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The Reciprocal Interaction of NK Cells with Plasmacytoid or Myeloid Dendritic Cells Profoundly Affects Innate Resistance Functions

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A reciprocal activating interaction between NK cells and dendritic cells (DC) has been suggested to play a role in the functional regulation of these cells in immunity, but it has been studied only using in vitro generated bone marrow- or monocyte-derived DC. We report that human peripheral blood plasmacytoid DC (pDC) and myeloid DC are necessary to induce NK cell function depending on the type of microbial stimulus. pDC and myeloid DC are required for strongly increased NK cytolytic activity and CD69 expression, in response to inactivated influenza virus or CpG-containing oligonucleotides and poly(I:C), respectively. Secreted type I IFN is required and sufficient for the augmentation of NK cell cytolytic activity in the coculture with pDC or myeloid DC, whereas CD69 expression is dependent on both type I IFN and TNF. In addition, in response to poly(I:C), myeloid DC induce NK cells to produce IFN-γ through a mechanism dependent on both IL-12 secretion and cell contact between NK cells and myeloid DC, but independent of type I IFN. IL-2-activated NK cells have little to no cytolytic activity for immature myeloid DC and pDC, but are able to induce maturation of these cells. Moreover, IL-2-activated NK cells induce, in the presence of a suboptimal concentration of CpG-containing oligonucleotides, a strong IFN-α and TNF production. These data suggest that the reciprocal functional interaction between NK cells and other pDC or myeloid DC may play an important physiological role in the regulation of both innate resistance and adaptive immunity to infections.

Dendritic cells (DC) recognize, through different receptors, conserved molecular structures of pathogens (1). In response to these structures, DC release several pro-inflammatory cytokines at an early time during infection activating the defensive mechanisms of inflammation and innate resistance. These structures also signal DC to acquire a mature phenotype characterized by functional and migratory properties that allow them to mediate efficient Ag presentation and activation of naïve T lymphocytes, thus inducing primary adaptive immune responses (2). Two distinct subsets of DC can be found in human peripheral blood, plasmacytoid DC (pDC) and myeloid DC (3). These DC subsets are characterized by a distinct pattern of expression of TLR (4). Myeloid DC are characterized by expression of TLR4 and TLR3, mediating the response to LPS and poly(I:C), respectively, in contrast, pDC express TLR7 and produce IFN-α with production of IL-12. Myeloid DC are characterized by expression of TLR4 and subsets are characterized by a distinct pattern of expression of TLR (5). The Reciprocal Interaction of NK Cells with Plasmacytoid or Myeloid D

Materials and Methods

Cells and cell culture

PBMC obtained from healthy human donors (provided by Centro Trasfusionale, Policlinico GB Rossi, Verona, Italy) were separated by Ficoll-Paque density gradient centrifugation. pDC were obtained from PBMC by positive magnetic selection with blood DC Ag (BDCA)-4 beads (Miltenyi Biotech, Auburn, CA). We report that human peripheral blood plasmacytoid DC (pDC) and myeloid DC are necessary to induce NK cell function depending on the type of microbial stimulus. pDC and myeloid DC are required for strongly increased NK cytolytic activity and CD69 expression, in response to inactivated influenza virus or CpG-containing oligonucleotides and poly(I:C), respectively. Secreted type I IFN is required and sufficient for the augmentation of NK cell cytolytic activity in the coculture with pDC or myeloid DC, whereas CD69 expression is dependent on both type I IFN and TNF. In addition, in response to poly(I:C), myeloid DC induce NK cells to produce IFN-γ through a mechanism dependent on both IL-12 secretion and cell contact between NK cells and myeloid DC, but independent of type I IFN. IL-2-activated NK cells have little to no cytolytic activity for immature myeloid DC and pDC, but are able to induce maturation of these cells. Moreover, IL-2-activated NK cells induce, in the presence of a suboptimal concentration of CpG-containing oligonucleotides, a strong IFN-α and TNF production. These data suggest that the reciprocal functional interaction between NK cells and other pDC or myeloid DC may play an important physiological role in the regulation of both innate resistance and adaptive immunity to infections. The Journal of Immunology, 2005, 174: 727–734.

