Brief Definitive Report

Receptor-induced Death in Human Natural Killer Cells: Involvement of CD16

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Summary

Propriocidal regulation of T cells refers to apoptosis induced by inerleukin 2 (IL-2) activation with subsequent antigen receptor stimulation. We examined whether natural killer (NK) cells exhibited cytokine- and ligand-induced death similar to activated T cells. Peripheral NK cells were examined for ligand-induced death using antibodies to surface moieties (CD2, CD3, CD8, CD16, CD56), with and without prior activation of IL-2. Only those NK cells stimulated first with IL-2 and then with CD16 exhibited ligand-induced death; none of the other antibody stimuli induced this phenomenon. Next we examined various cytokines (IL-2, IL-4, IL-6, IL-7, IL-12, IL-13, interferon α and γ) that can activate NK cells and determined if CD16-induced killing occurred. Only IL-2 and IL-12 induced NK cell death after occupancy of this receptor by aggregated immunoglobulin or by cross-linking with antireceptor antibody. The CD16-induced death was inhibited by herbimycin A, indicating that cell death was dependent upon protein tyrosine kinases. Identical to T cells, the form of cell death for NK cells was demonstrated to be receptor-induced apoptosis. Overall these data indicate that highly activated NK cells mediate ligand-induced apoptosis via signaling molecules like CD16. Whereas the propriocidal regulation of T cells is antigen specific, this is not the case for NK cells due to the nature of the receptor. The clinical implications of this finding are considered.

Stimulation of T cells via their antigen receptor can induce proliferation or cell death. This programmed cell death or apoptosis can occur in thymocytes and lymphocytes (1–3) and is a mechanism of negative selection (4, 5). This also can occur in mature T cells and has been suggested to be a mechanism for tolerance. The precise mechanism in lymphocytes by which the "choice" is made to proliferate or die is not well understood (6–11). It has been demonstrated that high doses of antigen can lead to a paradoxical suppression of the immune response that is termed high zone tolerance. A proposed mechanism to explain this phenomenon is based on the recent finding that T cells that have previously been exposed to IL-2 and then stimulated via their TCRs undergo apoptosis (10). This has been termed propriocidal regulation and is thought to represent feedback inhibition of T lymphocytes by which selective killing of specific T cell clones might occur.

While NK cells fail to rearrange TCR subunits, they nonetheless share a number of features with T cells, including expression of surface molecules and secretion of some of the same cytokines (12–14). T and NK cells also are developmentally related; however, unlike T cells, many of the details of NK cell development are poorly characterized. Like T cells, NK cells respond dramatically to IL-2 (12, 13). After stimulation with this cytokine, NK cells proliferate, secrete other cytokines (like IFN-γ), and have expanded cytolytic activity (termed lymphokine-activated killing). Because of this last activity, IL-2 has been used clinically in the treatment of malignancies (15, 16).

We and others have documented many similarities between TCR-mediated signaling and Fc receptor (FcR)-mediated signaling (17-19). The NK FcR (CD16) utilizes a chain first described in association with the TCR, the ζ chain. Additionally the NK FcR activates a nonreceptor protein tyrosine kinase and ζ is phosphorylated on tyrosine residues as occurs with TCR cross-linking. Finally, inhibition of protein tyrosine kinases block both TCR- and FcR-mediated signaling (19).

Because of the similarities in NK- and TcR-mediated signaling and because both NK and T cells have dramatic responses to IL-2, we sought to determine if NK cells might undergo apoptosis in response to occupancy of the FcR if the cells had been preactivated with cytokines, like IL-2.

Materials and Methods

Cell Culture. Cells were cultured in RPMI 1640 supplemented with 2% fetal bovine serum, and 4 mM glutamine (Biofluids Inc., Rockville, MD) at 37°C in an atmosphere of 5% CO_2.

Isolation of Large Granular Lymphocytes. Large granular lymphocytes were obtained from buffy coats of leukophoresed normal,

healthy volunteers (20). Enriched fractions of NK cells were further purified from contaminating T cells using antibody depletion.

