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IFN-α Production by Plasmacytoid Dendritic Cells Stimulated with RNA-Containing Immune Complexes Is Promoted by NK Cells via MIP-1β and LFA-1

Niklas Hagberg,* Olof Berggren,* Dag Leonard,* Gert Weber,†,‡,† Yenan T. Bryceson,§ Gunnar V. Alm,¶ Maija-Leena Eloranta,* and Lars Rönnblom*

Several systemic autoimmune diseases display a prominent IFN signature. This is caused by a continuous IFN-α production by plasmacytoid dendritic cells (pDCs), which are activated by immune complexes (ICs) containing nucleic acid. The IFN-α production by pDCs stimulated with RNA-containing IC (RNA-IC) consisting of anti-RNP autoantibodies and U1 small nuclear ribonucleoprotein particles was recently shown to be inhibited by monocytes, but enhanced by NK cells. The inhibitory effect of monocytes was mediated by TNF-α, PGE₂, and reactive oxygen species, but the mechanisms for the NK cell-mediated increase in IFN-α production remained unclear. In this study, we investigated the mechanisms whereby NK cells increase the RNA-IC–induced IFN-α production by pDCs. Furthermore, NK cells from patients with systemic lupus erythematosus (SLE) were evaluated for their capacity to promote IFN-α production. We found that CD56dim NK cells could increase IFN-α production >1000-fold after RNA-IC activation, whereas CD56bright NK cells required costimulation by IL-12 and IL-18 to promote IFN-α production. NK cells produced MIP-1α, MIP-1β, RANTES, IFN-γ, and TNF-α via RNA-IC–mediated FcγRIIA activation. The IFN-α production in pDCs was promoted by NK cells via MIP-1β secretion and LFA-mediated cell–cell contact. Moreover, NK cells from SLE patients displayed a reduced capacity to promote the RNA-IC–induced IFN-α production, which could be restored by exogenous IL-12 and IL-18. Thus, different molecular mechanisms can mediate the NK cell-dependent increase in IFN-α production by RNA-IC–stimulated pDCs, and our study suggests that the possibility to therapeutically target the NK–pDC axis in IFN-α–driven autoimmune diseases such as SLE should be investigated. The Journal of Immunology, 2011, 186: 5085–5094.

Type I IFNs are pleiotropic cytokines with both antiviral activity and ability to enhance innate and adaptive immune responses (1, 2). The plasmacytoid dendritic cells (pDCs) are the main producer of type I IFNs and have the capacity to synthesize up to 10⁷ IFN-α molecules/cell within 12 h after activation (3). The IFN-α is produced by pDCs in response to DNA or RNA viruses (4), but can also be induced by synthetic TLR agonists, such as oligodeoxynucleotides (ODNs) (5) or nucleic acid-containing immune complexes (ICs) (6). In systemic autoimmune diseases, DNA- or RNA-containing IC (RNA-IC) formed by apoptotic or necrotic cellular material and autoantibodies can act as endogenous IFN-α inducers (6). The ICs are internalized to endosomes through high-affinity FcγRIIA (CD32A) on pDCs where they interact with TLR7 or TLR9. Subsequent activation of the MyD88 pathway and IFN regulatory factor-7 results in IFN-α production (3). Besides the direct antiviral properties, IFN-α acts as an immune adjuvant and promotes maturation of dendritic cells, polarization of Th cells to Th1 cells, and activation of cytotoxic T cells (1, 2). Moreover, IFN-α promotes B cell differentiation, Ab production, and Ig class-switching (7). This broad activation of the immune system is beneficial during infections, but can be deleterious if the IFN-α production is not properly downregulated after clearance of pathogens. Prolonged exposure of the immune system to IFN-α increases the risk for loss of self-tolerance and autoimmune reactions. In fact, development of autoimmune diseases, including systemic lupus erythematosus (SLE), has been frequently described in patients during long-term treatment with type I IFN (8, 9).

NK cells mainly contribute to the immune response by killing target cells, but they are also important in both the innate and the adaptive immune response by their capacity to produce a large number of cytokines (10–12). Several of the cytokines produced by NK cells have immunoregulatory functions and could potentially modulate the response of pDCs to different IFN-α inducers. Based on their expression of CD56 and the low-affinity FcγRIIA (CD16), the NK cells can be divided into two different subpopulations. The majority of NK cells found in peripheral blood are CD56dim/CD16+ NK cells (>90%). A minor population of
CD56<sup>bright</sup>/CD16<sup>−</sup> NK cells is also present in peripheral blood, but the majority of these cells reside in secondary lymphoid organs. Generally, the CD56<sup>bright</sup> NK cells have been considered most important for cytotoxicity, whereas the production of immunoregulatory cytokines has been assigned to CD56<sup>dim</sup> NK cells (13, 14).