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Biotec). Purified cells were >98% pDC, as evaluated by immunofluorescence with BDCA-2-FITC mAb. Myeloid DC were obtained from PBMC by depletion of CD19+ B lymphocytes and of CD14+ cells with anti-CD19 and anti-CD14 microbeads followed by positive selection with CD1b monoclonal antibody and antibiotin microbeads (Miltenyi Biotec). Purified cells were >98% CD1c+ as evaluated labeling cells with Streptavidin-PE. Monocyte-de-
dicated DC were obtained by 5-day culture of plastic adherent PBMC in media with 12 ng/ml IL-4 (10 U/mg; Schering Plough) and 50 ng/ml GM-CSF (1.1×10^5 U/mg; Schering Plough). NK cells were purified by negative magnetic depletion using anti-CD3, anti-CD4, anti-CD19, anti-
CD14, anti-CD45RO mAb, and anti-HLA-DR mAb, and goat anti-mouse IgG microbeads (Miltenyi Biotec) plus BDCA-4 microbeads. Purified cells were >90% CD16+CD56+ cells. For experiments of cytolytic activity of NK cells cultured with pDC or myeloid DC in the presence of the different stimulants, NK cell preparations were further purified by positive selection using anti-CD56 microbeads (Miltenyi Biotec). Purified CD4+ T lymphocytes were obtained from PBMC by negative magnetic depletion using anti-CD19, anti-CD14, anti-CD16, anti-CD8 mAb, goat-anti-mouse IgG microbeads, and anti-CD56 microbeads. NK cells (0.18×10^6/well) and pDC or myeloid DC (12.5% or as indicated) were cultured in round-bottom microtiter plates with or without the following stimuli: formaldehyde-inacti-
Vated human influenza virus (strain Beijing/262/95 kindly provided by N. Kuehn, Aventis Pasteur, Val de Reuil, France) at a final concentration of 40
ng/ml or as indicated (MWG Biotech); CpG2006 (TCGTTTTGTCGTT) 10 µg/ml (MWG Biotech); or poly(I:C) at 10 µg/ml (InvivoGen). All these compounds were endotoxin-free (<0.05 IU/mg).

Where indicated pDC were treated with virus for 18 h or pulsed with virus for 1 h and further incubated for additional 18 h. Untreated pDC were
kept on ice in 100% FCS. Cells or their supernatant were cultured with NK
virus for 1 h and further incubated for additional 18 h. Untreated pDC were
used immediately after purification.

To evaluate requirement for direct cell-cell contacts, NK cells and pDC or myeloid DC were cultured together or separated by a 0.4-µm porous membrane (Corning).

Cell-mediated cytotoxicity assay
At the end of an 18-h culture, NK cells cultured alone or with pDC or myeloid DC in the presence or in the absence of the different stimuli, or with virus-treated pDC were incubated at different E:T ratios in U-bottom

microtiter plates, with 6×10^3 Daudi cells loaded with 5 µM carboxy-
fluorescin diacetate succinimidyl ester CFDA-SE (Molecular Probes) for 15 min. After 4 h each sample was resuspended in a final volume of 500
µl of PBS, to which 50 µl of Flow Check fluorospheres (Beckman Coulter) were added. Using the cytofluorimeter, events were acquired for a fixed
time and the percentage of killing was calculated using the following equa-
tion: [(the number of CFDA-SE+ cells acquired in the control without NK
cells) – (the number of CFDA-SE- cells in the sample)]/[the number of
CFDA-SE- cells in the control]]×100 (14). The regularity of cytometer
flow was checked by the constancy of the Flow Check fluorospheres.

Immunofluorescence
Expression of the Ags CD83, CD80, and CD86 was detected on pDC and on myeloid DC by direct double immunofluorescence and flow cytometry using PE-conjugated anti-CD86 and anti-CD80 mAb (Immunotech; Beck-
man Coulter) with FITC-conjugated BDCA-2 for pDC or FITC-conjugated anti-DR for myeloid DC, and FITC-conjugated anti-CD83 mAb with PE-
conjugated BDCA-2 for pDC or PE-conjugated anti-DR for myeloid DC. Because CD83 and CD86 Ags were spontaneously up-regulated in my-
eloid DC in culture (data not shown), their evaluation was not included in this study. CD69 Ag expression was detected on CD16+ or CD3+ purified T lymphocytes after 18-h coculture with pDC or myeloid DC in the presence or in the absence of stimuli, or with virus-treated pDC or pDC kept on ice, by double staining with PE-Cy5-conjugated anti-
CD69 (Immunotech; Beckman Coulter) mAb and FITC-conjugated anti-
CD3 or anti-CD16 mAb.

DC recovery after an 18-h coculture with IL-2-activated NK cells was evaluated as a percentage of DC cultured in the absence of NK cells and in the presence of virus or medium, respectively, for pDC and myeloid DC or monocyte-derived DC, counting the number of BDCA-4+/H11001 cells for pDC, HLA-
DR+/polyIC for untreated and GM-CSF + LPS-treated myeloid DC, and by cytometry side scatter and forward scatter parameters for untreated and LPS-treated monocyte-derived DC, in a time unit, in the region of viable cells, as confirmed by propidium iodide incorporation.

