Response of Human Natural Killer (NK) Cells to NK Cell Stimulatory Factor (NKSF): Cytolytic Activity and Proliferation of NK Cells Are Differentially Regulated by NKSF

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Summary

Natural killer cell stimulatory factor (NKSF) is a 70-kD heterodimeric cytokine that was initially isolated from conditioned medium of human B lymphoblastoid cell lines. The effects of recombinant NKSF on the function of human peripheral blood NK cells were examined. NKSF directly augmented the cytolytic activity of freshly isolated NK cells. Both CD56^{dim} and CD56^{bright} NK cells demonstrated enhanced cytotoxicity after brief exposure to NKSF. In contrast, highly purified T lymphocytes did not exhibit major histocompatibility complex–unrestricted cytotoxicity after short-term culture with NKSF. Like interleukin 2 (IL-2), NKSF augmented the lysis of NK-sensitive, NK-resistant, and antibody-coated targets. Both NKSF and IL-2 induced marked upregulation of several NK cell adhesion molecules known to participate in cytolysis, including CD2, CD11a, and CD54. However, NKSF activates NK cells through a pathway distinct from that of IL-2, since the presence of anti-IL-2 receptor (anti-IL-2R) antibodies or IL-4 did not inhibit the effects of NKSF. NKSF by itself induced very little proliferation of resting NK cells. NK cells preactivated in vitro with IL-2 demonstrated enhanced proliferation to NKSF, but the degree of proliferation was always inferior to that induced by IL-2 alone. Moreover, NKSF strongly inhibited IL-2-induced proliferation of either resting or preactivated NK cells. This inhibition was not the result of decreased IL-2R expression, because NKSF-activated NK cells expressed higher levels of both IL-2Rs p75 and p55. Furthermore, NKSF did not inhibit the proliferation of mitogen-activated T cells, indicating a selective effect on NK cell proliferation. Human NK cells expanded in vivo by prolonged continuous infusions of IL-2 remained fully responsive to NKSF. Picomolar concentrations of NKSF were as effective as nanomolar concentrations of IL-2 in augmenting the cytolytic activity of NK cells expanded in vivo by IL-2. NKSF may play an important role in the regulation of human NK cell function, and its possible use as a therapeutic cytokine deserves further investigation.

N K cells are lymphocytes that can lyse certain tumor cells and virus-infected cells spontaneously and without MHC restriction (1, 2). Unlike T and B cells, NK cells do not productively rearrange either TCR or Ig genes (1, 3, 4) and do not appear to possess clonotypic, antigen-specific receptors (1, 2, 4). Although the receptors responsible for triggering NK cytolysis remain poorly characterized, the cytolytic activity of these lymphocytes can be regulated by several cytokines (1, 2). Sufficient concentrations of IL-2 dra-

matically enhance NK cytotoxicity and induce NK cell proliferation (5–7), and IL-2-activated NK cells appear to be largely responsible for the LAK phenomenon (8–10). Administration of high doses of IL-2, with or without adoptive transfer of LAK cells generated in vitro, can induce tumor regressions in experimental animals and patients with advanced cancer (11–14). However, high-dose IL-2 therapy is associated with substantial toxicity (15), and only a small minority of patients demonstrates objective response to such therapy (13,

14). Optimal IL-2-based immunotherapy may require modifications in the dose and schedule of IL-2 or the use of additional cytokines (14).

Natural killer cell stimulatory factor (NKSF)[1] is a 70-kD disulfide-linked heterodimeric glycoprotein composed of 35- and 40-kD subunits (16, 17). NKSF was initially purified from the medium of human B lymphoblastoid cell lines stimulated with phorbol ester (16). NKSF has several biologic activities in vitro, including the induction of IFN-γ secretion by T cells and NK cells, augmentation of MHC-unrestricted cytolytic activity of unfractionated PBMC, and enhancement of proliferation of mitogen-activated PBMC (16-18). The cDNA encoding NKSF is virtually identical to that of cytotoxic lymphocyte maturation factor or IL-12 (19-21). The effects of NKSF, or IL-12, on highly purified human peripheral blood NK cells have not been reported in detail. We describe the disparate effects of NKSF on NK cell cytolytic activity and proliferation, and the differential regulation of NK cell and T cell proliferation by NKSF. The possible implications of these results for anticancer immunotherapy are discussed.

Materials and Methods

Antibodies. Fluorochrome-conjugated murine mAbs were obtained from Coulter Immunology (Hialeah, FL), including T3 (CD3), T11 (CD2), Mo1 (CD11b), NKH1 (CD56), IL-2R1 (CD25), and isotype-matched control mAb. FITC-conjugated Leu-1 (CD5) was purchased from Becton Dickinson & Co. (Mountain View, CA). FITC-conjugated anti-ICAM-1 (CD54) was purchased from AMAC, Inc. (Westbrook, ME). T1 /24T6G12 (CD5), 2F12 (CD11a), MY4 /322A (CD14), B1 /H299 (CD20), and IL-2R p55 /1HT44H3 (CD25) were produced as ascites in BALB/c mice bearing hybridoma tumors. Murine mAb 2R-B, directed against the p75 subunit of the human IL-2R (22), was provided by Coulter Immunology.

Cell Lines. K562 (a myeloid cell line derived from a patient with chronic myelogenous leukemia in blast crisis), COLO 205 (a colon adenocarcinoma cell line), and P815 (a murine mastocytoma cell line) were all obtained from the American Type Culture Collection (Rockville, MD). Cell lines were maintained in continuous suspension cultures in RPMI 1640 (Gibco Laboratories, Grand Island, NY) supplemented with 10% FCS, 2 mM L-glutamine, 1 mM sodium pyruvate, penicillin, and streptomycin.