Previous studies have described a reciprocal cross-talk between NK cells and pDCs. For instance, IL-2– or IL-12–pulsed NK cells promote the ODN-induced IFN-α production by pDCs (15, 16). In turn, IFN-α augments the NK cell cytotoxicity, and NK cells cocultured with pDCs acquire increased cytolytic capacity. These NK cells are capable of killing autologous immature monocyte-derived dendritic cells, but do not kill pDCs (16). We recently described that NK cells are extremely potent in promoting the IFN-α production by pDCs stimulated with RNA-ICs consisting of U1 small nuclear ribonucleoprotein (snRNP) particles and anti-RNP autoantibodies (17). The enhancing effect on the IFN-α production by NK cells could be inhibited by monocytexes. Consequently, a complex cross-talk between NK cells and pDCs exists, in which both cells can affect the function of each other.

In the current study, we sought to gain insights regarding how NK cells promote IFN-α production by RNA-IC-activated pDCs. We found that MIP-1β secretion and LFA-1–mediated cell–cell contact by NK cells contributed to pDC-derived IFN-α production. Moreover, CD56<sup>dim</sup> NK cells could promote the pDC response via FcyRIIIA stimulation, whereas CD56<sup>bright</sup> NK cells required costimulation with IL-12 and IL-18 to enhance the IFN-α production. Finally, we evaluated pDC–NK cell interactions in a chronic autoimmune disease characterized by increased levels of circulating ICs. Remarkably, NK cells from patients with SLE had a reduced capacity to promote the RNA-IC–induced IFN-α production, which could be restored by stimulation of NK cells with IL-12 and IL-18.

Materials and Methods

Cells

Human NK cells and pDCs were isolated from peripheral blood of healthy blood donors (Department of Transfusion Medicine, Uppsala University Hospital) or patients with SLE (Rheumatology Unit, Uppsala University Hospital) by negative selection (pDC or NK cell isolation kit; Miltenyi Biotec, Auburn, CA). Isolated cells were stained with PE-conjugated anti–BDCA-4 (Miltenyi Biotec) or anti-CD56 (NCAM 16.2; BD Biosciences, San Jose, CA) Abs and analyzed by flow cytometry (FACScanCant II; BD Biosciences). NK cells and pDCs contained at least 95% CD56<sup>+</sup> and 90% BDCA-4<sup>+</sup> cells, respectively. The CD56<sup>dim</sup> and CD56<sup>bright</sup> subpopulations of NK cells were isolated using the CD56<sup>CD16</sup><sup>−</sup> and CD56<sup>CD16</sup><sup>+</sup> isolation kits (Miltenyi Biotec), respectively. Cells were cultured in 0.1 ml vol in 96-well flat-bottom plates (Nunclon; Nunc, Roskilde, Denmark) using macrophage–serum–free medium (Invitrogen, Carlsbad, CA) supplemented with HEPES (20 mM), penicillin (60 μg/ml), and streptomycin (100 μg/ml). When indicated, medium was supplemented with 10 ng/ml IL-12 and 100 ng/ml IL-18 (Peprotech, Rocky Hill, NJ); MIP-1β (CCL4; Peprotech), cycloheximide, or chloroquine (Sigma-Aldrich, St. Louis, MO) were used at concentrations of 30 μg/ml and 1 mg/ml, respectively. If not stated otherwise, 25 × 10<sup>3</sup> pDCs and 100 × 10<sup>3</sup> NK cells/well were used. In experiments comparing NK cells from SLE patients and healthy controls, 25 × 10<sup>3</sup> NK cells/well were used. All cell cultures were set up as duplicates and incubated at 37°C with 5% CO2.

Patients

All patients fulfilled ≥4 of the American College of Rheumatology criteria for SLE. Patients were 46 ± 14 y old and had a disease duration of 17 ± 14 y (mean ± SD). The disease activity was determined by the modified SLE disease activity index (18), which excludes anti-dsDNA and complement levels. Eighteen of the patients had a disease activity score of 0, and five patients had a disease activity score between 1 and 4. The study was approved by the local ethics committee, and informed consent was obtained from all patients and controls.

ICs and IFN-α inducers

U1 snRNP particles were purified from HeLa cells, as previously described (19). SLE-IgG was purified by protein G chromatography from a patient serum containing autoantibodies to Sm and SmD, U1-RNP A, U1-RNP C, ribosomal P Ag, histone, and dsDNA (20). If not otherwise stated, the U1 snRNP particles were used together with SLE-IgG at a final concentration of 2.5 μg/ml and 1 mg/ml in culture medium, respectively. Fab(′)2 fragments of the SLE-IgG were prepared using the Fab(′)2 preparation kit (Pierce, Rockford, IL), according to manufacturer’s instructions. Normal IgG (Octagam; Octapharma, Stockholm, Sweden) was aggregated by heating at 63°C for 60 min at a concentration of 50 mg/ml and then used at indicated concentrations. HSV was prepared by propagation in WISH cells and inactivation by UV light, as previously described (6). ODN2216 was purchased from Cybergene (Stockholm, Sweden).

