Fas Ligand Induction in Human NK Cells Is Regulated by Redox Through a Calcineurin-Nuclear Factors of Activated T Cell-Dependent Pathway

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Fas ligand (FasL) on cytotoxic lymphocytes is important for mediating apoptosis of activated lymphocytes and other target cells. We have reported that NK cell functions, such as proliferation, cell death, and killing activity, are subject to regulation by cellular redox status. Here, we report that expression of FasL protein and mRNA in activated NK cells is also regulated by redox. Ligation of CD16 on IL-2-preactivated NK cells resulted in reduction of intracellular peroxide level as well as induction of FasL expression. This CD16-induced FasL expression was suppressed by oxidative stress, including thiol deprivation or treatment with hydrogen peroxide (H₂O₂). Addition of thiol-reducing compounds, such as 1-cystine, 2-ME, or N-acetyl cysteine, restored FasL expression. These data suggest that CD16 stimulation requires cellular reducing status for FasL induction in NK cells. Because FasL gene activation following CD16 cross-linking is regulated by the NF of activated T cells (NFAT), we examined the effect of oxidative stresses on NFAT activation. Electrophoretic mobility shift assays revealed that both thiol insufficiency and H₂O₂ treatment suppressed DNA-binding activity of NFAT and that addition of thiol-reducing compounds reversed or even enhanced it. Furthermore, these oxidative stresses inhibited activity of calcineurin, a serine/threonine phosphatase that regulates NFAT activation. These results suggest that suppression of calcineurin and NFAT activation is a mechanism by which oxidative stress inhibits FasL induction in activated NK cells and further support the hypothesis that thiol-reducing compounds might be required for maintenance of optimal NK functions under physiologic oxidative conditions.

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Materials and Methods

Reagents and Abs

Human rIL-2 was provided by Amgen (Thousand Oaks, CA). Metalloproteinase inhibitor KB8301 was purchased from PharMingen (San Diego, CA). Anti-CD5 mAb was purified from ascites as previously described (15). Anti-CD22 mAb was purchased from Becton Dickinson (Mountain View, CA). Anti-CD36, anti-CD56-phycoerythrin (PE), anti-CD16-PE, and anti-CD22 mAbs were purchased from PharMingen. Anti-CD16 (clone 3G8), anti-CD36, anti-CD56-phycoerythrin (PE), anti-CD16-PE, and anti-CD14-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). Hydrogen peroxide (H₂O₂), 2-ME, N-acetyl cysteine (NAC), L-cysteine (L-Cys), cyclosporin A (CsA), and goat anti-mouse IgG (GaM) mAb were purchased from Sigma (St. Louis, MO).

Cell culture and isolation

Standard RPMI 1640 medium (Life Technologies, Grand Island, NY) contained 10% FCS (HyClone Laboratories, Logan, UT), 50 μg/ml of gentamicin sulfate, and 2 mM glutamine. RPMI 1640 medium without L-Cys and reduced type glutathione (CYS(−)) medium was obtained from KEMP Biotechnologies (Gaithersburg, MD). FCS for CYS(−) medium was diaлизed against PBS to remove small m.w. compounds, including thiols. CYS(+) medium was CYS(−) supplemented with 200 μM L-Cys, the...
concentration normally found in cell culture medium. Cells were cultured for 12 h in CYS(−) or CYS(+) medium with the indicated FCS and IL-2 supplementation before use to decrease intracellular thiol levels. Total amounts of protein and mRNA, as well as cell viability (>90%), did not differ significantly between purified NK cells cultured for 12 h in CYS(−) or CYS(+) medium.

Human NK cells were purified from buffy coats from healthy donors (Blood Bank, National Institutes of Health, Bethesda, MD) as described (17). In brief, mononuclear cells were obtained by centrifugation over lymphocyte separation medium (Oreganok Teknika, Durham, NC) and treated with 100 mg/10^6 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-CD36 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 FACSscan (Becton Dickinson). The population of contaminating T cells (CD56^−, CD3^+), B cells (CD20^+), or monocytes/macrophages (CD14^+) in each experiment was <10%, <5%, or <5%, respectively.

