IL-12 or IL-4 Prime Human NK Cells to Mediate Functionally Divergent Interactions with Dendritic Cells or Tumors

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In the course of inflammatory responses in peripheral tissues, NK cells may be exposed to cytokines such as IL-12 and IL-4 released by other cell types that may influence their functional activities. In the present study we comparatively analyzed purified human peripheral blood NK cells that had been exposed to either IL-12 or IL-4 during short (overnight) incubation. We show that although IL-12-cultured NK cells produced abundant IFN-γ, TNF-α, and GM-CSF in response to stimuli acting on the NKp46-activating receptor, IL-4-cultured NK cells did not release detectable levels of these cytokines. In contrast, IL-4-cultured NK cells produced significant levels of TNF-α and GM-CSF only when stimulated with PMA and ionomycin. In no instance could the production of IL-5 and IL-13 be detected. Importantly, IL-12-cultured, but not IL-4-cultured, NK cells displayed strong cytolytic activity against various tumor cells or immature dendritic cells (DCs). Moreover, only NK cells that had been cultured in IL-12 were able to induce substantial DC maturation. Our data suggest that NK cells exposed to IL-12 for a time interval compatible with in vivo responses may favor the selection of appropriate mature DCs for subsequent Th1 cell priming in secondary lymphoid organs. On the contrary, NK cells exposed to IL-4 do not exert DC selection, may impair efficient Th1 priming, and favor either tolerogenic or Th2-type responses. The Journal of Immunology, 2005, 174: 3992–3998.
by DCs, this effect is transient and followed by a state of refractoriness to additional stimulation. The exhaustion of IL-12 production has an impact on the T cell-polarizing process, because soon after stimulation, DCs prime strong Th1 responses, whereas at later stages, the same cells preferentially prime Th2 as well as nonpolarized T cells (22). Importantly, the polarizing effects were largely IL-12 and IL-4 dependent, because they were prevented by cytokine-specific neutralizing Abs.

In the present study we comparatively analyzed the function of freshly isolated peripheral blood NK cells cultured for a short time in the presence of either IL-12 or IL-4. It must be stressed that the use of short term cytokine-induced NK cells is likely to mimic the in vivo effect of relevant cytokines. Indeed, in inflammatory sites, these must be secreted within minutes to hours to exert influence on priming of both innate and adaptive immune responses (23, 24).

We show that these different short-term-cultured NK cell populations display major differences in both cytokolytic activity and cytokine production. Our results suggest that IL-12- or IL-4-induced NK cells that are generated during acute tissue inflammation may differentially contribute to the promotion of downstream adaptive responses, including DC selection and T cell polarization.

Materials and Methods

Monoclonal Abs

The following mAbs, produced in our laboratory, were used in this study: JT3A (IgG2a; anti-CD3), BAB281 and KL247 (IgG1 and IgM, respectively; anti-NKp46), Z231 (IgG1; anti-NKp44), Z25 and F252 (IgG1 and IgM, respectively; anti-NKp30), ON72 (IgG1; anti-NKG2D), c127 (IgG1; anti-CD16), c218 and FS280 (IgG1 and IgGa2a, respectively; anti-CD56), c227 (IgG1; anti-CD69), MAR93 (IgG1; anti-CD25), F35 (IgG1; anti-CD244), MA127 (IgG1; anti-NTBA), MAR206 (IgG1; anti-CD122), KRA236 (IgG1; anti-CD226), c284 (IgG1; anti-CD11a), Z270 and Z199 (IgG1 and IgG2b, respectively; anti-NKG2A), GL183 (IgG1; anti-KIR2DL2/3), EB6 (IgG1; anti-KIR2DL1), and Z27 (IgG1; KIR3DL1). Anti-CD83 (IgG2b) PE-conjugated, anti-CD86 (IgG2b), a mixture of PC5-conjugated anti-CD56 mAb and FITC-conjugated anti-CD3 mAb, were purchased from Beckman Coulter.

Perforin and granzyme B expression analyses in NK cells were performed using purified anti-perforin mAb (Ancell) and purified anti-granzyme B mAb (Alexis Biochemicals), respectively, after cells were fixed in 1% paraformaldehyde and permeabilized.