Preparation of Recombinant Human NKSF and Anti-NKSF Heteroantiserum. Cos-1 cells or CHO cells were transfected with cDNAs encoding both the NKSF p35 and p40 chains by a method previously described (23). Conditioned medium of transfected Cos cells was used as the source of rNKSF in most experiments. This preparation contained 2,500 U/ml of NKSF activity, as determined in a PHA blast proliferation assay. 1 U/ml NKSF is approximately equivalent to 3.5 pM (16). For some experiments, rNKSF was purified from the supernatants of transfected CHO cells by a method similar to that previously described for the purification of natural NKSF (16). A neutralizing rabbit antiserum to NKSF was prepared as previously described (18).

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[1] *Abbreviations used in this paper:* ADCC, antibody-dependent cell-mediated cytotoxicity; CAM, cell adhesion molecule; NKSF, natural killer cell stimulatory factor.

Commercial Cytokines and Reagents. All commercial cytokines were recombinant human proteins. IL-2 (sp act, 1.5 × 10^7 U/mg protein) and IFN-α were provided by Hoffmann-La Roche, Inc. (Nutley, NJ); 1 U/ml of IL-2 is approximately equivalent to 4.4 pM. IL-4 was provided by Schering-Plough Corp. (Bloomfield, NJ) and IFN-γ (2.5 × 10^7 U/mg) was purchased from Genzyme (Cambridge, MA). PHA was purchased from Burroughs Wellcome Co. (Greenville, NC), PMA from Sigma Chemical Co. (St. Louis, MO), and ionomycin from Calbiochem-Behring Corp. (La Jolla, CA).

Isolation and Culture of Lymphocytes. PBMC were isolated on a Ficoll-diatrizoate gradient from heparinized venous blood or from cytopheresis buffy coats obtained from normal volunteer donors. Adherent mononuclear cells were depleted by incubation on plastic petri dishes for 1 h at 37°C. Enriched NK cells were obtained by incubating PBL with a mixture of excess T1 /24T6G12, B1 /H299, and MY4 /322A mAbs, and then separating antibody-bound cells using immunomagnetic beads (Advanced Magnetics, Cambridge, MA). The resulting T cell, B cell, and monocyte-depleted PBL consisted of 65 ± 14% CD56$^+$ cells (mean ± SD of nine experiments) and 11 ± 7% CD3$^+$ cells; only 2 ± 1% of these cells coexpressed CD56 and CD3. Highly purified CD5$^-$CD56$^+$ NK cells and CD5$^+$CD56$^-$ T cells were isolated from PBL by standard methods (24) using an EPICS 752 flow cytometer (Coulter Electronics, Hialeah, FL). CD56^{dim} and CD56^{bright} NK cell subsets were obtained by flow cytometry as described previously (25). The CD56 mAb used does not affect NK cell proliferation or cytolytic activity (26-28). Lymphocytes were incubated in either 25-cm^2 tissue culture flasks (Becton Dickinson Labware, Lincoln Park, NJ) or 96-well U-bottomed microtiter plates (Flow Laboratories, McLean, VA) at a concentration of 1-2 × 10^6/ml in 5% CO$_2$ humidified air at 37°C. Culture medium was RPMI 1640 supplemented with 15% heat-inactivated, pooled human AB serum, 2 mM L-glutamine, 1 mM sodium pyruvate, penicillin, streptomycin, and gentamycin. Recombinant cytokines were added to some cultures at the concentrations indicated.

Immunofluorescence Studies. Samples of cells were stained directly or indirectly with fluorochrome-conjugated mAb, washed, fixed in 1% formaldehyde, and analyzed by flow cytometry as previously described (28). Because cultured lymphocytes often demonstrated high background fluorescence, such cells were centrifuged through a Ficoll gradient to remove dead cells and debris before staining.

Cytotoxicity Assays. For NK activity assays, target cells were incubated for 1 h at 37°C with 150 μCi of Na^{51}CrO$_4$, washed twice, and plated at 3,000 or 5,000 cells per well in 96-well U-bottomed microtiter plates. Effector cells were then added to triplicate wells to yield various E/T ratios in a final volume of 200 μl. After a 4-h incubation at 37°C, the microtiter plates were centrifuged and well supernatants were collected using a cell harvester (Skatron, Inc., Sterling, VA). Supernatant radioactivity was assayed using an automated gamma counter (LKB Wallac, Turku, Finland). For antibody-dependent cell-mediated cytotoxicity (ADCC) assays, P815 cells were preincubated with medium or with a 1:100 dilution of polyclonal rabbit anti-mouse lymphocyte serum (Accurate Chemical & Scientific Corp., Westbury, NY) and then labeled with ^{51}Cr for 1 h at 37°C. Chromium release was determined as described for NK activity assays. For all cytotoxicity assays, maximum chromium release was determined by addition of 1% NP-40 detergent and minimum release by addition of medium alone to target cells. Spontaneous release was generally <10% and never exceeded 20% of maximum release for the experiments reported. Specific cytotoxicity was calculated using a standard formula (29).

