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Lipopolysaccharide-Mediated Mast Cell Activation Induces IFN-γ Secretion by NK Cells

Katja Vosskuhl, Tim F. Greten, Michael P. Manns, Firouzeh Korangy,1 and Jochen Wedemeyer1

Mast cells (MCs) that are well known for their important effector function in IgE-associated immune responses play a key role in innate immune defenses. In this study, we investigate the interaction between MCs and NK cells in vitro and in vivo. We show that mouse bone marrow-derived cultured MCs activated with LPS, polyinosinic-polycytidylic acid, or CpG can stimulate NK cells to secrete increasing concentrations of IFN-γ. MCs induce a 20-fold increase in IFN-γ release from NK cells after LPS stimulation. This enhancement of IFN-γ secretion is cell contact dependent and TNF-α independent. Furthermore, we show that this interaction is in part mediated by OX40 ligand on MCs. NK cell-mediated cytotoxicity was not affected by the presence of MCs.

NK cells are cytotoxic lymphocytes that circulate through the blood, secondary lymphatic organs, and peripheral nonlymphatic tissues like liver and spleen to seek transformed or pathogen-infected cells (25, 26). NK cells can regulate both innate and adaptive immune responses through interaction with other immune cells. They are part of the innate immune system and are recruited to the site of infection by various chemokines. The ability of NK cells to produce IFN-γ is linked with their cytotoxic activity and activation of APCs, which can initiate T cell responses (27–29).

Different mediators that can be released by MCs, such as IL-4, IL-12, and TNF-α, are capable of inducing NK cell activation (7, 30, 31) and therefore could potentially facilitate a MC-NK cell interaction. In addition to possible immune modulation by cytokines, direct receptor–ligand interactions might facilitate cell–cell interactions. Therefore, we hypothesized that MCs activated via their TLRs (TLR3, -4, and -9) can modulate NK cell function in the context of innate immunity.

In this study, we show that MCs enhance cytokine release by NK cells in vitro upon stimulation by TLR3, -4, and -9. LPS injection into C57BL/6 (wild-type [WT]) and MC-deficient C57BL/6 Kitwsh/wsh (Wsh) mice confirmed the MC-NK cell interaction in vivo. Modulation of NK cell activity by MCs has important implications in understanding the complex interactions of the effector cells of the innate immune system.

Materials and Methods

Mice

C57BL/6 (WT) mice were purchased from Charles River Laboratories (Sulzfeld, Germany). C57BL/6 TNF−/− (TNF−/−) mice were kindly provided by Dr. Steinbrink (Mainz, Germany). BALB/c mice were purchased from Janvier (Le Genest-St-Ise, France), and C57BL/6 Kitwsh/wsh (Wsh) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Mice were kept in community cages at light periods of 12 h and were fed water and mouse chow ad libidum. All animal experiments were approved by the local authorities.

Preparation of bone marrow-derived cultured MCs

Bone marrow was aseptically flushed from femurs of 9–12-wk-old female C57BL/6, BALB/c, or TNF−/− mice and cultured in complete DMEM ([Biochrom, Berlin, Germany] supplemented with 10% heat-inactivated
FBS [Biochrom], 20% IL-3 containing WEHI-3 cell-conditioned medium, 1% penicillin/streptomycin [PAA Laboratories, Pasching, Austria], and 200 μM α-monomethylglycero [Sigma-Aldrich, Seelze, Germany]), and washed, and resuspended in complete DMEM. Medium was changed twice a week. After 6 wk, 98% of the cells were identified as MCs using May-Grünwald-Giemsa staining and FACS analysis of FcεRI (IgE anti-DNP detected with anti-IgE) and anti-CD117 (c-kit) expression.

