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Activation of Naive NK Cells in Response to *Listeria monocytogenes* Requires IL-18 and Contact with Infected Dendritic Cells

Jessica Humann and Laurel L. Lenz

The mechanisms for NK cell activation during infection by intracellular bacterial pathogens are not clearly defined. To dissect how *Listeria monocytogenes* infection elicits NK cell activation, we evaluated the requirements for activation of naive splenic NK cells by infected bone marrow-derived dendritic cells (BMDCs). We found that NK cell activation in this setting required infection of BMDCs by live wild type bacteria. NK cells were not activated when BMDCs were infected with a live hemolysin deficient (Δhly) strain. Neutralization of IL-12, TNF-α, or caspase-1 each dramatically reduced NK cell IFN-γ production in response to live wt *L. monocytogenes* infection. Addition of recombinant IL-18, but not IL-1β, reversed the effects of caspase-1 inhibition. Recombinant IL-18 also restored NK cell activation by BMDCs infected with Δhly *L. monocytogenes*, which produced IL-12 but not IL-18. IL-18 acted on NK cells because MyD88 expression was required in responding NK cells, but not infected BMDC. However, secreted cytokines were not sufficient for activation of naive NK cells by infected BMDCs. Rather, NK cell activation additionally required contact between infected BMDCs and NK cells. These data suggest that the activation of NK cells during *L. monocytogenes* infection requires both secreted cytokines and ligation of NK activating receptors during direct contact with infected DCs. The *Journal of Immunology*, 2010, 184: 5172–5178.

Natural killer cells play an important role in innate immune responses to tumors, viruses, and bacteria. Host cells lacking MHC I, such as tumor cells, are targeted by NK cells because of missing self-recognition (1). NK cells also recognize upregulated self and nonself molecules induced in response to stress or infection, such as the NKG2D ligands RAE1 and MULT1 in mice (2). Certain viral proteins also activate NK cells. For example, the MCMV viral protein m157 is presented on the surface of virally infected cells and is recognized by the NK receptor Ly49H (2). In addition to lysis of infected and tumor cells, NK cells produce IFN-γ. IFN-γ promotes inflammatory and antibacterial responses by inducing other inflammatory chemokines and cytokines and by eliciting NO and reactive oxygen species in IFN-γ-responsive cells (3).

Several cytokines are known to promote NK cell effector mechanisms. For example, viral induction of type I IFNs (IFN-α and -β) promotes NK cell cytotoxicity (4). Dendritic cell trans-presentation of IL-15 has also been implicated recently in the priming of NK cells to become fully activated for lysis and IFN-γ secretion (5). In addition, IL-12 promotes NK and Th1 type T cells to produce IFN-γ, in part via induction of the transcription factor Tbet (6). TNF-α and TLR signaling through IRFs and NF-κB enhances NK cell production of IFN-γ in concert with IL-12 to enhance NK cell activation (7, 8). NF-κB is also activated by IL-1β and IL-18 through an MyD88-dependent pathway (9, 10). IL-1β and IL-18 are synthesized as procytokines that are processed into their active forms by caspases 1 and 11 (11). Caspase-1-deficient mice exhibit decreased IFN-γ levels in response to infection with the bacterial pathogen *Listeria monocytogenes* (12), further implicating IL-1β, IL-18, or possibly other recently identified caspase-1 substrates in NK cell responses to *L. monocytogenes* infection (13).

*L. monocytogenes* is a facultative intracellular bacterium that replicates within the host cell cytosol and uses host actin machinery to spread from cell to cell (14). *L. monocytogenes* requires the hemolysin LLO to escape from phagosomes and enter the host cell cytosol. Mice infected with LLO-deficient *L. monocytogenes* (Δhly) *L. monocytogenes* do not produce IFN-γ (15). Mice lacking the ability to produce or respond to IFN-γ fail to control *L. monocytogenes* expansion and succumb to normally sublethal doses as early as 4 d after systemic inoculation (16, 17). Early production of IFN-γ is thought to promote a Th1 response required for efficient clearance of the pathogen (18). Cells involved in the early production of IFN-γ include NK and T cells (19), with the NK cells being the major source of this cytokine during the first 2 d of infection in C57BL/6 mice (20). However, previous data from our laboratory and others suggest that activation of NK cells by *L. monocytogenes* can promote virulence (20, 21). It is thus important to determine how NK cells are activated during infection with live wild type (wt) *L. monocytogenes*.

