the efficient generation of IL-12p70 by DCs. Flow cytometric analysis of intracellular IFN-γ in NK cells from these chimeric mice 9–14 h after Lpn infection showed that only WT but not IL-18R−/− or MyD88−/−-Ly5.1− NK cells produced IFN-γ (Fig. 3E). The failure of IL-18R−/−-Ly5.1− NK cells in these chimeras to produce IFN-γ indicates firstly that IL-18 acts directly on the NK cells, and, secondly, that the reduced levels of IL-12p70 in IL-18−/− or IL-18R−/− mice are a downstream effect rather than the cause of this failure. Although our data do not exclude the possibility that NK cells recognize Lpn directly, they clearly demonstrate a strict in vivo dependence on IL-18 signaling for IFN-γ production by NK cells in Lpn-infected mice.

± SD of three mice per group (*, p = 0.0062; ***, p = 0.0029). D. The influx of neutrophils into spleens triggered by Lpn infection was assessed by cytometric counting. Numbers of CD11b[high]Ly6G[high] cells falling into a leukocyte gate from animals injected with PBS (open bars) or Lpn (filled bars) are shown. Results are presented as mean ± SD of three mice per group. E. WT mice were immunized with Lpn or left untreated, and intracellular IL-12p40 in splenocytes was measured 9–12 h later. Stainings for IL-12p40 and Gr-1 (left panels) or CD11c (right panels) on splenocytes falling into a leukocyte gate are shown. Numbers indicate the percentage of cells falling into the respective quadrant gates. Results are representative of two (A–D) and >20 (E) independent experiments with three mice per group.
FIGURE 3. The requirement for MyD88 on NK cells in Lpn-induced IFN-γ can be attributed to the role of IL-18. A, WT, IL-1R−/−, IL-18R−/−, or MyD88−/− mice were immunized with Lpn. Nine to twelve hours later, production of IFN-γ in splenic NK cells was assessed. Dot plots of stainings for IFN-γ and DX5 of splenocytes falling into a leukocyte scatter gate are shown. Numbers in box gates indicate the percentage of total NK cells (DX5−) staining positive or negative for IFN-γ. B, The concentration of IFN-γ, IL-12p70, and TNF-α in the sera from the same experimental animals as in A was measured by cytometric bead array (n.d.: not detectable). Results are presented as mean ± SD of three mice per group. C, The influx of neutrophils into spleens triggered by Lpn infection was assessed by cytometric counting. Numbers of CD11bhighLy6Ghigh cells falling into a leukocyte gate from animals
Neutrophils are the principal source of IL-18 upon Lpn infection

Our observation that the phenotype of neutrophil-depleted mice infected with Lpn largely resembled that of IL-18R⁻/⁻ mice suggests that a lack of IL-18 in neutrophic mice is responsible for the failure of NK cells to produce IFN-γ. We therefore hypothesized that IL-18 might be produced by neutrophils. IL-18 is initially synthesized as pro-IL-18 and requires cleavage by the protease caspase-1 into the mature form to be secreted (30, 31). Any cell processing pro-IL-18 into mature IL-18 is therefore characterized by the presence of active caspase-1. Interestingly,
caspase-1 is an integral part of inflammasomes that have been implicated in the recognition of many intracellular pathogens including *Lpn* (32). We therefore assayed diverse leukocyte populations ex vivo (6–12 h postinfection) for caspase-1 activity using fluorescently labeled YVAD, a peptide selectively binding active caspase-1. As shown in Fig. 4, A and B, active caspase-1 was detectable primarily in neutrophils and to a lesser extent in monocytes but surprisingly not in DCs. No appreciable signal for active caspase-1 was detected in other leukocyte populations (unpublished observation). Based on our findings, we hypothesized that the requirement for caspase-1 activity for the processing of IL-18 should result in a defective IFN-γ response by NK cells in *Lpn*-infected caspase-1−/− mice. We immunized caspase-1−/− mice with *Lpn* and analyzed the induction of IFN-γ in NK cells 9–14 h later. Two thirds of the NK cells in *Lpn*-infected WT mice produced IFN-γ, while virtually none stained positive in caspase-1−/− mice (Fig. 4C). These mice cannot efficiently clear *Lpn* (33); however, it is important to note that the defective IFN-γ response in *Lpn*-infected caspase-1−/− mice is not the result of an inability of these mice to detect *Lpn*. This is exemplified by the normal recruitment of neutrophils (Fig. 4D) as well as the intact TNF-α and IL-12p40 response in these mice (Fig. 4E). Taken together, these findings suggest that monocytes and neutrophils from *Lpn*-infected mice could process pro-IL-18 (and pro-IL-1β) into the mature cytokine. To directly identify the cellular source of IL-18, we stained endogenous neutrophil granulocytes in the host mice, as the transferred neutrophils contributed less than 0.5% to the total neutrophil population (unpublished observation). However, when purified neutrophils from IL-18−/− rather than WT mice were transferred into *Lpn*-infected IL-18−/− host mice, the NK cells remained unable to produce IFN-γ. Importantly, neutrophils purified from IL-12p40−/− mice were as efficient as neutrophils from WT mice in restoring the production of IFN-γ, corroborating that the neutrophils are not a critical source of IL-12. Taken together, these data provide evidence that in *Lpn*-infected mice the bulk of IL-18, but not IL-12, is produced by neutrophils and that these cells are indispensable for the production of IFN-γ by NK cells.