NK cell supernatants

Cell-free supernatants from NK cells cultivated 14–16 h were harvested at 1000 × g for 7 min. When indicated, supernatants were either dialyzed against culture medium using a membrane with m.w. cutoff 6–8 kDa (Spectrum Labs, Rancho Dominguez, CA) or ultrafiltered through a Microcon YM-100 centrifugal filter unit (m.w. cutoff 100 kDa; Millipore, Billerica, MA). If not otherwise stated, NK supernatants were used at a final concentration of 50% (v/v).

Immunoassays

Concentrations of GM-CSF, IL-1β, IL-3, IL-6, IL-8 (CXCL8), IL-12p70, IL-17, IL-18, IFN-γ, IP-10 (CXCL10), MCP-1 (CCL2), MIP-1α (CCL3), MIP-1β, RANTES (CCL5), TNF-α, and TNF-β in cell culture supernatants were determined using a magnetic bead-based multiplex assay, according to the manufacturer’s instructions (Biospicea; Bio-Rad, Hercules, CA). The IFN-α concentration was measured with a dissociation-enhanced lanthanide fluorimunoassay, as previously described (21).

Neutralization of cytokines in NK cell supernatants

Supernatants from RNA-IC–stimulated NK cells were diluted 2.5-fold and incubated with indicated Abs for 2 h at 4°C. Affinity-purified goat polyclonal anti–IFN-γ (10 μg/ml), anti–MIP-1α (10 μg/ml), anti–MIP-1β (50 μg/ml), anti–RANTES (10 μg/ml) Abs, or normal goat IgG (R&D Systems, Minneapolis, MN) were used. Anti–TNF-α mAb (MAB210; R&D Systems) and isotype-matched Ab IgG1 (DakoCytomation, Glostrup, Denmark) were used at a concentration of 5 μg/ml. The concentrations of Abs needed for neutralization were estimated based on the concentrations of cytokines in the culture medium determined by the multiplex immunoassay. Ab-treated supernatants were added to RNA-IC–stimulated pDCs at a concentration of 50% (v/v). The stimulatory activity of NK supernatants was calculated by subtracting the basal production of IFN-α by RNA-IC–stimulated pDCs from the IFN-α produced when NK supernatants were added. Percentage of NK–RNA-IC supernatant activity was defined as the ratio between IFN-α production in cultures supplemented with supernatants treated with neutralizing Ab and supernatants treated with control Ab.

Blocking of cell surface receptors

NK cells were preincubated with mAbs blocking FcyRII (KB61; Santa Cruz Biotechnology, Santa Cruz, CA) or FcyRII (3G8; Molecular Probes, Invitrogen) for 30 min at 37°C before stimulated with RNA-IC. Supernatants were harvested, ultrafiltered, and added to RNA-IC–stimulated pDCs. Anti-CD11a mAb at the optimal concentration 1 μg/ml (BCA-1; British Bio-Technology, Oxford, UK) was used to block FcαRI. Anti-CD11c mAb (BCA-3; British Bio-Technology) and control isotype-matched IgG1 (DakoCytomation) and IgG2a (BD Pharmingen, San Diego, CA) were also used at 1 μg/ml.

Immunofluorescence microscopy

Purified pDCs and NK cells were cultivated in 96-well plates (black/gray tissue culture–treated imaging plate; BD Falcon, San Jose, CA) with RNA-IC or medium for 20 h. The supernatants were carefully aspirated and the wells were rinsed with 1% human serum albumin in PBS. The cells were stained for 45 min with Alexa Fluor 647–conjugated anti-CD56 or mouse IgG1 isotype control (Miltenyi Biotec) and rinsed several times with PBS before analyzed with a Zeiss LSM Meta 510 fluorescence microscope using Zen 2009 software (Carl Zeiss, Jena, Germany).
Both CD56dim and CD56bright NK cells promote the RNA-IC–induced IFN-α production by pDCs

Initially, we compared the capacity of NK cells to enhance the IFN-α production by pDCs stimulated with RNA-IC, ODN2216, or HSV. RNA-IC was a much weaker IFN-α inducer than ODN2216 when pDCs were cultured alone (p = 0.0003 and p = 0.0156, respectively; Fig. 1). Addition of NK cells increased the IFN-α production by all three stimuli used with a median increase for RNA-IC, ODN2216, or HSV stimulation of 90-, 3-, and 3-fold, respectively (Fig. 1). Mock-stimulated pDCs or pDC–NK cell cocultures produced no IFN-α (data not shown). Thus, the NK cell enhancement of IFN-α production was remarkably potent with RNA-IC as stimuli in comparison with ODN2216 or HSV stimulation. In this study, we explored the mechanisms by which NK cells rendered the relatively weak IFN-α inducer, RNA-IC, a strong IFN-α inducer.

