Cutting Edge: Differentiation of Human NK Cells into NK1 and NK2 Subsets

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Human NK cells cultured in the presence of IL-12 or IL-4 differentiate into cell populations with distinct patterns of cytokine secretion similar to Th1 and Th2 cells. NK cells grown in IL-12 (NK1) produce IL-10 and IFN-γ, whereas NK cells grown in IL-4 (NK2) produce IL-5 and IL-13. Although these NK cell subsets do not differ in cytokine activity, NK1 cells express higher levels of cell surface CD95 (Fas) Ag than NK2 cells and are more sensitive to Ab or chemically induced apoptosis. Like Th1 cells, NK1 cells accumulate much higher levels of the IL-12Rβ2-chain mRNA and are significantly more responsive to IL-12 than NK2 cells at the level of activation of STAT4 transcription factor. The identification of NK cell subsets that are analogous to T cell subsets suggests a new role for NK cells in innate inflammatory responses and in their effect on adaptive immunity. The Journal of Immunology, 1998, 161: 5821–5824.

NK cells were considered non-MHC-restricted cytotoxic cells producing type 1 cytokines, such as IFN-γ, TNF-α, granulocyte-macrophage CSF (GM-CSF), lymphotoxin (LT), and IL-8 (1). The early migration of IFN-γ-producing NK cells into the inflammatory sites and draining lymph nodes is important in the generation of a Th1 immune response (2). The production of IFN-γ by NK cells has been shown to be enhanced by IL-12, which favors a Th1 response, and inhibited by IL-4, which favors a Th2 response (3). Until the recent demonstration that NK cells can produce IL-5, a Th2 response cytokine (4), the cytokine repertoire of NK cells was thought to be restricted to a type 1 pattern. Secretion of IL-5 by NK cells raised the possibility that NK cells might differentiate into distinct sub-sets. We report here that NK cells differentiate into two distinct subsets reminiscent of Th1 and Th2 cells, although neither subset produces IL-2 or IL-4. NK2 cells have decreased expression and function of the IL-12R, which is a hallmark of Th2 cells (5). There is no difference in cytokine activity of either subset, yet NK1 do express higher levels of CD95 and are more susceptible to apoptotic induction.

Materials and Methods

NK subset generation

NK cells were generated from 13 donors as described (6). PBL (2.5 × 10^7/ml) were cultured in 24-well plates with 5 × 10^5 γ-irradiated RPMI 8866 cells in the presence of either 1 ng IL-12 + anti-IL-4 mAb (4F2/5A4) or 50 ng/ml IL-4 + anti-IL-12 mAb (C8.6). At day 8, NK cell subsets were purified by negative selection using mAb anti-CD3, -CD4, -CD21, -CD19, -CD4 to >98% purity as determined by CD16 or CD56 staining. NK cells were stimulated for 18 h with 20 ng/ml PMA and 50 μM ionomycin (10^6 cells/ml), and cell-free supernatants were tested by RIA for IL-4, IL-5, IL-10, IL-13, IFN-γ, GM-CSF, LT, and TNF-α levels. To determine reversibility of NK subsets, NK cells from 4 donors were washed and placed in the reversing condition (or kept in the same condition) for 4 days, purified, and stimulated as above.

RNase protection assay (RPA)

RNase protection using 10 μg/lane total mRNA was performed using PharMingen (San Diego, CA) multitemplate probe kits (hck1, hApo3, hApo4, and probes generated in our lab). RNA intensity was normalized against internal glyceraldehyde-3-phospate dehydrogenase (GAPDH) band to adjust for loading or RNA harvesting errors.

Electrophoretic mobility shift assay (EMSA)

Nuclear extracts, obtained from purified NK1 and NK2 cells, were incubated overnight in medium with anti-IFN-γ mAb (B133.3), followed by incubation for 20 min with medium alone or with added IL-12. End-labeled gamma-activated site (GAS) DNA probe (5′-AGCTTGATT TCCCGAAGGATTCC-3′), derived from the FosYR1 (CD64) promoter (50,000 cpm/sample), was mixed with 4 μg of nuclear extract for 30 min at room temperature. Samples were fractionated on a 4% polyacrylamide gel for 1 h at 200 V. When supershifts were performed, nuclear extracts were preincubated for 30 min at 4°C with 2 μg/ml of monoclonal Abs (anti-STAT1, anti-STAT4, Santa Cruz Biotechnology, Santa Cruz, CA).