Cytotoxicity Assay. Evaluation of cell death was accomplished using two methods. (*a*) Trypan blue exclusion. Cells were evaluated in the presence of 0.4% trypan blue visually. (*b*) Flow cytometry. Cells were evaluated for exclusion of propidium iodide (PI) on the FACSort® Flow Cytometer (Becton Dickinson & Co., Mountain View, CA). Using this method, cells also could be stained with FITC and evaluated for subset markers.

Cytokines and Treatments. The following cytokines were used: IL-2 (1,000 IU/ml); IL-4 (500 U/ml); IL-6 (1,000 U/ml); IL-7 (1,000 U/ml); IL-12 (50 U/ml); IL-13 (100 ng/ml); IFN-α (1,000 U/ml); and IFN-γ (1,000 U/ml). All cytokines except IL-12 and IL-13 were provided by the Biological Response Modifiers Program Repository. IL-12 was provided by Genetics Institute (Cambridge, MA). IL-13 was provided by Dr. P. Ferrara (Sanofi Elf Bio Recherches, Lebege, France). Lymphocytes (5×10^6 cells) were treated for 48 h with cytokines, washed with HBSS, then treated with antibodies (10 μg/ml) with or without goat anti-mouse IgG (20 μg/ml) or with aggregated Ig (50 μg/ml) for 0–4 h at 37°C. At various times, the cells were analyzed for cell death or apoptosis.

FACS® Tunnel Assay (Apoptosis). Labeling of nicked DNA was assayed using a modified nick translation assay on the flow cytometer (21). Briefly, cells were fixed with formaldehyde, permeabilized with methanol, and the degree of nuclear damage was evaluated by performing a nick translation assay using FITC-dUTP (Boehringer Mannheim Corp., Indianapolis, IN). Cells were enumerated on a FACSort® Flow Cytometer (Becton-Dickinson & Co., Mountain View, CA).

Results

TCR-dependent apoptosis after IL-2 activation has been proposed as a mechanism of extrathymic tolerance. Because of the documented similarities between T and NK cells, we first sought to determine if a similar phenomenon occurs in NK cells. NK cells were tested immediately after purification or after 48 h of IL-2 activation for induction of cell death. When cells were treated with CD3 (as a negative control), CD56, or CD16, no cell death was observed with these ligands even if these antibodies were cross-linked with goat anti-mouse (GaM) (Fig. 1 *A*). However, if the cells were first activated with IL-2, subsequent cross-linking of the FcR with anti-CD16 antibody and GaM induced a rapid cell death as determined by trypan blue exclusion (Fig. 1 *B*). Anti-CD16 alone induced cell death with slower kinetics, but of similar magnitude at time points of ≥2 h. Neither CD3 nor CD56 induced any cell death, regardless of whether or not GaM was used to cross-link these antibodies. Similar to activated T cells, these data indicated that activated but not resting NK cells could be induced to death by strong surface receptor activation.

To utilize a quantitative procedure to measure cell death, flow cytometric analysis (using PI uptake) was used with a panel of monoclonal antibodies (CD3, CD8, CD2, CD56, CD16) plus GaM on purified NK cells (Fig. 1 *C*). Again after preactivation with IL-2, CD16 cross-linking induced cell death. Because NK cells also can be activated by other surface molecules such as CD2 (22–24), we next examined if this ligand-induced killing was specific for CD16 or if it could occur with other receptor molecules. As shown in Fig. 1 *C*, CD2 failed to induce cell death. When the flow cytometry was performed using GaM-FITC (to enumerate the antibody-positive cells and to cross-link the receptor), ≥75% of the CD16+ cells were dead at 2 h (not shown). The cell death by trypan blue exclusion (not shown) and flow cytometry