Detection of IFN-γ, IFN-α, and TNF production
These cytokines were quantitated in cell culture supernatants by ELISA
kits (Duoset for IFN-γ and TNF; Bender MedSystems; and PeliKine Compact (CLB Systems) for TNF).

Results
Different stimuli selectively induce either pDC or myeloid DC to activate NK cell functions
Although inactivated influenza virus, poly(I:C), CpG2216, or CpG2006 strongly enhanced NK cell-mediated cytotoxicity against the NK-resistant Daudi cell line when added to PBMC (Refs. 6, 16, 17 and data not shown), culture for 18 h in the presence of these stimuli did not endow purified human peripheral blood NK cells with cytolytic activity (Fig. 1A). Also,
no cytolysis was induced in NK cells cocultured with up to 5% pDC or myeloid DC in the absence of other stimuli. However, NK cells cocultured with as few as 0.2% of autologous pDC in the presence of virus (Fig. 1A, middle panel) or of myeloid DC in the presence of poly(I:C) (Fig. 1A, right panel) acquired a strong cytolytic activity. At a concentration of pDC of 5%, poly(I:C), CpG2216, and CpG2006 also induced strong NK cell cytolytic activity. Unlike what was observed with pDC, coculture of NK cells with up to 5% myeloid DC in the presence of virus, CpG2216, or CpG2006 did not induce lytic activity.

Purified NK cells cultured in medium or in the presence of the different stimuli did not produce IFN-γ (data not shown). High IFN-γ production was observed in the supernatant fluid of NK cells cocultured with ≥1% myeloid DC in the presence of poly(I:C) but not of virus, CpG2216, CpG2006, or in the absence of any stimulus (Fig. 1, B and C). In contrast, IFN-γ was almost undetectable in supernatants of NK cells cultured with pDC independently of the stimuli used (Fig. 1B).

**Activation of NK cells by virus-stimulated pDC depends on type I IFN production**

Plasmacytoid DC either cultured for 18 h in the presence of virus and washed (virus-preactivated pDC) or continuously cultured in the presence of virus (Fig. 2, A–C) were similarly effective in inducing cytolytic activity in cocultured NK cells. Virus-preactivated pDC also induced a strong CD69 expression in NK cells (Fig. 2E), but not in T lymphocytes (data not shown).

To evaluate whether soluble factors play a role in cytolytic activity and CD69 expression induced in NK cells by virus-preactivated pDC, cells were cocultured together in the same wells, or separated by a porous membrane in transwell plates. Cytolytic activity in NK cells cocultured with as few as 0.2% of autologous pDC in the presence or in the absence of poly(I:C) was comparable to those induced by virus-preactivated pDC, indicating that soluble factors accounted for the activation of NK cells. NK cells cultured across a transwell plate with virus-preactivated pDC were able, depending on the NK cell to DC ratio, to either lyse immature monocyte-derived DC (data not shown) or to induce their maturation as detected by CD86 expression (Fig. 2D) similar to what we have previously shown for IL-2-activated NK cells (13).

We evaluated the role of type I IFN in the induction of CD69 expression and cytolytic activity in NK cells cultured with virus-activated pDC or with their supernatant fluids. A mixture of Abs neutralizing IFN-α, IFN-β, and IFN-αβ receptor reduced the cytolytic activity induced in NK cells by pDC in the presence of virus (Fig. 2C) and by virus-pretreated pDC (Fig. 2B), and completely inhibited the cytolytic activity induced by the supernatant fluid derived from virus-activated pDC (Fig. 2B). The Abs also completely abrogated CD69 expression induced in NK cells by IFN-α (Fig. 2H), but only reduced CD69 expression induced in NK cells by virus-activated pDC (Fig. 2F) and by their supernatant fluid (Fig. 2G). Whereas TNF by itself did not induce cytolytic activity in NK cells, it increased the cytolytic activity (data not shown) and CD69 expression (Fig. 2F) induced by recombinant IFN-α. The increased cytolytic activity induced by TNF was abrogated by a TNF-neutralizing mAb (data not shown). pDC produced TNF when activated by different stimuli (see Fig. 4). Addition of

![FIGURE 2](http://www.jimmunol.org/)

