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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

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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.

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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 FcγRIIIA (CD16), the NK cells can be divided into two different subpopulations. The majority of NK cells found in peripheral blood are CD56dimCD16– NK cells (>90%). A minor population of
CD56<sup>bright</sup><sub>CD16<sup>b</sup></sub> 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 acquired 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 monocystes. 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 FcγRIIIA 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 (FACSCanto 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>dim</sup>CD16<sup>bright</sup> isolation kits (Miltenyi Biotec), respectively. Cells were cultured in 0.1 mL vol in 96-well flat-bottom plates (NuncNun; Nunc, Roskilde, Denmark) using macrophage-serum–free medium (Invitrogen, Carlsbad, CA) supplemented with HEPS (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. 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, 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. F(ab′)2 fragments of the SLE-IgG were prepared using the F(ab′)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 (Bio-Plex; Bio-Rad, Hercules, CA). The IFN-α concentration was measured with a dissociation-enhanced lanthanide fluoroimmunoassay, 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 super- natants 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 FcγRII (KB61; Santa Cruz Biotechnology, Santa Cruz, CA) or FcγRIII (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 (B2-1; British Bio-Technology, Oxford, U.K.) was used to block FcγRII. Anti-CD11c mAb (B2-9; 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/clear 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).
Statistical analysis

Data were analyzed using GraphPad Prism software 5.0 (GraphPad Software, San Diego, CA). Differences between groups were analyzed by Mann–Whitney U or Wilcoxon signed rank test. A p value <0.05 was considered significant.

Results

NK cells promote the IFN-α production by RNA-IC-, ODN2216-, or HSV-stimulated 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 HSV or 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 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 RNA-IC–stimulated IFN-α production (Supplemental Fig. 1). A dose–response study demonstrated that the supernatants from RNA-IC–stimulated 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.
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 (Ctrl). A–E, The IFN-α concentration in supernatants was determined after 20 h with an immunoassay. F–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 < 0.01.

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 FcγRI, 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).
FIGURE 4. RNA-IC activates NK cells via FcyRIIIA to produce soluble factors that enhance the RNA-IC-induced IFN-α production by pDCs. A. Supernatants from NK cells stimulated with RNA-IC formed with either intact or F(ab′)2 fragments of SLE-IgG were added to RNA-IC–stimulated pDCs. B, NK cells were preincubated with indicated concentrations of mAbs against FcyRIII or FcyRII before stimulation with RNA-IC. Supernatants were harvested and ultrafiltrated to remove the mAbs before being added to RNA-IC–stimulated pDCs. C, NK cells were preincubated for 30 min in the presence of indicated concentrations of chloroquine before being stimulated with RNA-IC. Supernatants were harvested, dialyzed, and added to RNA-IC–stimulated pDCs. D, NK cells were stimulated with RNA-IC or HAIG at indicated concentrations. Supernatants were harvested and ultrafiltrated to remove RNA-IC or HAIG before being added to RNA-IC–stimulated pDCs. A–D, IFN-α concentrations were measured with immunoassay after 20 h. Data (mean ± SD of duplicate wells) are representative of at least two independent experiments.

Thus, FcyRIIIA ligation on NK cells is sufficient to induce the production of soluble factor(s) that can promote the IFN-α production by pDCs.

NK cell-derived MIP-1β enhances the IFN-α production by pDCs

To identify the NK cell-derived factor(s) responsible for the stimulation of pDCs, we analyzed the supernatants from NK cells stimulated by RNA-IC, HAIG, or IL-12 and IL-18 for 17 different cytokines (Fig. 5A). The cytokine profiles from RNA-IC– and HAIG-stimulated NK cells were indistinguishable. Of the cytokines examined after FcyR stimulation, MIP-1β was produced in the highest amount (>15 ng/ml), and substantial amounts of MIP-1α, RANTES, TNF-α, and IFN-γ were also produced. These findings are consistent with previous reports (23).

The cytokine secretion profile between IL-12/IL-18– and FcyR-stimulated NK cells was quite similar with MIP-α, MIP-1β, RANTES, and TNF-α produced in comparable levels. However, IL-12/IL-18 stimulation led to a >500-fold increase in IFN-γ production compared with RNA-IC stimulation. Furthermore, IL-12/IL-18 stimulation induced secretion of GM-CSF and TNF-β, which were weakly or not produced upon FcyR activation of NK cells (Fig. 5A).

Having identified several cytokines secreted by RNA-IC– or IL-12/IL-18–stimulated NK cells, we investigated whether any of these contributed to the stimulatory effect seen on IFN-α production by pDCs. Inhibition with neutralizing Abs targeting IFN-γ, MIP-1α, 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. 6D). 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 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 donors 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 CD56bright NK cells and/or a downregulation of CD16 on CD56dim NK cells. When comparing the mean fluorescent intensity of CD16 on CD56dim NK cells, no significant difference between patients with SLE and healthy controls was seen (data not shown).
However, NK cells from patients with SLE contained a higher proportion of CD56 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-$\alpha$
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 epithelial pathology. Our main findings were that NK cells activated by IC via FcγRIIA 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 used were 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 costimulation to fully activate the IFN-α production. These results explain why CD56bright NK cells, which lack FcγRIIA, are unresponsive to RNA-IC, but can be activated by IL-12 and IL-18.