**Discussion**

Neutrophils use a variety of intra- and extracellular means to combat microbial infection. However, these direct bactericidal mechanisms do not account for all roles of neutrophils in the protection of the host from microbial infection. Some microbial pathogens are resistant to these direct microbialid control mechanisms; however, neutrophils are nevertheless crucially involved in the control of these pathogens (3). In the present study, we used experimental murine infection with *Lpn* to investigate the mechanisms by which neutrophils exert a protective, but not directly bactericidal role in microbial infection. *Lpn* are resistant to killing by neutrophils yet, paradoxically, the absence or malfunction of neutrophils renders the host highly susceptible to legionellosis. Accordingly, mice rendered neutropenic by administration of either of two depleting Abs were unable to control *Lpn* infection. A 100-fold higher susceptibility to lethal *Lpn* infection in neutrophil-depleted mice was previously attributed to the failure to induce IL-12 and hence IFN-γ production by Th1 cells (10). Although these two cytokines are indeed absent from the serum of *Lpn*-infected neutropenic mice, it seems very unlikely that the inability to control the bacteria is due to a defective Th1 response, as the very rapid kinetics of IFN-γ production in *Lpn*-infected mice is incompatible with the time required for the differentiation of naïve CD4+ T cells to Th1 cells. Rather, the vast majority of IFN-γ is derived from NK cells (12). In this study, we demonstrate that *Lpn*-induced IL-12 is not derived from neutrophils but from conventional DCs. This is in contrast to an earlier report which claims that the neutrophil granulocytes are the main IL-12 producers and consequently that depletion of neutrophils would directly abrogate IL-12 production (10). However, a study with human neutrophils confirms our finding that neutrophils do not produce IL-12 upon *Lpn* stimulation (34).
this study, we show that the failure of neutrophenic mice to produce IL-12 upon Lpn infection can be explained as a secondary effect of the striking absence of IFN-γ from NK cell in neutrophenic mice (17, 18). In line with these results, the concentration of IL-12p70 in the serum of IFN-γ−/− or NK cell-depleted mice infected with Lpn is reduced compared with that in WT mice (12). Thus, as Lpn is resistant to direct neutrophil bactericidal functions, the role of neutrophils in Lpn-infected mice is to provide vital help to NK cells. A recent report on murine Haemophilus influenzae infection provides indirect evidence for a potential link from Gr-1high cells to NK cells via IL-18 (35). Although we cannot exclude a role for neutrophil-derived IL-15 in Lpn infection, we demonstrate for the first time that the neutrophils confer their accessory role for NK cell activation by the production of IL-18. The neutrophil-produced IL-18, together with the DC-produced IL-12, is a nonredundant activator of IFN-γ production by NK cells, which is essential for the control of Lpn infection. It has been suggested that neutrophils may have the potential to produce IL-18 under noninfectious conditions when stimulated via an autocrine IL-18-dependent loop or IL-15 (36, 37). There, neutrophil-produced IL-18 was implicated in inflammatory diseases such as rheumatoid arthritis. The role of neutrophils as cytokine producers is best described in toxoplasmosis where IL-12 and TNF-α are produced (38, 39), and as a source of TNF-α and various chemokines in inflammatory conditions. Our data do not exclude the possibility that non-neutrophils could potentially contribute some IL-18 in Lpn-infected mice, as other cell types such as macrophage, DCs, epithelial cells, etc. have been described to produce the mature cytokine. In line with that, residual Lpn-induced IL-18 is detectable in neutrophil-depleted mice. Nevertheless, the amount of IL-18 secreted by non-neutrophils in Lpn-infected mice is clearly insufficient to activate NK cells for the production of IFN-γ.

Our findings are particularly relevant in the context of clinically important infections, in which the pathogen is resistant to direct bactericidal neutrophil function, yet the control of the infection is dependent on neutrophils (1). For example, Mycobacterium tuberculosis resides mainly in macrophages and is therefore inaccessible for neutrophils. Nevertheless, neutrophils play a protective role in infection with M. tuberculosis (40). In fact, ablation of neutrophils before mycobacterial infection results in an increased bacterial burden, resembling what we describe in the present study using the Lpn model. Interestingly, IFN-γ is not induced in M. tuberculosis-infected mice in the absence of neutrophils. As the NK cells are the main producers of IFN-γ upon M. tuberculosis infection, this suggests that the absence of a neutrophil-derived factor, possibly IL-18, is responsible for the blunted IFN-γ response in neutrophenic mice infected with M. tuberculosis. Of note, IL-18-deficient mice generate diminished responses to M. tuberculosis, a defect that has also been described in several other microbial infections (41, 42). A similar mechanism described in the present study can be postulated to play a role in the protection from infection with Burkholderia pseudomallei, the causative agent of melioidosis. Similar to the results described here, experimentally induced neutropenia causes massively increased bacterial burdens and the failure to produce NK cell-derived IFN-γ (43). It would be interesting to determine whether this defect can also be attributed to the lack of IL-18 in neutrophil-depleted mice. Intriguingly, earlier reports suggest that neutrophils play an inhibitory rather than activatory role in NK cell biology (44, 45). Cytotoxic NK cell activity is suppressed by reactive oxygen species generated by the neutrophil myeloperoxidase upon stimulation with phorbol esters in vitro. It is unclear, however, whether the capacity of neutrophils to dampen NK cell activity also affects the production of IFN-γ in the context of a microbial infection.

In summary, we show here a requirement for neutrophils in the induction of IFN-γ by NK cells in Lpn-infected mice, which can be attributed to their role as the principal source of IL-12. Thus, we propose a novel central role of neutrophils as essential IL-18 producers in bactericial infections, which is strictly required by NK cells for IFN-γ production upon Lpn and very likely other microbial infections.

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Disclosures
The authors have no financial conflict of interest.

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