Cytolytic activity and CD69 expression induced in NK cells by activated pDC are dependent on type I IFN. A and E, pDC were kept on ice or cultured for 18 h with virus and washed before coculture with purified NK cells together or separately by a transwell. B, F, and G, pDC were pulsed for 1 h with virus, washed and further incubated in medium. After 18 h, pDC (B and F) or their supernatant fluids (B and G) were added to purified NK cells in the presence or in the absence of a mixture of anti-IFN-αβ Abs (25 U/ml) and anti-TNF mAb. C, NK cells were cultured with or without pDC in the presence of virus ± anti-IFN-αβ Abs or anti-IL-12 mAb. D, NK cells were incubated with medium or IL-2 (500 U/ml), or cultured in transwell with pDC that had been pulsed for 1 h with virus. After 18 h NK cells were washed and further cultured for 18 h with monocyte-derived DC at a 2:1 ratio. H and I, NK cells were incubated with or without IFN-α (500 U/ml) in the presence or in the absence of the mixture of anti-IFN-αβ Abs (50 U/ml) or of TNF or anti-TNF mAb (I). Cytolytic activity against Daudi targets (A–C), CD69 expression on transwell, as defined by cytofluorimetric side scatter and forward scatter parameters (D) and CD69 expression on CD16+ NK cells (E–I) were evaluated after 18 h. Mean fluorescence intensity for each histogram (D–I) is reported by number in brackets. Similar results were obtained in at least three different experiments.
the TNF-neutralizing mAb to the mixture of type I IFN-neutralizing Abs, however, did not further reduce the cytolytic activity induced by virus-pretreated pDC or by their supernatant fluid (data not shown). In contrast, the anti-TNF mAb, added to the mixture of type I IFN-neutralizing Abs, strongly reduced CD69 expression induced by virus-pretreated pDC (Fig. 2F) and by their supernatant fluid (Fig. 2G). A neutralizing anti-IL-12 mAb had no effect on the augmentation of cytolytic activity (Fig. 2C) or on CD69 expression (data not shown).

CD69 expression and cytolytic activity induced by myeloid DC in NK cells depend on secreted type I IFN, whereas IFN-γ production depends on IL-12 and cell-to-cell contact

Myeloid DC in the presence of poly(I:C) induced a strong CD69 expression in NK cells (Fig. 3A) in addition to increasing their cytolytic activity (Figs. 1A and 3B). Both activating effects were observed when NK cells and myeloid DC were either cultured together or separated by a porous membrane (Fig. 3, A and B, left panels), indicating that soluble factors mediated the activation.

To evaluate the relevance of type I IFN among possible soluble factors responsible for activation of NK cells by myeloid DC in the presence of poly(I:C), cells were cultured in the presence of the mixture of type I IFN-neutralizing Abs. These Abs strongly reduced CD69 expression (Fig. 3A, right panel) and almost completely inhibited the cytolytic activity (Fig. 3B, right panel) induced in NK cells. An IL-12-neutralizing mAb did not have any effect on cytolytic activity (Fig. 3B, right panel) or CD69 expression (data not shown), whereas addition of a TNF-neutralizing mAb to the mixture of type I IFN-neutralizing Abs further reduced CD69 expression induced in NK cells by myeloid DC in the presence of poly(I:C) (Fig. 3A, right panel).

The IFN-γ production induced in NK cells by coculture with myeloid DC plus poly(I:C) was drastically reduced by the IL-12-neutralizing mAb, whereas the type I IFN-neutralizing Abs had almost no effect (Fig. 3C, right panel). IFN-γ production was completely inhibited when NK cells and myeloid DC, in the presence of poly(I:C), were separated by a porous membrane in a transwell plate (Fig. 3C, left panel) indicating that both soluble factors such as IL-12 and cell-to-cell interactions were required for IFN-γ induction by poly(I:C).

**FIGURE 3.** Cytolytic activity and CD69 expression induced in NK cells by myeloid DC in the presence of poly(I:C) is dependent on type I IFN, whereas IFN-γ production is dependent on IL-12 secretion and cell contact. NK cells were cultured in the presence or in the absence of myeloid DC (10% in A and B, left, and C or with 1.25% in B, right) with or without poly(I:C). Cells were cocultured together or separately across a transwell (A–C, left), in the presence or in the absence of a mixture of anti-IFN-α/β Abs (A–C, right), anti-IFN-α/β Abs plus anti-TNF mAb (A, right), or anti-IL-12 mAb (B and C, right). CD69 expression on CD16+ NK cells, mean fluorescence intensity in brackets (A), cytolytic activity (B) are shown, and are results from one of two similar experiments, and IFN-γ production (mean ± SE, n = 3) (C) were evaluated after 18 h.