Both CD56dim and CD56bright NK cells promote the RNA-IC–induced IFN-α production by pDCs

To determine whether the capacity of NK cells to stimulate pDCs was restricted to a subpopulation of NK cells, CD56dim or CD56bright NK cells were cocultivated with RNA-IC–stimulated pDCs. We found that the stimulatory effect of NK cells on pDCs in the RNA-IC–containing cultures was concentration dependent and almost entirely confined to the CD56dim NK cells (Fig. 2A). The lack of pDC stimulation by the CD56bright NK cells could either be an intrinsic property or the result of inadequate stimulation of these cells by RNA-IC. To explore this, we examined the effect of costimulation with IL-12 and IL-18, which can induce the production of several cytokines by NK cells (22). Addition of IL-12 and IL-18 to the RNA-IC–stimulated pDC and NK cell cultures rendered the CD56bright NK cells as efficient as CD56dim NK cells in enhancing the IFN-α production by pDCs (Fig. 2B). To determine whether NK cells produced soluble factors that could induce IFN-α production in the absence of RNA-IC stimulation of pDCs, the RNA-ICs were removed from supernatants by ultrafiltration. Such supernatants, or supernatants from IL-12/IL-18–stimulated NK cells alone, induced no IFN-α production by pure pDCs, but enhanced their response to added RNA-IC (Supplemental Fig. 1). A dose–response study demonstrated that the supernatants from RNA-IC–activated NK cells stimulated the pDCs in a concentration-dependent manner (Fig. 3C). Notably, the stimulatory effect on pDCs was always superior when direct cell contact between pDCs and NK cells was allowed than when supernatants from RNA-IC–stimulated NK cells were used.
pendent experiments. This difference could be explained by the fact that the final concentrations of NK supernatants only were 50% (v/v) of the total cell culture volume, thus resulting in lower concentrations of factors activating pDCs. However, it is also possible that direct receptor–ligand interactions between pDCs and NK cells increase the IFN-α-promoting capacity of NK cells.

Time–course studies showed that the factor(s) stimulating pDCs first appeared in NK cell supernatants between 30 min and 4 h after RNA-IC activation. The stimulatory effect of the supernatants increased up to 8 h after addition of RNA-IC to the NK cell cultures and thereafter remained relatively stable throughout the time course studied (Fig. 3D). Due to the rapid release of the pDC-activating factor(s), we asked whether NK cells released preformed activating factors or whether new protein synthesis was necessary. Therefore, NK cells were treated with the translation inhibitor cycloheximide before the addition of RNA-IC to the cell cultures. Supernatants were harvested and dialyzed to remove cycloheximide. To confirm that cycloheximide was removed during dialysis, cycloheximide was added to harvested RNA-IC–stimulated NK cell supernatants that were dialyzed and added to pDCs. Addition of cycloheximide prior to RNA-IC stimulation, but not after harvest, completely abolished the stimulatory effect of the NK cell supernatants (Fig. 3E), demonstrating the requirement for new protein synthesis.

In conclusion, RNA-IC or IL-12/IL-18 stimulation of NK cells rapidly induces the production of soluble factors that have a marked stimulatory effect on the IFN-α production by RNA-IC–stimulated pDCs. Still, receptor–ligand interactions in the context of cell–cell contact might also contribute to the stimulatory effect.

**FIGURE 3.** NK cells stimulated with RNA-IC or IL-12 and IL-18 produce soluble factors that promote the RNA-IC–induced IFN-α production by pDCs. IFN-α production in RNA-IC–stimulated pDC cultures supplemented with the following. A. Medium (medium; n = 15), supernatants (sup.) 50% (v/v) from RNA-IC–stimulated NK cells (NK-RNA-IC sup.; n = 15), supernatants 50% (v/v) from NK cells stimulated with medium (NK-mock sup.; n = 15), or NK cells (n = 11). n, number of blood donors used. Horizontal scale bars show the median IFN-α production. B. Medium or supernatants from mock-, RNA-IC–, or IL-12/IL-18–stimulated NK cells (NK-IL12/18 sup.). C. Increasing concentrations (% v/v) of supernatants from mock- or RNA-IC–stimulated NK cells. D. Supernatants from mock- or RNA-IC–stimulated NK cells, harvested at indicated time points after stimulation. E. Supernatants obtained from RNA-IC–stimulated NK cells cultured in the presence of indicated concentrations of cycloheximide. As a control for dialysis efficiency, cycloheximide (50 μg/ml) was added to supernatant after harvest, followed by dialysis (Crl). A–E. The IFN-α concentration in supernatants was determined after 20 h with an immunoassay. B–E. Data (mean ± SD of duplicate wells) are representative of at least two independent experiments. A. Statistical analysis comparing medium and NK cell supernatants was performed using Wilcoxon signed rank test. Comparison of supernatants from RNA-IC–stimulated NK cells and NK cells was performed using Mann–Whitney U test. **p < 0.001, ***p < 0.001.