**NK cell isolation**

Splenocytes were isolated from 9–12-wk-old female WT mice by crushing the spleens following 40-μm filtration and red blood lysis with red blood lysis buffer [150 mM {NH₄}Cl, 9.9 mM KHCO₃, 0.1 mM EDTA] for 8 min. The resulting single-cell suspension was blocked with CD16/CD32 and stained for CD49b and CD3. NK cells were sorted by CD49b-positive, CD3-negative staining using a MoFlo (DakoCytomation, Glostrup, Denmark) and Summit software (Fort Wayne, IN).

**Abs, flow cytometry, and cell sorting**

Cells were subjected to immunofluorescence staining and analyzed with an FACSCan (BD Biosciences, Heidelberg, Germany) and FlowJo software (Tree Star, Ashland, OR). Anti-CD49b, anti-CD3, anti-CD48, anti–Qa-1b, anti-CD54, anti-CD11a, anti-CD18, anti-IgE, anti-CD117, anti-CD23, anti-CD16/CD32, and anti-CD69 as well as streptavidin allophycocyanin and isotype controls were purchased from BD Pharmingen (Heidelberg, Germany); anti-OX40L, anti-CD48, anti–B7H1, anti-CD70, anti-ICAM, anti-ICOSL, and isotype controls were blocked with mouse IgG isotype Abs, flow cytometry, and cell sorting (DakoCytomation, Glostrup, Denmark) and Summit software (Fort Wayne, IN).

ELISA

IFN-γ and TNF-α concentrations in the cell culture supernatant were determined using duo-set ELISA kits according to the manufacturer’s manual (R&D Systems).

**Coculture**

Bone marrow-derived cultivated MCs (BMCMCs) and NK cells, each 0.5 × 10⁶ cells, were cocultured for 24 h in round-bottom 96-well plates (#3799, Corning, Wiesbaden, Germany) at 37°C and 5% CO₂. Cells were incubated in 150 μl complete RPMI medium (RPMI 1640 [InviGen, Karlsruhe, Germany] supplemented with 10% heat-inactivated FBS [Biochrom], 1% penicillin/streptomycin [PAA Laboratories], 200 μM α-monomethylglycero [Sigma-Aldrich] and additionally added 600 U/ml IL-2 and 1.0, 0.1, or 0.01 μg/ml LPS (Sigma-Aldrich), 50 or 100 μg/ml poly I:C (Sigma-Aldrich), 100 nM CpG, and CpG control as indicated. For some experiments, BMCMCs were precultured for 24 h with 1 μg/ml IgE anti-DNP and 1 × 10⁵ cells/ml before coculturing with NK cells and stimulation with 100 ng/ml LPS for 24 h. For verification of BMCMC functionality, cells precultured with 1 μg/ml IgE anti-DNP were stimulated for 6 h with 10⁻⁶ M Calcium-Ionomorph I (Sigma-Aldrich) and 10⁻⁶ M PM (Sigma-Aldrich) or 500 ng/ml DNP, or cells were cultured alone as control. Following incubation, cells and supernatant were removed from the plate and separated by centrifugation. Supernatants were frozen at −80°C until ELISA analysis, whereas cells were directly used for FACS analysis.

For kinetic experiments, BMCMCs and NK cells, each in triplicate. Target cells were cultured alone for maximum release. After a 4-h incubation, 100 μl/well was harvested, and an equal volume of scintillation liquid was added. Assays were counted with a Wallac 1450 MicroBeta Trilux (PerkinElmer, Rodgau-Jügesheim, Germany). Cytotoxicity rate was calculated by experimental release/target maximum release × 100.

**In vivo LPS**

WT or Wsh mice were injected i.p. with 100 μg LPS per 25 g body weight in a volume of 200 μl PBS. Mice were controlled every 3 h and sacrificed 14 h postinjection. Peritoneal cells, isolated by peritoneal lavage, spleen, and inguinal and axillary lymph nodes were collected. A single-cell suspension of splenocytes and lymphocytes was prepared by crushing the spleen and lymph nodes through a 40-μm nylon mesh. Cells were stained for NK cells and intracellular IFN-γ-like described above and analyzed by FACS.