Intracellular staining for IFN-γ

NK subsets were generated as described and stimulated for 4 h with 20 ng/ml PMA + 50 μM ionomycin and monensin. These cells were stained with anti-CD56-phycocerythrin (PE) surface labeling and fixed with 4% paraformaldehyde for 30 min, then treated with 0.1% saponin and intracellular stained with anti-IFN-γ-PE-FTTC in the presence or absence of 1 μg/ml exogenous rIFN-γ.

CD95 surface expression and NK cell apoptosis

Cultured NK1 and NK2 cells were stained with anti-CD95-FTTC (CH-11), anti-CD16 (3G8) biotin/PE-Cy5 streptavidin and anti-CD3-PE (OKT3).
Cultured NK1 and NK2 cells were treated for 4 h with medium, anti-CD16 (3G8), or 20 ng PMA + 50 μM ionomycin. These cells were stained with anti-CD56-FITC and propidium iodine (PI), followed by flow cytometry. Alternatively, cells were stained with anti-CD56-PE, fixed in 1% paraformaldehyde/PBS solution for 15 min at room temperature, permeabilized with 0.1% Triton X-100 at 4°C for 2 min, and resuspended in 50 μl aldehyde/PBS solution for 15 min at room temperature, permeabilized with 0.1% Triton X-100 at 4°C for 2 min, and resuspended in 50 μl terminal deoxynucleotidyltransferase-mediated UTP end labeling (TUNEL) reaction mixture (Boehringer Mannheim, Indianapolis, IN) for 1 h at 37°C.

Results and Discussion

We generated NK cells by coculturing PBLs with irradiated RPMI 8866 cells for 8 days in the presence of either recombinant IL-12 and an anti-IL-4 mAb (NK1 condition) or recombinant IL-4 and an anti-IL-12 mAb (NK2 condition). Upon stimulation of purified NK cells with either plastic-bound anti-CD16 or PMA/ionomycin, cells generated under NK1 conditions produced high levels of IFN-γ, GM-CSF, TNF-α, and LT. Cells grown under NK2 conditions produced similar levels of TNF-α, GM-CSF, and LT, but significantly less IFN-γ, and significantly higher levels of IL-5 and IL-13 (Fig. 1). RPA confirmed the protein findings and showed skewed patterns of cytokine mRNA accumulation in NK1 and NK2 cells. Neither subset expressed detectable mRNA for IL-2, IL-4, IL-14, IL-15, or IL-9 (Fig. 2).

The overall phenotypes induced by the NK1 and NK2 priming conditions cannot be reversed in short-term cultures, as NK cells that were grown in either NK1- or NK2-inducing conditions for 8 days and then cultured in the reverse conditions for an additional 4 days retained the cytokine expression profiles established during the 8-day priming (Fig. 1). However, some modulation of cytokine production was evident. In agreement with previous studies of T and NK cells (4, 7), IL-5 production by NK2 cells was partially inhibited by IL-12, whereas IL-13 production was enhanced. The production of either IL-5 or IL-13 was not increased above basal levels in NK1 cells by any of the culture conditions tested. Culturing NK cells, primed in either NK1 or NK2 conditions for an additional 4 days with IL-12, enhanced IFN-γ production in both subsets with higher levels being produced by the restimulated NK1 cells. Together, these results suggest that NK1 and NK2 cells became unresponsive to the differentiation stimulus of IL-4 and IL-12, respectively, and, in short-term culture, are stable in their patterns of cytokine production but are susceptible to the transient effects of IL-12 (8). IL-10, generally considered to be a type 2 cytokine, was produced at low to undetectable levels by both NK1 and NK2 cells during primary culture; however, the secondary stimulation with IL-12 enhanced IL-10 production in NK1 cells (Fig. 1).