**Flow cytometric analysis of expression of FasL and intracellular peroxide levels**

Quantitation of surface FasL expression was assessed by flow cytometric analysis. Cells were treated for 6 h with 10 μM KB8301 (PharMingen), a metalloprotease inhibitor, to inhibit FasL release (18), washed with FACS buffer (PBS with 1% FCS and 0.1% Na₂SO₄), and incubated with biotin-conjugated anti-human FasL mAb (PharMingen) or biotin-conjugated mouse IgG1 (PharMingen) for 20 min on ice. After washing with FACS buffer, cells were incubated with streptavidin PE (Becton Dickinson) for 20 min on ice. The fluorescence intensity was measured by FACSscan.

Intracellular peroxide levels were assayed as described (19). Briefly, cells were cultured in medium with 5 μM 2′,7′-dichlorofluorescin diacetate (DCFH-DA; Molecular Probes, Eugene, OR) for 15 min at 37°C following the indicated treatments and harvested for flow cytometry. In experiments designed to confirm whether NK cells contain intracellular peroxides, cells were stained first with anti-CD56- and anti-CD16-PE mAbs to identify NK cells, or with CD14-PE mAb to identify monocyte/macrophages before assay for peroxides with DCFH-DA.

**RT-PCR analysis of FasL and IFN-γ mRNA**

FasL and IFN-γ mRNA expression was determined by RT-PCR using [32P]dCTP as previously described (17, 20). 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 glyceraldehyde-3-phosphate dehydrogenase (G3PDH; Sigma, St. Louis, MO) and 32 P-labeled dsDNA probe in 20 μl of binding buffer, cells were incubated with streptavidin PE (Becton Dickinson) for 20 min at room temperature with 1 μM 32 P-labeled phosphopeptide, a phosphatase type 1 and 2A inhibitor. Reaction mixtures contained 1 μl of antisera against murine NFAT1 (Upstate Biotechnology, Lake Placid, NY), which cross-reacts with human NFAT1, was added to nuclear extracts and incubated on ice for 30 min before the binding reaction.

**Calcineurin assay**

Calcineurin activity was assayed as previously described (24). Hypotonic lysates from 10^7 purified NK cells were assayed following their ability to dephosphorylate a 32 P-serine-labeled 19-amino acid peptide substrate (Sigma) in the presence of okadaic acid (Sigma), a phosphatase type 1 and 2A inhibitor. Reaction mixtures contained 20 μl of lysates, 500 nM okadaic acid, 5 μM 32P-labeled phosphopeptide, and 40 μl of assay buffer containing 20 mM Tris (pH 8.0), 100 mM NaCl, 6 mM MgCl₂, 0.1 mM CaCl₂, 0.5 mM DTT, 0.1 mg/ml of BSA. After 15 min at 30°C, reactions were terminated by the addition of 0.5 ml 100 mM potassium phosphate buffer (pH 7.0) containing 5% trichloroacetic acid. Free inorganic phosphate was isolated using a Dowex cation-exchange resin (AG50W-X8 100–200 mesh; Bio-Rad, Hercules, CA) and quantitated by scintillation counting. Variation between duplicates was <10%. The number of picomoles of phosphate released was calculated by using the sp. act. (cpm in 300 pmol 32P-labeled phosphopeptide) measured on the day of the assay. The protein content of cell lysates was determined with a Bio-Rad Protein Assay Kit, and calcineurin activity was expressed as picomoles of released 32P per minute per milligram of lystate protein.