**IL-2-activated NK cells interact with pDC and myeloid DC**

As previously reported (13, 19, 20), IL-2-activated NK cells were cytolytic for immature, but not mature monocyte-derived DC (Fig. 4A). However, IL-2-activated NK cells did not lead to any loss in the recovery of autologous or allogeneic pDC cocultured with them (Fig. 4A), indicating that pDC, unlike immature monocyte-derived DC, are resistant to NK cell-mediated cytotoxicity.

Upon culture together with IL-2-activated, but not with untreated NK cells, pDC strongly increased CD83 expression (Fig. 4B, left panel). This increased expression was not observed when cells were cultured separately porous membrane in a transwell plate (Fig. 4B, right panel). No significant increase in CD86 or CD80 expression was observed (data not shown).

IFN-α production was undetectable in supernatant fluids of pDC cultured alone or with untreated NK cells, and almost undetectable in supernatants of pDC cultured with IL-2-activated NK cells (Fig. 4, C and D). The class A CpG ODN CpG2216 added to purified pDC strongly induced IFN-α production at 10 μg/ml, as expected, but not at submicrogram concentrations. However, IFN-α production was strongly enhanced when pDC were cultured with IL-2-activated NK cells in the presence of suboptimal concentrations of CpG2216 that did not induce any detectable IFN-α in pDC alone (Fig. 4, C and D). IFN-α production in response CpG2216 was only slightly increased when pDC were cultured with resting NK cells or with IL-2-treated T lymphocytes. The class B CpG ODN CpG2006 added to pDC induced a very modest IFN-α production at all the concentrations used. Interestingly, in the presence of IL-2-activated NK, pDC stimulated by 0.2–0.75 μg/ml CpG2006 produced high levels of IFN-α (Fig. 4C). A strong TNF production was also observed in supernatants of pDC cocultured with IL-2-activated NK cells in the presence of suboptimal amounts of CpG2216 (Fig. 4D). When IL-2-activated NK cells were cultured with pDC separated by a porous membrane, IFN-α (Fig. 4E) and TNF (data not shown) were no longer detected in supernatant fluids.

The presence in culture of IL-2-activated NK cells only reduced slightly the recovery of freshly separated peripheral blood allogeneic myeloid DC at the highest NK cell to DC ratio (Fig. 5A). A loss of both autologous and allogeneic immature myeloid DC, however, was observed when DC were maintained for 18 h at 4°C, and the cytotoxic effect of NK cells was more pronounced on allogeneic than autologous myeloid DC (data not shown). Coculture with IL-2-activated NK cells induced a strong expression of CD80 maturation Ag in myeloid DC (Fig. 5, A–C), comparable to the CD80 expression induced by treatment of myeloid DC with GM-CSF and LPS (Fig. 5B, left panel) or poly(I:C) (data not shown).
CD80 expression was not increased when myeloid DC were cultured with IL-2-activated NK cells separated by a porous membrane in a transwell plate (Fig. 5B, right panel). NK cells exposed to virus-preactivated pDC were able to induce CD80 expression on myeloid DC similarly to IL-2-activated NK cells (data not shown). Untreated NK cells or either untreated or IL-2-activated CD4+ CD56+ T lymphocytes did not induce CD80 expression (Fig. 5, B and C).

Discussion

In this paper we analyze the cross-talk between NK cells and the two major populations of human peripheral blood-derived DC. We found that ex vivo isolated pDC or myeloid DC induce functional activation of NK cells in response to distinct microbial stimuli or compounds mimicking them and that pDC are instrumental for the NK cell response to inactivated influenza virus. Reciprocally, we found that IL-2-activated NK cells interact with pDC and with myeloid DC inducing their maturation.

As low as 0.2% pDC, in the presence of inactivated influenza virus or pre-exposed to it, induces in NK cells a strong cytolytic activity against the NK cell-resistant Daudi target cells. This enhancement of NK cell-mediated cytotoxicity is dependent on secretion of type I IFN. In addition to their increased cytotoxic activity against target cell lines, NK cells activated by pDC upregulated the expression of the CD69 activation Ag and were also able either to lyse or to induce maturation of monocyte-derived DC and peripheral blood myeloid DC. pDC have been shown to be identical with the natural type I IFN producing cells, the rare HLA-DR-positive accessory cells in human peripheral blood responsible for type I IFN production in response to most viruses (7, 8). The essential role of IFN-α produced by these HLA-DR-positive accessory cells for the cytolytic activity of NK cells against virus-infected target has been previously demonstrated (9) and these accessory cells have been differentiated from classical peripheral blood DC by conventional gradient separation methods. In this study we definitely discriminate the unique role of highly purified pDC in induction of cytolytic activity of NK cells in response to viral stimulation because the other population of HLA-DR-positive accessory cells in peripheral blood, myeloid DC, although very efficient in inducing cytolytic activity in cocultured NK cells in response to poly(I:C), do not induce cytolytic activity in response to virus.