*Proliferation Assays.* Sorted lymphocyte subsets were plated at

30,000 cells per well in 96-well U-bottomed microtiter plates in medium alone or medium containing various stimuli as indicated. Unless otherwise noted, 1 μCi of [^{3}H]TdR was added to each well after 4 d of culture. Samples were collected 18 h later using a cell harvester (Cambridge Technology, Watertown, MA), and [^{3}H]TdR incorporation was measured using a liquid scintillation counter (Packard Instrument, Downers Grove, IL). For preactivation experiments, the fold increase in counts per minute associated with preactivation was calculated as follows: (cpm of preactivated cells cultured in NKSF − cpm of preactivated cells cultured in medium)/(cpm of resting cells cultured in NKSF − cpm of resting cells cultured in medium). NK cells were preactivated for 18 h with 10 ng/ml PMA, 1 μM ionomycin, or 1,000 U/ml IL-2, and T cells were preactivated with 5 μg/ml PHA as indicated.

Results

Effects of NKSF on the Cytolytic Activity of Human NK Cells. rNKSF augmented the cytolytic activity of enriched peripheral blood NK cells in a dose-dependent manner. Although enhanced cytolytic activity was evident after stimulation of NK cells with as little as 0.1 U/ml of NKSF, optimal enhancement required NKSF concentrations in the range of 1–10 U/ml ($\sim$3.5–35 pM; data not shown). These results are in close agreement with those obtained previously using purified natural NKSF and unfractionated PBL (16). Incubation with comparable dilutions of supernatants from mock-transfected Cos cells did not augment the cytotoxicity of NK cells, and the NKSF-induced enhancement of NK cytolysis could be completely abrogated by a neutralizing heteroantiserum to NKSF (data not shown). The augmentation of NK cytotoxicity is thus a specific effect of rNKSF and not due to contaminating factors in the Cos cell supernatants.

To establish whether NKSF acts directly on NK cells, highly purified NK cells were obtained by cell sorting. After stimulation with rNKSF, sorted CD56$^+$CD5$^-$ NK cells demonstrated substantial lysis of the NK-resistant cell line COLO (Fig. 1 *A*). These results demonstrate that NKSF can augment NK cytotoxicity by directly stimulating NK cells, and that indirect effects of NKSF on T lymphocytes or other PBMC are not required. Furthermore, unlike NKSF-induced secretion of IFN-γ (18), the enhancement of NK cytotoxicity by NKSF does not seem to require the presence of nonadherent, MHC class II–positive accessory cells. In as much as resting CD56^{dim} and CD56^{bright} NK cells differ somewhat in their cytolytic activities (30, 31), we also isolated these two subsets of NK cells and stimulated each with rNKSF. NKSF augmented the MHC-unrestricted cytotoxicity of both CD56^{dim} and CD56^{bright} NK cells comparably (Fig. 1, *B* and *C*). Finally, sorting experiments confirmed that purified CD5$^+$CD56$^-$ T lymphocytes did not exhibit "anomalous killing" after short-term culture with NKSF (data not shown).

In addition to MHC-unrestricted natural killing (NK activity), NK cells can lyse antibody-coated target cells by ADCC (1, 2). Furthermore, IL-2-activated NK cells can lyse targets that are relatively resistant to killing by unstimulated NK cells (1, 2). It is not clear whether such LAK activity represents a distinct cytolytic pathway or merely reflects augmented natural killing. The effects of NKSF on all of these types of NK
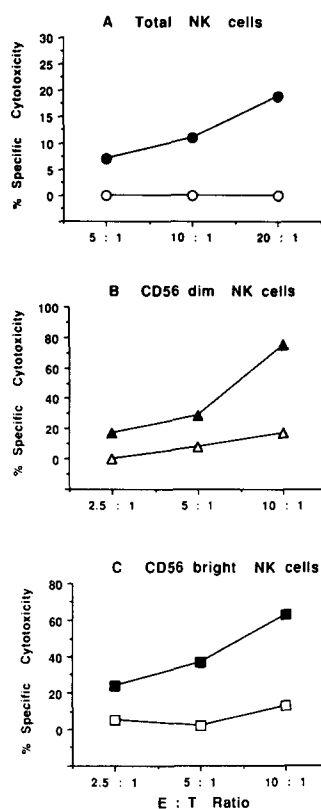

Figure 1. Effect of NKSF on cytolytic activity of highly purified NK cells. Sorted CD56$^+$CD5$^-$ NK cells (*A*), CD56^{dim} NK cells (*B*), and CD56^{bright} NK cells (*C*) were incubated for 18 h with medium alone (*open symbols*) or NKSF 4 U/ml (*filled symbols*) and then tested for cytotoxicity against COLO (*A*) or K562 (*B* and *C*) at the E/T ratios indicated. Symbols represent means of triplicate wells. Experiment using total NK cells was repeated twice with similar results.

cytotoxicity were examined, and compared with the effects of other cytokines known to activate NK cells. NKSF consistently augmented the lysis of NK-sensitive, NK-resistant, and antibody-coated targets (Fig. 2). NKSF enhanced K562 killing in a manner comparable with IL-2 (Fig. 2 *A*), although optimal concentrations of NKSF appeared to be slightly inferior to optimal concentrations of IL-2 in the induction of LAK activity (Fig. 2 *B*). Both NKSF and IL-2 stimulated MHC-unrestricted killing of K562 and COLO more effectively than did IFN-α and IFN-γ. However, IL-2, NKSF, and IFN-α all augmented ADCC similarly (Fig. 2 *C*).

