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*J Immunol* 1999; 163:1473-1480; http://www.jimmunol.org/content/163/3/1473

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Human NK Cells Express Endothelial Nitric Oxide Synthase, and Nitric Oxide Protects Them from Activation-Induced Cell Death by Regulating Expression of TNF-α

Keizo Furuke,* Parris R. Burd,* Judith A. Horvath-Arcidiacono,* Kotaro Hori,† Howard Mostowski,* and Eda T. Bloom1†*

Although NO appears important in rodent immune responses, its involvement in the human immune system is unclear. We report that human NK cells express constitutive endothelial NO synthase mRNA and protein, but not detectable levels of inducible NO synthase. They produce NO following activation by coculture with target cells or cross-linking with anti-CD16 mAb, and production is increased in the presence of IL-2. N-monomethyl-L-arginine (L-NMA), a NOS inhibitor, partially inhibited NK cell lysis of four different target cells (<40% inhibition at 500 μM L-NMA), but not granule release following coculture with target cells, or Fas ligand induction following cross-linking with anti-CD16 mAb. However, L-NMA augmented apoptosis of NK cells induced by activation through CD16 ligation or coculture with K562. An NO donor, S-nitroso-N-acetylpenicillamine (SNAP), suppressed apoptosis of NK cells induced by CD16 cross-linking or coculture with target cells, suggesting that endogenous NO production is involved in protection of NK cells from activation-induced apoptosis, thereby maintaining NK activity. SNAP also suppressed, and L-NMA enhanced, expression of TNF-α, reported to be involved in activation-induced NK cell death, in response to CD16 cross-linking. Suppression of anti-CD16-induced apoptosis by SNAP was reversed by the addition of rTNF-α. DNA-binding activity of the transcription factor, NF-AT, which is involved in TNF-α induction upon ligation of CD16, was inhibited by SNAP and enhanced by L-NMA. Our results suggest that down-regulation of TNF-α expression, possibly due to suppression of NF-AT activation, is a mechanism by which endogenous NO protects NK cells from activation-induced apoptosis, and maintains lytic capacity.


Nitric oxide has been implicated in a number of physiological functions, including smooth muscle relaxation, platelet inhibition, neurotransmission, and macrophage-mediated cytotoxicity (1, 2). The wide variety of effects is achieved from its interactions with target molecules via a rich oxidation-reduction (redox) potential (3). Synthesis of NO from L-arginine is catalyzed by the enzyme NO synthase (NOS),2 which includes two constitutive isozymes, endothelial NOS (eNOS) and neuronal NOS, and an inducible isozyme, termed iNOS (4). iNOS is induced by stimuli such as bacterial endotoxins or cytokines and, in mice, has been shown to participate in immune defense against bacterial, fungal, viral, or parasitic pathogens through production of large amounts of NO by macrophages (5). However, although human iNOS is also reported to be induced in monocytes and/or macrophages by LPS, IFN-γ, or HIV infection, the levels of NO production appear relatively low compared with that in rodent systems (6–8). Thus, a physiological role for NO in the human immune system has not been identified. Activity of constitutive NOSs is Ca2+ dependent, transient, and responsible for NO production at very low levels in comparison with that of iNOS (4, 9). The low levels of NO produced by the constitutive isozymes are believed to function in signaling for regulation of vascular tonus or neurotransmission (4, 9).

NK cells play a crucial role in immune surveillance by recognizing and eliminating virus-infected or neoplastic target cells (10–12). We previously reported that NK cell functions are regulated by the intracellular redox status (13, 14). Thiol-reducing compounds, such as cysteine and glutathione, are required for optimal proliferation and killing activity of NK cells in response to IL-2. Conversely, a variety of oxidative stresses is known to mediate apoptosis in many systems (15, 16) and facilitates Fas-induced apoptosis in NK cells (17). NO-releasing agents have been reported to inhibit human NK cell activity (18, 19). Production of NO by murine NK cells has been reported (20), whereas NO production by human NK cells has not been confirmed. In the present study, we showed that human NK cells express eNOS mRNA and protein and identified a potential physiological role for NO production and mechanism of action in human NK cells.