One challenge in dissecting the requirements for NK cell activation during in vivo infection is defining whether various cytokines primarily act on NK cells or other specific cell types. Although cell culture models can be used to shed light on this issue, previous in vitro studies have largely used NK cells expanded or cultured with IL-15 or IL-2 prior to stimulation with killed infectious agents (8, 22, 23). To determine the factors necessary for activation of naïve NK cells during infection with live *L. monocytogenes* and to characterize the respective effects of such factors on NK cells or other cell
types, we developed a novel in vitro NK cell activation assay. Using fresh NK cells isolated from naive mouse spleens and infected bone marrow–derived dendritic cells (BMDCs), we were able to reproducibly induce activation of a large fraction of NK cells. With this assay system we confirmed that cytokines such as IL-12 and IL-18 are essential for potent NK cell activation by L. monocytogenes infection. We further demonstrated that LLO expression by L. monocytogenes is required to trigger NK cell activation via activation of caspase-1 and the subsequent production of IL-18. However, our findings also revealed that cytokines alone are not sufficient to drive NK cell activation by live L. monocytogenes infection. Rather, we found that cell contact between naive NK cells and infected BMDCs was essential for efficient L. monocytogenes-induced NK cell production of IFN-γ. This requirement for NK cell contact with infected DCs is not explained by MHC I downregulation or IL-15 NK cell activating ligand(s).

Materials and Methods

Mice

Rag1−/− IL-15Rα−/− and MyD88−/− mice crossed onto the B6 background for at least 10 generations were used as sources for bone marrow stem cells to cultivate BMDCs, to isolate spleen NK cells, and for in vivo mouse infections. C57BL/6 mice were obtained from The Jackson Laboratory (Bar Harbor, ME). Rag1−/− IL-15Rα−/− mice were obtained from Dr. Steve Jameson (University of Minnesota). MyD88−/− mice were obtained from Dr. Christina Leslie (National Jewish Health). Bone marrow and spleens from IL-12Rβ2−/− mice were obtained from Dr. Ross Kedl (University of Colorado). Mice were housed in the National Jewish Health Biological Resource Center, and all animal studies were approved by the National Jewish Health Institutional Animal Care and Use Committee.

Mouse infections

Female C57BL/6 and MyD88−/− mice between 8 and 10 wk of age were used for all in vivo experiments. Mice were infected with 3 × 10⁶ CFU of log-phase mouse-passaged L. monocytogenes strain 10403S via intraperitoneal injection. At the indicated times postinfection, sera and spleens were harvested for analysis. Spleens were processed into single-cell suspensions for staining and flow cytometry.

Adoptive cell transfers

NK cells were isolated from C57BL/6 mouse spleens via nylon-wool column enrichment followed by depletion of CD3, CD19, CD11c, and CD11b cells using PE-conjugated Abs (BD Biosciences, San Jose, CA) and anti-PE magnetic beads (Miltenyi Biotech, Auburn, CA). The resulting NK cell population was ∼85% pure; 1.5 × 10⁶ purified NK cells were injected into recipient MyD88−/− mice at the indicated time before infection.

Cell culture

For BMDCs, bone marrow cells were flushed from both femurs of one mouse and cultured for 7 d in RPMI 1640 media supplemented with 10% FBS, 1% sodium pyruvate, 1% l-glutamine, 1% penicillin/streptomycin, 2-ME, and ∼2% GM-CSF (B78hi hybridoma supernatants). During culture, media were changed at days 2 and 4. Nonadherent cells were plated on day 7 for use in experiments in vitro.

Infection and stimulation of cultured BMDCs

Following overnight culture in antibiotic-free media, BMDCs were infected with L. monocytogenes strain 10403S or an isogenic L. monocytogenes strain with an in-frame deletion of the hly gene coding for LLO (Δhly). Strains were originally obtained from Dr. Daniel Portnoy (University of California–Berkeley). BMDCs were infected with wt L. monocytogenes at a multiplicity of infection (MOI) of 1 and Δhly L. monocytogenes at an MOI of 20, unless otherwise indicated. LPS was used in some experiments at 10 ng/ml. At 1 h postinfection, fresh media and 10 µg/ml gentamicin were added to each well to kill extracellular bacteria.