Memory T cells respond to IL-12 or IL-4 with a heterogeneous cytokine secretion profile (9). In contrast, clones derived from naïve T cells in the presence of either IL-12 or IL-4 display a type 1 or type 2 cytokine production phenotype, respectively (9). Since there are no reliable techniques for isolating naïve NK cells from peripheral blood, we examined cytokine production in NK cells isolated from cord blood and cultured under NK1 and NK2 conditions. Cord blood-derived NK1 cells produced high levels of
IFN-γ, although somewhat lower than their peripheral blood counterparts, while the NK2 cells produced nearly undetectable levels of IFN-γ, IL-5, and IL-13 (Fig. 3 inset). Consistent with the protein secretion data, IFN-γ intracellular staining of peripheral blood-derived NK2 cells revealed a subpopulation (15–45%) of high IFN-γ-producing NK cells, whereas IFN-γ intracellular staining in cord blood-derived NK2 cells was just above baseline (Fig. 3). These data suggest that at least a portion of peripheral blood, but not cord blood, NK cells have been activated and primed in vivo for IFN-γ production, and this ability is maintained even in the NK2 growth conditions.

IL-12 induces cytolytic activities and transcription of cytokotoxicity-associated genes, such as perforin and granzyme B, in cytotoxic T and NK cells (10). However, analysis of the cytotoxic ability of the NK cell subsets revealed no detectable differences between NK1 and NK2 populations in either perforin or granzyme mRNA levels (Fig. 2). In addition, no significant difference was observed between NK1 and NK2 cell-mediated cytotoxic activity against NK-sensitive K562 cells or in redirected lysis of anti-CD16-coated P815x2 mastocytoma cells (not shown). With the exception of CD95 (Fas) Ag, which was expressed at higher levels on the NK1 than NK2 cell surface (Fig. 4), no significant differences in the expression of a variety of surface proteins were observed. However, CD95 mRNA accumulation did not differ in NK1 and NK2 cells, suggesting translational or posttranslational regulation. Apoptosis, induced by either anti-CD16 cross-linking or PMA/ionomycin, and evaluated by either PI uptake or TUNEL, was more extensive in NK1 than NK2 cells (Fig. 4). IL-12 has been reported to increase NK cell susceptibility to anti-CD16-induced apoptosis (11), possibly due to the ability to up-regulate CD95 expression (12).

The mechanism by which T cells differentiate relates, in part, to a decreased responsiveness of Th2 cells to IL-12 due to the lack of expression of the IL-12Rβ2-chain (13). Similarly, levels of IL-12Rβ2-chain mRNA were lower in NK2 than NK1 cells, while levels of IL-12Rβ1-chain mRNA were equivalent in both subsets (Fig. 2). This decreased level of IL-12Rβ2 is important in light of the role of this receptor chain in generating the high-affinity binding sites necessary for IL-12 signaling (14). IL-12 ligand binding assay revealed a threefold decrease in the number of high-affinity binding sites on NK2 as compared with NK1 cells, whereas no difference in the number of low-affinity binding sites was observed (data not shown). IL-12R signaling induces a rapid phosphorylation of JAK2 and Tyk2 kinases followed by phosphorylation of STAT1, STAT3, and STAT4 (15). STAT4 phosphorylation is induced only by IL-12 and IFN-α (16). EMSAs of NK1 and NK2 nuclear extracts after IL-12 stimulation demonstrated a marked decrease in the induction of IFN-γAS binding protein in NK2 cells compared with NK1 cells (Fig. 2 inset). Supershift experiments confirmed that the IL-12-inducing GAS-binding activity was primarily due to STAT4 (not shown). These results indicate that NK1 and NK2 subsets show IL-12R modulation similar to those of their T cell counterparts.

Our data demonstrate that NK cells can differentiate into cells with NK1 and NK2 phenotypes, similar to those described in T cells. The importance of NK cells in generating an appropriate Th1 response in vivo is well documented (2). It remains to be determined whether NK2 populations exist in vivo and whether they play an important role in the inflammatory outcome of infections. Because NK cells do not produce IL-4, it is unlikely that NK2 cells are directly involved in Th2 cell generation. However, NK2 cells could contribute to a general type 2 response with production of IL-5 and IL-13. Eosinophilia in patients treated with IL-2 and/or IL-4 has been associated with both elevated IL-5 and NK cell infiltration (17, 18). Recently, it has been shown that IL-5 production by NK cells contributes to eosinophil infiltration in a mouse
model of allergic inflammation (19). Further studies are needed to define the exact role of NK cell subsets in inflammation.

References