Materials and Methods

Reagents, Abs, and media

Human rIL-2 and rIL-12 were provided by Amgen (Thousand Oaks, CA) and Genetics Institute (Cambridge, MA), respectively. Human rTNF-α was purchased from Genzyme (Cambridge, MA). N-monomethyl-L-arginine, monooacetate salt (t-NMA) was purchased from Calbiochem-Novabiochem (La Jolla, CA). S-nitroso-N-acetylpenicillamine (SNAP) was obtained from Sigma (St. Louis, MO). Metalloprotease inhibitor KB8301 was purchased from PharMingen (San Diego, CA). Anti-CD5 mAb was purified...
from ascites, as described previously (14). Anti-CD22 mAb was purchased from PharMingen. Anti-CD16 (clone 3G8), anti-CD36, anti-CD16 PE, and anti-CD56 PE mAbs were purchased from Immunotech (Westbrook, ME). Anti-CD16 FITC mAb and anti-CD56 mAb as an isotype control for 3G8 were purchased from Becton Dickinson (Mountain View, CA). Goat anti-mouse IgG (GaM) mAb was purchased from Sigma.

RPMI 1640 media with or without phenol red or without arginine were purchased from Becton Dickinson (Mountain View, CA). Goat anti-mouse IgG (GaM) mAb was purchased from Sigma.

**Cells and purification of human NK cells**

Human NK cell lines, YT and NK3.3, were kindly provided by Dr. J. Yodoi (Kyoto University, Kyoto, Japan) and Dr. J. Kornbluth (University of Arkansas, Little Rock, AR), respectively.

Human NK cells were purified from buffy coats from healthy donors (Blood Bank, National Institutes of Health, Bethesda, MD), as described (17). Briefly, mononuclear cells were obtained by centrifugation over lymphocyte separation medium (LSM; Organon Teknika, Durham, NC) and treated with 100 mg/106 cells of sterilized carbonyl iron (Sigma) in standard RPMI 1640 medium at 37°C for 30 min, followed by exposure to a magnetic field to remove monocytes. Cell suspensions were centrifuged over 47.5% Percoll to obtain low density large granular cells. NK-enriched mononuclear cells were further treated with anti-CD5, anti-CD22, and anti-CD56 mAbs at 4°C for 2–4 h, incubated with GaM-coated magnet beads (PerSeptive Biosystems, Framingham, MA) at 4°C for 30 min, and exposed to a magnetic field to eliminate residual T cells, B cells, and monocytes. The purity of NK cells (CD16+ and/or CD56+) in each experiment was always 85–95%, as assessed by flow cytometry (FACScan; Becton Dickinson). Of the population defined as CD56+ and/or CD16+ cells, 10% expressed CD16 alone, 30% expressed CD56 alone, and greater than 50% expressed both markers.

A human glioblastoma cell line, A172, stimulated with IFN-γ and LPS, was used as a positive control for human iNOS mRNA and protein (21).

**RT-PCR analysis of RNA**

Human iNOS, eNOS, and TNF-α mRNAs were identified by semiquantitative RT-PCR, as described previously (22). Total RNA was isolated from cells using TRIzol (Life Technologies), according to the manufacturer’s instructions. The sequences of the primer pairs in this experiment were as follows: human β-actin as an internal control (5′-ATG TGG CAT TTC ACC TTC TAC A-3′, 5′-GTT TCG TGG ATG CCA GAC TAC-3′), human iNOS (5′-TGG GCC ACC TTC AGT CCA GTG ACA-3′, 5′-GCT CAT CTC CCA GTG GTT AGG AGG-3′), human eNOS (5′-GCA TCC CTA CTC CCA GGC CCA-3′, 5′-TCC CGG GCA TGG AAC TGC AGC-3′), and human TNF-α (5′-TCA GTG GTA GGT GGA TCT G-3′, 5′-TCA GCC AGC GTG ACT AC-3′), Amplification reactions were performed in a thermocycler (GeneAmp PCR system 9600; Perkin-Elmer, Norwalk, CT), as follows: 94°C, 3 min, followed by 21 (β-actin), 40 (NOSs), or 30 (TNF-α) cycles (94°C, 1 min; 57°C, 1.5 min; 72°C, 2 min). Aliquots of each amplification were analyzed by electrophoresis in 6% (β-actin and NOSs) or 8% (TNF-α) acrylamide Tris-borate gels. The lanes were normalized to β-actin mRNA levels.