**Results**

**Time course of increase in FasL expression in IL-2-preactivated NK cells by anti-CD16 Ab**

It has been reported that FasL mRNA can be detected in IL-2-preactivated NK cells within 6 h following treatment with anti-CD16 Ab (6). Therefore, we first examined the time course of cell surface FasL protein expression, as determined by flow cytometry, on IL-2-preactivated NK cells following ligation of CD16. NK cells cultured with IL-2 for 3 days expressed FasL only weakly (mean fluorescent intensity (MFI) < 10). As shown in Fig. 1, however, FasL expression was increased within 6 h and peaked between 6–24 h following addition of anti-CD16 mAb, although time points of the peak FasL expression varied among cells from individual donors. We confirmed that the fluorescence intensity was specific for FasL using biotin-conjugated mouse IgG1 as a negative control. The induction of FasL was specific for CD16

**FIGURE 1.** Time course of CD16-induced FasL expression in IL-2-pre-treated NK cells. Purified NK cells were cultured with 100 U/ml of rIL-2 for 3 days, treated with 10 μg/ml of anti-CD16 or an isotype control anti-CD56 mAb plus 10 μg/ml of GaM for the indicated time periods, and harvested for flow cytometric analysis of FasL expression. Data are expressed as means ± SD of the MFI obtained on NK cells from three different donors.
Intracellular peroxide levels are known to reflect the generation of reactive oxygen species (ROS) (25) and to be increased under conditions of oxidative stress (26). Therefore, we next measured intracellular peroxide levels as an indicator of whether CD16 ligation affects intracellular redox status in IL-2-preactivated NK cells. We found that intracellular peroxides could be detected in CD56+ and/or CD16+ NK cells, which represented >85% of the cell population, and in CD14+ monocyte/macrophages, representing only 3% of the cell population (Fig. 2A). The amount of intracellular peroxide was strongly reduced following treatment with anti-CD16 mAb (Fig. 2B), although no effect was seen with anti-CD56 mAb treated with 100 μM H2O2 for 2 h increased contents of intracellular peroxides in IL-2-preactivated NK cells (Fig. 2). These results suggest that ROS production is suppressed or levels are quenched following CD16 cross-linking in NK cells, and that both thiol deprivation and H2O2 treatment result in oxidative stress through increasing intracellular ROS levels.

**Ligation of CD16 decreased intracellular peroxide level in IL-2-preactivated NK cells**

To clarify whether cellular redox status plays a role in FasL induction following CD16 cross-linking, we next assessed the effects on FasL induction of manipulating cellular redox status in activated NK cells using thiol deprivation with CYS(-) medium or by adding back thiol-reducing compounds. NK cells were treated with IL-2 in standard medium for 60 h, followed by culture in CYS(+) or CYS(-) medium in the presence or absence of 50 μM 2-ME or 10 mM NAC. FasL expression was suppressed by oxidative stress, including thiol deprivation or H2O2 treatment, and restored by addition of thiol-reducing compounds, such as l-Cys, 2-ME, or NAC. Data are MFI ± SD from three different donors.

**Inhibition of FasL induction in anti-CD16-treated NK cells by H2O2 treatment or by thiol deprivation**

To evaluate the effects of oxidative stress on FasL expression, we determined the effect of H2O2 treatment on FasL cell surface expression in anti-CD16-treated NK cells. When IL-2-pretreated NK cells were exposed to 0.1–100 μM H2O2 for 12 h, FasL expression induced by anti-CD16 mAb was reduced in a dose-dependent manner (Fig. 3A). Cell viability was not significantly affected at the same concentrations of H2O2 (data not shown).