Upregulation of NK Cell Adhesion Molecules (CAM) by NKSF. We have previously shown that IL-2 upregulates several NK cell adhesion molecules, and that increased levels of CAM may mediate enhanced lysis of NK-resistant targets by IL-2-activated NK cells (28). We therefore studied the effects of NKSF on the expression of CAM by NK cells. NKSF-activated NK cells demonstrated marked upregulation of several adhesion molecules, including CD2, CD11a, CD54, and CD56 (Fig. 3). Indeed, the increased levels of adhesion molecules induced by NKSF were very similar to those induced by IL-2. As shown previously for IL-2 (28), levels of CD11b were not upregulated by NKSF (data not shown); the increases in CAM were therefore specific and did not merely reflect an increased size of activated NK cells. Unlike IL-2 and NKSF, IFN-α induced little change in CAM expression by NK cells (data not shown); IFN-α also stimulated NK cytolytic activity to a much lesser degree than did IL-2 or NKSF.

Role of IL-2 in the Stimulation of NK Cells by NKSF. Since
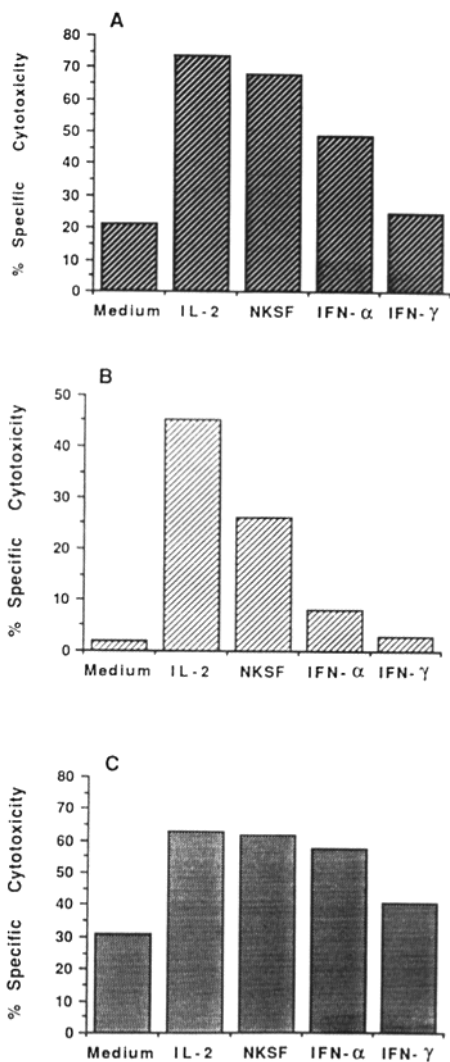
Figure 2. Augmentation of NK cell cytolytic activities by stimulatory cytokines. Enriched NK cells were incubated for 18 h with medium alone, 230 U/ml IL-2, 4 U/ml NKSF, 1,000 U/ml IFN-α, or 1,000 U/ml IFN-γ as indicated and tested for cytotoxicity against K562 (*A*), COLO (*B*), or antibody-coated P815 (*C*). Means of triplicate wells from a single representative experiment for each target cell line are shown. Results were confirmed in at least four experiments. The E/T ratio was 5:1 for K562 and P815⁺ Ab, and 20:1 for COLO. Background killing of P815 in the absence of antibody (2% for medium alone, 17% for IL-2, 6% for NKSF, 2% for IFN-α, and 0% for INF-γ) has been subtracted.

the effects of NKSF and IL-2 were quite similar in several respects, we wished to determine whether IL-2 was involved in NKSF-induced activation of NK cells. A combination of mAbs to both IL-2R p55 and p75 abolished the augmentation of NK killing induced by IL-2, as expected (Fig. 4 *B*). In contrast, these mAbs did not significantly inhibit the activation of NK cells by NKSF (Fig. 4 *A*). Furthermore, simultaneous incubation of NK cells with both IL-4 and IL-2 markedly abrogated the enhancement of NK cytotoxicity induced by IL-2 alone, whereas the presence of IL-4 did not affect NK cell activation by NKSF (data not shown). IL-4 also selectively inhibited the upregulation of NK cell CAM

by IL-2, but not by NKSF (data not shown). Thus, NKSF appears to stimulate NK cells through a distinct pathway that is independent of IL-2.

Effects of NKSF on Proliferation of Resting NK Cells. The observation that B lymphoblastoid cell lines supported human NK cell growth provided a rationale for the initial isolation and purification of NKSF (16). The effects of NKSF on NK cell proliferation were therefore examined. Highly purified NK cells demonstrated little proliferation to concentrations of NKSF as high as 250 U/ml (data not shown). Compared with IL-2 alone, NKSF alone induced much less NK cell proliferation (Table 1). This disparity did not appear to be due to differences in the kinetics of proliferation induced by NKSF and IL-2, since NK cells stimulated with NKSF for up to 12 d did not exhibit significantly greater proliferation (data not shown). Furthermore, NKSF strongly inhibited the proliferation of NK cells induced by IL-2 (Table 1). Such inhibition was consistently seen in every experiment performed. The proliferative responses of CD56^dim and CD56^bright NK cells to IL-2 are quite different: CD56^bright NK cells consistently exhibit much greater proliferation than do CD56^dim NK cells stimulated with IL-2 (25, 30, 31). It was therefore possible that NKSF also affected IL-2-induced proliferation of these two subsets differently. When CD56^dim and CD56^bright NK cells were isolated by flow cytometry and tested independently, however, we found that NKSF inhibited the proliferation of each subset by ~80–90% (Table 1). This effect was not due to nonspecific inhibitory factors, because: (*a*) neutralizing heteroantiserum to NKSF largely reversed the inhibition; (*b*) highly purified CHO-derived rNKSF also inhibited proliferation; and (*c*) supernatant from mock-transfected Cos cells did not significantly inhibit proliferation (data not shown).