Isolation of NK cells

Spleens were harvested from uninfected naïve mice and processed into single cell suspensions. Cell suspensions were added to nylon wool columns and incubated for 1 h at 37°C. Column elutions were collected, and cells were stained for NK.1 (PK136) and CD3 (145-2C11). NK cells composed ∼6% of the cell population collected from the columns, compared with 2% from total spleen preparations. For pure NK cells, nylon wool nonadherent (NWNA) cells were sorted using NK1.1 (PK136) and CD3 (145-2C11) Abs on a MoFlo XDP sorter (Beckman Coulter, Brea, CA) to achieve >97% NK cell purity. All Abs were obtained from BD Biosciences and eBioscience.

Coculture of NK cells and BMDCs

BMDCs were infected with the indicated L. monocytogenes strains. At 2 h postinfection, NK cells were added to the infected BMDCs at a ratio of 0.1:1 and allowed to incubate in coculture for up to 19 h. At the indicated time points, cells and/or supernatants were collected for analysis. For cell staining, NK1.1 (PK136), CD3 (145-2C11), and IFN-γ (XMG1.2) Abs were used. Cells were fixed and permeabilized using saponin and paraformaldehyde buffers. ELISAs were performed using kits for murine IL-1β, TNF-α, IL-12p70, and IFN-γ (BD Biosciences), and IL-18 (MBL International, Woburn, MA). Anti-IL-12 (C17.8), anti–TNF-α (XT.11), rIL-18 (MBL International), rIL-1β (R&D Systems, Minneapolis, MN), control mouse IgG2a (BD Biosciences), control caspase inhibitor FA-FMK (R&D Systems), caspase-1–specific inhibitor Z-WHD-FLMk (R&D Systems), and pan-caspase inhibitor ZVAD-FMK (R&D Systems) were used at the indicated concentrations. Anti-mouse IL-18 (93-10C) (MBL International) and anti-mouse ICAM (R&D Systems) were also used at the indicated concentrations.

Statistics

All conditions were tested in triplicate in each experiment, and each experiment was repeated three times. Data shown are a representative experiment. Statistics were done for each experiment using a Student t test. Significance was determined to be p < 0.05.

Results

Coculture of naïve NK cells and BMDCs infected with live, cytosolic L. monocytogenes elicits IFN-γ production

We previously showed that up to 60% of splenic NK1.1+CD3− NK cells produce IFN-γ within 24 h of wt L. monocytogenes infection in C57BL/6 (20). We sought to evaluate the mechanism for this bulk activation of NK cells. We used freshly isolated naïve splenic lymphocytes (NWNA) as our source of NK cells. CD11b+CD11c+ BMDCs were used as the infected cell population (Fig. 1A). BMDCs were infected with wt L. monocytogenes at an MOI of 1. Postinfection, NWNA cells were added into the culture. At 19 h postinfection, the cultures were analyzed for IFN-γ production by both intracellular cytokine staining and ELISA of cell supernatants. BMDCs infected with wt L. monocytogenes triggered >40% of the NK cells in the NWNA population to produce IFN-γ (Fig. 1B, 1C). However, heat-killed wt L. monocytogenes (data not shown) and LLO-deficient L. monocytogenes (Δhly L. monocytogenes) used at an MOI of 20 failed to induce significant IFN-γ in parallel cocultures. IFN-γ production by the NK cells peaked by 12 h of coculture with infected BMDCs (Fig. 1D). These data indicate that cytosolic L. monocytogenes infection of BMDCs is both necessary and sufficient to activate naïve splenic NK cells to produce IFN-γ during in vitro cocultures.

