Jpn. J. Cancer Res. 85, 853–861, August 1994

Induction Mechanism of Human Blood CD8$^+$ T Cell Proliferation and Cytotoxicity by Natural Killer Cell Stimulatory Factor (Interleukin-12)

Roustem Nabioullin, Saburo Sone,[1] Akihiko Nii, Takashi Haku and Takeshi Ogura

Third Department of Internal Medicine, The University of Tokushima School of Medicine, 18-15 Kuramoto-cho 3-chome, Tokushima 770

Natural killer cell stimulatory factor (NKSF/IL-12) has been found to induce cytotoxic activity of human blood T cells. In the present study, the effect of NKSF on induction of cytotoxic CD8$^+$ T cells in the presence or absence of monocytes was examined. Highly purified lymphocytes (>99%) and monocytes (>90%) were isolated by centrifugal elutriation from peripheral blood of normal donors. Then, CD8$^+$ cells were isolated with antibody-bound magnetic beads from purified lymphocytes. The cytotoxicity of CD8$^+$ cells was measured by ^{51}Cr release assay for 4 h. NKSF enhanced the proliferative response of CD8$^+$ cells stimulated with suboptimal concentrations of interleukin-2 (IL-2), but rather inhibited their proliferative and cytotoxic responses on stimulation with an optimal concentration of IL-2. NKSF stimulated CD8$^+$ cells to produce interferon γ (IFNγ) irrespective of the presence of added IL-2, and this effect was augmented by co-cultivation with monocytes. Blood monocytes upregulated induction of cytotoxic CD8$^+$ cells stimulated with NKSF alone, and this effect was abolished by addition of antibody against IFNγ, but not of antibody against tumor necrosis factor α. Induction of NKSF-inducible cytotoxic CD8$^+$ cells was inhibited by addition of transforming growth factor β, but not of IL-4. These observations suggest that *in situ* induction of NKSF-stimulated cytotoxic CD8$^+$ cells may be regulated by complex cytokine networks, depending on the participation of monocytes.

Key words: CD8$^+$ cell — Monocyte — IL-12 — Natural killer cell stimulatory factor — IFNγ

CTL[2] constitute one of the major effector cell populations of the immune system involved in the control of tumor cells and virally infected cells, most probably by lysis of infected and transformed cells.[1,2] Much attention has been paid to the effector mechanism by which CTL kill tumor cells. In addition to IL-2,[3] other cytokines such as IL-4,[4] IL-5,[5] IL-6[6] and IL-7[7] have been found to potentiate specific human cytotoxic T cell responses. Induction of cytotoxic T cell activity is regulated by the interaction of numerous cytokines, many with overlapping properties.[8,9]

NKSF is a recently described heterodimeric cytokine with ability to augment NK activity and induce IFNγ production.[10] These actions have been mainly defined by assays on freshly isolated peripheral blood lymphocytes or purified populations of NK cells.[11-13] Both T and NK cells also produce a message for IFNγ following overnight stimulation in the presence of NKSF.[14] Although it seems that the induction of IFNγ production requires accessory cells,[12] the induction of NK activity appears to be a direct action of NKSF on CD56$^+$ cells not requiring accessory cells.[15] In contrast, NKSF was found to cause proliferation of PHA-activated CD8$^+$ cells.[16] Little is known, however, about the effect of NKSF on generation of cytotoxic CD8$^+$ T cells, or its regulatory mechanism. In this study we found that the ability of CD8$^+$ cells to proliferate and to generate cytotoxic activity in response to NKSF depended on the time of addition of blood monocytes, and that the induction of cytotoxic CD8$^+$ cells by NKSF was suppressed by TGFβ, but not by IL-4. We also found that in the presence of monocytes, NKSF enhanced IL-2-stimulated CD8$^+$ cell proliferation, but inhibited the generation of cytotoxic activity induced by an optimal concentration of IL-2.

MATERIALS AND METHODS

Cell lines Cell lines of human Burkitt lymphoma (Daudi), and human lung small cell carcinoma (N291) were obtained from the American Type Culture Collection (Rockville, MD). A human lung adenocarcinoma cell line (PC-9) and lung small cell carcinoma cell line (H-69) were kindly supplied by Dr. Y. Hayata (Tokyo Medical College, Tokyo) and Dr. Y. Shimosato (National Cancer Center Research Institute, Tokyo), respectively. Cell lines were maintained in culture in RPMI 1640 medium (Nissui Pharmaceutical Co., Tokyo) supplemented with 10% heat-inactivated fetal bovine serum (GIBCO Laboratories, Auckland, New Zealand) and gentamicin (Schering-Plough, Osaka), designated as

[1] To whom correspondence should be addressed.

[2] Abbreviations: CTL, cytotoxic T lymphocytes; IFNγ, interferon γ; IL-2, interleukin-2; NK, natural killer; NKSF, NK cell stimulatory factor; TGF, transforming growth factor; TNF, tumor necrosis factor.

complete RPMI 1640 (CRPMI 1640) medium, at 37°C in a humidified atmosphere containing 5% CO_2. For cytotoxicity assays, cultured target cells were used in the exponential growth phase.

Reagents Recombinant human NKSF (natural killer cell stimulatory factor; IL-12) was supplied by Genetics Institute (Tokyo). Recombinant human IL-2 was a gift from Takeda Pharmaceutical Co. (Osaka), and had a specific activity of 3.5×10^4 U/mg as assayed on IL-2-dependent murine NKC3 cells.[17] One unit determined by this method is equivalent to approximately 400 Japanese reference units (JRU). The anti-human IFNγ antibody used was a polyclonal rabbit antiserum prepared against purified human IFNγ and was a gift from Otsuka Pharmaceutical Co., Tokushima. Recombinant human IL-4 (specific activity, 10^6 U/mg protein) was a gift from Ono Pharmaceutical Co., Osaka. Human TGFβ (lot No. A153023) was purchased from R & D Systems, Minneapolis, MN. A monoclonal antibody specific to human TNFα (IgM type, neutralizing activity 2.2×10^5 U/ml) was a gift from Hayashibara Institute, Okayama. None of these materials contained detectable endotoxins, as judged by *Limulus* amebocyte assay (sensitivity limit, 0.1 ng/ml) (Seikagaku Kogyo Co., Tokyo).