A requirement for secreted type I IFN was also observed for induction of CD69 expression in NK cells by both pDC and myeloid DC, although for optimal induction TNF also was required. Type I IFN secreted by both pDC and myeloid DC in response to viral stimulation because the other population of HLA-DR-positive accessory cells in peripheral blood, myeloid DC, although very efficient in inducing cytolytic activity in cocultured NK cells in response to poly(I:C), do not induce cytolytic activity in response to virus.

FIGURE 4. IL-2-activated NK cells activate pDC. A, pDC from different donors (a, b, and c) or immature or LPS-treated monocyte-derived DC (donor d) were cultured with autologous or allogeneic IL-2-activated NK cells from different donors (a, b, and c) at the indicated NK cell to DC ratios. B and C, pDC were cultured in medium alone or with untreated or IL-2-activated NK cells (1:2 ratio) together (B, left) or separated by a transwell (B, right), in the presence of different concentrations of CpG2216 (C, top) (mean ± SE, n = 4) or CpG2006 (C, bottom) Results are from two different experiments. D, pDC were cultured with IL-2-activated NK cells or IL-2-treated CD4+ T lymphocytes (triplets of histograms represent 1:4, 1:2, 1:1 ratios) in the presence of different concentrations of CpG2216. At the CpG2216 concentration of 4 μg/ml only 1:4 and 1:2 ratios were performed. E, pDC were cultured with IL-2-activated NK cells (1:2 ratio) together or separated by a transwell, in the presence of 0.2 μg/ml CpG2216. Results are from two different experiments. After 18 h of culture pDC and monocyte-derived DC recovery (A), calculated as a percentage of DC cultured, respectively, with virus or medium alone, in the absence of NK cells, was evaluated by BDCA-4-FITC mAb for pDC or cytometric side scatter and forward scatter parameters for monocyte-derived DC, in the region of viable cells; CD83 expression (B) and mean fluorescence intensity shown in brackets on BDCA-4+ cells was evaluated by double immunofluorescence; IFN-α (C and D, top and E) and TNF (D, bottom) production were tested in the supernatants.
The capacity of pDC and myeloid DC to activate NK cell functions depends on the selectivity of the stimulus for receptors expressed on the DC population. The ability of pDC to produce type I IFN in response to virus is presently attributed to their high expression of TLR9 recognizing DNA viruses and TLR7 recognizing RNA viruses such as influenza virus (4, 22–25). Productive infection is not required for IFN production by pDC and inactivated virus is effective (26). Both the TLR9 ligands CpG2216 and CpG2006, which have been reported to induce IFN-α and IFN-β production by pDC, although at different amounts and with different kinetics (27), endow these cells with the ability to activate NK cells. The maximal amounts of IFN-α produced by high concentrations of pDC stimulated by CpG2216 are comparable to those produced by pDC stimulated by virus but it is much lower in culture with low pDC concentration (data not shown). Accordingly, in the presence of CpG2216, the number of pDC required to stimulate NK cytolytic activity is higher than in the presence of virus.

Myeloid DC express TLR3, but not TLR9 (4). Indeed, we found that myeloid DC, even at a very low DC to NK cell ratio, induce cytolytic activity and IFN-γ production in NK cells when stimulated by the TLR3 ligand poly(I:C), but not by TLR9 ligands. Thus, in the presence of a selective stimulus, myeloid DC efficiently activate NK cells. pDC, which have been reported to be TLR3-negative (4), in the presence of poly(I:C), consistently produced a very low amount of IFN-α, up-regulated CD83 expression (data not shown) and, at high concentration, induced NK cell cytolytic activity. Although it cannot be excluded that a residual presence of a small contamination with other accessory cells could account for this finding, it is possible that TLR3 is up-regulated on pDC upon activation or that a TLR-independent stimulatory pathway mediates the poly(I:C) effect on pDC (26, 28).

Activation of NK cells required the presence of either pDC or myeloid DC, depending on the stimulus, whereas purified NK cells alone could not respond to any of the stimuli used. However, activation of purified NK cells by poly(I:C) has been previously reported (29). The explanation of these contrasting results might rest in the NK purification procedure used in this study that includes a careful depletion of HLA-DR^+ cells before enrichment of CD56^+ cells: indeed, we observed that even a minimal contamination with accessory cells renders the NK cell preparations responsive to poly(I:C).