Cell staining revealed that NWNA cell preparations contained NK cells (∼6%), T cells (∼70%), a few contaminating B cells, and monocytes (Fig. 1A and data not shown). Depletion of CD3+ cells from NWNA using magnetic bead separation did not affect IFN-γ production in cocultures with infected BMDCs (data not shown), suggesting that T cells did not significantly contribute to the IFN-γ produced in response to infected BMDCs. To confirm that only NK cells within the NWNA populations produced IFN-γ in response to live wt L. monocytogenes infection, we sorted NWNA cells into three populations: NK1.1+CD3− (NK cells), CD3+ (T cells), and CD3− NK1.1+ (other cells). All sorted populations were >97% pure for their respective cell type (Fig. 2A and data not shown).
FIGURE 1. NK cells produce IFN-γ in vitro in response to coculture with L. monocytogenes-infected BMDCs. A, CD11c⁺CD11b⁺ BMDCs were derived from bone marrow cells cultured in GM-CSF. Spleen cells were processed via nylon wool columns to enrich for lymphocytes. These NWWA cells were cocultured with wt L. monocytogenes or Δhly L. monocytogenes-infected BMDC for 19 h. B and C, NK cells produced IFN-γ only in response to wt L. monocytogenes infection as measured by intracellular staining and ELISA. D, Kinetics of IFN-γ production by NWWA responding to wt L. monocytogenes in infected BMDCs as measured by ELISA.

not shown). Each sorted cell type was then cocultured with wt L. monocytogenes-infected BMDCs for 19 h. Neither CD3⁺ nor CD3⁻NK1.1⁻ cells produced IFN-γ in coculture. In contrast, sorted NK1.1⁺CD3⁻ cells populations produced significant amounts of IFN-γ (Fig. 2B). Furthermore, addition of purified CD3⁺ or CD3⁻NK1.1⁺ cells to the sorted NK cells did not affect the ability of NK cells to produce IFN-γ (Fig. 2B). Because NK cell activation did not require help from T cells or other cell populations present in the NWWA preparations, we used this in vitro system to further study the requirements for activation of naive NK cells during live L. monocytogenes infection.

Proinflammatory cytokines produced by infected BMDCs contribute to NK cell activation

Several proinflammatory cytokines previously implicated in the induction of IFN-γ by NK cells were also produced by L. monocytogenes-infected cocultures. As measured by ELISA, wt L. monocytogenes infection of BMDCs induced secretion of nanogram concentrations of IL-12p70, TNF-α, and IL-1β, and picogram concentrations of IL-18 by 19 h of coculture with NWWA cells (Fig. 3A–D). In contrast, Δhly L. monocytogenes infection of BMDCs, which failed to induce IFN-γ (Fig. 1C), induced high levels of IL-12p70 and TNF-α, but less IL-1β and no IL-18 (Fig. 3A–D).

LPS stimulation of BMDCs induces a similar proinflammatory cytokine profile to Δhly L. monocytogenes infection of BMDCs (24). We thus compared LPS stimulation of BMDCs with L. monocytogenes infection of BMDCs to determine which cytokines were necessary for NK activation. After 19 h of stimulation, LPS induced high levels of IL-12p70, but TNF-α and IL-1β concentrations were low when compared with wt L. monocytogenes infection (Fig. 3A–D). However, there was a substantial level of TNF-α (nanogram concentrations) after 6 h of LPS treatment (data not shown). LPS stimulation of BMDCs also poorly induced IFN-γ (Fig. 3E). These data indicate that neither LPS nor a noncytosolic L. monocytogenes infection were sufficient to induce naive NK cells to make IFN-γ.

To further determine the relative importance of BMDC-derived cytokines for naive NK cell activation, we inhibited IL-12 and TNF-α using neutralizing mAbs at a concentration of 10 μg/ml. We blocked caspase activation with the pan-caspase inhibitor Z-VAD-FMK. In each case, IFN-γ production was significantly attenuated (Fig. 4). Neutralization of IL-12 did not affect the levels of TNF-α produced by infected BMDCs, and vice versa (data not shown). These results suggest that IL-12, TNF-α, and caspase-1 activation are all involved in naive NK cells production of IFN-γ. However, eliminating individual cytokines or caspases failed to prevent NK cell activation.