Isolation and culture of human peripheral blood monocytes Leukocyte concentrates were collected from peripheral blood (200 ml) of healthy donors in an RS-6600 rotor of a Kubota KR-400 centrifuge, and mononuclear cells were separated from the leukocyte concentrates in lymphocyte separation medium (Litton Bionetics). Then monocytes were separated from the mononuclear cell samples by centrifugal elutriation in a Hitachi SRR6Y elutriation rotor.[18,19] A fraction containing more than 95% of the total monocyte population was obtained at a speed of 2000 rpm and flow rate of 20 ml/min. More than 90% of these cells were monocytes as determined by nonspecific esterase staining and morphological examination, and more than 97% were viable, as judged by the trypan blue dye exclusion test. This fraction was washed twice with phosphate-buffered saline, and resuspended in RPMI 1640 medium supplemented with 5% heat-inactivated fetal bovine serum and gentamycin, designated as CRPMI 1640, at a concentration of 5×10^5 monocytes per ml. These cells were plated for 1 h in 96-well Microtest III plates (Falcon, Oxnard, CA), and nonadherent cells were removed by two washes with warm RPMI 1640 medium. At this stage the purity of the monocytes was >99% as judged from their morphology and nonspecific esterase staining.

Isolation of $CD8^+$ cells using antibody-coated immunomagnetic beads The sterile anti-CD8 antibody-coated immunomagnetic beads (Dynabeads M-450; Dyna Inc., Great Neck, NY) were washed twice with ice-cold CPMRI 1640, using a magnet to collect the beads. Then $CD8^+$ cells were isolated by using anti-CD8 antibody-coated beads as described previously.[20] Briefly, the bead pellet (3×10^7/ml) was suspended in 5 ml of the cell mixture (1×10^7 cells/ml) and incubated on ice with gentle agitation every 5 min for 30 min. Then the tube was inverted to resuspend the beads and placed in a magnetic holder for 2 min. The supernatant was pipetted off and the pellet was resuspended in 25 ml of cold RPMI 1640 and replaced in the magnet. This washing procedure was repeated, and the beads were suspended in 50 ml of CRPMI 1640 at 37°C for 16 h. The beads were then removed from the cultures by vigorously agitating the mixture in each plate with a Pasteur pipette and placing the mixture in a magnetic holder. After 2 min, the supernatant was pipetted off and saved for replating. By this procedure more than 99% of the $CD8^+$ cells were isolated, as judged by FACScan analysis (data not shown). $CD8^+$ cells with or without monocytes were plated at 10^5 cells/well into a 96-well Microtest III plates (Falcon) and incubated for 4 days in medium with or without IL-2 at 37°C.

Cytotoxicity assay Cell-mediated cytotoxicity was assayed by measuring ^{51}Cr release in a 4 h test period, as described previously.[21] Briefly, 10^5 $CD8^+$ cells were incubated in CRPMI 1640 with NKSF and/or IL-2 in the presence or absence of 10^4 monocytes in 96-well plates for 4 days. In some experiments, anti-TNFα monoclonal antibody or anti-IFNγ antiserum was added. After 4 days, the supernatants were removed and frozen for measurement of cytokines, while the remaining cells were washed and their cytotoxicity against ^{51}Cr-labeled Daudi cells was measured at an effector/target ratio (E/T) of 10:1. Preliminary experiments indicated that at this point there was no difference in the number of lymphocytes treated with IL-2 and/or NKSF in the presence or absence of monocytes (data not shown). Percentage cytotoxicity was calculated as 100 × (experimental cpm − spontaneous cpm)/(maximum cpm − spontaneous cpm). The spontaneous releases observed with different target cells ranged from 5 to 15% (total lysis).

Cell proliferation assay For proliferation assay, purified $CD8^+$ cells were dispensed in triplicate wells in flat-bottomed plates, and incubated for 4 days with various concentrations of NKSF and/or IL-2. Eighteen hours before the end of incubation ^3H-TdR (6.7 Ci/mmol: Amersham, Arlington Heights, IL) was added at 0.5 mCi/well. After incubation, the cells were harvested on a glass fiber filter with a cell harvester, MASH II, and radioactivity was measured by counting the samples in a β-counter.

Quantitative measurements of cytokines Enzyme immunoassays (EIAs) for human IFNγ, IL-2, TNFα and TNFβ were performed essentially as described in detail previously.[18] Sensitivity limits of the EIAs for IFNγ, IL-

2, TNFα and TNFβ were 20 pg/ml, 20 pg/ml, 20 pg/ml and 1 U/ml, respectively.

Statistical analysis The statistical significance of differences between groups was analyzed by means of Student's t test (two-tailed).

RESULTS

Induction by IL-12 of cytotoxic CD8$^+$ T cells in the presence of monocytes First, we examined whether induction by NKSF of tumor cytotoxicity mediated by CD8$^+$ T cells was dependent on monocytes. For this, CD8$^+$ T cells were incubated for 4 days in medium with NKSF (10 or 100 U/ml) in the presence or absence of monocytes at densities of 0.05×10^5 to 2×10^5 cells. Representative results of 3 different experiments are shown in Fig. 1. CD8$^+$ cells that had been incubated in NKSF-free medium with or without monocytes did not show significant cytotoxicity. Blood monocytes alone, irrespective of NKSF treatment, were not cytotoxic (data not shown). When blood CD8$^+$ T cells were treated with NKSF alone in the absence of monocytes, low levels of tumor cytotoxicity were observed. Under the same experimental conditions, full induction of cytotoxic CD8$^+$ cells by NKSF was dependent on the density of monocytes added.