NKSF is a potent stimulus for IFN-γ secretion by both T cells and NK cells, and is synergistic with IL-2 in inducing IFN-γ (16–18). Like IFN-α and IFN-β, IFN-γ has been reported to have antiproliferative effects in some experimental systems (32). Thus, the inhibition of IL-2-induced NK cell proliferation by NKSF could be mediated in part by autocrine secretion of IFN-γ. Although NKSF inhibited IL-2-induced proliferation of CD56^dim NK cells by 89 ± 7% (mean ± SD of seven experiments), 1,000 U/ml of rIFN-γ failed to significantly inhibit NK cell proliferation (2 ± 8% inhibition in five experiments). Furthermore, highly purified NK cells have been reported to produce IFN-γ very poorly due to the absence of requisite accessory cells (18). Therefore, it is unlikely that IFN-γ plays a major role in NKSF-induced inhibition of NK cell proliferation.

Some investigators have suggested that induction of IL-2R p55 expression by activated NK cells is required for IL-2-induced proliferation (33–35). We evaluated the possibility that NKSF inhibited such proliferation by decreasing NK cell expression of IL-2R. As previously described for NK cells stimulated with IL-2 alone (33, 34), NK cells stimulated with either NKSF alone or NKSF together with IL-2 express increased levels of IL-2R p55 (Fig. 5). Furthermore, NKSF appears to upregulate the expression of IL-2R p75 by NK cells

FRESH   IL-2   NKSF

CD 2 - FITC / CD 11a - FITC / CD 54 - FITC vs CD 56 - PE
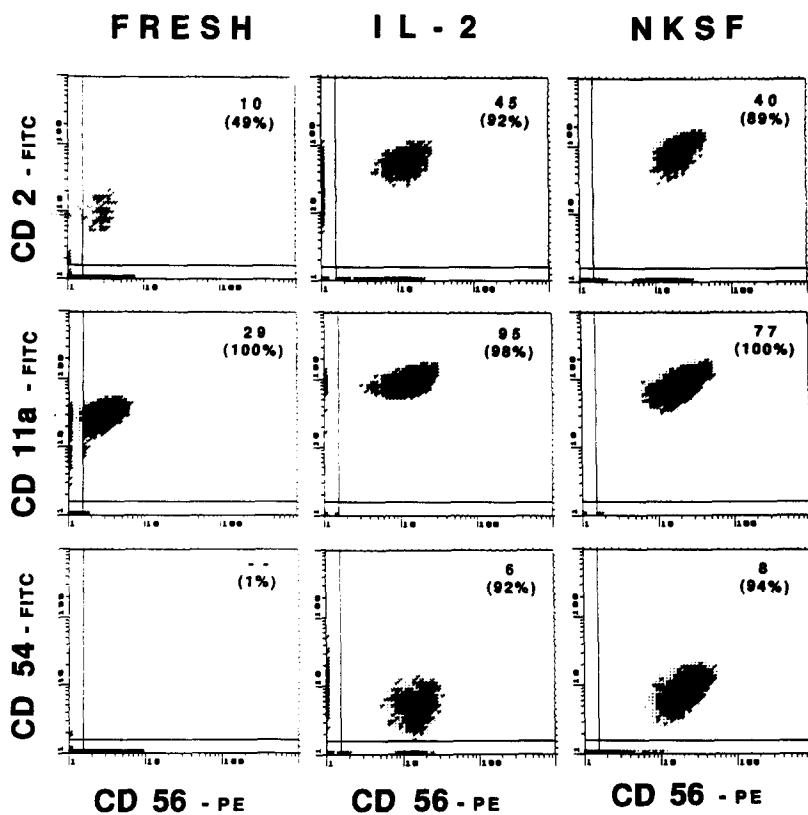
Figure 3. Upregulation of NK cell adhesion molecules by IL-2 and NKSF. Enriched NK cells were stained with CD56-PE and FITC-conjugated mAb recognizing CD2, CD11a, or CD54 immediately after isolation (FRESH) and after 6 d of culture with 1,000 U/ml IL-2 or 4 U/ml NKSF as indicated. Two color immunofluorescence analysis was performed as previously described (28). Logarithm of red fluorescence is displayed on the x-axis and logarithm of green fluorescence on the y-axis. Quadrant settings, distinguishing positive from background fluorescence, were determined by staining with isotype-matched control mAb (not shown). Numbers in the upper right corner of each histogram are the mean fluorescence intensity of FITC staining for the relevant CAM, determined as previously described (28); numbers in parentheses are the percentage of CD56+ cells that coexpresses the indicated CAM. Results are from one representative experiment out of three performed.

A NKSF Stimulation

B IL - 2 Stimulation
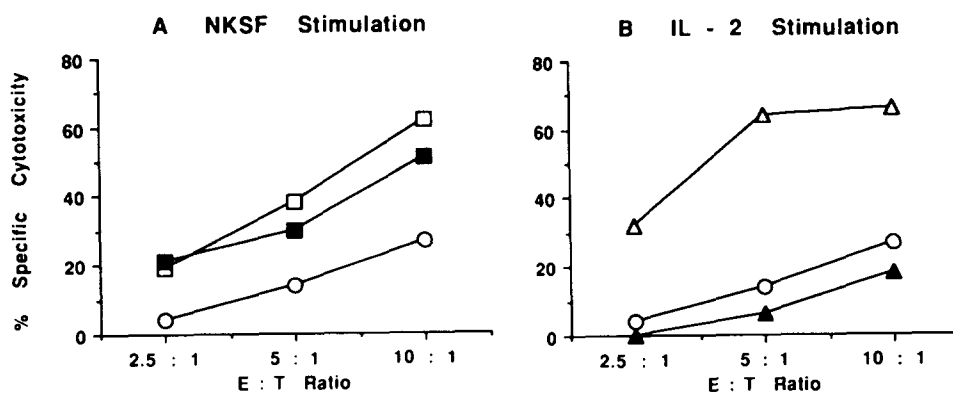
Figure 4. Effects of anti-IL-2R mAb on augmentation of NK cytotoxicity by cytokines. Enriched NK cells were incubated for 18 h with 2 U/ml NKSF (A, squares) or 230 U/ml IL-2 (B, triangles) in the presence (filled symbols) or absence (open symbols) of saturating amounts of mAb to both the p55 and p75 subunits of the IL-2R. Cytotoxicity against K562 was tested at the indicated E/T ratios. Symbols represent means of triplicate wells. Lysis of K562 by NK cells incubated for 18 h in medium alone is also shown (open circles). Data are from one representative experiment out of three performed.