**Statistics**

For statistical analysis, GraphPad Prism software (GraphPad Software, La Jolla, CA) and Excel (Microsoft, Redmond, WA) were used. All data are presented as mean ± SEM. Student t test was used to test for statistical significance. A p value <0.05 was considered significant.

**Results**

**NK cells secrete IFN-γ after coculture with LPS-stimulated BMCMCs**

To confirm that the differentiated BMCMCs are functional, we analyzed the release of TNF-α (Supplemental Fig. 1A) and β-hexosaminidase (Supplemental Fig. 1B) upon stimulation with ionomycin/PMA or IgE/DNP. Maturity was determined by May-Grünwald-Giemsa staining (Supplemental Fig. 1C), FACS analysis of FcεRI, and c-kit surface expression (Supplemental Fig. 1D).

To investigate MC-NK cell interactions, we cocultured them for 24 h and measured TNF-α release for MC activation and IFN-γ release for NK cell activation by ELISA. Furthermore, we examined CD69 surface expression as a marker of NK cell activation by FACS analysis.

Coincubation of splenic NK cells with BMCMCs without additional stimulants did not induce a significant cytokine release by either MCs or NK cells (Fig. 1A, 1B). As expected, LPS induced significant MC activation as shown by high levels of TNF-α release, whereas no detectable TNF-α release was induced by LPS in NK cell cultures. Furthermore, LPS stimulation significantly induced IFN-γ secretion in NK cell cultures, but had no effect on IFN-γ secretion by MCs. As shown in Fig. 1A, the addition of LPS induced an ~20-fold increase in IFN-γ secretion as compared with NK cell cultures alone (4746.62 ± 864.90 versus 236.65 ± 81.36). This effect was dose and time dependent with the highest
IFN-γ levels after 24 h (Fig. 1C) and 1 μg/ml LPS. In contrast, TNF-α release by MCs was not affected by coincubation with NK cells and did not increase in comparison with TNF-α release by MCs alone after LPS stimulation (Fig. 1B). In addition, the β-hexosaminidase release of BMCMCs was not significantly altered in the presence of LPS or NK cells (Supplemental Fig. 1B). Similar results have been shown by Supajatura et al. (12). These experiments confirm that TLR4-mediated MC stimulation by LPS induces IFN-γ secretion from NK cells. In contrast, degranulation of BMCMCs and TNF-α secretion were not influenced by NK cells.

Intracellular IFN-γ staining confirmed that NK cells were the major source of IFN-γ (Fig. 1D), whereas BMCMCs did not secrete any IFN-γ (Fig. 1E) after coculture with NK cells and stimulation with LPS. However, the CD69 surface expression on NK cells revealed no significant differences between NK cells cultured alone and NK cells cocultured with BMCMCs (Fig. 1F).

Stimulation of MCs via TLR3 and TLR9 amplifies IFN-γ release from NK cells

MCs can be activated through various pathways. Therefore, we analyzed whether MC activation via FcεRI, c-kit, TLR3, or TLR9 could influence the IFN-γ release by NK cells. Stimulation of BMCMCs via FcεRI using IgE/DNP had no effect on the IFN-γ release by NK cells. Similarly, BMCMC stimulation via c-kit using stem cell factor had no significant effect on the IFN-γ release by NK cells in the cocultures (Fig. 2A, 2B). However, activation of BMCMCs via TLR3 using poly I:C or via TLR9 using CpG did significantly amplify IFN-γ secretion by splenic NK cells (Fig. 2C, 2D), though this amplification was significantly lower for poly I:C than the effect observed after LPS stimulation.