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 FcγRIIA 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 FcγRIIA 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, FcγRIIA ligation of NK cells was sufficient to induce the factors stimulating pDCs. These results explain why CD56bright NK cells, which lack FcγRIIA, 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 FcγRIIA 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...
MIP-1β alone, we cannot exclude that other soluble factors also are stimulatory for pDCs, including the other CCR5-stimulating chemokines MIP-1α and RANTES that were produced at lower levels by the NK cells.

Formation of aggregates containing both NK cells and pDCs was observed when the cells were stimulated with RNA-IC. Because NK cells were clearly superior in promoting pDC function compared with supernatants from RNA-IC–stimulated NK cells, we explored whether cell adhesion was involved in the increased IFN-α production. Indeed, inhibition of LFA-1 by Abs in pDC–NK cell cocultures had a strong negative impact on the IFN-α production. Besides a direct effect of LFA-1 signaling to pDCs, several other possible mechanisms for the LFA-1–mediated increase in IFN-α exist. For example, cell adhesion results in much higher local concentrations of cytokines and can also facilitate interactions between other activating cell surface receptors. In addition, co-stimulation of FcγRIIA and LFA-1 has previously been shown to increase the cytokine production by NK cells (23). In support of this, we noted a prominent increase in the production of the cytokines IP-10, MIP-1α, MIP-1β, RANTES, IL-8, IL-6, TNF-α, and IFN-γ when pDCs and NK cells were cocultivated. Abs to LFA-1 reduced the production of these cytokines to various extents. Consequently, an LFA-1–dependent cross-talk between pDCs and NK cells increases the production of several cytokines of importance for the inflammatory response and activation of the adaptive immune system. This included chemokines such as MIP-1β, which promotes the production of IFN-α by pDCs.

Patients with SLE have interferogenic ICs (38) together with an increased expression of type I IFN-regulated genes (39, 40), referred to as the IFN signature. In addition, increased serum levels of IL-12 (41), IL-18 (42), and MIP-1β (43), as well as an up-regulation of LFA-1 (44), have been reported in patients with SLE. Thus, all components for an efficient pDC–NK cell interaction are available to generate an ongoing IFN-α production, which will sustain the autoimmune process. Our observations could therefore be relevant for the understanding of the pathogenesis of IC-mediated autoimmune diseases such as SLE. Prior studies have shown reduced numbers of circulating NK cells and impaired NK cell cytotoxicity in SLE patients (45, 46). However, the regulatory function of NK cells from patients with SLE on IFN-α production has to date not been studied. We found that even though NK cells from SLE patients could increase the IFN-α production strongly, they were less stimulatory than NK cells from healthy donors when cocultivated with RNA-IC–stimulated pDCs. The weaker response to RNA-IC by SLE–NK cells could not be explained by a difference in FcγRIIA expression on CD56dim NK cells. However, consistent with data from Schepis et al. (47), the patients with SLE had an increased proportion of CD56bright NK cells in the blood. Thus, NK cells not responding to ICs are more prevalent in the circulation of patients with SLE than healthy individuals. An important observation was, however, that addition of IL-12 and IL-18 rendered the NK cells from patients with SLE as efficient as NK cells from healthy donors in promoting the IFN-α production. Whether costimulation with IL-12 and IL-18 better reflects the actual in vivo situation for patients with SLE is not known, but given the fact that these cytokines are upregulated in these patients, this might be the case. Interestingly, MIP-1β production was also decreased in NK cells from patients with SLE. 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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Disclosures
The authors have no financial conflicts of interest.

References
**Supplementary Figure 1.** The soluble factors produced by RNA-IC- or IL-12/IL-18-stimulated NK cells do not induce IFN-α by pDCs. Supernatants from A, RNA-IC- (NK-RNA-IC sup.) or B, IL-12/IL-18-stimulated (NK-IL12/18 sup.) NK cells were added to RNA-IC- or non-stimulated pDCs. When indicated supernatants from RNA-IC-stimulated NK cells were ultra-filtrated through a filter with MWCO of 100 kDa to remove RNA-IC. IFN-α production was determined after 20 h with an immunoassay. Data are presented as mean+SD from duplicate wells.

**Supplementary Figure 2.** Blocking of LFA-1 does not affect cytokine production by pDCs or NK cells cultured separately (A) pDCs were either cultured together with NK cells and stimulated with RNA-IC or cultured alone and stimulated with RNA-IC, HSV (10 %) or ODN 2216 (3μg/ml). RNA-IC-stimulated pDCs were supplemented with IFNα2β (500 U/ml, IntronA;Schering-Plough). Cells were cultured in the presence or absence of 1 μg/ml anti-CD11a mAb and IFN-α was measured in 20 h cell culture supernatants by immunoassay. (B) NK cells were stimulated with RNA-IC in the presence or absence of anti-CD11a mAb (1 μg/ml) for 16 h. Concentrations of cytokines in the culture supernatants, except for IFN-α, were determined using a multiplex bead based immunoassay and the IFN-α concentration was determined by a separate immunoassay. Data (mean+SD) from two different blood donors are shown.