Table 1. *Effects of NKSF on Proliferation of Resting NK Cells*

Culture conditions*	Proliferation ([³H]TdR incorporation)		
	Total NK cells	CD56ᵈⁱᵐ NK cells	CD56ᵇʳⁱᵍʰᵗ NK cells
Medium alone	291 ± 30‡	298 ± 46	297 ± 44
2 U/ml NKSF	1,906 ± 524	674 ± 103	2,043 ± 1,050
230 U/ml IL-2	27,180 ± 7,864	11,783 ± 1,811	45,827 ± 17,017
IL-2 + NKSF	3,862 ± 1,499	1,824 ± 958	8,795 ± 6,202

* Sorted total NK cells, CD56ᵈⁱᵐ NK cells, and CD56ᵇʳⁱᵍʰᵗ NK cells were cultured with medium alone or with cytokines as indicated. Proliferation assays were performed as described in Materials and Methods.
‡ Mean ± SD of triplicate cpm from two (total NK cells) or four (NK cell subsets) representative experiments.
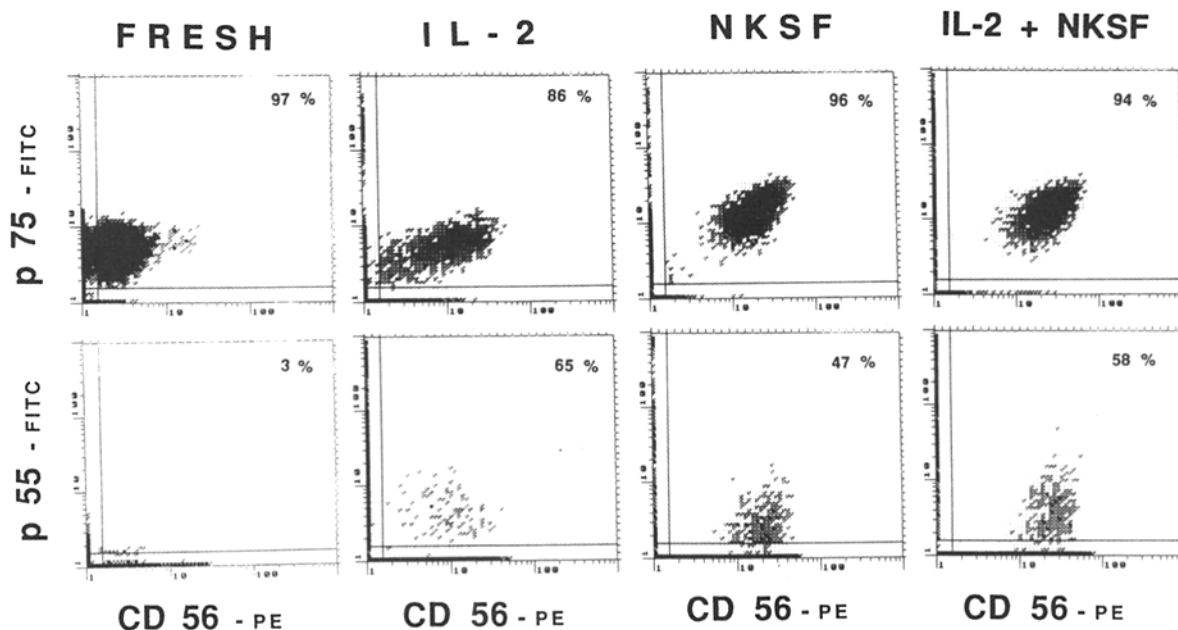
Figure 5. Expression of IL-2R subunits by NK cells activated with cytokines. Enriched NK cells were stained with CD56-PE and FITC-conjugated anti-IL-2R p75 (*top*) or anti-IL-2R p55 (*bottom*) immediately after isolation (FRESH) or after 4 d of culture with 230 U/ml IL-2, 4 U/ml NKSF, or both cytokines. Numbers in the upper right corner of each histogram represent the percentage of CD56+ cells that coexpresses the indicated IL-2R subunit. Results are from one representative experiment out of three performed.

(Fig. 5). Thus, NKSF does not seem to inhibit IL-2-induced NK cell proliferation by interfering with IL-2R expression.