**BMCMC-mediated IFN-γ secretion by NK cells is TNF-α independent**

NK cells express transmembrane (tm) TNFR1 and tmTNFR2 (32). TNFR1 is known to be activated by tmTNF as well as by soluble TNF; however, TNFR2 can only be activated by tmTNF (33, 34). We therefore studied the role of TNF-α as a potential activator of NK cells in the context of MC-NK cell coactivation. We generated TNF-α–deficient (TNF−/−) BMCMCs and analyzed IFN-γ secretion after coculture of TNF−/− BMCMCs with WT splenic NK cells. The increase in IFN-γ secretion was not significantly altered after coculture of TNF−/− BMCMCs with NK cells,
confirming a mostly TNF-α–independent interaction (Fig. 3A). Similar results were obtained when TNFR1- and TNFR2-blocking Abs were added to the cocultures (Fig. 3B).

Direct cell–cell contact is essential for IFN-γ secretion by NK cells

As TNF-α had no influence on the IFN-γ secretion by NK cells, we analyzed whether this effect is mediated by soluble factors or whether a direct cell–cell interaction is required. Supernatant from BMCMCs cultures stimulated with LPS were added to splenic NK cells. After 24 h, there was no increase in IFN-γ secretion by splenic NK cells (Fig. 4A). These data were further confirmed by transwell experiments. In contrast to NK cells cocultured with BMCMCs and stimulated with LPS, the separation of NK cells from BMCMCs using transwells did not result in the amplification of IFN-γ secretion (Fig. 4B). These experiments confirmed that direct cell–cell interaction between BMCMCs and splenic NK cells is essential for the LPS-dependent mast cell–mediated activation of NK cells.

Blocking OX40L on BMCMCs reduces IFN-γ secretion by NK cell

Our next goal was to identify receptors and ligands that mediate the MC-NK cell interactions. As shown above, the interaction of BMCMCs and NK cells is mediated via a direct cell–cell contact. Thus, we explored potential receptor ligand interactions using different blocking Abs to identify possible molecules involved in this interaction. B7-H1 (CD274) as well as OX40L and CD153, both members of the TNF super family, are expressed on BMCMCs (17) and could potentially mediate a direct effect via their receptors expressed on NK cells. We used the anti-OX40L, anti-CD48, anti–B7-H1, anti-ICAM, anti-ICOSL, anti–Qa-1b, and anti-CD153 Abs and determined the IFN-γ concentration in the supernatants after 24 h coculture. Anti-ICOSL (Fig. 5A), anti-CD70 (Fig. 5B), anti-CD153 (Fig. 5C), anti-CD48, anti–B7-H1, anti-ICAM, and anti–Qa-1b (data not shown) had no effect on IFN-γ release in comparison with isotype control Abs, whereas OX40L blockage (Fig. 5D) revealed a significant decrease in IFN-γ secretion by NK cells. The OX40L

FIGURE 2. IFN-γ release of NK cells is amplified by BMCMCs stimulated via TLR3 or TLR9. BMCMCs were incubated with or without 1 μg/ml anti-DNP IgE for 24 h. After washing, BMCMCs and NK cells were cocultured for 24 h with or without 1000 pg/ml stem cell factor (A), 100 ng/ml DNP (B), 50 and 100 μg/ml poly I: C (C), or 100 nM CpG or CpG control (D). IFN-γ levels in the supernatant were determined by ELISA. Data are presented as mean ± SEM. Figures show representative results from at least three independent experiments using different batches of BMCMCs and NK cells. *p < 0.05.

FIGURE 3. Mast cell-mediated IFN-γ release of NK cells is TNF-α independent. A, BMCMCs or TNF−/− BMCMCs were incubated with NK cells for 24 h without LPS or with 1.0 or 0.1 μg/ml LPS. B, NK cells were coincubated with BMCMCs in the presence of 1 μg/ml LPS and 20 μg/ml anti-TNF1 or anti-TNFR2 or both of them as indicated for 24 h. IFN-γ levels in the supernatant were determined by ELISA. Data are presented as mean ± SEM and show results from at least four independent experiments using different batches of BMCMCs and NK cells.