In a parallel experiment we examined the effect of the time of NKSF addition on induction of cytotoxic CD8$^+$ cells in the presence of monocytes. NKSF was added to cultures of CD8$^+$ cells plus monocytes on the days indicated in Fig. 2 and incubation was continued until day 4. Addition of NKSF to the cultures at the onset of

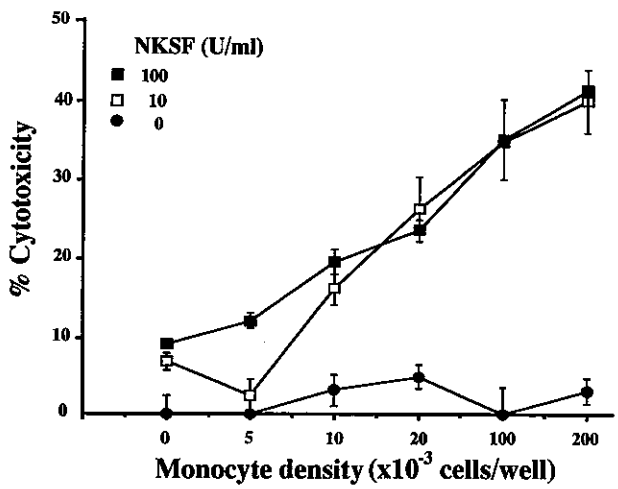

Fig. 1. Effect of monocyte density on induction of cytotoxic CD8$^+$ cells by NKSF. CD8$^+$ cells (10^5/well) were incubated for 4 days in medium with or without NKSF in the presence or absence of monocytes at the indicated densities, and then their cytotoxic activities were assayed on Daudi cells at an E/T ratio of 10:1, as described in "Materials and Methods." Bars show SDs of means for triplicate cultures.

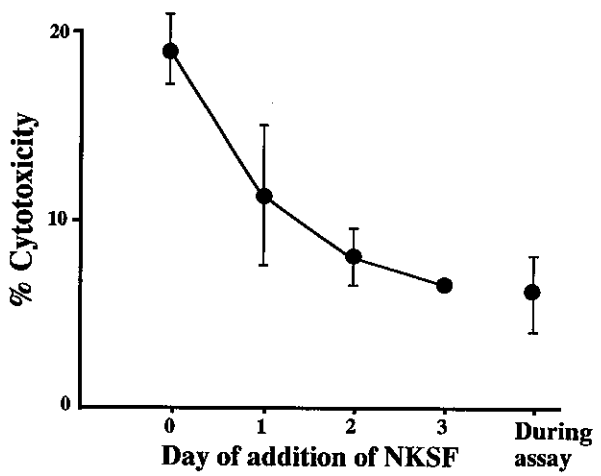

Fig. 2. Effect of time of NKSF addition on induction of cytotoxic CD8$^+$ cells in the presence of monocytes. CD8$^+$ cells (10^5/well) were incubated for 4 days in the presence of monocytes (10^4/well). NKSF was added on the indicated days and cytotoxicity was assayed on day 4. Bars show SDs of means for triplicate cultures. Data are representative of three experiments.

Table I. Antitumor Activity of NKSF-induced Cytototoxic CD8$^+$ Cells against Various Lung Cancer Cells

Treatment	Addition of monocytes	Percent specific cytotoxicity			
		Daudi	PC-9	H-69	N291
Medium	−	1.2±0.5[a]	1.5±1.0	0	0.2±0.7
	+	14.1±4.8[b]	2.9±3.0	4.9±1.5[b]	6.6±0.9[b]
NKSF	−	3.1±0.8	1.6±1.0	0	0.7±1.6
10 U/ml	+	26.6±3.5[b]	14.5±3.8[b]	11.8±0.2[b]	28.1±1.8[b]

a) Mean±SD for triplicate cultures.
b) Significantly different from the value without monocytes ($P<0.05$).

incubation resulted in the greatest induction of cytotoxic activity, and on its addition at later times, its augmenting effect became progressively less.

Antitumor spectrum of NKSF-induced cytotoxic CD8$^+$ cells We examined the antitumor activities of NKSF-induced CD8$^+$ cells against various human lung cancer cells. For this, CD8$^+$ cells (10^5/well) with or without monocytes (10^4/well) were incubated for 4 days in medium with NKSF (10 U/ml) before addition of various target cells at an E/T ratio of 10:1. Monocytes (10^4/well) alone that had been treated with or without NKSF did not show cytotoxicity to any of the target tumor cells (data not shown). The results in Table I show that CD8$^+$ cells that had been co-cultured with monocytes without NKSF were significantly cytotoxic to Daudi cells, H-69 and N291 cells, and that in the presence of monocytes, NKSF induced cytotoxic activity of CD8$^+$ cells against all the target cells examined.

Effect of NKSF on proliferation and cytotoxicity of immunomagnetically purified CD8$^+$ T cells in response to IL-2 To determine the effect of NKSF on proliferation of CD8$^+$ T cells, we incubated CD8$^+$ T cells purified immunomagnetically from normal blood lymphocytes for 4 days in medium with various amounts of NKSF in the presence or absence of IL-2, and then determined their proliferative responses by measuring their ^{3}H-TdR uptake. Purified CD8$^+$ T cells proliferated dose-dependently in response to IL-2, and addition of NKSF (1–100 U/ml) to cultures of CD8$^+$ cells plus IL-2 resulted in a significant increase in CD8$^+$ cell proliferation (Fig. 3).