NK cell purification and culture in the presence of cytokines

PBMC were obtained from heparinized blood by density gradient centrifugation over Ficoll (Sigma-Aldrich). NK cells were purified by NK Cell Separation Cocktails (Rosette Sep; StemCell Technologies). The purity of NK cells was >96%, as assessed by flow cytometric analysis of cells stained with a mixture of CD56-PC5 and CD3-FITC (Beckman Coulter). CD13 contamination in purified NK cells was <1%.

Such freshly purified NK cells were resuspended in RPMI 1640 medium, supplemented with 2 mM glutamine, 50 µg/ml penicillin, 50 µg/ml streptomycin, and 10% heat-inactivated FCS (PAA Laboratories) in the presence of either 0.5 ng/ml IL-12 (PeproTech) or 20 ng/ml IL-4 (PeproTech). Cells were plated at 10⁶ cells/ml in flat-bottom, 24-well tissue culture plates (Costar; Corning). After overnight (20-h) culture, NK cells were directly assessed by flow cytometric analysis and cytokolytic activity or were stimulated for 4 h with purified KL247 (IgM; anti-NKp46 mAb), which was used as a plate-bound protein (at a concentration of 5 µg/ml), or with phosphor ester (PMA) and Ca²⁺ was used as a plate-bound protein (at a concentration of 5 × 10⁵ cells/ml in ice-cold medium with 50 µg/ml anti-IFN-γ/CD45 Ab-Ab conjugates (Miltenyi Biotec). Cells were suspended in 37°C medium to a final concentration of 5 × 10⁵ cells/ml and were allowed to secrete IFN-γ for 40 min at 37°C. After capturing secreted cytokines at their surface, cells were centrifuged at 300 × g for 5 min at 4°C and resuspended at a concentration of 10⁶ cells/ml in ice-cold PBS containing 0.5% BSA and 5 mM EDTA (BioSource International; from Sigma-Aldrich). The cells were then stained with 5 µg/ml PE-conjugated anti-IFN-γ for 10 min at 4°C and analyzed by flow cytometry.

Results

Induction of activation markers on the surface of NK cells after exposure to IL-12 or IL-4

Highly purified peripheral blood NK cells were cultured overnight in the presence of IL-12 or IL-4, then analyzed for the expression of various markers, including CD56, Nkp46, Nkp30, NKG2D, CD11a, CD226 (Fig. 1a). The two cell types were cocultured in RPMI 1640 medium for 24 h. Before coculture, iDCs were removed from their GM-CSF/IL-4-containing medium by washing with RPMI 1640 medium. IL-12- and IL-4-treated NK cells, before culture with iDCs, were extensively washed to remove IL-12 and IL-4, respectively.

Induction of activation markers on the surface of NK cells after exposure to IL-12 or IL-4

Highly purified peripheral blood NK cells were cultured overnight in the presence of IL-12 or IL-4, then analyzed for the expression of various markers, including CD56, Nkp46, Nkp30, NKG2D, CD11a, CD226 (Fig. 1a, b). In other experiments, NK cells cultured with IL-12, but not in those cultured in IL-4, in which the levels of expression of this molecule remained substantially unchanged compared with freshly isolated NK cells (Fig. 1). The level of surface expression of the other markers analyzed in these experiments remained substantially similar in IL-12- and IL-4-cultured NK cells. Most NK cells cultured in the presence of IL-12, but only some of those exposed to IL-4 (Fig. 1b) expressed the CD69 activation marker. In IL-12-exposed NK cells, CD69 was expressed by both CD56ⁱˡᵃᵗ and CD56ⁱˡ:checked cells, whereas in NK cells exposed to IL-4, it was confined to a fraction of CD56ⁱˡᵃᵗ cells. Although not shown, the acquisition of CD69 by cytokine-cultured NK cells did not correlate with the expression of KIRs or NKG2A. Regarding the expression of Nkp44 (26), no major differences between the two cell types could be detected, although this molecule was slightly more expressed in NK cells that had been cultured with IL-12. However, Nkp44 was essentially confined to the CD56ⁱˡᵃᵗ subset in both IL-12- and IL-4-cultured NK cells (Fig. 1b). Finally, low levels of expression of CD25 were detected in both cell types (Fig. 1b).
Cytokine release by NK cells exposed to IL-12 or IL-4

The same NK cells, after culture in the presence of IL-12 or IL-4, were evaluated for cytokine release after 4-h stimulation with plastic-adherent anti-NKp46 mAb.