The ability of myeloid DC in the presence of poly(I:C) to induce in NK cells a strong IFN-γ production that is drastically reduced by an IL-12-neutralizing mAb is consistent with the capacity of myeloid DC to produce IL-12 p70 when cultured with poly(I:C) (Ref. 30 and data not shown). However, in addition to secreted IL-12, IFN-γ production required direct cell contact between myeloid DC and NK cells. IFN-γ was almost undetectable in supernatant fluid from NK cells cultured with freshly isolated pDC that, at least in response to the stimuli used in the present study, do no produce IL-12 (Ref. 31 and data not shown). In other studies, it has been shown that several days culture in IL-3 may endow pDC with the ability to produce IL-2 and to promote IFN-γ production (31, 32).

NK cells did not produce IFN-γ when cultured with pDC in the presence of stimuli such as virus or CpG2216 that induce very high levels of type I IFN. Neutralizing anti-IFN-αβ Abs also did not affect IFN-γ produced by NK cells cultured with myeloid DC in the presence of poly(I:C). Thus, type I IFN does not appear to play a major role in inducing IFN-γ production by human NK cells either alone or in combination with IL-12, even in the presence of pDC or myeloid DC.

The capacity of activated NK cells to interact with in vitro generated human monocyte-derived DC inducing their functional maturation has been reported (13, 14, 33). In this study we show that IL-2-activated NK cells profoundly affect the maturation and functions of pDC and myeloid DC, freshly isolated from peripheral blood.

The functional maturation of pDC cultured with IL-2-activated NK cells was revealed by increased expression of CD83 and by a dramatic up-regulation of IFN-α and TNF production in the presence of suboptimal CpG ODN concentrations. The maturation of myeloid DC was revealed by increased expression of CD80 Ag. The maturation of both pDC and myeloid DC was induced by IL-2-activated NK cells in the absence of any detectable cell lysis. The low myeloid DC lysis observed after culture only with elevated numbers of NK cells might suggest a possible regulatory role depending on the level of activation and the number of NK cells, in analogy to previous reported results with immature monocyte-derived DC (13, 14).
The reason of the different susceptibility to NK cell-mediated lysis of immature monocyte-derived DC compared with freshly isolated pDC and myeloid DC is not clear at the moment. It is possible that peripheral blood myeloid DC are in a more mature stage than monocyte-derived DC and, thus, resistant to lysis. However, blood DC are likely precursor of tissue-associated DC and, physiologically, in an immature state. In addition to an intrinsic susceptibility to lysis, differential expression of certain MHC class I alleles, as in the case of the different resistance between immature and mature monocyte-derived DC, as well as the expression of adhesion molecules or of target molecules for activating NK cell receptors, for example the ligand for the Nkp30 receptor that has been shown to be involved in NK cell-mediated lysis of monocyte-derived DC (15), may be possible candidates as mechanisms for myeloid DC resistance to NK cell-mediated lysis.

Direct DC-to-NK cell contact is necessary for CD83 expression and IFN-α production from pDC, and for CD80 expression in myeloid DC. The surface molecules and possibly soluble factors involved in the cellular interaction between DC and IL-2-activated NK cells are at present unknown and preliminary experiments with Ab against a few candidate molecules have not yet provided definitive results.

pDC cultured with IL-2-activated NK cells up-regulated the expression of CD83 mature Ag and synergized with microbial stimuli for IFN-α production, but did not up-regulated CD80 or CD86 expression. It would be suggestive to explain the activation pattern of pDC exposed to IL-2-activated NK cells by the existence of different stimulatory pathways leading to differential induction of pDC maturation and/or IFN-α production. Indeed, pDC treated with optimal concentration of the class A CpG ODN CpG2216, a strong IFN-α inducer, increase the expression of CD83, but not CD80 or CD86 maturation Ags, whereas pDC treated with the class B CpG ODN CpG2006, a poor IFN-α inducer, up-regulate expression of all the three maturation Ags (Ref. 27 and data not shown). The molecular mechanisms underlying the different activity of class A and class B CpG ODN remain obscure, but it is intriguing that in the presence of IL-2-activated NK cells both class of ODN become equivalent in their ability to induce production of IFN-α at very low concentrations.