**Immunoblot analysis**

iNOS and eNOS protein levels were assayed by immunoblot. A total of 5 × 105 cells was lysed with lysis buffer (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM EGTA, 50 μg/ml leupeptin, 50 μg/ml aprotinin, 50 μM p-nitrophenyl p′-guanidino-benzoate). Equivalent amounts of total cellular protein (50 μg/lane) were separated on 7.5% SDS-polyacrylamide gels and blotted to polyvinyl difluoride membranes (Millipore, Bedford, MA). After blocking with 1.25% BSA, 1.25% chicken egg albumin, 25 mM Tris-HCl (pH 7.5), 150 mM NaCl, and 3 mM KCl, the membrane was incubated with rabbit anti-iNOS or iNOS Abs (Calbiochem-Novabiochem) and then with donkey HRP-conjugated anti-rabbit IgG Ab (Amersham, Arlington Heights, IL) at room temperature for 1 h. Protein bands were visualized using enhanced chemiluminescence detection reagents (Amersham) and exposure to XAR film (Eastman Kodak, Rochester, NY) for 10–30 s.

**Measurement of NO oxidation products, nitrite plus nitrate**

Because NO in oxygen-containing solutions is chemically unstable and undergoes rapid oxidation to nitrite, and cellular components catalyze its further oxidation to nitrate, production of NO was determined by measuring the formation of the stable oxidation products of NO, nitrite, and nitrate, as described (23). Cells were treated as described in Results at 104/ml. Nitrate in cell culture supernatants was reduced to nitrite by incubation of the samples for 30 min with nitrate reductase (0.1 U/ml; Sigma) in the presence of 100 μM nicotinamide adenine dinucleotide phosphate (NAPDH) and 10 μM flavin adenine dinucleotide. Remaining NAPDH was oxidized with lactate dehydrogenase (10 U/ml) in the presence of 10 mM sodium pyruvate. Total nitrite concentration was then determined by using a procedure based on the Griess reaction (24). The baseline level of this assay was 0.1 nmol/106 cells according to the levels detected in media alone.

**Cytotoxicity assay**

The percentage of apoptotic cells was measured by fluorescence microscopy, after staining with acridine orange (AO) and ethidium bromide (EB), as described (26).

The JAM test of Matzinger (27) was used to quantify target-induced DNA fragmentation in [3H]TdR-labeled NK cells, with some modifications as described (28), except that NK cells were pulsed with 0.2 μCi of [3H]TdR per 5 × 105 cells in standard medium at 37°C for 12 h, and labeled cells were cocultured with or without K562 cells at E:T (NK:K562) ratios of 3:1, with the indicated treatment at 37°C for 4 h. The percentage of DNA fragmentation was calculated as: percentage of DNA fragmentation = (S − R)/S × 100; S = cpm retained in the absence of effector cells, R = cpm retained in the presence of effector cells.

**Granule exocytosis assay**

Granule exocytosis by NK cells upon coculture with target cells was measured by BLT-esterase assay, as described, with modifications (29). In brief, 50 μl of 2 × 105 cells/ml IL-2-activated NK cells were cultured with 50 μl of 2 × 105 cells/ml K562 cells in the absence or presence of the indicated concentrations of l-NMA for 4 h at 37°C. Esterase was measured in supernatants, and total esterase content was obtained in supernatants following incubation of cells with 1% Nonidet P-40 for 4 h at 37°C. Percentage of secretion of esterase was calculated as: percentage of secretion = 100 × (E − B)/(T − B); E = mean absorbance of the supernatants from NK cells incubated with K562 cells, B = mean absorbance of the supernatants from NK cells incubated without K562 cells, and T = total esterase content.