We also examined the effect of addition of IL-2 on induction of cytotoxic CD8$^+$ cells by NKSF in the presence or absence of monocytes. For this, magnetically

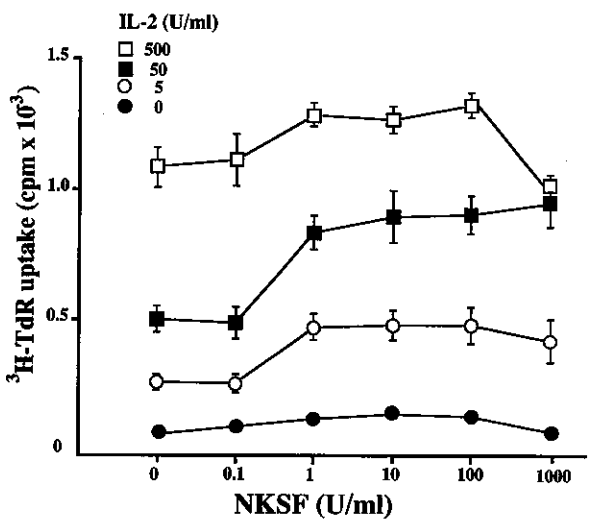

Fig. 3. Dose-dependence of the effect of NKSF on proliferation of purified CD8$^+$ T cells in the presence of monocytes in response to IL-2. CD8$^+$ cells (10^5/well) and monocytes (10^4/well) were incubated with the indicated concentrations of NKSF and/or IL-2 (5, 50 or 500 U/ml). ^{3}H-TdR incorporation was determined after incubation for 4 days. Data are representative of three separate experiments. Bars show SDs of means for triplicate cultures.

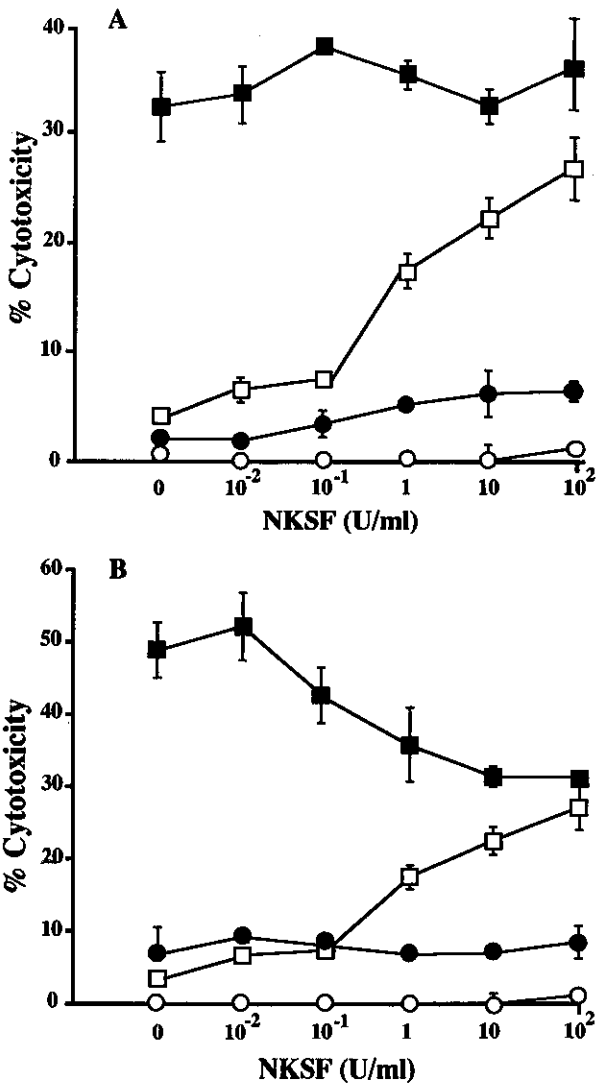

Fig. 4. Dose-dependence of effect of NKSF on induction of cytotoxic CD8$^+$ T cells in the presence or absence of monocytes in response to IL-2. CD8$^+$ cells (10^5/well) with (squares) or without monocytes (10^4/well) (circles) were incubated for 4 days with or without the indicated concentrations of NKSF in the absence (open symbols) or presence of 5 U/ml (A) or 500 U/ml (B) of IL-2 (closed symbols). Then, their cytotoxic activity against Daudi cells was determined at an E/T ratio of 10:1. Data are representative of three separate experiments. Bars show SDs of means for triplicate cultures.

isolated CD8$^+$ cells with or without monocytes were incubated for 4 days in medium with or without a suboptimal (5 U/ml) or an optimal (500 U/ml) concentration of IL-2 in the presence or absence of NKSF at concentrations of 0.01 to 100 U/ml, and then their cytotoxic activities were measured. As shown in Fig. 4, induction of cytotoxic CD8$^+$ cells by both suboptimal and optimal doses of IL-2 was augmented by addition of monocytes. Similarly, monocytes augmented the induction of cytotoxic CD8$^+$ cells by NKSF at concentrations of more than 1 U/ml. The maximal cytotoxicity mediated by NKSF-stimulated CD8$^+$ cells was consistently lower than that of those stimulated with IL-2. Under similar experimental conditions, NKSF and a suboptimal concentration of IL-2 did not have synergistic effects on the induction of the cytotoxic activity. On the contrary, NKSF suppressed the induction of cytotoxic CD8$^+$ cells by an optimal concentration of IL-2 in the presence of monocytes. In a parallel experiment, we compared the abilities of CD8$^+$ cells plus monocytes of 6 different donors stimulated for 4 days with optimal concentrations of IL-2 and NKSF to kill Daudi cells. The maximum cytotoxic activity of NKSF-stimulated CD8$^+$ cells varied from 11.0% to 66.1% (mean 39.3%) of those stimulated with IL-2 (data not shown).