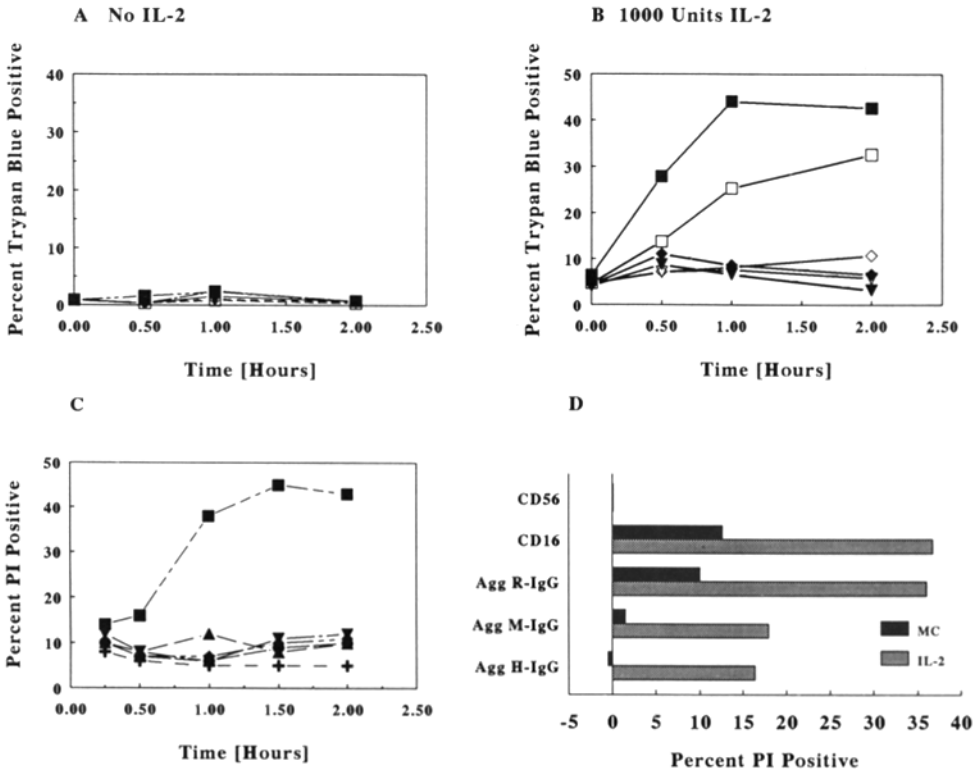

Figure 1. Receptor-induced death of NK lymphocytes. (*A* and *B*) Purified populations of CD56+ NK cells (≥70% CD56+) were tested immediately after purification (*A*) or activated for 48 h with 1,000 U of IL-2 (*B*). The cells were treated with CD3 (▽, ▼ +GaM); CD56 (◇, ◆ +GaM); or CD16 (□, ■ + GaM), followed by goat anti-mouse Ig. Values represent trypan blue evaluation of cell death and are representative of four or more experiments. (*C*) IL-2–activated NK cells were assayed by flow cytometry using GaM IgG-FITC. The cells were washed and treated with GaM alone (+); CD3 (▲); CD8 (●); CD2 (▼); CD56 (◆); or CD16 (■), followed by goat anti-mouse Ig. Values represent evaluation of cell death and are representative of four experiments. (*D*) Using CD16 (10 μg/ml) or aggregated Ig (50 μg/ml), the induced cell death of control (■) or IL-2–activated (■) NK cells were determined at 3 h by flow cytometry. Values are representative of two experiments.

using PI was similar in percent cell death. Thus although both CD16 and CD2 have both been reported (22–24) to be activating for NK cells, only CD16 was able to induce death. In addition, to ensure that occupancy of the receptor with a more physiologically relevant ligand resulted in cell death, aggregated Ig (rabbit, mouse, and human) were examined (Fig. 1 *D*). All aggregated Igs tested induced death. The kinetics of the rabbit Ig were more rapid than either mouse or human Ig, but all achieved similar levels at later time points (not shown).

Because propriocidal regulation of T cells involves apoptosis, we next determined if the anti-CD16–induced death in NK cells also involved genomic DNA degradation. Cells were activated for 48 h in IL-2, washed, then treated with CD3, CD56, or CD16 and GaM for 2 h. Nuclear degradation was determined by flow cytometry measuring incorporation of FITC-UTP. Fig. 2 *A* shows that significant nuclear degradation occurred after treatment with CD16, but not with other antibodies.