**Flow-cytometric analyses for expression of FasL and NOS**

Cell surface FasL expression was quantitated by flow-cytometric analysis, as described previously (30). Briefly, cells were treated with a metallopeptase inhibitor, KB8301, which blocks FasL cleavage resulting in high levels of cell surface FasL expression (31), for 6 h, washed with FACS buffer (PBS with 1% FCS and 0.1% NaN3), and incubated with biotin-conjugated anti-human FasL mAb (PharMingen) or biotin-conjugated mouse IgGl (PharMingen) for 20 min on ice. After washing with FACS buffer, they were incubated with streptavidin PE (Becton Dickinson), and fluorescence intensity was measured by FACSscan.

NOS expression was examined in NK cells by double staining with Abs to surface and intracytoplasmic Ags. Cells were stained with anti-CD16 PE and anti-CD56 PE or a control mouse IgG PE (Sigma), followed by fixation with 4% paraformaldehyde in FACS buffer (1% FCS and 0.1% sodium azide in PBS) for 20 min on ice. Cells were washed with FACS buffer, permeabilized with saponin (0.1% saponin in FACS buffer), vortexed gently, incubated with rat IgG (Sigma) for 20 min on ice to block nonspecific binding and then with anti-NO Abs, anti-iNOS Abs, or a control rabbit IgG (Sigma) for 30 min on ice, and finally washed with saponin solution. Next they were stained with FITC-conjugated anti-rabbit Ab (Sigma) in saponin solution for 30 min on ice, washed twice with saponin solution. Fluorescence was analyzed by FACSscan.

**Electrophoretic mobility shift assay**

Nuclear extracts were prepared from cells cultured with the indicated treatment, as described by Schreiber et al. (32). We used the sequence for human IL-2 distal NF-AT site (GGA GGA AAA ACT GTT TCA TAC AGA AGG) (33) as a probe to measure NF-AT
amide gels in 0.25% 0.1% bromophenol blue, followed by electrophoresis on 5% polyacrylamide gels. mRNA. Human NK cells constitutively express eNOS, but do not express iNOS, mRNA.

Briefly, nuclear extracts (5 μg per reaction volume) were incubated for 20 min at room temperature with 1 μg poly(dI-dC) (Amersham Pharmacia Biotech) and 0.5 ng 32P-labeled dsDNA probe in 20 μl of binding buffer (10 mM Tris-HCl, pH 7.5), 50 mM NaCl, 30 mM KCl, 5 mM MgCl2, 10% glycerol, and 0.05% Nonidet P-40. The reaction was stopped with 2 μl of 0.1% bromophenol blue, followed by electrophoresis on 5% polyacrylamide gels in 0.25× Tris-buffered EDTA (pH 8.4) under nondenaturing conditions. Five nanograms of unlabeled cold probe were added at the beginning of the reaction as a competitor. For supershift, antisera against murine NF-AT1 (Upstate Biotechnology, Lake Placid, NY), which cross-reacts with human NF-AT1, were added to nuclear extracts and incubated on ice for 30 min before the binding reaction.

Statistical analysis
The one-tailed Student’s t test was used for data analysis.

Results
Human NK cells expressed eNOS, but not iNOS, mRNA and protein
We assessed the expression of eNOS and iNOS mRNA in freshly purified and IL-2-stimulated NK cells. RT-PCR analysis revealed that human NK cells express eNOS, but not iNOS, mRNA (Fig. 1), and that constitutive levels of eNOS mRNA were maintained following culture in the presence of IL-2, although levels decreased in the absence of IL-2. No induction of iNOS mRNA could be detected following IL-2 treatment (Fig. 1). CD16 cross-linking, or coculture with target cells (data not shown).