To clarify whether cellular redox status plays a role in FasL induction following CD16 cross-linking, we next assessed the effects on FasL induction of manipulating cellular redox status in activated NK cells using thiol deprivation with CYS(-) medium or by adding back thiol-reducing compounds. NK cells were treated with IL-2 in standard medium for 60 h, followed by culture in CYS(+) or CYS(-) medium in the presence of anti-CD16 or anti-CD56 mAb plus 10 μg/ml of GaM for 12 h. FasL expression determined by flow cytometry was suppressed by oxidative stress, including thiol deprivation or H2O2 treatment, and restored by addition of thiol-reducing compounds, such as l-Cys, 2-ME, or NAC. Data are MFI ± SD from three different donors.
pounds. In contrast, mRNA encoding for IFN-g2-ME, or NAC (Fig. 4). In contrast, the augmentation of IFN-g produced by thiol deprivation were reversed by the addition of L-Cys, with H2O2 in a dose dependent manner and completely suppressed NK cells as determined by EMSA. As shown in Fig. 5, activation of NFAT transcription factor activity in anti-CD16-treated (11, 27), we examined the effect of either thiol deprivation or H2O2 treatment on DNA-binding activity of NFAT in anti-CD16, but this level was unaffected by manipulation of redox conditions. IL-2-preactivated NK cells were cultured in CYS(+) or CYS(-) medium for 9 h, then treated as indicated (described in Fig. 3) for 3 h. RT-PCR analysis for FasL, IFN-g, or G3PDH mRNA is depicted and is a representative of three independent experiments. Quantitative presentation (percentage of NFAT DNA-binding activity in each sample compared with the sample treated with anti-CD16 in CYS(+) medium, lane 2) is also shown using data obtained by PhosphoImage (Molecular Dynamics, Sunnyvale, CA) analysis.

shown to increase intracellular reduced type glutathione in NK cells (15) (Fig. 3B).

Taken together, these findings show that oxidative stress may generally suppress FasL induction in activated NK cells and that reducing conditions are necessary for optimal FasL expression.

Suppression of FasL mRNA levels and NFAT transcription factor activity in anti-CD16-treated NK cells by thiol deprivation or H2O2 treatment

We next asked whether FasL mRNA expression was also suppressed by oxidative stress. Semiquantitative RT-PCR revealed that the increase in FasL mRNA following treatment with anti-CD16 mAb was completely suppressed by thiol deprivation or H2O2 treatment (Fig. 4). The diminished FasL mRNA levels induced by thiol deprivation were reversed by the addition of L-Cys, 2-ME, or NAC (Fig. 4). In contrast, the augmentation of IFN-g mRNA levels induced by CD16 cross-linking was not affected by manipulations of cellular redox status, including thiol deprivation, H2O2 treatment, or addition of 2-ME or NAC (Fig. 4), suggesting that events downstream of CD16 activation are not uniformly subject to regulation by redox.

Because the transcription factor NFAT has been reported to participate in the regulation of FasL expression in activated T cells (11, 27), we examined the effect of either thiol deprivation or H2O2 treatment on DNA-binding activity of NFAT in anti-CD16-treated NK cells as determined by EMSA. As shown in Fig. 5, activation of NFAT following ligation of CD16 was inhibited by treatment with H2O2 in a dose dependent manner and completely suppressed by thiol depletion. The suppressed DNA-binding activity of NFAT by thiol deprivation was restored by addition of L-Cys or NAC and even augmented by 2-ME treatment compared with CYS(+), indicating that thiol-reducing compounds are required for optimal activation of NFAT. This result also suggests that oxidative stress inhibits CD16-induced FasL expression at the level of, or upstream to, NFAT activation.

Thiol deprivation and addition of H2O2 suppressed calcineurin phosphatase activity in vitro