Stimulations of IFNγ and TNFα productions by CD8$^+$ T cells by NKSF and/or IL-2 NKSF has been found to induce IFNγ production by NK cells and T cells.[11] We examined whether NKSF induced cytokine production by CD8$^+$ cells in the presence or absence of monocytes. For this, CD8$^+$ cells (10^5/well) with or without monocytes (10^4/well) were incubated for 4 days in medium with or without NKSF (1, 10 and 100 U/ml), and then the supernatants were harvested for quantitative measurements of IFNγ, IL-2, TNFα and TNFβ. Of these four cytokines only IFN-γ was produced in significant quantity by CD8$^+$ cells stimulated with NKSF (more than 10 U/ml) in the presence of monocytes (data not shown).

In a parallel experiment, we examined the effect of IL-2 plus NKSF on IFNγ and TNFα production by CD8$^+$ cells in the presence or absence of monocytes (Table II). Significant IFNγ production by CD8$^+$ cells was induced by the synergistic action between NKSF and IL-2 at suboptimal concentrations. Addition of monocytes (10^4/well) to cultures of CD8$^+$ cells (10^5/well) resulted in a marked increase in IFNγ production by CD8$^+$ cells stimulated with NKSF plus IL-2. Under these experimental conditions CD8$^+$ cells with or without NKSF and/or IL-2 did not produce a detectable amount of TNFα (data not shown).

Effect of anti-IFNγ antiserum on induction of cytotoxic CD8$^+$ cells by NKSF CD8$^+$ cells were incubated with monocytes for 4 days in medium with various concentrations of NKSF in the presence or absence of anti-IFNγ antiserum before addition of labeled target Daudi cells. As shown in Fig. 5, in the presence of monocytes, cytotoxic CD8$^+$ cells were induced by NKSF dose-dependently. Addition of anti-IFNγ antiserum to the cultures of CD8$^+$ cells and monocytes with NKSF resulted in significant suppression of the induction of cytotoxic CD8$^+$ cells (Fig. 5A). Under the same conditions, anti-TNFα antibody did not affect the induction of cytotoxic CD8$^+$ cells by NKSF (Fig. 5B).

Effects of IL-4 and TGFβ on induction of cytotoxic CD8$^+$ cells by NKSF in the presence of monocytes IL-4 and TGFβ have been found to suppress various functions of monocytes.[19, 22-24] Therefore, we examined whether IL-4 or TGFβ affected the induction of cytotoxic CD8$^+$ cells by NKSF in the presence of monocytes (Fig. 6). IL-4 did not suppress NKSF-induced generation of cytotoxic CD8$^+$ cell activity. In 2 out of 5 different donors, significantly higher cytotoxic activity was observed with the IL-4 plus NKSF combination. In contrast, TGFβ (1 ng/ml) almost completely inhibited NKSF-induced generation of cytotoxic CD8$^+$ cells in the presence of monocytes.

Table II. IFNγ Production by CD8$^+$ Cells Stimulated with NKSF and/or IL-2 in the Presence or Absence of Monocytes

NKSF (U/ml)	Production of IFNγ (pg/ml)					
	Medium		IL-2 (5 U/ml)		IL-2 (500 U/ml)	
	Medium	Monocytes	Medium	Monocytes	Medium	Monocytes
0	<20	<20	<20	<20	<200[a]	3220[a]
0.01	<20	<20	<20	53	<200	nd[b]
0.1	<20	<20	28	415	<200	5680
1	<20	<20	57	893	<200	11110
10	<20	72	69	3618	<200	38620
100	<20	489	nd	4436	500	nd

a) Samples of culture supernatants were diluted 10-fold with phosphate-buffered saline, and used for quantitative measurement of IFNγ.
b) Not done.

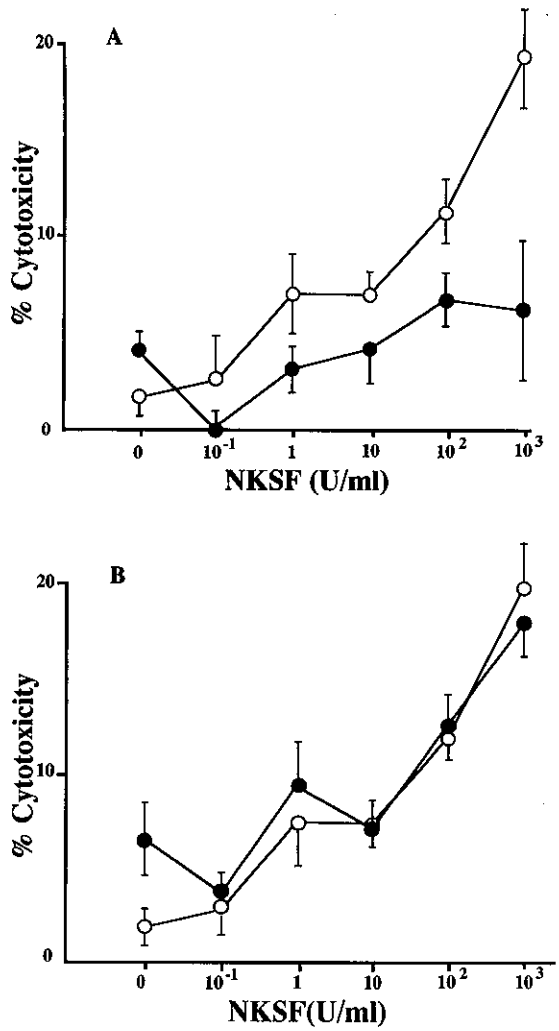

Fig. 5. Effect of addition of anti-IFNγ antiserum or anti-TNFα antibody on induction by NKSF of cytotoxic CD8$^+$ T cells in the presence of monocytes. CD8$^+$ cells (10^5/well) with monocytes (10^4/well) were incubated in medium with or without the indicated concentrations of NKSF in the presence (closed circles) or absence (open circles) of anti-human IFNγ antiserum (A) or monoclonal anti-human TNFα antibody (B). After 4 days, their cytotoxic activities against Daudi cells were assayed at an E/T ratio of 10:1. Bars show SDs of means for triplicate cultures. Data are representative of two independent experiments