In addition, we examined the expression of eNOS and iNOS proteins by immunoblot analysis. As shown in Fig. 2A, eNOS protein was found in freshly isolated NK cells, and its level was not significantly affected by culture with IL-2 for 3 or 6 days, although eNOS protein levels were reduced after 3 days of culture in the absence of IL-2. Because NK cells cultured without IL-2 for 6 days were less than 50% viable, it was not possible to obtain appropriate quantities of mRNA or proteins from these cells. These results suggest that IL-2 is required for maintenance of constitutive level of eNOS protein. To confirm that eNOS was indeed a product of the phenotypic NK cells in the cell preparations, cells that had been surface stained with anti-CD56 PE and anti-CD16 PE were permeabilized and treated with anti-eNOS Ab, anti-iNOS Ab, or a control rabbit IgG. The data show that 89% of the total cells, and 99% of the CD56+ and/or CD16+ cells express intracytoplasmic eNOS.

NO was produced by human NK cells stimulated by coculture with target cells or CD16 cross-linking
Accumulation of nitrite, a downstream end product of NO, has been reported in murine and rat NK cells in culture medium after coculture with target cells (20, 35). Therefore, we examined nitrite/nitrate accumulation in culture media of human NK cells after a variety of treatments, including incubation with or without IL-2 and/or IL-12 for 3 days, coculture with K562 target cells for 24 h...
that treatment with L-NMA partially inhibited killing activity of NK cells, as determined by 
proliferation, viability, and cytotoxicity of IL-2-activated NK cells. constitutionally NOSs are associated with transient and low NO upon increased by IL-2. It is also consistent with the fact that the concentration of nitrite in the supernatants of NK cells stimulated by coculture with K562 cells or by CD16 cross-linking, and levels were further augmented if IL-2 was present together with the K562 or CD16 cross-linking treatments (Fig. 3). The concentration of nitrite in the supernatants of NK cells stimulated by coculture with K562 cells or by CD16 cross-linking, although increased over baseline, was still only in the range of 0.5–1.2 nmol/10^6 cells. This observation suggests that the constitutive enzyme, eNOS, is activated in NK cells by coculture with target cells or by CD16 ligation, and that this activation is further increased by IL-2. It is also consistent with the fact that the constitutive NOSs are associated with transient and low NO upon Ca^{2+} elevation (4, 9), but compatible with the absence in human NK cells of iNOS, which is associated with greater NO production, at least in rodent systems (5).

**Treatment with l-NMA or culture in Arg(−) medium partially suppressed cytotoxicity of human NK cells**

To evaluate whether NO has a role in NK cell function, we examined the effects of l-NMA, a competitive inhibitor of NOS, on proliferation, viability, and cytotoxicity of IL-2-activated NK cells. l-NMA (500 μM) had no significant effect on IL-2- and/or IL-12-induced proliferation of NK cells, as determined by [3H]TdR incorporation, and l-NMA did not affect viability of NK cells following IL-2 treatment for 3 days (data not shown). However, as shown in Fig. 4, the results from 51Cr release assays demonstrated that treatment with l-NMA partially inhibited killing activity of four different target cells by IL-2-activated NK cells in a dose-dependent manner (<40% inhibition at 500 μM l-NMA), and that cytotoxicity of NK cells cultured in Arg(−) medium was reduced by 40% as compared with that in standard medium (data not shown). These results were obtained consistently, although absolute levels varied among different donors. NK cells exert cytotoxicity through two major mechanisms, namely release of granules containing perforin and granzymes and lysis of Fas^+ target cells through ligation with FasL on the NK cell surface (36, 37). However, neither granule exocytosis nor induction of Fas ligand by activation was altered by treatment with l-NMA (Fig. 5, A and B), suggesting that NO production may not be required for function of the lytic mechanisms in NK cells.