Enhanced NKSF-induced Proliferation of NK Cells Preactivated In Vitro by IL-2. NKSF does not induce the proliferation of resting PBL, but can support the proliferation of T cells preactivated with mitogens or phorbol esters (16, 21). Therefore, we examined the effect of NKSF on the proliferation of NK cells preactivated in vitro. Preincubation with PMA or ionomycin for 18 h failed to enhance consistently NKSF-induced NK cell proliferation (data not shown). In contrast, NKSF induced about ninefold greater proliferation (fold increase in cpm, 8.8 ± 5.5; mean ± SD of four experiments) of CD56^{dim} NK cells preincubated with IL-2 for 18 h compared with CD56^{dim} NK cells preincubated with medium alone for 18 h. Nevertheless, NKSF-induced proliferation of such preactivated NK cells was still quite inferior to the proliferation induced by IL-2 alone (Fig. 6 *A*). Similar to its

effects on freshly isolated NK cells, NKSF inhibited by ~80–90% the proliferation of preactivated NK cells cultured in IL-2 (Fig. 6 *A*). However, NKSF did not inhibit the proliferation of PHA-activated T cells to IL-2 (Fig. 6 *B*). Furthermore, as previously reported (16, 17), we found that NKSF could augment the proliferation of PHA-activated T cells in the absence of exogenous IL-2 (Fig. 6 *B*). Our results thus suggest that NKSF specifically and selectively interferes with the ability of IL-2 to signal proliferation in NK cells.

Effects of NKSF on NK Cells Preactivated In Vivo by IL-2. In attempts to induce prolonged activation of NK cells in vivo, we have administered human rIL-2 by continuous intravenous infusion for 90 d to patients with advanced cancer (36). Progressive and selective expansion of peripheral blood NK cell numbers have been consistently seen in patients receiving IL-2 at doses of 6.0 × 10^5 U/m^2/d. We examined the in vitro effects of NKSF on these in vivo preactivated
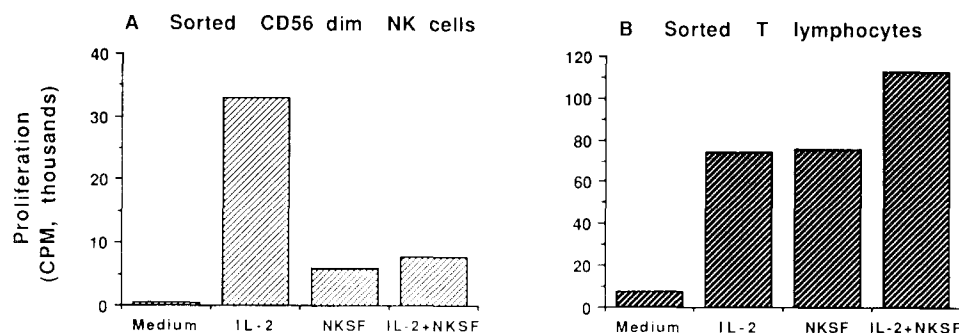
Figure 6. Effects of NKSF on proliferation of lymphocytes preactivated in vitro. Sorted CD56^{dim} NK cells preactivated with 1,000 U/ml IL-2 (*A*) or sorted CD5+CD56− T cells preactivated with 5 μg/ml PHA (*B*) for 18 h were washed twice and then incubated in medium alone, 230 U/ml IL-2, 2 U/ml NKSF, or both cytokines for 4 d. Mean cpm of triplicate wells from a single experiment are shown. Experiment assessing NK cell proliferation was repeated three times with similar results.

NK cells. As we have described previously (36), PBL freshly isolated from these patients can mediate modest lysis of the NK-resistant cell line COLO, and this killing is markedly enhanced by brief exposure to exogenous IL-2 in vitro (Fig. 7). Furthermore, overnight incubation with 4 U/ml of NKSF augmented the cytolytic activity of in vivo expanded NK cells as effectively as incubation with 230 U/ml of IL-2 (Fig. 7). We also examined the effects of NKSF on the proliferation of NK cells preactivated in vivo with IL-2. NKSF by itself induced a modest proliferation of sorted NK cells from these patients, but NKSF inhibited IL-2-induced NK cell proliferation (data not shown). These results are very similar to those seen with NK cells from normal donors preactivated in vitro with IL-2. Thus, NK cells expanded in vivo by chronic administration of IL-2 remain fully responsive to NKSF.
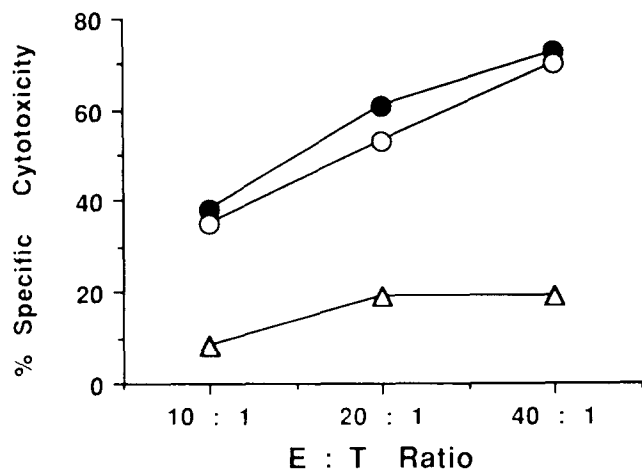
Discussion

NKSF is a novel heterodimeric cytokine that was originally isolated from the medium of EBV-transformed human B lymphoblastoid cell lines (16). Although NKSF has been previously reported to augment the MHC-unrestricted cytolytic activity of normal PBL (16, 19), the direct effects of NKSF on human NK cells have not been described in detail. Our results conclusively demonstrate that resting NK cells can respond to NKSF in the absence of any other stimuli. The presence of accessory cells, reportedly obligate for NKSF-induced IFN-γ secretion (18), does not appear to be required for augmentation of NK cytotoxicity. Both the CD56^{dim} and the CD56^{bright} NK cell subsets demonstrate enhanced cytolytic activity after stimulation with NKSF. Furthermore, NKSF augments all known NK cytolytic activities, including lysis of NK-sensitive targets (NK activity), lysis of NK-resistant