FIGURE 4. Mast cell-mediated IFN-γ release of NK cells is dependent on direct interaction of BMCMCs and NK cells. A, NK cells were either coincubated with BMCMCs in the presence of 1 μg/ml LPS or with the supernatant of BMCMCs that had previously been stimulated for 24 h with 1 μg/ml LPS. B, BMCMCs and NK cells were either cocultured or separated by a transmembrane (0.4 μm). Cells were stimulated for 24 h with LPS or medium control. IFN-γ levels in the supernatant were determined by ELISA. Data are presented as mean ± SEM and show results from at least three independent experiments using different batches of BMCMCs and NK cells. *p < 0.05; **p < 0.01; ***p < 0.005.
Ab did not alter the TNF-α secretion from BMCMCs upon stimulation with LPS (Supplemental Fig. 2). OX40L expression was confirmed by FACS analysis and revealed an increase in OX40L surface expression poststimulation with LPS for 24 h (Fig. 5E). However, the increase in OX40L expression was NK cell independent. Although the OX40L expression did not differ between C57BL/6 and BALB/c BMCMCs (Supplemental Fig. 2B), there was no influence of blocking OX40L on BALB/c BMCMCs on NK cell-mediated IFN-γ secretion (Supplemental Fig. 2C).

Cytotoxicity of NK cells is not altered in the presence of BMCMCs

In addition to the release of cytokines, such as IFN-γ, NK cell activation can alter their cytotoxic effect on target cells, such as tumor cells or virus-infected cells. We investigated the effect of BMCMCs on NK cell-mediated cytotoxicity. Therefore, we used the NK cells cocultured with MCs after 24-h stimulation with LPS and incubated them for 4 h with chromium-labeled YAC cells as targets. In contrast to cytokine responses, the cytotoxicity of NK cells cocultured with BMCMCs was not significantly affected at all effector/target ratios tested (Fig. 6A). These data were confirmed by FACS analysis of the intracellular granzyme B accumulation (Fig. 6B).

Intracellular IFN-γ production by NK cells is reduced in mast cell-deficient mice after LPS injection

To verify the effect of MCs on NK cells in vivo, we injected LPS i.p. into WT and Wsh mice. After 14 h, intracellular IFN-γ expression in NK cells from peritoneal lavage, lymph nodes, and spleen was determined by FACS analysis. Mast cell-deficient Wsh mice showed a significant reduction in intracellular IFN-γ in splenic NK cells (Fig. 7) as compared with WT mice. NK cells from peritoneal cells and lymph nodes revealed a reduction of IFN-γ production by guest on September 15, 2017 http://www.jimmunol.org/ Downloaded from
in Wsh mice as well; however, the difference was not statistically significant (Fig. 7).

Discussion
In this study, we show a novel role for MCs, in which LPS-stimulated murine MCs enhance IFN-γ secretion by NK cells significantly in a cell contact-dependent manner. These in vitro data were confirmed by i.p. injection of LPS into mast cell-deficient Wsh and WT mice. LPS-induced intracellular IFN-γ production was decreased in splenic, peritoneal lavage, and lymph node-derived NK cells in mast cell-deficient mice in comparison with WT mice. However, this reduction was only significant for splenic NK cells.

NK cells as important regulatory cells of the immune system play a central role in eliminating virus-infected cells as well as in controlling tumor cell growth. Our data suggest that MCs as important effector cells of the innate immune system can influence NK cell activity and regulate their function.

One of the most important aspects of MCs concerning acquired immunity is their FcεRI that leads to a release of preformed lipid and chemical mediators (stored in granules) via Ag-mediated crosslinking of receptor-bound IgE, causing an allergic (acute) reaction (35–40). However, IgE/Ag-mediated stimulation of MCs did not show any changes in IFN-γ secretion of NK cells in our experimental setup. Therefore, relevance of the MC-NK cell interactions for IgE-mediated acquired immune mechanisms can be excluded.