Previously, FcR-mediated signaling has been shown to be dependent upon protein tyrosine kinase activity. Therefore, we next determined whether kinase inhibitors could block the CD16-induced death. NK cells were activated for 48 h in IL-2. The protein tyrosine kinase inhibitor, herbimycin A, was added at −6 h, since it requires pretreatment for several hours to mediate inhibition (19), then was removed during the assay. Staurosporin, a less specific but more rapidly acting kinase inhibitor than herbimycin A, was added during the assay period. Fig. 2 *B* shows the effect of several doses of both inhibitors. Herbimycin strongly inhibited FcR-dependent cell death at both 3 and 1 μM concentrations. Staurosporin only potently inhibited at 10^{-7} M, a concentration that also blocks tyrosine kinase activity (25).

Since IL-2 previously has been shown to induce a rapid activation of NK cells and now has been shown to prime NK cells for CD16-induced killing, we examined a panel of additional cytokines to determine if any other cytokines had the capacity of priming NK cells in a manner similar to IL-2. Cells were treated for 48 h with medium, IL-2, IL-4, IL-6, IL-7, IL-12, IL-13, IFN-α, and IFN-γ, then analyzed by cross-linking cells with CD2, CD3, CD56, and CD16. As seen in Fig. 1, untreated NK cells demonstrated some low level susceptibility, but only IL-2 and IL-12 were able to strongly prime NK cells for susceptibility to CD16-induced killing (Table 1). IFN-α resulted in a modest increase. The remainder of the cytokines were not different from the low level of background killing induced when cells were cultured in medium. The pattern was similar when aggregated Ig was used as a stimulus rather than anti-CD16 (data not shown). In parallel experiments (not shown) both IL-2 and IL-12 resulted in anti-CD16–induced apoptosis, resulting in parallel nuclear degradation to that shown in Table 1.

In addition, since CD16 involves engagement of the FcγRIII, we determined if antibody-coated target cells would induce similar events. Aliquots of medium- and IL-2–pretreated cells were cultured with the NK-resistant target (FeMX) for 3 h then analyzed visually for cell death of effectors (Table 1). Neither the total population of NK cells nor the target-bound effectors were induced by antibody-coated targets to death.