IL-2-activated NK cells up-regulated CD80 expression on myeloid DC, but did not lead to detectable production of IL-12 (data not shown). IL-12 p70 production is induced in myeloid DC by poly(I:C). In preliminary experiments, IL-12 production observed when myeloid DC are stimulated by poly(I:C) was not increased by coculture with IL-2-activated NK cells, and this result might be consistent with our finding that IL-12 p70 production in PBMC stimulated by poly(I:C) does not require exogenous IFN-γ, differently from other stimuli, such as R848 or LPS (G. Carra, unpublished observations). In the same experiments, in cocultures of IL-2-activated NK cells and myeloid DC stimulated by poly(I:C), increased IFN-α production was observed. Evaluation of the production of IL-12 p70, IFN-α as well as TNF after stimulation with different doses of stimuli such as R848 or LPS, other than poly(I: C), will be necessary to clarify the role of IL-2-activated NK cells on cytokine production by myeloid DC.

There are several lines of experimental evidence indicating that NK cells may have an important role not only in the early innate resistance to pathogens but also in favoring an effective immune response, particularly Th1-type response. Mouse NK cells produce IFN-γ early in Leishmania major, Listeria monocytogenes, and Chlamydia trachomatis infection and are needed both for innate resistance and for generation of a protective Th1 response (34–36). The ability of IL-12 to induce a Th1 response in vivo in the mouse and in vitro in humans has been shown to be optimal if NK cells are present (37, 38). Although other studies have not always confirmed an absolute requirement for NK cells in Th1 responses (39), in some cases, for example in NK cell-deficient mice immunized with keyhole limpet hemocyanin, the Th1-dependent IgG2a response was deficient even if T cell production of IFN-γ was elevated (40). In murine models has been demonstrated in vivo that NK cells activated by MHC class I<sup>+</sup> tumor cells prime DC to induce a protective CD8<sup>+</sup> T cell response and NK cells are required for CD8α<sup>+</sup> DC survival during murine cytomegalovirus infection (41, 42). Because NK cells have been identified primarily as circulating cells and DC as cells present in tissue or secondary lymphoid organs, the site of possible interaction between the two cells has been an unresolved issue raising doubts on the physiologic relevance of DC-NK cell interaction. It is becoming evident, however, that NK cell and DC in vivo migration may be similarly regulated and in particular DC and pDC have similar regulation of chemokine responsiveness (43). Thus, DC and NK cells may meet in inflamed or stressed tissues (44) and colocalization and direct contact between NK cells and DC was directly demonstrated in atopic skin lesions infected by the yeast Malassezia (45). NK cells are now known to be present in large number in secondary lymphoid organ parafollicular T cell zone, particularly as immature or CD56<sup>neg</sup>CD16<sup>+</sup> cells with low cytotoxic activity and high ability to produce cytokines (46, 47), indicating the secondary lymphoid organs in addition to inflamed tissues as a site of interaction for DC and NK cells (48).

Our results on the differential reciprocal interactions between human NK cells and ex vivo derived peripheral blood pDC or myeloid DC have by necessity been obtained using in vitro experimental conditions that may not fully reproduce the actual in vivo context. However, the use of freshly ex vivo isolated DC subsets rather than in vitro differentiated DC might contribute to clarify mechanisms underlying an immune response to infection by viruses or other pathogen in humans. In the first stages of an immune response, type I IFN produced by pDC, activated by viral or bacterial stimuli, may augment NK cell cytotoxic activity endorsing them with the ability to lyse infected cells, tumor cells. NK cells may further be activated by activation molecules, e.g., NKG2D ligands, expressed on infected cells or by down-modulation of HLA class I Ags (41, 49). Activated NK cells may lyse-infected cells providing antigenic material for a specific immune response but also may induce maturation of myeloid DC favoring naïve T lymphocytes priming. The enhanced type I IFN production by pDC, induced by activated NK cells, may increase myeloid DC recruitment, survival, and cross-presentation of Ag to CD8<sup>+</sup> T lymphocytes further contributing to the engagement of an acquired immune response (50–52). In response to TLR ligands including poly(I:C), a mimic of viral double-stranded RNA, produced by virally infected cells and released after their lysis, myeloid DC produce IL-12 and induce NK cells to produce IFN-γ. IFN-γ enhances, in turn, IL-12 production from accessory cells, thus favoring the development of a Th1-like T cell response (53). IL-2, released by Ag-specific T lymphocytes at the adaptive stage of the immune response, activates NK cells that, even in the presence of suboptimal levels of microbial stimuli, induce production of type I IFN and other proinflammatory cytokine by pDC and maturation of both pDC and myeloid DC, contributing to enhance a protective secondary T cell immune response.

References