**l-NMA augmented activation-induced apoptosis of NK cells, and an NO donor, SNAP, suppressed it in a dose-dependent fashion**

Because there is increasing evidence that low levels of NO protect cells from apoptosis (38–41), we examined the effects of l-NMA and SNAP, which is reported to release nanomolar concentrations of free NO at 0.1–10 mM concentrations (42), on apoptosis of IL-2-pretreated NK cells. We used IL-2 pretreatment because we have observed that apoptosis is easier to demonstrate in IL-2-pre-treated NK cells than in freshly isolated NK cells (28, 43). As shown in Fig. 6A, SNAP suppressed target cell-induced DNA fragmentation in a dose-dependent manner. In addition, SNAP also inhibited apoptosis induced by cross-linking with anti-CD16 mAb in a dose-dependent fashion (Fig. 6B). Furthermore, we found that l-NMA significantly enhanced apoptosis induced by either coculture with K562 cells or CD16 cross-linking (Fig. 6, A and B). However, treatment with SNAP or l-NMA had no effect on DNA fragmentation of freshly isolated NK cells or IL-2-pre-activated NK cells in the absence of target cells (data not shown). These data suggest that endogenous NO production is involved in protection...
of NK cells from apoptosis induced by stimulation through CD16 or with target cells. The enhancement of apoptosis in NK cells by l-NMA may explain the finding that l-NMA moderately decreased cytotoxicity of NK cells.

l-NMA increased TNF-α mRNA expression following ligation of CD16, and SNAP decreased it

Jewett et al. reported that endogenous TNF-α production by NK cells results in functional anergy and apoptosis in anti-CD16-treated NK cells (44). Therefore, we examined whether treatment with l-NMA or SNAP alters TNF-α production by anti-CD16-treated NK cells, using treatment with anti-CD56 mAb as a control. When 500 μM l-NMA was added to IL-2-pretreated NK cells 4 h before RNA isolation, induction of TNF-α mRNA following CD16 cross-linking was enhanced, as shown by RT-PCR analysis (Fig. 7). Conversely, treatment of IL-2-pretreated NK cells with 1 mM SNAP for 4 h suppressed expression of TNF-α mRNA following treatment with anti-CD16 mAb (Fig. 7). PhosphorImager (Molecular Dynamics, Sunnyvale, CA) analyses of TNF-α mRNA showed that l-NMA treatment induced approximately a 7-fold increase in expression of TNF-α mRNA as compared with anti-CD16 alone, and that SNAP reduced expression by one-fifth as compared with anti-CD16 alone. Treatment with Ab to CD56, used as a control, had no effect on TNF-α mRNA expression, and SNAP or l-NMA did not affect TNF-α mRNA expression in anti-CD56 mAb-treated NK cells (data not shown). These results suggest that NO is involved in regulation of TNF-α tran-
scription in activated NK cells. We next investigated the effect of addition of rTNF-α on SNAP-induced suppression of apoptosis of anti-CD16-treated NK cells. The result, shown in Fig. 8, demonstrated that NK cells underwent apoptosis following treatment with rTNF-α in a dose-dependent fashion, although the percentage of apoptosis was lower than that induced by CD16 ligation even with 100 ng/ml rTNF-α. The data further show that 10 ng/ml rTNF-α completely reversed the SNAP-induced (1 mM) suppression of apoptosis in anti-CD16-treated NK cells. Together, these data support the conclusion that the effect of SNAP is through suppression of TNF-α, and suggest the possibility that inhibition of TNF-α expression by NO is a mechanism by which NO can protect activated NK cells from apoptosis.

SNAP inhibited and l-NMA enhanced DNA-binding activity of the NF-AT in anti-CD16-treated NK cells

TNF-α gene transcription involves activation of a transcription factor, NF-AT, in T cells as well as NK cells (34, 45). Because we found that inhibition or replacement of NO affected TNF-α gene expression in activated NK cells, we hypothesized that NO may
inhibit NF-AT activation in anti-CD16-treated NK cells. DNA-binding activity of NF-AT in nuclear extracts from NK cells stimulated with anti-CD16 mAb in the absence or presence of SNAP or L-NMA was examined by EMSA. As shown in Fig. 9, a band corresponding to a NF-AT-DNA complex, which was completely inhibited by cold probe, was induced by treatment with anti-CD16 mAb. The band was supershifted by anti-NF-AT1 Ab, identifying the band as containing an NF-AT1-DNA complex. The DNA-binding activity of NF-AT induced by treatment with anti-CD16 mAb was suppressed by SNAP and enhanced by L-NMA. This demonstrates that NO suppresses NF-AT activation following ligation of CD16 and suggests that the suppression of NF-AT by NO may be responsible for down-regulation of TNF-α production.