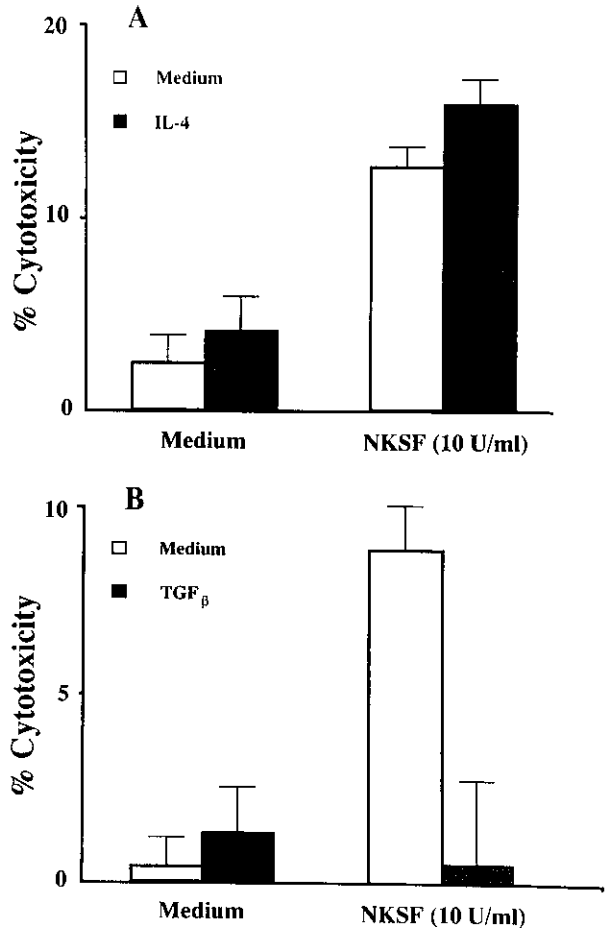

Fig. 6. Effect of IL-4 and TGFβ on induction of cytotoxic CD8$^+$ cells by NKSF in the presence of monocytes. CD8$^+$ cells (10^5/well) plus monocytes (10^4) were incubated for 4 days with or without NKSF in the presence or absence of IL-4 (100 U/ml) or TGFβ (1 ng/ml). Then, the cells were used for tumor cytotoxicity assay as described in "Materials and Methods." Bars show SDs of means for 5 different experiments (A) and for 2 different experiments (B).

DISCUSSION

NKSF (IL-12) is a novel heterodimeric cytokine that was recently isolated from the culture medium of Epstain-Barr virus-transformed human B lymphoblastoid cell lines.[10] Although NKSF produced endogenously by the B cell line and normal human blood mononuclear cells has been reported to induce proliferation of activated NK and T cells,[24,25] the effects of NKSF on human CD8$^+$ T cells have not been described in detail. The present study clearly demonstrated that resting human CD8$^+$ cells alone fail to respond to NKSF, but that they can proliferate in response to NKSF in the presence of monocytes. The presence of monocytes was also required for NKSF-induced CD8$^+$ cell-mediated cytotoxicity. This finding is in contrast to a previous report[13] that augmentation of NK activity by NKSF does not require accessory cells. Furthermore, CD8$^+$ cells stimulated with NKSF in the presence of monocytes were also nonspecifically cytotoxic to all the allogeneic lung cancer cell lines used as target cells (Table I).

The present finding that maximal induction of cytotoxic CD8$^+$ cells by NKSF requires continuous interaction with autologous monocytes (Fig. 2) suggests that NKSF is required for differentiation of cytotoxic precursors into effector CD8$^+$ cells. NKSF can affect the functional state of cytotoxic effector cells independently of its ability to stimulate proliferation. When monocytes were present, the maximal cytotoxic activity of CD8$^+$ cells induced by an optimal dose of IL-2 was much greater than that induced by an optimal dose of NKSF. IFNγ is known to be required for induction of cytotoxic T cells by IL-2.[26,27] NKSF also triggered CD8$^+$ cells to secrete IFNγ, but not TNFα, TNFβ or IL-2. In this regard, it would be interesting to examine whether the effect of NKSF on the induction of cytotoxic CD8$^+$ cells in the presence of monocytes is mediated by other induced cytokines. We obtained evidence for a role of IFNγ, but not TNFα, in NKSF-induced cytotoxic activity between CD8$^+$ cells and monocytes (Fig. 5). This enhancing effect was almost completely abolished by a specific IFNγ-neutralizing antiserum, but was not affected by an anti-TNFα antibody. These findings suggest that NKSF stimulates CD8$^+$ cells in the presence of monocytes to produce IFNγ, which may be required to augment the cell-to-cell interaction between CD8$^+$ cells and monocytes for differentiation of CD8$^+$ cells to become cytotoxic effector cells.

A high concentrations, IL-2 induces maximal proliferation of T cells in the presence of monocytes irrespective of a CD3/TCR co-signal.[28] In contrast, NKSF mediates cytotoxic T cell proliferation only when the cells have recently been co-stimulated with alloantigen or anti-CD3 antibody.[29,30] In the present study IL-2 induced proliferation of CD8$^+$ cells dose-dependently, but NKSF alone did not induce their proliferation.

Interestingly, NKSF at concentrations of 1 to 100 U/ml augmented IL-2-induced proliferation and IFNγ-producing ability of CD8$^+$ cells. In contrast, NKSF and IL-2 did not have synergistic effects in induction of cytotoxic CD8$^+$ cells, but instead NKSF dose-dependently suppressed the induction of cytotoxic CD8$^+$ cells by an optimal concentration of IL-2. The mechanism of the latter effect by NKSF is unknown: conceivably NKSF functions in control of the induction of IL-2-inducible killer activity from CD8$^+$ cells, and so can either enhance or inhibit IL-2-induced killer activation depending on the relative concentrations of the two cytokines. This observation seems to be in accordance with a previous finding that high concentrations of IL-2 and NKSF tended to inhibit cytotoxicity mediated by monocyte-depleted blood mononuclear cells[31] and by purified CD8$^+$ cells stimulated with immobilized anti-CD3 antibody in cultures.[30] These findings raise another possibility, that some other unidentified cytokine(s) produced by activated CD8$^+$ cells and/or monocytes might be responsible for suppression of the cytotoxic CD8$^+$ cell induction through a cytokine network. In contrast, Robertson *et al.*[13] showed that NKSF acted directly on sorted CD56$^+$ cells, greatly augmenting their NK activity, but inhibited the proliferative response to a high concentration of IL-2.