Ligation of FcγRIIIA on NK cells induces soluble factors that promote the IFN-α production by pDCs

Activation of NK cells by RNA-IC to produce soluble factors that promote the pDC production of IFN-α could be due to either the stimulation of FcγRs, RNA-sensing receptors, or costimulation of both receptors. To determine which part(s) of the RNA-IC was essential for the NK cell activation, the cells were stimulated with RNA-IC consisting of U1 snRNP particles and either intact SLE-IgG or F(ab’2) fragments of the SLE-IgG. RNA-ICs, lacking the Fc portion of the IgG, were unable to trigger NK cells to produce the pDC-stimulating factor(s) (Fig. 4A). Because NK cells primarily express FcγRIIIA, we examined whether blocking this receptor would inhibit the activation of NK cells by RNA-IC. Indeed, blocking of FcγRIII, but not FcγRII, resulted in a dose-dependent reduction of the stimulatory effects by supernatants from RNA-IC–activated NK cells (Fig. 4B). Next, we investigated whether the TLR-activating property of the RNA-IC was essential for the production of the factor(s) stimulating pDCs. To this end, we used chloroquine, which inhibits the endosomal acidification and thereby interferes with the endosomal TLR signaling. Precipitation of NK cells with chloroquine before RNA-IC stimulation did not affect the production of the factor(s) that activated pDCs (Fig. 4C). Furthermore, we investigated whether ICs without RNA content could trigger NK cells to produce stimulatory factors for pDCs. Heat-aggregated normal IgG (HAIG) or RNA-IC were used to activate NK cells, and after removal of ICs the supernatants were added to pDCs together with RNA-IC. The supernatants from HAIG-stimulated NK cells were as efficient as supernatants from RNA-IC–stimulated NK cells in enhancing the IFN-α production by pDCs (Fig. 4D).
production by pDCs. Inhibition with neutralizing Abs targeting IFN-γ, MIP-1β, RANTES, and TNF-α revealed that inhibition of MIP-1β could suppress approximately half of the stimulatory effect from the supernatants of RNA-IC–stimulated NK cells (Fig. 5B). No significant decrease in the supernatant activity with the other Abs was observed. Moreover, addition of rMIP-1β to RNA-IC–stimulated pDCs resulted in a dose-dependent increase in IFN-α production (Fig. 5C).

Together, these data indicate that the chemokine MIP-1β contributes to the stimulatory effect on IFN-α production seen in supernatants from RNA-IC–stimulated NK cells.

**LFA-1 is involved in the NK cell-mediated enhancement of IFN-α production by RNA-IC–stimulated pDCs**

Upon RNA-IC stimulation of cocultivated NK cells and pDCs, large cell aggregates were observed. Immunofluorescent staining showed that the aggregates consisted of both pDCs and NK cells (Fig. 6A). These aggregates were not formed in the absence of RNA-IC or when NK cells and pDCs were cultivated separately (data not shown). Because addition of NK cells to RNA-IC–stimulated pDCs consistently induced a stronger IFN-α production than supernatants from RNA-IC–stimulated NK cells, we asked whether receptor–ligand interactions between NK cells and pDCs further enhanced the IFN-α production. Because FcyRIIIA engagement of NK cells is known to induce a higher affinity conformational state of LFA-1 (24) and chemokines such as MIP-1α, MIP-1β, and RANTES induce activation of its ligands (25), we hypothesized that LFA-1 could be involved in the stimulation of pDCs mediated by NK cells. When neutralizing Abs to the α-chain of LFA-1 (CD11a) were added to pDC–NK cell cocultures, the IFN-α production was reduced considerably (Fig. 6B). In fact, inhibition of cell–cell contact via anti-CD11a reduced the stimulatory effect of NK cells to that seen with supernatants from RNA-IC–stimulated NK cells (Fig. 6C). In contrast, neither Abs to CD11c nor isotype-matched Abs resulted in a decreased IFN-α production (Fig. 6B). Inhibition of CD11a in cultures of pDCs without NK cells, stimulated with HSV, ODN2216, or RNA-IC, did not affect the IFN-α production (Supplemental Fig. 2A), and blocking CD11a when stimulating NK cells with RNA-IC did neither affect the concentrations of the cytokines analyzed (Supplemental Fig. 2B) nor affect the stimulatory property of the culture supernatant on IFN-α production by pDCs (data not shown).