Role of IL-18 in L. monocytogenes-induced NK cell activation

Given that IL-18 was produced only during wt L. monocytogenes infection of BMDCs, and only wt L. monocytogenes potently induced IFN-γ production by naive NK cells, we hypothesized that caspase-1 activation and release of IL-18 might act as a limiting factor in naive NK cell activation. To test this, we pretreated wt L. monocytogenes-infected BMDCs with the irreversible caspase-1-specific inhibitor Z-WHED-FMK. Z-WHED-FMK treatment of infected BMDCs reduced IL-1β levels in cell culture supernatants as measured by ELISA (data not shown). The BMDCs were washed after inhibitor treatment and incubated with NWWA cells. Inhibition of caspase-1 activity in the BMDCs prior to coculture reduced NWWA IFN-γ production to basal levels (Fig. 5A). Furthermore, addition of recombinant IL-18 reversed the effects of Z-WHED-FMK on IFN-γ production, whereas addition

FIGURE 2. Purified NK cells are solely responsible for IFN-γ production in response to L. monocytogenes-infected BMDCs. A, NWWA cells were isolated as in Fig. 1 and sorted into three populations: NK1.1⁺CD3⁻ (NK), CD3⁺NK1.1⁻ (T), and CD3⁻NK1.1⁻ (other), each population >90% pure. B, Sorted NK cells produce IFN-γ in response to coculture with wt L. monocytogenes-infected BMDCs. T cells or other cells alone do not produce IFN-γ. Addition of either of the other sorted populations does not enhance IFN-γ production in cocultures.
of Δhly L. monocytogenes to induce NK cell IFN-γ. Indeed, when recombinant IL-18 was added to Δhly L. monocytogenes infected cocultures, IFN-γ secretion by NWWA cells was restored to levels seen with wt L. monocytogenes infected BMDCs (Fig. 5B). Hence, caspase-1 dependent release of IL-18 is crucial for NK cell IFN-γ production and the failure of Δhly L. monocytogenes to induce IL-18 secretion explains why infection with this attenuated L. monocytogenes strain fails to activate naive NK cells.

**MyD88 signaling in NK cell activation**

MyD88 is a well-known adaptor molecule for many TLRs. In addition, IL-1R and IL-18R use MyD88 as an adaptor molecule for downstream signaling (8). MyD88−/− mice exhibit reduced levels of IL-12 and TNF-α and fail to make IFN-γ in response to L. monocytogenes infection (10). We sought to directly test whether the reduced IFN-γ production in MyD88−/− mice was due to a requirement for TLR or IL-18 signaling in DCs, naive NK cells, or both. We thus infected C57BL/6 and MyD88−/− BMDCs with wt L. monocytogenes and cocultured them with either C57BL/6 or MyD88−/− NWWA. Wt L. monocytogenes infection induced similar IFN-γ production by NWWA cells regardless of MyD88 expression by the infected BMDCs (Fig. 5C). Conversely, NWWA cells lacking MyD88 did not produce IFN-γ in response to wt L. monocytogenes-infected BMDCs, even when BMDCs expressed MyD88 (Fig. 5C). These data reveal that signaling via MyD88 is necessary only in responding NK cells during infection, likely because of its role in the NK cell response to IL-18 produced by infected dendritic cells.

To further examine the requirement and sufficiency of MyD88 signaling in NK cells during in vivo L. monocytogenes infection, we established an adoptive transfer system using MyD88−/− mice. On day 0, we isolated NK cells from the spleens of C57BL/6 mice by negative selection, labeled them with CFSE, and transferred them into recipient MyD88−/− mice. The purity of the transferred NK cell population was ∼84% (data not shown). Recipient and control mice were infected with L. monocytogenes on day 1, and all mice were analyzed on day 2. At the time of harvest, we detected a significant population of CFSE-labeled NK cells in the spleens of infected mice that had received the adoptive transfer (Fig. 6A), indicating that the transferred cells were able to populate this organ. Analysis of the CFSE staining levels in the transferred NK cells suggested that these cells failed to proliferate within the first day of the L. monocytogenes infection (Fig. 6B). However, the sera of infected MyD88−/− mice that had received C57BL/6 NK cells exhibited a significantly increased IFN-γ concentration when compared with control MyD88−/− mice (Fig. 6C). These findings confirm that expression of MyD88 by naive NK cells is both necessary and sufficient to permit their activation during L. monocytogenes infection. Furthermore, our data suggest the requirement for MyD88 is due to its role in the response to IL-18 rather than IL-1β or TLR agonists.