Previously, MCs were primarily known for their important effector role in IgE-mediated acquired immune responses. However, it was not before the discovery of TNF-α release from MCs and their important role in septic peritonitis that the immunomodulatory capabilities of MCs were discovered. So far, TNF-α released by MCs remains one of the key cytokines to activate and interact with other cell types both in innate as well as in acquired immune mechanisms (41). TNF-α is necessary for neutrophil recruitment in models of septic peritonitis as well as in late-phase allergic reaction (6, 12, 13). Furthermore, MC release of TNF-α is relevant for dendritic cell migration and enhances T cell activation (16–19). Surprisingly, our data show that MC-NK cell interaction is completely TNF-α independent; the use of TNF-α−/− MCs had no effect on the amplification of IFN-γ released by NK cells. Therefore, this shows one of the first TNF-α−/− independent regulatory functions of MCs. Our data also suggest that although MCs affect cytokine release by NK cells their cytotoxicity is not affected. However, there are reports describing differentially regulated cytotoxicity and cytokine production in human (42, 43) as well as in murine (44, 45) NK cells.

The mechanism by which MCs enhance NK cell function is not clear. In our study, the mast cell-dependent increase in IFN-γ secretion by NK cells can only be elicited if cells are stimulated via their pattern recognition receptors TLR3, -4, or -9. Therefore, we suggest that the MC-NK cell interaction will be relevant to pathophysiological processes within the context of innate immunity. Previous studies have shown that the expression of TLR4 by MCs is mandatory to promote optimal host defense in experimental septic peritonitis in mice (13). In addition to MCs, NK cells have been implicated in models of sepsis. NK cells are a major source of IFN-γ in endotoxin-induced sepsis (46–50). Etogo et al. (46) have recently described that NK cell numbers and activation increase in the peritoneal cavity after cecal ligation and puncture.

We hypothesize that within the context of sepsis, MCs are activated by LPS via their TLR4 and stimulate NK cells to release increased amounts of IFN-γ. IFN-γ in turn might stimulate macrophages and can enhance direct antimicrobial pathways (51). Furthermore, IFN-γ can promote Ag processing and presentation in macrophages. This is important in developing adequate acquired immune defenses against host invasion. Injection of LPS in WT and Wsh mice, resulting in a reduced NK cell IFN-γ production in Wsh mice, supports our findings from the in vitro studies. Our data also confirm the findings of Echtenacher et al. (4) that MCs seem to have a protective role in sepsis. In contrast, there is another study (52) describing a detrimental role of MCs during sepsis. However, the mechanisms and functional relevance of in vivo interaction of MCs and NK cells have to be analyzed in further experiments.

In addition to LPS, other activators relevant for innate immune responses; namely, CpG and poly I:C showed a significant increase in IFN-γ secretion. However, LPS was by far the strongest activator in our experimental setting.

Blocking of OX40L on C57BL/6 BMCMCs results in decreased IFN-γ secretion by NK cells, which suggests a significant role of the OX40-OX40L interaction within this setting. The OX40-OX40L interaction has been shown to be important for MC-associated activation of T cells (17) and dendritic cell-mediated NK cell activation (53). This is in agreement with recent publications describing the functional presence of OX40L on human and murine MCs (17, 54, 55) and OX40 on murine NK cells (53). However, we could neither show an increased expression of OX40L on BALB/c BMCMCs in comparison with C57BL/6 BMCMCs as described before (17) nor expression of OX40 on all MCs. We were also not able to block IFN-γ secretion in all of the tested mouse strains. We assume that the interaction shows a considerable complexity and might be influenced by additional mediators.

In summary, our findings have revealed a cell-proximity and TLR-dependent mechanism through which MCs can enhance the IFN-γ secretion of NK cells in vitro. Additionally, we could show that MCs increase the IFN-γ production of splenic NK cells in vivo after LPS injection. These observations clearly demonstrate for the first time that MCs can directly modulate NK cell function. Further work is required to elucidate the mechanism by which MCs activate NK cells to better understand the regulation of the innate immunity.

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Disclosures
The authors have no financial conflicts of interest.

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