IL-4 and TGFβ are known as immunoregulatory cytokines. These cytokines have suppressive effects on production of cytokine by monocytes and macrophages.[22-24] Interestingly, addition of an optimal concentration of TGFβ, but not of IL-4, to cultures of CD8$^+$ cells plus NKSF in the presence of monocytes resulted in almost complete suppression of cytotoxic cells (Fig. 6). This difference between IL-4 and TGFβ may be due to the different effects of IL-4 and TGFβ on CD8$^+$ cells. Indeed, TGFβ was recently found to suppress perforin production of by CD8$^+$ cells.[32] Thus, TGFβ may suppress NKSF-inducible cytotoxic CD8$^+$ cells in two ways: 1) by inhibiting accessory functions of monocytes and 2) by inhibiting the stimulatory effects of NKSF on perforin production by CD8$^+$ cells. Studies on these possibilities are in progress.

Further studies on the regulations of NKSF-inducible cytotoxic CD8$^+$ cells may contribute to our understanding of the complex cytokine networks, allowing us to generate more potential cytotoxic CD8$^+$ T cells *in vivo* for cancer immunotherapy in humans.

ACKNOWLEDGMENTS

The authors thank Mr. Y. Ohmoto for measurements of cytokines. This work was supported by grants from the Ministry of Education, Science and Culture and the Ministry of Health and Welfare of Japan.

(Received January 24, 1994/Accepted May 7, 1994)

REFERENCES

1) Young, J. D. Killing of target cells by lymphocytes: mechanistic view. *Physiol. Rev.*, **69**, 250–314 (1989).
2) Podack, E. R. Molecular mechanism of lymphocyte-mediated tumor cell lysis. *Immunol. Today*, **6**, 21–27 (1985).
3) Kern, D. E., Gillis, S., Okada, M. and Henney, C. S. The role of interleukin-2 (IL-2) in the differentiation of cytotoxic T cells: the effect of monoclonal antibody and absorption with IL-2 dependent T cell lines. *J. Immunol.*, **127**, 1323–1328 (1981).