**NK-DC contact is required for IFN-γ production in response to infection**

The data above indicate roles for IL-12, TNF-α, IL-1β, and IL-18 in the activation of naive NK cells by wt L. monocytogenes infection. To test whether these secreted cytokines were sufficient for L. monocytogenes-induced NK cell activation, we transferred conditioned media from infected BMDCs onto uninfected BMDCs cultured with NWWA cells. Surprisingly, the transfer of conditioned media from infected BMDCs failed to induce IFN-γ production and secretion (Fig. 7A). Furthermore, when a 0.5-μM membrane was used to separate infected BMDCs from responding NWWA cells for...
IL-18 is necessary for IFN-γ production by naive NK cells. A, Wt L. monocytogenes-infected BMDC cocultures were treated with the caspase-1 specific inhibitor Z-WEHD-FMK at 100 μM. Caspase-1 inhibition abrogated IFN-γ induction. Addition of 500 pg/ml exogenous IL-18 restored IFN-γ induction, but 2.5 ng/ml of exogenous IL-1β did not. Specific inhibition of IL-18 (1 μg/ml of αIL-18) significantly reduced IFN-γ induction in cocultures infected with wt L. monocytogenes. B, Addition of recombinant IL-18 to Δhly L. monocytogenes-infected cocultures restored IFN-γ induction to levels seen with wt L. monocytogenes infection. C, Wt L. monocytogenes induction of IFN-γ requires MyD88 expression on NWNA. C57BL/6 and MyD88−/− BMDC were infected with wt L. monocytogenes and cocultured with either C57BL/6 or MyD88−/− NWNA. Only cocultures with MyD88−/− NWNA failed to induce IFN-γ in response to L. monocytogenes infection.

Given a previous report suggesting that cell contact was required in DC priming of NK cells by IL-15 (25), we asked whether IL-15 trans-presentation might account for the contact-dependent interaction in our infection system. Consistent with the previous work, we found that IL-15 trans-presentation was important for NK cell priming, because infected IL-15Rα−/− BMDC induced lower amounts of NK cell IFN-γ production than did control C57BL/6 BMDC. However, wt L. monocytogenes infection of the BMDCs still activated NK cell IFN-γ production well above the background level seen with the control Δhly L. monocytogenes infection (Fig. 8A). Thus, IL-15Rα expression by BMDCs contributed to priming of naive NK cells in our system, but failed to fully account for the contact-dependent induction of IFN-γ. To examine cell contact requirements further, we asked whether blocking cell–cell interactions using an mAb against ICAM reduced IFN-γ production in the cocultures. We found that blocking ICAM reduced NK cell activation and IFN-γ production to a low level. However, such blockade failed to fully abrogate production of IFN-γ, which was still modestly but significantly higher in the anti-ICAM treated wt L. monocytogenes-infected cultures than that seen in the Δhly L. monocytogenes infected cultures for this experiment (Fig. 8B). These data suggest that either ICAM-specific dendritic cell–NK cell interactions are directly involved in the activation of naive NK cells or that αICAM interferes with the ability of naive NK cells to interact with the infected BMDCs in a manner that permits NK cell receptors to bind ligands induced by the wt L. monocytogenes infection.

Discussion

Our results help to clarify the mechanisms for activation of naive NK cells early after L. monocytogenes infection. We show that infection of BMDCs with live wt L. monocytogenes potently activates naive NK cells to release IFN-γ. The mechanism for such activation involves the activation of caspase-1 and the release of IL-18 by infected BMDCs. Caspase-1 activation in response to L. monocytogenes infection is known to require the pore-forming LLO protein (26). Our data show that Δhly L. monocytogenes LLO protein (26). Our data show that Δhly L. monocytogenes infection of BMDCs is not sufficient to trigger activation of naive NK cells. However, the Δhly L. monocytogenes infection.
infection is sufficient when exogenous IL-18 is added. We also show that TNF-α and caspase-1 activity contribute to NK cell activation in this system. Although inhibition of either of these molecules impairs IFN-γ production, neither is essential for wt L. monocytogenes-induced NK cell activation.