Cytofluorometric analysis revealed that NK cells exposed to IL-12 homogeneously released high levels of IFN-γ. Thus, as shown in Fig. 2a, both CD16\textsuperscript{bright} and CD16\textsuperscript{dim} NK cell subsets released comparable amounts of IFN-γ in response to anti-NKp46 mAb. Accordingly, because KIR\textsuperscript{+} cells are normally confined to the CD16\textsuperscript{bright} NK cell population, no substantial differences existed in IFN-γ production between KIR\textsuperscript{+} and KIR\textsuperscript{−} or between NKp46\textsuperscript{+} and NKp46\textsuperscript{−} cell subpopulations (not shown). These data indicate that virtually all NK cells, when cultured for a short time period in the presence of IL-12, can acquire the ability to release IFN-γ in response to NKp46-mediated triggering. In contrast, in line with previous results (27), most IFN-γ-producing cells were confined to the CD56\textsuperscript{bright} subset in freshly isolated NK cell populations (not shown). Thus, it appears that after short term exposure to IL-12, NK cells acquire a more general capability of releasing IFN-γ compared with freshly isolated NK cells, because both CD56 subsets become capable of releasing similar levels of this cytokine in response to NKp46 triggering. In contrast, upon culture in the presence of IL-4, NK cells (derived from the same donor) released either small amounts or no IFN-γ. Remarkably no IFN-γ-releasing cells could be detected even within the CD16\textsuperscript{bright} NK cell population after culture in the presence of IL-4. Thus, the ability (or lack thereof) to release large amounts of IFN-γ appears to represent a clear-cut criterion to distinguish NK cells exposed to IL-12 or IL-4.

It has previously been reported that exposure of NK cells to IL-2 (Fig. 2a) results in increases in their IFN-γ production (28). Accordingly, anti-NKp46 mAb-mediated triggering of NK cells cultured overnight in the presence of IL-2 resulted in levels of IFN-γ release comparable to those observed after culture with IL-12. In certain donors, a small subset of NK cells expressing a virtually CD56\textsuperscript{−} phenotype could be detected (see Fig. 2a). However, these cells expressed classical NK cell markers, including CD16, NKp30, and NKp46 and released cytokines upon stimulation with anti-NKp46 mAb.

Culture supernatants derived from the same NK cells (donor A) were also analyzed by ELISA for the presence not only of IFN-γ, but also of TNF-α, GM-CSF, IL-5, and IL-13. As shown in Fig. 2b, IL-12–cultured, but not IL-4–cultured, NK cells released high levels of IFN-γ, TNF-α, and GM-CSF. In NK cells exposed to IL-12, the release of these cytokines was potentiated in the presence of IL-2 (28), whereas it was inhibited by the addition of IL-4 (Fig. 2b). IL-4 could also strongly inhibit cytokine production by NK cells that were cultured in the presence of IL-2 (29). Finally, although not shown, NKp46–mediated NK cell stimulation did not result in detectable levels of IL-5 or IL-13 release by NK cells cultured in the presence of IL-12 or IL-4.

In another series of experiments, NK cells were stimulated with PMA and ionomycin. Under these strong stimulating conditions, IL-4–cultured NK cells were able to release both TNF-α and GM-CSF, whereas the levels of IFN-γ remained generally low. Even
under these conditions, IL-4-cultured NK cells failed to release detectable levels of IL-5 or IL-13 (Fig. 2c).

**Antitumor cytolytic activity of IL-12- or IL-4-cultured NK cells**

NK cells that had been cultured in the presence of IL-12 or IL-4 were also assessed for their ability to kill different tumor target cells. As shown in Fig. 3, NK cells exposed to IL-12 were strongly cytolytic against target cells such as K562 (HLA class I− erythroleukemia), FO-1 (HLA class I− melanoma), and LCL 721.221 (HLA class I− EBV cell line), whereas NK cells cultured in IL-4 displayed poor lytic activity against these cell lines. These target cells were used because of their high susceptibility to NK-mediated killing and also because their recognition is known to be mediated via distinct sets of triggering receptors (26). For example, killing of FO-1 is primarily due to NK cell activation via Nkp46 and NKG2D, whereas killing of LCL 721.221 involves Nkp46, 2B4 (CD244), and NTB-A.