Figure 7. Cytolytic activity of NK cells expanded in vivo by IL-2. PBMC (∼75% CD56⁺CD3⁻) from a patient who had received rIL-2 6 × 10⁵ U/m²/d by continuous intravenous infusion for 12 wk were isolated and cryopreserved. These cells were subsequently thawed, incubated for 18 h with medium alone (*open triangles*), 230 U/ml IL-2 (*filled circles*), or 4 U/ml NKSF (*open circles*) and tested for cytotoxicity against COLO. Experiment was repeated once with PBMC from a different patient with identical results.

targets (LAK activity), and lysis of antibody-coated targets (ADCC). NKSF appears to be more efficient than either IL-2 or the IFNs in augmenting NK cytotoxicity; marked enhancement of NK lytic activity requires nanomolar concentrations of IL-2 or IFN but only picomolar concentrations of NKSF (16). However, optimal concentrations of IL-2 (∼5-10 nM) consistently induce greater cytolytic activity against NK-resistant targets than do optimal concentrations (∼1-10 pM) of NKSF (Fig. 2 and reference 16). Like IL-2-activated NK cells (28), NKSF-activated NK cells demonstrate increased expression of several CAM, including CD2, CD11a, and CD54. In as much as these structures have been shown to participate in NK cell adhesion to and lysis of target cells (28, 37, 38), NKSF could in part augment NK cytolytic activity by upregulating these or other adhesion molecules.

NKSF and IL-2 appear to activate human NK cells through distinct pathways that interact in complex ways. The effects of NKSF on NK cells do not require direct participation of IL-2, since anti-IL-2R mAbs that completely abrogate IL-2-induced NK cytotoxicity have little effect on NKSF-induced killing. Furthermore, IL-4 coincubation selectively inhibited the augmented cytolytic activity induced by IL-2 but not by NKSF. Nonetheless, IL-2 pretreatment rendered NK cells more responsive to the weak proliferative signal induced by NKSF. Increased IL-12 binding sites have apparently been detected on a subset of human PBMC stimulated with IL-2 (21), and hence, upregulation of NKSF receptors could mediate the increased NKSF-induced proliferation of IL-2-activated NK cells. Conversely, we found that NKSF potently inhibits NK cell proliferation in response to IL-2, despite stimulating increased NK cell expression of both IL-2R p55 and p75. Further study is needed to elucidate the mechanisms underlying these complicated interactions between effects of NKSF and IL-2 on human NK cells.

In contrast to its effects on NK cells, NKSF did not inhibit the proliferation of PHA-activated T cells to IL-2. NKSF thus appears to interfere selectively with the proliferative signal induced by IL-2 in NK cells. NKSF is composed of two subunits, p40 and p35, which are encoded by distinct genes (17, 20). The p40 chain, which has significant homology to the IL-6R (39), does not appear to be biologically active by itself; it is not clear whether the p35 chain alone has bioactivity (17). It has been suggested that NKSF may actually represent a soluble heterodimer comprised of a cytokine (p35) and part of its receptor (p40), and that either p35 or the p35/p40 complex might interact with additional cell surface receptor subunits (39). The differential effects of NKSF on the proliferation of activated NK cells and activated T cells could reflect differences in the NKSF receptor subunits expressed by the two cell types. Alternatively, identical receptors for NKSF may activate different second messenger pathways in T cells and NK cells upon ligand binding. Understanding of the disparate effects of NKSF on NK cells and T cells will require better characterization of the NKSF receptor and detailed examination of the biochemical and genetic alterations induced by NKSF.

Treatment with IL-2, with or without adoptive transfer

of activated autologous lymphocytes, has produced tumor regressions in some patients with advanced renal cell carcinoma and melanoma (13, 14). However, successful immunotherapy using IL-2 has generally required administration of high doses of the cytokine and has been associated with substantial toxicity (13–15). Several groups have undertaken modifications of the original high-dose IL-2 regimen, in attempts to enhance antitumor activity and to limit toxicity (36, 40–42). We have administered prolonged continuous intravenous infusions of IL-2 to patients with advanced cancer (36). IL-2 was well tolerated in doses as high as 6.0×10^5 U/m²/d for 90 consecutive days. Such therapy produced marked expansion of peripheral blood NK cells, and the PBL of these patients demonstrated enhanced killing of NK-sensitive and NK-resistant targets. The cytolytic activity of these in vivo preactivated NK cells can be further augmented by brief in vitro incubation with high concentrations of IL-2 (36). In the present study, we found that picomolar concentra-

tions of NKSF were as effective as nanomolar concentrations of IL-2 in augmenting the cytolytic activity of NK cells expanded in vivo. Furthermore, the effects of NKSF on proliferation of these NK cells were very similar to the effects of NKSF on normal NK cells preactivated in vitro with IL-2. Thus, NK cells expanded in vivo by chronic exposure to low concentrations of IL-2 seem to remain fully responsive to NKSF. It is conceivable that IL-2 and NKSF could be used together for anticancer immunotherapy. Since NKSF by itself induces very little NK cell proliferation, a rational strategy might include low-dose continuous infusions of IL-2 to expand NK cells in vivo, followed by administration of NKSF to augment NK cell cytolytic activity. NKSF administered in low doses might prove less toxic than high doses of IL-2, while producing similar activation of NK cytotoxicity. Further evaluation of NKSF as a potential therapeutic cytokine appears warranted.

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