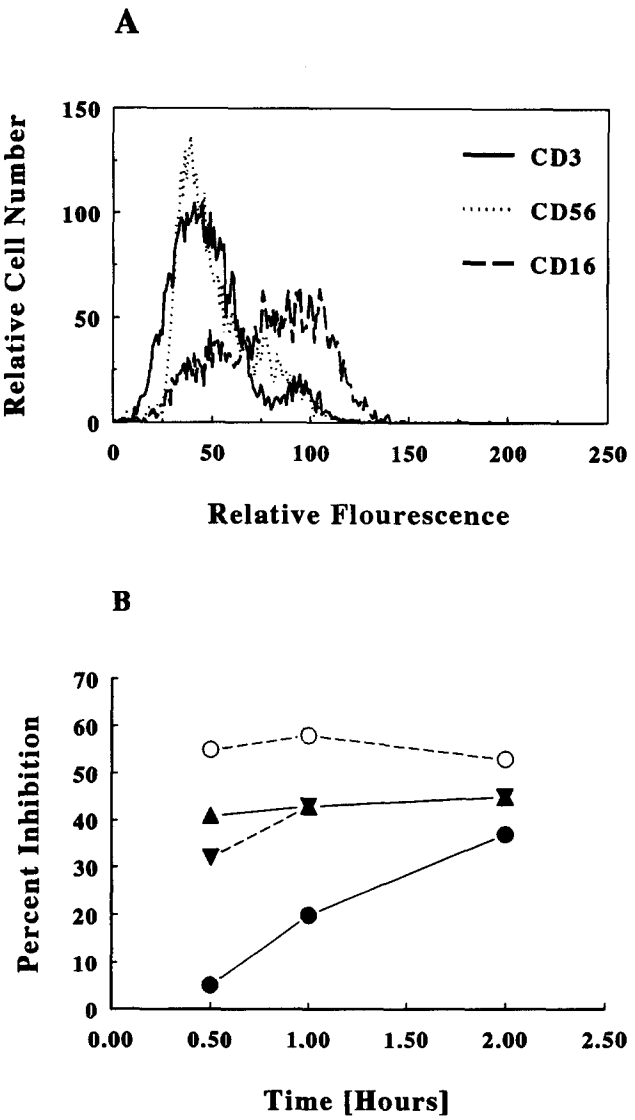

Figure 2. (*A*) Examination of nuclear degradation in FcR-mediated cell death. Purified populations of CD56+ cells (80% CD56+ and 55% CD16+) were activated for 48 h with 1,000 U, the cells were washed and treated with CD3 (———), CD56 (····), or CD16 (– – –) followed by goat anti-mouse Ig for 2 h. Cells were assayed for nuclear degradation by measurement of FITC-UTP incorporation. (*B*) Effect of kinase inhibitors on CD16-induced death of IL-2–activated NK cells. Purified populations of CD56+ cells (≥70% CD56+) were activated for 48 h with 1,000 U, the cells were washed and treated with CD16 followed by goat anti-mouse Ig. Values represent evaluation of percent inhibition of CD16+ cell death using a GaM-FITC, where the CD16 plus GaM induced 23%, 37%, and 57% death of the CD16+ cells at 0.5, 1.0, and 2 h, respectively. Cells were treated with herbimycin A at 3 μM (○) or 1 μM (▼) and staurosporin at 10^{-7} M (▲) or 10^{-8} M (●). Herbimycin A treatment began at −6 h and removed from the assay, while staurosporin was added at −30 min and not washed from the assay.

Discussion

TCR engagement and the resulting apoptosis has been

Table 1. *Ability of Various Cytokines to Prime NK Cells for CD16-induced Killing*

	None*	IL-2	IL-4	IL-6	IL-7	IL-12	IL-13	IFN-α	IFN-γ
Medium	0.8	0.6	0.8	0.6	0.3	0.2	0.2	0.5	0.4
CD2	−6.3	−3.3	−4.6	−8.2	−2.1	3.3	−5.9	−3.4	−5.2
CD3	0.9	2.0	1.4	1.4	0.6	3.2	0.7	1.4	2.9
CD56	2.0	3.4	1.5	0.5	−0.6	3.4	0.3	3.1	1.2
CD16	9.9	33.3	9.3	13.8	13.5	35.6	13.7	19.6	9.9

	NT	IL-2
Targets‡	Total/Bound	Total/Bound
FeMX	<1/ND§	1.0/1.3
FeMX/Ab	3.0/0.9	1.9/1.8

* Cells were incubated for 48 h with indicated cytokine at the concentration indicated in Materials and Methods. Cells were analyzed for killing by PI exclusion using a flow cytometer. Values represent percent PI-positive (dead) cells.
‡ NK cells cultured with medium or IL-2 were mixed for 3 h with FeMX targets (untreated or coated with rabbit anti-FeMX antibody) at a 1:1 ratio. Cells were enumerated for cell death by visual enumeration of total lymphocytes or by counting only target-bound effectors. The percent dead were based on cell counts of ≥100 cells.
§ ND indicates not determined due to lack of sufficient binding.