Because both inhibition of CD11a and MIP-1β led to a reduction in IFN-α production, we asked whether these effects were additive. First, we examined whether the IFN-α production could be reduced by inhibiting MIP-1β in cocultures of RNA-IC–stimulated pDCs and NK cells where cell contact was allowed. Indeed, blocking MIP-1β in pDC/NK cell cocultures decreased the IFN-α production (Fig. 6C). Neutralization of MIP-1β in CD11a-blocked cocultures led to a slight further reduction in IFN-α production (Fig. 6C), suggesting an additive effect of blocking these molecules.

Taken together, these data demonstrate an important role for LFA-1–dependent cell–cell contact and MIP-1β in NK cell–mediated enhancement of IFN-α production by RNA-IC–stimulated pDCs.

**Cocultivation of pDCs and NK cells increases the production of several cytokines in an LFA-1–dependent manner**

Having seen the LFA-1–mediated increase in IFN-α production when pDCs and NK cells were cocultivated, we asked whether other cytokines also were regulated via interactions with pDCs and NK cells. Initially, supernatants from RNA-IC–stimulated pDCs, NK cells, or cocultivated pDCs and NK cells were analyzed for the same cytokines as in Fig. 5A. Interestingly, cocultivation of pDCs and NK cells resulted in a 2-fold increase in MIP-1β (Fig. 6D). Furthermore, secretion of MIP-1α, RANTES, and IFN-γ was...
increased 6-, 4-, and 12-fold, respectively. When RNA-IC–stimulated pDCs were cultured alone, small amounts of IL-6 and IL-8 were produced. Addition of NK cells caused a strikingly increased production of these cytokines. The production of IP-10 and TNF-α was also increased upon cocultivation. Because IFN-α and IFN-γ can induce/enhance the expression of several of these cytokines, e.g., IP-10 and MIP-1α (26, 27), the up-regulation of these cytokines could be the result of increased IFN-α/IFN-γ expression rather than a direct effect of LFA-1 ligation. None of the soluble factors analyzed was found exclusively in the supernatants from cocultivated pDCs and NK cells.

To determine to what extent the increased levels of these cytokines were dependent on LFA-1 ligation, the effect of CD11a inhibition was examined. Inhibition of CD11a led to a clear decrease of IL-6, IL-8, IP-10, MIP-1α, MIP-1β, and RANTES (Fig. 6D), indicating that the increased production of these factors was partially dependent on LFA-1, either directly or secondarily to other cytokines dependent on LFA-1. The levels of IFN-γ and TNF-α showed a more modest decrease, suggesting that the increased production of these cytokines could possibly result from other receptor–ligand interactions or cross-talk between pDC and NK cells via soluble factors that are not dependent on LFA-1.

Thus, in addition to IFN-α, the production of several inflammatory cytokines is increased in an LFA-1–dependent manner when RNA-IC–stimulated pDCs and NK cells are cultured together.

The enhancing capacity of RNA-IC–stimulated NK cells from patients with SLE is diminished, but is restored by costimulation with IL-12 and IL-18

Because patients with SLE have interferogenic ICs in circulation and display a prominent increase in the expression of IFN-α–regulated genes, we investigated whether the stimulatory capacity of NK cells on IFN-α production by RNA-IC–stimulated pDC was altered in SLE. NK cells from SLE patients or healthy controls were cocultivated together with RNA-IC–stimulated pDCs from healthy blood donors. We found that NK cells from patients with SLE could strongly increase the RNA-IC–induced IFN-α production by pDCs (p < 0.0001; median increase = 16-fold; Fig. 7A), but the stimulatory capacity of the SLE–NK cells was significantly lower compared with NK cells from healthy controls when stimulated with RNA-IC (p < 0.0001; Fig. 7A). However, the stimulatory capacity of NK cells from patients with SLE was increased to the same level as NK cells from healthy donors when costimulated with RNA-IC and IL-12 and IL-18 (p = 0.0961; Fig. 7A).

The phenotypes of the NK cells were analyzed to determine whether the weaker stimulatory capacity of RNA-IC–stimulated SLE–NK cells could be explained by a higher proportion of CD56<sup>bright</sup> NK cells and/or a downregulation of CD16 on CD56<sup>dim</sup> NK cells. When comparing the mean fluorescent intensity of CD16 on CD56<sup>dim</sup> NK cells, no significant difference between patients with SLE and healthy controls was seen (data not shown).