The precise roles of IL-12 and IL-18 in activation of naive NK cells are not clear. Mice deficient in both IL-12 and IL-18 have significantly impaired NK cell IFN-γ activity compared with mice deficient in either cytokine alone (27). Furthermore, previous in vitro studies have indicated that IL-18 can act in synergy with IL-12 to induce NK cell IFN-γ production by L. monocytogenes and other stimuli (9, 23, 28). One study suggested that IL-12 induces IFN-γ transcription while IL-18 stabilizes these transcripts and promotes translation of IFN-γ (9). Our results reveal that MyD88 signaling is essential in naive NK cells, likely owing to its role in the response of these cells to IL-18. Although L. monocytogenes infection of MyD88−/− mice fails to induce NK cell IFN-γ production (10), we show that transfer of C57BL/6 NK cells is sufficient to partially restore IFN-γ production. MyD88 expression by NK cells was also necessary and sufficient for their IFN-γ production in our coculture system. In contrast to MyD88, our in vitro data reveal that IL-12R expression is required on both DCs and NK cells for efficient induction of IFN-γ (data not shown). We speculate that IL-12 may likewise have roles on both infected and responding cells during the activation of naive NK cells during in vivo L. monocytogenes infection.

An additional conclusion from our studies is that soluble pro-inflammatory cytokines are not sufficient for activation of naive NK cells in response to wt L. monocytogenes infection. Although naive NK cells secrete IFN-γ in our hands after extended culture in recombinant IL-12 and IL-18 (data not shown), the rapid NK cell IFN-γ production seen in response to live wt L. monocytogenes additionally requires direct cell contact between the infected BMDCs and responding NK cells. Cell contact has also recently been implicated as a requirement for activation of human NK cells by macrophages infected with Salmonella (22) and RBCs infected with Plasmodium (29). In the case of in vivo infection, cell contact may explain why only 30–60% of splenic NK cells are activated in response to wt L. monocytogenes (20). Cell contact likely occurs within the lymphoid follicles of the spleen, because recent work suggests that NK cells cluster near DCs and L. monocytogenes organisms in these areas prior to producing IFN-γ (30). The requirement for cell contact might be a way for the immune system to localize NK cell activation within the lymphoid follicles. Such localization might help reduce immunotoxicity and damage to neighboring uninfected cells and could indicate an important role for NK cells in regulating the function of other lymphocytes.

The specific mechanisms of cell contact in infection-induced NK cell activation are still unclear. Some previous studies have suggested that IL-12 is secreted across a synapse between NK cells and DCs (31). It has also been published that NK/DC interactions can result in the directed release of IL-18 to NK cells (32). Such
directed release of cytokines might increase their effective concentrations in cultures in which NK cells and DCs are allowed to intimately associate with one another. Thus, one possible mechanistic explanation for the cell contact requirement to activate NK cells is that the low level of IL-18 produced by L. monocytogenes infection (<500 pg/ml in vitro) is more efficiently presented to NK cells that are in close proximity to the infected DCs. Numerous receptor–ligand interactions have been implicated in the activation of NK cells. NKGD2 is expressed by almost all NK cells responding to L. monocytogenes infection (data not shown) and is associated with cytokine production and cytotoxicity in NK cells (33–37). However, our studies argue against a role for NKGD2 in NK cell activation, because blocking NKGD2 had no effect on IFN-γ production in response to wt L. monocytogenes infection (data not shown). Adhesion molecules such as ICAM have been associated previously with NK cell activation during Mycobacterium tuberculosis infection (38), and we also found that blocking cell contact with an Ab to ICAM1 significantly reduced NK cell IFN-γ production in our coculture system. These findings suggest that proximity between infected and responding cells is not sufficient for NK cell activation. Rather, NK cell activation in response to wt L. monocytogenes infection requires an association between the NK cell and the infected BMDCs that can be disrupted by blockade of ICAM1. Whether the actual activating stimulus received by the NK cell is due to ligation of ICAM1 or other specific receptor-ligand pairs remains to be determined. Our findings reveal that regulation of the NK cell response involves a complex combination of priming, proinflammatory cytokine stimulation, and contact-dependent firing in response to contact with L. monocytogenes infected DCs. Our in vitro assay system can be used as a tool to further examine the precise mechanisms of NK cell activation by this, and possibly other, intracellular pathogens.

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Disclosures
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References