Discussion

The present study demonstrates that human NK cells constitutively express eNOS mRNA and protein, and the constitutive level of eNOS expression is maintained in the presence of IL-2 in vitro. NK cells that have been precultured in IL-2 and then activated for 24 h by coculture with target cells or by treatment with anti-CD16 mAb and GaM Ab to cross-link the CD16 receptor, produce low levels of NO (~0.5 nmol/10^6 cells of NO oxidation products, nitrite plus nitrate), and this level is increased by including IL-2 in the 24-h culture with the activation stimuli, up to 1.2 nmol/10^6 cells of nitrite/nitrate. These findings show that NK cells produce a constitutive NOS, namely eNOS, and suggest that the enzymatic activity eNOS is activated in NK cells by the same stimuli that normally activate these cells. In contrast, NK cells cannot be induced to express iNOS, the primary NOS produced by rodent macrophages (5), by treatment with IL-2, CD16 ligation, or exposure to target cells, nor do NK cells produce the higher levels of NO associated with NO synthesis in human macrophages, reported at 5–30 nmol/10^6 cells of nitrite alone (46, 47). Recently, Salvucci et al. reported that human NK cells express iNOS mRNA and protein upon stimulation with IL-12 and TNF-α, and that an inhibitor of iNOS increased lytic activity against NK-sensitive target cells (48). However, in our system using NK cells stimulated with IL-2 and/or IL-12, iNOS mRNA and protein could not be detected by RT-PCR and immunoblot analysis, respectively. The absence of iNOS expression in our system is consistent with our finding that NK cells produced only small amounts of NO upon stimuli, such as coculture with target cells or CD16 cross-linking. We also investigated whether NK cells produce neuronal NOS, an enzyme that exhibits similar regulation and activity to eNOS. However, the data to date have been difficult to interpret because of technical difficulties and lack of appropriate positive controls.

We further showed that NO suppresses apoptosis of IL-2-pre-treated NK cells induced by coculture with K562 cells or CD16

FIGURE 7. Increase in anti-CD16-induced TNF-α mRNA expression by l-NMA and its reduction by SNAP. A, Purified NK cells were activated with 100 IU/ml rIL-2 for 3 days, followed by treatment with 10 μg/ml anti-CD16 or anti-CD56 mAb and 20 μg/ml GaM Ab in the absence or presence of 500 μM l-NMA or 1 mM SNAP for 4 h. Total RNAs were isolated and subjected for RT-PCR analysis. A representative of tests on cells from three different donors is shown. B, Quantitative presentation of A using data obtained by PhosphorImage analysis.
cross-linking. This effect on apoptosis is associated with alteration in lytic activity, such that suppression of NO production partially inhibits NK lytic activity without affecting known lytic mechanisms, namely granule exocytosis and FasL expression. Stimulation of NK cells with either K562 or anti-CD16 mAb has been shown to elicit intracellular Ca\(^{2+}\) elevation (49). Constitutive eNOS is known to be activated for short periods of time by Ca\(^{2+}\)-elevating stimuli and to be responsible for the low levels of NO that are involved in homeostatic processes, such as in blood vessels (4, 9). Moreover, addition of exogenous NO appears to regulate activity of Janus kinase 3 immunoprecipitated from an NK cell line (50). Our findings, showing that an inhibitor of NOS, L-NMA, enhanced apoptosis and inhibited cytotoxicity by human NK cells, suggest that NO produced through eNOS activation is involved in suppression of activation-induced cell death and maintenance of cytotoxicity mediated by NK cells. Therefore, our data suggest that eNOS may play a homeostatic role in the maintenance of a minimum level of activity by activated NK cells through inhibiting their apoptosis.