In most instances, the ability of NK cells to kill given target cells directly correlates with the surface density of natural cytotoxicity receptors (NCR) (26, 30). In this case, however, although the level of surface expression of these different triggering receptors was similar in IL-12- and IL-4-cultured NK cells (Fig. 1), the ability to kill tumor cells was significantly different. Moreover, IL-12- and IL-4-cultured NK cells expressed similar surface densities of NKG2D (Fig. 1a), 2B4 (CD244), NTB-A, and CD2 (not shown). Finally, DNAM-1 (CD226) and LFA-1 (CD11a) were also expressed in similar amounts (Fig. 1a), thus ruling out possible differences in the expression of molecules involved in cell adhesion and activation.

Another parameter that we took into consideration was the content of perforin and granzyme B, because low cytolytic activity may also reflect low amounts of these proteases (31). However, as shown in Fig. 4, no significant differences could be detected in the expression of these cytoplasmic proteases between highly cytolytic (cultured in IL-12) and poorly cytolytic (cultured in IL-4) NK cells.

**NK cells exposed to IL-12 or IL-4 display different abilities to kill myeloid iDCs**

Because IL-12- or IL-4-induced NK cells may be generated in peripheral tissues upon the release of these cytokines during inflammatory responses to pathogens, we also evaluated their ability to exert cytolytic activity against monocyte-derived iDCs. To this aim, IL-12- or IL-4-induced NK cells were assessed for their ability to lyse allogeneic iDCs generated by culturing peripheral monocytes for 6 days in the presence of GM-CSF and IL-4 (4, 7).

As shown in Fig. 5a, in this case also, IL-12-cultured NK cells displayed significantly higher cytolytic activity compared with IL-4-cultured NK cells. Moreover, in agreement with previous reports on polyclonal NK cell populations generated in the presence of IL-2 (4), NK cells exposed to IL-12 were inhibited in their lytic activity against iDCs by anti-Nkp30 mAb (Fig. 5b).

**Different capabilities of IL-12- or IL-4-cultured NK cells to respond to Abs against activating NK receptors**

The NK-mediated killing of tumors or iDCs involves the engagement of various triggering NK receptors by ligands expressed on target cells (26). In particular, as mentioned above, the NK cell-mediated killing of iDCs requires the engagement of Nkp30 by still undefined ligands on DCs (1, 4). Thus, by redirected killing assays against the FcγR-positive P815 target cells, we analyzed the ability of IL-12- or IL-4-induced NK cells to respond to mAb specific for different triggering NK receptors, including Nkp30. As shown in Fig. 6, the cytolytic activity of IL-12-induced NK cells against P815 targets was triggered by different mAb (anti-Nkp46, anti-Nkp30, and anti-CD16). On the contrary, IL-4-cultured NK cells were poor responders to all stimuli. These data indicate that the defective cytolytic activity of IL-4-cultured NK cells against both tumor and iDCs may be consequent to a defect in cell activation in response to stimuli acting on the major triggering NK receptors.

**Involvement of NK cells exposed to IL-12 in DC maturation**

Previous studies indicated that activated NK cells promote DC maturation by mechanisms that are at least in part dependent on...
TNF-α release (5, 6). In this study we analyzed the ability of NK cells exposed to IL-12 or IL-4 to promote DC maturation, as assessed by the surface acquisition of CD83 and CD86 molecules. Thus, iDCs were cocultured for 24 h with IL-12- or IL-4-induced NK cells, then analyzed by cytofluorometric analysis. As shown in Fig. 7, substantial increments in the expression of CD83 and CD86 were detected when iDCs were cocultured with NK cells that had been exposed to IL-12, but not with those exposed to IL-4 (or to culture medium alone). Finally, DC maturation induced by IL-12-induced NK cells was clearly contact-dependent, as revealed by experiments in which separation of NK and DC by a permeable membrane (Transwell) did not allow DC maturation (Fig. 7, lower panels).