**FIGURE 5.** NK cells promote the IFN-α production by pDCs via MIP-1β. A, NK cells were stimulated with medium only (mock), RNA-IC, HAIG, or IL-12 and IL-18 for 16 h. Concentrations of the cytokines in the culture supernatants, except for IFN-α, were determined using a bead-based multiplex immunoassay. The IFN-α concentration was determined by a separate immunoassay. Data (mean + SD) from four different donors for RNA-IC– and mock-stimulated NK cells and two donors for HAIG- and IL-12/IL-18–stimulated NK cells are shown. Because the exogenously added IL-12 and IL-18 cannot be distinguished from IL-12 or IL-18 potentially produced by NK cells, data from these cytokines in the IL-12/IL-18–stimulated NK cell supernatants were excluded. B, Supernatants (sup.) from RNA-IC–stimulated NK cells were preincubated with neutralizing Abs to MIP-1α, MIP-1β, IFN-γ, RANTES, TNF-α, or corresponding control Abs. Supernatants were subsequently added to RNA-IC–stimulated pDCs. The IFN-α production by pDCs was determined after 20 h, and the results were expressed as percentage of activity (%NK-RNA-IC sup. activity) of Ab-treated versus control Ab-treated NK supernatants, as described in Materials and Methods. Values represent mean + SD from two to five experiments, as indicated in the scale bars. C, MIP-1β in indicated concentrations or supernatant from RNA-IC–stimulated NK cells (NK-RNA-IC sup.) was added to the RNA-IC–stimulated pDC cultures. IFN-α production was determined after 20 h with an immunoassay. Data (mean + SD from duplicate wells) are representative of four experiments.
However, NK cells from patients with SLE contained a higher proportion of CD56\textsuperscript{bright} NK cells compared with healthy donors ($p = 0.0442$; Fig. 7C), which could explain the reduced stimulatory capacity of RNA-IC–stimulated SLE–NK cells.

Having seen the IFN-$\alpha$–promoting effect of MIP-$1\beta$, we asked whether MIP-$1\beta$ production was affected in NK cells from patients with SLE. Indeed, RNA-IC– or IL-12/IL-18–stimulated NK cells from patients with SLE produced significantly lower amounts of MIP-$1\beta$ compared with healthy donors ($p = 0.0008$ and $p = 0.04$, respectively; Fig. 7D). Even though MIP-$1\beta$ production was increased when SLE–NK cells were cocultured with RNA-IC–stimulated pDCs, the amount of MIP-$1\beta$ produced was still significantly lower compared with cocultures of pDCs with NK cells from healthy donors ($p = 0.0068$; Fig. 7E). Notably, there was no significant difference in MIP-$1\beta$ production between NK cells from SLE patients and healthy donors when cocultures of pDCs and NK cells were stimulated with both RNA-IC and IL-12 and IL-18 ($p = 0.31$; Fig. 7E).

In conclusion, circulating NK cells from SLE patients have the ability to promote IFN-$\alpha$ production by pDCs, but are dependent on IL-12/IL-18 activation to execute a normal stimulation.

**Discussion**

We previously showed that NK cells strikingly enhanced the IFN-$\alpha$ production by pDCs upon stimulation by RNA-IC (17). In the present investigation, we verified that pDCs alone produced relatively little or no IFN-$\alpha$ in response to RNA-IC and that addition of NK cells increased the amount of IFN-$\alpha$ produced by pDC, often >1000-fold. In contrast, HSV and ODN2216 were strong inducers of IFN-$\alpha$ in pDCs in the absence of NK cells, and addition of NK cells only caused a 3-fold increase in the IFN-$\alpha$ production. Such a NK-mediated increase in IFN-$\alpha$ production by pDCs stimulated with ODNs has been described before (15, 16), but the mechanisms responsible for this stimulatory effect of NK cells have not been revealed. In the current study, we therefore investigated the mechanisms whereby NK cells enhance the IFN-$\alpha$
FIGURE 7. NK cells from SLE patients have decreased capacity to enhance the IFN-α production by RNA-IC–stimulated pDCs, which is restored by co-stimulation with IL-12 and IL-18. A, pDCs from healthy blood donors were cultured either alone or together with NK cells from patients with SLE or healthy controls and stimulated with RNA-IC in the absence (n = 23) or presence of IL-12 and IL-18 (n = 19). **, number of blood donors used. IFN-α was measured in 20-h cell culture supernatants by immunoassay. B and C, Isolated NK cells from patients with SLE or healthy controls were stained for CD16 and CD56 and analyzed with flow cytometry. Cells were gated into CD56dim and CD56bright NK cells, as shown in B, and the frequency of CD56bright NK cells in patients with SLE and healthy controls was compared. D and E, NK cells from patients with SLE or healthy controls were cultured alone or together with pDCs from healthy blood donors. NK cells were stimulated with RNA-IC or IL-12, and IL-18 (n = 9) and pDC–NK cocultures were stimulated with RNA-IC in the absence or presence of IL-12 and IL-18 (n = 10). A and C–E, Horizontal scale bars show the median value. Statistical analysis comparing RNA-IC stimulation with and without addition of IL-12 and IL-18 was performed using Wilcoxon signed rank test, and comparison of cells from healthy individuals and patients with SLE was performed using Mann–Whitney U test. ***p < 0.001, **p < 0.01, *p < 0.05.