Although NO has been reported to inhibit apoptosis in various cells, including human EBV-transformed B cells (38), human T cells (39), rat hepatocytes (40), sheep endothelial cells (41), and human eosinophils (51), the underlying mechanism remains unclear. It is reported that endogenous TNF-α is involved in and required for apoptosis in NK cells activated with anti-CD16 mAb (44) or cytokines including IL-2 and IL-15 (52). We hypothesized that inhibition of transcription of TNF-α may be a mechanism of suppression of apoptosis in activated NK cells by NO. Our data showed that inhibition of NOS activity augmented TNF-α production, and SNAP, an NO donor, decreased it, suggesting that NO indeed suppresses TNF-α mRNA expression in anti-CD16-treated NK cells. This hypothesis was supported by the demonstration that addition of rTNF-α reversed suppression by NO of apoptosis in anti-CD16 mAb-treated NK cells. A nuclear factor, NF-AT, is known to be involved in TNF-α induction following CD16 cross-linking in NK cells (34). Our study revealed that SNAP treatment suppressed DNA-binding activity of NF-AT in NK cells activated by anti-CD16 mAb, suggesting further that NO inhibits TNF-α induction in activated NK cells by suppression of transcription through NF-AT activation. The nuclear translocation of NF-AT is known to be regulated by the serine/threonine phosphatase, calcineurin, which contains Fe in its active site (53, 54). Because NO is highly reactive with transition metals, including Fe (3), it is possible that NO may modulate NF-AT activity through an effect on calcineurin.

Although NF-AT has been recently reported to be involved in FasL expression in activated human T cells (55), our data did not show any alteration by L-NMA in FasL expression in anti-CD16-activated NK cells. Because apoptosis of NK cells can also occur in part through Fas/FasL interaction (28, 56), NO may be involved in modulation of this pathway. In fact, Fas-induced apoptosis of Jurkat T cells is reported to be blocked by treatment with SNAP (39), and a disruption by NO of Fas receptor signaling in human eosinophils has been proposed (51), and NOS activity has been reported to inhibit Fas-induced apoptosis in a number of human leukocytic cell lines (57). However, our data suggest that NO may not play a major role in the Fas-FasL-mediated pathway of NK cell-mediated cytotoxicity because FasL expression was not altered by L-NMA treatment. In addition, we observed that L-NMA treatment had no effect on apoptosis of purified NK cells induced by agonist anti-Fas Ab (data not shown).

A number of reports demonstrate that NO inhibits the activation of NF-κB, which regulates transcription of a number of immunologically crucial genes (42, 58). Therefore, we investigated the effect of SNAP on DNA-binding activity of NF-κB in anti-CD16 mAb-treated NK cells. Activation of NF-κB in NK cells in response to anti-CD16 mAb occurred only after 4 h of stimulation with anti-CD16 mAb, much more slowly than in response to other stimuli, such as TNF-α and PMA, and was inhibited by SNAP (data not shown). This delayed kinetics of NF-κB activation may be explained by the hypothesis that NF-κB may be activated after CD16 ligation through synthesis of endogenous TNF-α following NF-AT activation.

This is a first report that human NK cells express eNOS and that NO modulates NF-AT activation. In addition, our results suggest a physiological role for NO production, i.e., protection of NK cells from apoptosis to maintain a level of cytotoxic activity. Finally, our data suggest a possible mechanism by which this protection occurs, namely inhibition of TNF-α transcription by inactivation of NF-AT. We hypothesize that endogenous NO production may be important in vivo to maintain a basal level of activated NK cell activity by protecting such cells from apoptosis (Fig. 10).

**Acknowledgments**

We thank Amgen for supplying human rIL-2, Dr. J. Yodoi for the kind gift of the YT cell line, and Dr. J. Kornblith for providing the NK3.5 cell line. We thank Drs. Giovanna Tosato and Mark Hayes for critical review of the manuscript.

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