4) Spits, H., Yssel, H., Paliard, X., Kastelein, R., Figdor, C. and de Vries, J. E. IL-4 inhibits IL-2-mediated induction of human lymphokine-activated killer cells, but not the generation of antigen-specific cytotoxic T lymphocytes in mixed leukocyte cultures. *J. Immunol.*, **141**, 29–36 (1988).
5) Takai, Y., Wong, G., Clarke, S. C., Burakiff, S. J. and Herrmann, S. B cell stimulatory factor-2 is involved in the differentiation of cytotoxic T lymphocytes. *J. Immunol.*, **140**, 508–512 (1988).
6) Alderson, M. R., Sassenfeld, H. M. and Widmer, M. B. Interleukin 7 enhances cytolytic T lymphocyte generation and induces lymphokine-activated killer cells from human peripheral blood. *J. Exp. Med.*, **172**, 577–587 (1990).
7) Ochoa, A., Gromo, G., Barbara, B. J., Sondel. P. M. and Bach, F. H. Long term growth of lymphokine-activated killer (LAK) cells: role of anti-CD3, β-IL-1, interferon-γ and -β. *J. Immunol.*, **138**, 2728–2733 (1978).
8) Owen-Schaub, L. B., Gutterman, J. U. and Grimm, E. A. A synergy of tumor necrosis factor and interleukin-2 in the activation of human cytotoxic lymphocytes: effect of tumor necrosis factor-α and interleukin-2 in the generation of human lymphokine-activated killer cell cytotoxicity. *Cancer Res.*, **48**, 788–792 (1988).
9) Sone, S., Yanagawa, H., Nii, A. and Ogura, T. Induction of interleukin 2-activated killer effector activity and its regulatory mechanism. *Gann Monogr. Cancer Res.*, **40**, 81–93 (1993).
10) Kobayashi, M., Fitz, L., Ryan, M., Hewick, R. M., Clark, S. C., Chan, S., Loudon, R., Sherman, F., Perussia, B. and Trinchieri, G. Identification and purification of natural killer cell stimulatory factor (NKSF), a cytokine with multiple biologic effects on human lymphocytes. *J. Exp. Med.*, **170**, 827–845 (1989).
11) Chan, S. H., Perussia, B., Gupta, J. W., Kobayashi, M., Pospisil, M., Young, H. A., Wolf, S. F., Young, D., Clark, S. C. and Trinchieri, G. Induction of interferon γ production by natural killer cell stimulatory factor: characterization of the responder cells and synergy with other inducers. *J. Exp. Med.*, **173**, 869–879 (1991).
12) Chehimi, J., Starr, S. E., Frank, I., Rengaraju, M., Jackson, S. J., Llanes, C., Kobayashi, M., Perussia, B., Young, D., Nickbarg, E., Wolf, S. F. and Trinchieri, G. Natural killer (NK) cell stimulatory factor increases the cytotoxic activity of NK cells from both healthy donors and human immunodeficiency virus-infected patients. *J. Exp. Med.*, **175**, 789–796 (1992).
13) Robertson, M. J., Soiffer, R. J., Wolf, S. F., Manley, T. J., Donahue, C., Young, D., Hermann, S. H. and Ritz, J. Response of human natural killer (NK) cells to NK cell stimulatory factor (NKSF): cytolytic activity and proliferation of NK cells are differently regulated by NKSF. *J. Exp. Med.*, **175**, 779–788 (1992).
14) Chan, S. H., Kobayashi, M., Santoli, D., Perussia, B. and Trinchieri, G. Mechanisms of IFN-γ induction by natural killer cell stimulatory factor (NKSF/IL-12). Role of transcription and mRNA stability in the synergistic interaction between NKSF and IL-2. *J. Immunol.*, **148**, 92–98 (1992).
15) Gately, M. K., Wilson, D. E. and Wong, H. L. Synergism between recombinant interleukin 2 (IL-2) and IL-2-depleted lymphokine containing supernatants in facilitating allogeneic human cytolytic T lymphocyte responses *in vitro*. *J. Immunol.*, **135**, 1274–1281 (1986).
16) Gately, M. K., Desai, B. B., Woltizky, A. G., Quinn, P. M., Dwyer, C. M., Podlaski, F. J., Familletti, P. C., Sinigaglia, F., Chizonnite, R., Gubler, U. and Stern, A. S. Regulation of human lymphocyte proliferation by a heterodimeric cytokine, IL-12 (cytotoxic lymphocyte maturation factor). *J. Immunol.*, **147**, 874–882 (1991).
17) Kato, K., Yamada, T., Kawahara, K., Onda, H., Asano, T., Sugino, H. and Kakimura, A. Purification and characterization of recombinant human interleukin-2 produced in *Escherichia coli*. *Biochem. Biophys. Res. Commun.*, **130**, 692–699 (1985).
18) Sone, S., Yanagawa, H., Nishioka, Y., Orino, E., Bhaskaran, G., Nii, A., Mizuno, K., Heike, Y., Ogushi, F. and Ogura, T. Interleukin-4 as a potent down-regulator for human alveolar macrophages capable of producing tumor necrosis factor-α and interleukin-1. *Eur. Respir. J.*, **5**, 174–181 (1992).
19) Orino, E., Sone, S., Nii, A. and Ogura, T. IL-4 up-regulate IL-1 receptor antagonist gene expression and its production in human blood monocytes. *J. Immunol.*, **149**, 925–931 (1992).
20) Sugihara, K., Sone, S., Shono, M., Nii, A., Munekata, M., Okumura, K. and Ogura, T. Enhancement by monocytes of perforin production and its gene expression by human $CD8^+$ T cells stimulated with interleukin-2. *Jpn. J. Cancer Res.*, **83**, 1223–1230 (1992).
21) Sone, S., Utsugi, T., Nii, A. and Ogura, T. Effect of human alveolar macrophages on the induction of lymphokine (IL-2)-activated killer cells. *J. Immunol.*, **139**, 29–34 (1987).
22) Hart, P. H., Fitti, G. F., Burgess, D. R., Whitty, G. A., Piccoli, D. S. and Hamilton, J. A. Potential antiinflammatory effects of interleukin 4: suppression of human monocyte tumor necrosis factor α, interleukin 1, and prostaglandin E_2. *Proc. Natl. Acad. Sci. USA*, **86**, 3803–3807 (1989).
23) Nishioka, Y., Sone, S., Orino, E., Nii, A. and Ogura, T. Down-regulation by interleukin 4 of activation of human alveolar macrophages to the tumoricidal state. *Cancer Res.*, **51**, 5526–5531 (1991).
24) Chantry, D., Turner, M., Abney, E. and Feldman, M. Modulation of cytokine production by transforming growth factor-β. *J. Immunol.*, **142**, 4295–4300 (1989).
25) D'Andrea, A., Rengaraju, M., Valiante, N. M., Chehimi, J., Kubin, M., Aste, M., Chan, S. H., Kobayashi, M., Young, D., Nickbarg, E., Chizzonite, R., Wolf, S. F. and Trinchieri, G. Production of natural killer cell stimulatory factor (interleukin-12) by peripheral blood mononuclear cells. *J. Exp. Med.*, **176**, 1387–1398 (1992).

26) Farrar, W. L., Johnson, H. M. and Farrar, J. J. Regulation of the production of immune interferon and cytotoxic T lymphocytes by interleukin 2. *J. Immunol.*, **126,** 1120–1125 (1981).
27) Itoh, K., Shiba, K., Shimizu, Y., Suzuki, R. and Kumagai, K. Generation of activated killer (AK) cells by recombinant interleukin 2 (IL-2) in collaboration with interferon γ (IFN-γ). *J. Immunol.*, **134,** 3124–3129 (1985).
28) Roosnek, E. E., Brouwer, M. C., Kipp, J. B. and Aarden, L. A. Monocyte-dependent induction of human peripheral T cells by recombinant interleukin 2. *Eur. J. Immunol.*, **16,** 35–40 (1986).
29) Bertagnolli, M. M., Lin, B.-Y., Young, D. and Herrmann, S. H. IL-12 augments antigen-dependent proliferation of activated T lymphocytes. *J. Immunol.*, **149,** 3778–3783 (1992).
30) Mehrotra, P. T., Wu, D., Crim, J. A., Mostowski, H. S. and Siegel, J. P. Effects of IL-12 on the generation of cytotoxic activity in human CD8$^+$ T lymphocytes. *J. Immunol.*, **151,** 2444–2452 (1993).
31) Gately, M. K., Wolitzky, A. G., Quinn, P. M. and Chizzonite, R. Regulation of human cytolytic lymphocyte responses by IL-12. *Cell. Immunol.*, **143,** 127–142 (1992).
32) Smyth, M. J., Strobl, S. L., Young, H. A., Ortaldo, J. R. and Ochoa, A. G. Regulation of lymphokine-activated killer activity and pore-forming protein gene expression in human blood CD8$^+$ lymphocytes. Inhibition by transforming growth factor-β. *J. Immunol.*, **146,** 3289–3297 (1991).