production by pDCs stimulated with RNA-IC consisting of purified U1 snRNP particles and autoantibodies to them. Such RNA-ICs are found in several autoimmune diseases (28), and are thought to be endogenous IFN-α inducers, responsible for the ongoing activation of type I IFN production, and thus of etiopathogenic significance. Our main findings were that NK cells activated by IC via FcγRIIIA or by the cytokines IL-12 and IL-18 used soluble factors, including the chemokine MIP-1β, and LFA-1–mediated cell–cell interactions to stimulate IFN-α production in pDCs. Because the RNA-ICs were used dependent on NK cell costimulation to fully activate the IFN-α production, they appear to be incomplete activators of pDCs and represent a new class of type I IFN inducers. This class may include other similar interferogenic IC formed in autoimmune diseases (29, 30), as well as ICs containing viruses or their components (31). Our observations could therefore have implications for the understanding of both the clearance of certain pathogens via pDC–derived type I IFN and the mechanisms leading to IFN-α–driven autoimmune diseases, such as SLE.

The classical view of NK cells is that CD56dim NK cells are cytolytic, whereas CD56bright NK cells have immunoregulatory functions via cytokine secretion (13). Unexpectedly, we found the CD56dim–, but not the CD56bright–NK cell subset to be responsible for promoting the IFN-α production by RNA-IC–stimulated pDCs. However, the view of CD56dim NK cells producing negligible amounts of cytokines is based on investigations using exogenous cytokines such as IL-12, IL-15, and/or IL-18 as stimuli of NK cells. In contrast, Fauriat et al. (23) recently demonstrated that the CD56dim NK cells are also efficient cytokine producers when stimulated via receptors involved in target cell recognition, such as FcγRIIIA and 2B4 (CD244). In line with these data, addition of IL-12 and IL-18 to RNA-IC–stimulated pDC–NK cell cocultures rendered the CD56bright NK cells as efficient as CD56dim NK cells in enhancing the IFN-α production. Thus, our results suggest at least two pathways whereby NK cells are activated to promote the RNA-IC–induced IFN-α production by pDCs, namely via IL-12/IL-18 and RNA-IC. This dual mechanism for activating NK cells to promote the pDC function may guarantee a sufficiently strong IFN-α response during both innate and adaptive immune activation.

We showed that RNA-IC stimulated NK cells via FcγRIIIA to a de novo synthesis of soluble factors that caused a prominent costimulation of the IFN-α production in pure pDC cultures. The possibility that endosomal TLRs or other RNA-sensing receptors were involved in activating NK cells was excluded because inhibition of endosomal TLRs using chloroquine had no effect and ICs without RNA content were as efficient as RNA-IC. Thus, FcγRIIIA ligation of NK cells was sufficient to induce the factors stimulating pDCs. These results explain why CD56bright NK cells, which lack FcγRIIIA, are unresponsive to RNA-IC, but can be activated by IL-12 and IL-18.

After activation by RNA-IC, HAIG, or IL-12 and IL-18, NK cells produced IFN-γ, TNF-α, MIP-1α, MIP-1β, and RANTES. MIP-1β was abundantly secreted after RNA-IC stimulation of NK cells and was found to directly enhance the IFN-α production by RNA-IC–stimulated pDCs. MIP-1β binds to CCR5, which is expressed by many different types of immune cells, including pDCs (32). The mechanisms whereby signals from CCR5 might integrate with signals from RNA-sensing TLR and FcγRIIIA and enhance IFN-α production by pDCs remain to be elucidated. However, PI3K-, p38 MAPK–, and Rac-signaling pathways could be involved as they are activated via CCR5 (33) and have been implicated in IFN-α production by pDCs (34–37). Because supernatants were considerably more potent activators of pDCs than...
by a difference in FcγR response to RNA-IC by SLE–NK cells could not be explained when cocultivated with RNA-IC–stimulated pDCs. The weaker function of NK cells from patients with SLE on IFN-α-cell cytotoxicity in SLE patients (45, 46). However, the regulatory function of IL-12 and IL-18 rendered the NK cells from patients with SLE or healthy controls was seen. But when cocultures of pDCs and NK cells were stimulated with both RNA-IC and IL-12/IL-18, no significant difference between NK cells from patients with SLE or healthy controls was seen. Thus, the IFN-α–promoting effect of NK cells in circulation seems to be more cytokine dependent in patients with SLE compared with healthy individuals.

In conclusion, our study shows that NK cells via different mechanisms have the ability to strongly promote the RNA-IC–induced IFN-α production by pDCs. This pDC–NK cell cross-talk may be of importance in the defense against viral infections mediated by type I IFN. The results also suggest that NK cells may be one important, and to date largely neglected, actor in the complex process that leads to a continuous type I IFN production in systemic autoimmune diseases. Further studies to clarify the molecular basis and role of the pDC–NK cell cross-talk in type I IFN–driven autoimmune diseases are therefore warranted.

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**References**

NK cells promote IFN-α production by pDCs