postulated as an important process in thymic education and extrathymic tolerance (4, 5, 8). The balance between activation and apoptosis is likely due to a variety of signals received by T cells. These signals can determine the fate of cells between death and proliferation. One such factor appears to be preexposure to IL-2. This aspect of T cell physiology can be exploited clinically (11). In this study, we have examined whether similar signals would result in activation-induced death in NK cells. While NK cells do not have a well-defined antigen receptor, they do have a receptor that can bind immune complexes. Indeed our studies indicate that only cross-linking the NK FcR was able to mediate ligand-induced death, whereas other molecules on NK cells such as CD2, CD8, and CD56 were incapable. This ligand-induced death did not occur unless the NK cells were activated with IL-2 or IL-12 before receptor cross-linking. In addition, similar to reports with T cells, cell death involved apoptosis. The CD16-induced apoptosis observed with NK cells is of interest, as the NK FcR like the TCR of T cells, is comprised of a ζ chain (17, 18) that is rapidly phosphorylated after receptor engagement. CD2 has been shown to trigger NK cells for cytolytic and secretory functions (22–24). Previous studies have demonstrated that CD16 is a potent ligand that releases granule serine proteases (23), known to be a major component of NK cell granules.

It is interesting that only IL-2 and IL-12 were able to activate/prime NK cells for CD16- or aggregated Ig–induced apoptosis. Many of the other cytokines tested are able to activate NK cytotoxicity (IL-6, IL-7, IFN-α, and IFN-γ), or induced gene transcription (IL-6, IL-7, IL-13, IFN-α, and IFN-γ). The exact nature of the activation signal required for receptor-induced killing is unknown; however, both IL-2 and IL-12 induce NK cell proliferation, a demonstrated requirement for TCR-induced apoptosis. The inability of the antibody-coated tumor targets to induce a similar cell death using identical effectors suggest that cell–cell interactions deliver a different qualitative and probably quantitative signal to the NK cells than total receptor cross-linking using CD16 or aggregated Ig. Cell–cell interactions involve numerous other ligands, including integrins, and engage only a small percentage of the cell membrane. Possibly these other interactions regulate the receptor process to avoid effector cell damage. Alternatively, the stimulus induced by an Ig-bearing target causes ligation of relatively few receptors and simply may be a suboptimal stimulus. Further elucidation of the mechanism of CD16-induced cell death would provide a better understanding of the lack of antibody-coated targets to induce similar events.

These findings clearly have relatively important implications clinically as IL-2 has been used extensively in the treatment of various malignancies. This treatment causes dramatic expansion of NK cells with increased lytic activity (15, 16). These results imply that high dose IL-2 therapy could cause in vivo apoptotic death of NK cells. If IL-2–activated NK cells encounter immune complexes, they could be neutralized by the processes described in this study. This possibility will need to be clarified in future studies.

At the present time, the physiologic role of FcR-dependent apoptosis in IL-2/IL-12–activated NK cells is unclear. Whether NK cells are selected during ontogeny is still unclear, though there is expanding evidence supporting the recognition of self and nonself by NK cells. The role of such processes in positive and negative selection and tolerance in NK cells are less defined than in T cells. Since both CD2 and CD8, which react with NK cells, failed to demonstrate activation-induced cell death, it is interesting to speculate that the association of ζ-chain with the CD16 (17, 18) is required for this phenomenon. To date, no other cell surface receptors in NK

cells have been associated with the ζ-chain. CD16 expression has been shown to occur early in fetal thymus development and could play an important role in the differentiation of T cells and NK cells during ontogeny. It is conceivable that this receptor might function in a negative selection for both NK and T cells. The physiological role of this process in the periphery is less clear and one possibility is that it is vestigial. Alternatively, like T cells, this may be a mechanism to dampen an exuberant immune and inflammatory response. Unlike T cells, though, where such a process would be proposed to prevent and regulate self-reactive cells, one could postulate that with NK cells this process might be involved in regulation of inflammation involving NK cells. In this case the term propriocidal regulation would not be apt, as NK cells do not produce large quantities of IL-2 or IL-12. Nonetheless, the finding that activated NK cells undergo apoptosis in response to FcR-mediated stimulation has implications both clinically and, perhaps, for NK cell ontogeny. It also underscores the similarities in function of T cells and NK cells.

We are grateful to Drs. Dan Longo and Jonathan Ashwell for critical review and to Susan Charbonneau and Joyce Vincent for secretarial and editorial assistance.

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Received for publication 5 August 1994 and in revised form 11 Sepember 1994.

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