Discussion
In this study we show that after a short exposure to IL-12 or IL-4, NK cells display marked differences not only in their ability to release cytokines, but also in their cytolytic activity against tumors or immature DC. Moreover, they display a significant difference in the ability to promote DC maturation. These findings may have important implications in the early events that modulate innate immune responses and, possibly, in the regulation of the subsequent T cell priming in secondary lymphoid compartments (1, 11, 32).

It has been proposed that NK cells and DCs may mutually shape their phenotype and function during interactions that initiate at inflammatory sites and subsequently proceed in lymph nodes. During the early phases of inflammation, NK cells may be recruited to inflamed tissues in response to the proinflammatory chemokines IL-8 and fractalkine (1). Once there, NK cells would undergo a process of priming consequent to pathogen-derived signals acting on their TLRs (10) or to recognition of cellular ligands on tumor or virus-infected cells. Another signal that promotes NK cell activation is mostly DC dependent and involves DC-derived cytokines such as IL-12 released in response to triggering by pathogen-associated products. Thus, NK cells can up-regulate their cytolytic function and are induced to proliferate and release cytokines such as TNF-α and IFN-γ that, in turn, can promote the maturation program of DCs that have captured the Ag (33–35). In the present study we show that these events are initiated by danger signals promoting DC responses characterized by the release of higher levels of IL-12. In contrast, in the absence of IL-12, NK cells that respond to type 2 cytokines, released by DC2, eosinophils, or mast cells, are unable to properly regulate DC function and survival in inflamed tissues or secondary lymphoid compartments.

It is now clear that the process of NK cell activation in response to danger signals may differ in magnitude depending on the nature of the pathogen. Along this line we showed recently that in the presence of IL-12, NK cell cytotoxicity and cytokine release are strongly up-regulated when the danger signal can directly stimulate both immature DC and NK cells (10). For example poly (I.C) not only promotes the release of IL-12 by DC, but also primes (TLR3+) NK cells. In contrast, LPS promotes IL-12 release by (TLR4+) DC, but does not prime (TLR4+) NK cells (8). Importantly, NK cells stimulated with poly (I.C) in the presence of IL-12 acquire cytolytic activity against immature DC that is considerably higher than that elicited by NK cells exposed to IL-12 alone (10). Poly (I.C)-primed NK cells also release higher levels of IFN-γ compared with NK cells cultured in IL-12 alone. Thus, the NK functional phenotype, induced by DC-derived IL-12, is further reinforced when NK cells are simultaneously primed by stimuli acting via TLR expressed by both NK and DC.

In contrast, this functional NK phenotype can be partially acquired as a consequence of Ag uptake by iDCs. Thus, NK cells cultured in the presence of DC and LPS acquire the ability to lyse iDCs (5, 6). Together, the above findings are in line with the concept that one of the functions of the NK cell is to keep in check the quality of DC undergoing maturation and to control the amplitude of DC responses (1, 2). As previously shown, this function is mostly based on the ability of NK cells to selectively kill DCs that do not express optimal amounts of HLA-E at the cell surface (7). Because HLA-E expression is down-regulated in iDCs, but is progressively acquired in DCs undergoing maturation, NK cells would operate a positive selection of those DCs that undergo an optimal maturation program (1, 2). Certain danger signals fail to induce IL-12 production by DCs. In some instances they appear to suppress type 1 cytokines upon induction of type 2 responses in T cells. Pathogen-associated signals that promote Th2-like responses include schistosome egg Ags and hyphae of C. albicans as well as Porphyromonas gingivalis (36–38). Little is known about the ability of NK cells to directly respond to these stimuli. However, we show that exposure to IL-4 can seriously compromise the ability of NK cells to mediate cytolytic activity and cytokine release.
Previous studies proposed the classification of human NK cells into NK1 and NK2 based on the cytokine profile (e.g., type 1 and type 2, respectively) that was acquired after culture in the presence of IL-12 or IL-4, respectively (12, 13). Under the long term culture conditions used in these studies, it is conceivable that certain cell subsets might have been selected that produced type 2 (IL-5/IL-13) cytokines, undetectable in our study. Along this line, more recent studies indicated that type 2 cytokines are selectively produced by a small subset of NK cells expressing CD161, but lacking CD56, CD16, and NKP46 (18). In agreement with these studies, we did not detect the production of type 2 cytokines in NK cells cultured in IL-12 or IL-4 that homogeneously expressed the CD56<sup>+/++</sup> phenotype (see Fig. 1). Our observations are also in line with recent studies by the Caligiuri group showing that no IL-5 production can be induced in classical (CD56<sup>++/+</sup>, CD16<sup>++/−</sup>, and NKP46<sup>++/−</sup>) NK cells, and that in these NK cells, IL-13 production can only be induced upon simultaneous exposure to IL-15 and IL-18 (39).