Activation of NFAT is known to be regulated by a CsA-sensitive, calcium/calmodulin-dependent serine/threonine phosphatase, calcineurin (28). Therefore, we examined whether oxidative stress affects calcineurin activity in IL-2-preactivated NK cells. Culture in CYS(-) medium for 12 h markedly suppressed calcineurin activity compared with standard medium (Fig. 6A). However, treatment of IL-2-preactivated NK cells with H2O2 had only a marginal effect on calcineurin activity, even with 1 mM H2O2. Because the calcineurin assay reaction mixture contained DTT, we next investigated the effect of incubation with H2O2 in vitro of lysates from IL-2-preactivated NK cells, which had not been treated with H2O2, as a more sensitive means to assess changes in calcineurin activity. As shown in Fig. 6B, calcineurin activity in IL-2-preactivated NK cells was inhibited by H2O2 in a dose-dependent fashion. These results demonstrated that oxidative stress, including thiol deprivation or treatment with H2O2, suppressed calcineurin activity in NK cells in vitro, and thereby suggests that suppression of calcineurin activity is a mechanism by which oxidative stress inhibits NFAT.
The present study demonstrated that FasL expression following CD16 ligation on NK cells was suppressed by thiol deprivation or H₂O₂ treatment, on calcineurin activity. A, IL-2-preactivated NK cells were cultured in CYS(+) or CYS(−) medium for 11 h, then treated with H₂O₂ (0.1–1 mM) or CsA (10 μM) as a positive control for 1 h, and harvested for calcineurin assay. Means ± SD among the data from three different donors were shown. The data indicated that thiol deprivation suppressed calcineurin activity in IL-2-preactivated NK cells. B. The indicated concentrations of H₂O₂ or 10 μM CsA were added to the reaction mixtures with lysates from IL-2-preactivated NK cells, and calcineurin activity was assayed. A representative of three repeated experiments is presented. The results demonstrate that calcineurin activity in lysates from IL-2-preactivated NK cells was inhibited by addition of H₂O₂ in vitro.

Activation of FasL is well known to be regulated by a serine/threonine phosphatase, calcineurin (28). Since superoxide dismutase protects calcineurin from inactivation (34) and a thiol reducing enzyme, thioredoxin, inhibits activity of CsA and FK506, inhibitors for calcineurin (35), we hypothesized that cellular redox status affects calcineurin activity, resulting in regulation of NFAT activity. The evidence that the active site of calcineurin contains Fe and Zn ions (36), both of which are redox-sensitive, supports this possibility. Our study demonstrated that lysates from thiol-depleted NK cells contained lower calcineurin activity than those from normal NK cells, suggesting that calcineurin activity also requires thiol-mediated reducing conditions. However, calcineurin activity in lysates from H₂O₂-treated NK cells was not significantly altered. We also demonstrated that thiol deprivation was more potent for increasing intracellular peroxide levels than H₂O₂ treatment. In the calcineurin assay, sufficient doses of a reducing reagent, DTT (0.5 mM), were required to measure calcineurin activity in vitro. Therefore, it is possible that DTT reversed an H₂O₂ effect on calcineurin activity during the assay, although it was not sufficient to reverse the effect in cells cultured under conditions of thiol deprivation. To determine whether H₂O₂ treatment can indeed inhibit calcineurin activity, we performed an experiment in which H₂O₂ was directly added into the reaction mixture with the lysate from IL-2-preactivated NK cells, and the result showed that H₂O₂ inhibited calcineurin activity in the lysate in a dose-dependent fashion. Taken together, these findings suggest that oxidative stress, including thiol deprivation or H₂O₂ treatment, inhibits FasL expression induced in activated NK cells through suppression of activation of calcineurin-NFAT pathway. However, thiol deprivation appears to induce oxidative stress more efficiently than addition of H₂O₂, since reduced calcineurin activity following thiol deprivation was observed even in the presence of DTT in the reaction.

Recent studies on Fas/FasL interaction have revealed that FasL-induced apoptosis is involved in a number of pathological conditions, including eliminating virus-infected or malignant cells, tumor tolerance from cytotoxic lymphocytes, graft rejection, acute hepatitis, and autoimmune disease (1, 2, 37–39). Moreover, since ROS have been implicated in the pathophysiology of these disorders (40), oxidative stress appears to colocalize with sites of Fas/FasL interaction. Although there have been several reports on the redox regulation in Fas receptor expression (41, 42) and on the involvement of ROS in FasL-induction in T cells activated through TCR-stimulation (43, 44), this is the first study showing the redox control of FasL expression in NK cell activation and exploring the mechanism of that control. Our results may provide not only means to maintain optimal NK functions but also possible therapeutics for such pathological conditions.

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References