In the evaluation of the physiological relevance of our present study, it is important to note that the window of time available for an innate response to take place or for a Th precursor to become committed has been estimated to be only few hours or few days, respectively (23, 24). Therefore, in vivo, the relevant cytokines must be secreted and exert their functional effect within a short interval after the onset of an infection. For these reasons, our studies were focused on the effect of a short exposure of freshly isolated NK cells to classical type 1 or type 2 cytokines.

Our results indicate that although virtually all NK cells exposed to IL-12 (independent of their KIR, NKG2A, CD16, or CD56 phenotype) secrete IFN-γ, only a minor subset of NK cells cultured in IL-4 release small amounts of this cytokine. Both NK cell types were found to secrete TNF-α in response to PMA and ionomycin, suggesting that NK cells exposed to IL-4 are potentially capable of inducing some degree of DC maturation. However, this function appears to be restricted to IL-12-cultured NK cells (see Fig. 7). This reflects the fact that IL-12-cultured (but not IL-4-cultured) NK cells can release TNF-α in response to activating stimuli acting on triggering NK receptors, including NCRs (i.e., NKP46). Preliminary data support the idea that NCRs are primarily involved in the TNF-α-dependent DC maturation induced by NK cells. The marginal responses of IL-4-cultured NK cells to stimuli acting on triggering NK receptors may justify the observed inefficiency of these cells in promoting DC maturation (see Fig. 7).

Another clear-cut difference between the two cell types was their different ability to mediate target cell killing. Thus, although NK cells exposed to IL-12 displayed strong lytic activity against all the various tumor target cell lines analyzed, NK cells exposed to IL-4 were consistently poorly cytolytic. It is of note that functional differences exist between IL-12- and IL-4-induced NK cells in their ability to kill tumors as well as iDCs. Thus, a major functional event occurring during NK/iDC interactions is strongly impaired in NK cells exposed to IL-4. Together, these data suggest that NK cells exposed to IL-12, but not those exposed to IL-4, may contribute to the proposed quality control process resulting in the selection of DCs undergoing pathogen-driven maturation. Accordingly, only IL-12-exposed NK cells would be able to limit the number of iDCs available for generating suitable numbers of mDCs. The lack of NK-mediated DC killing has been proposed to result in altered numbers of DC reaching complete maturation (1, 23, 40, 41). Thus, NK cells, after exposure to IL-4, might favor an abnormal process of DC maturation, possibly resulting in tolerogenic signals or in Th2 responses (41).

Stimuli, such as LPS, that promote the release of type 1 cytokines by DCs (DC1 responses) have been shown to induce, after a certain time interval, a period of DC refractoriness (DC exhaustion) that may result in subsequent DC2-like responses (22, 42). Because IL-12 is produced only transiently, its availability in lymph nodes would require a continuous influx of recently activated DCs drained from peripheral inflamed tissues. Under these conditions, T cell priming will be biased toward Th1. However, at later stages, exhausted DCs could outnumber the active ones, thus creating conditions for DC2-mediated development of Th2 (22). The production of IL-4 by Th2 cells is likely to provide an additional mechanism that negatively regulates IL-12-dependent IFN-γ production by NK cells in lymph nodes (11, 31, 43). In conclusion, our data suggest that, depending on the type of cytokines released by resident or recruited cells at early stages of an inflammatory response, NK cells may differentially contribute to the quality and magnitude of innate immune responses (44–47). This, in turn, is likely to have a marked impact on the subsequent adaptive immune response, as recently reported in the case of immune responses to Borrelia pertussis (41).


