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Cytokine Secretion Is Distinct from Secretion of Cytotoxic Granules in NK Cells

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NK cells are renowned for their ability to kill virally infected or transformed host cells by release of cytotoxic granules containing granzymes and perforin. NK cells also have important regulatory capabilities chiefly mediated by secretion of cytokines, such as IFN-γ and TNF. The secretory pathway for the release of cytokines in NK cells is unknown. In this study, we show localization and trafficking of IFN-γ and TNF in human NK cells in compartments and vesicles that do not overlap with perforin or other late endosome granule markers. Cytokines in post-Golgi compartments colocalized with markers of the recycling endosome (RE). REs are functionally required for cytokine release because inactivation of REs or mutation of RE-associated proteins Rab11 and vesicle-associated membrane protein-3 blocked cytokine surface delivery and release. In contrast, REs are not needed for release of perforin from preformed granules but may be involved at earlier stages of granule maturation. These findings suggest a new role for REs in orchestrating secretion in NK cells. We show that the cytokines IFN-γ and TNF are trafficked and secreted via a different pathway than perforin. Although perforin granules are released in a polarized fashion at lytic synapses, distinct carriers transport both IFN-γ and TNF to points all over the cell surface, including within the synapse, for nonpolarized release. The Journal of Immunology, 2010, 184: 4852–4862.

As a subset of cytotoxic lymphocytes, NK cells recognize and destroy malignant and virally infected cells (1, 2). NK cells respond to signals generated by activating and inhibitory receptors on their surfaces to constrain killing to appropriate target cells. Upon making contact with an appropriate target, NK cells release the membrane-disrupting protein, perforin, and proteolytic serine proteases, the granzymes, from secretory granules (3). The pathway for perforin release has been well studied, mainly in CTLs, which have similar cytotoxic granules and lytic capacity to NK cells. Upon target cell contact, granules cluster around the microtubule-organizing center (MTOC), which realigns to face the synapse (4). This facilitates polarized delivery of the granules to the synapse and ensures that damaging granule contents are released only at the synapse. In contrast to CTLs, cytotoxic granules in NK cells are preformed before cell activation, and so their maturation and release initially have to be carefully constrained and then enabled upon target cell contact (5). In NK cells also, polarity required for effective lytic function is established more slowly and is uniquely sensitive to cytoskeletal dynamics (6).

A second essential function of NK cells, especially early in viral infections, is to release antiviral cytokines, such as IFN-γ and TNF-α (TNF) (7, 8), as immunodefensive agents that additionally serve to activate resident inflammatory cells and recruit other cells (9). These NK cell-sourced cytokines also regulate dendritic cells, T cells, and B cells (10) and regulate TNF-mediated apoptosis of the NK cells and neighboring cells to delimit immune responses (11). Thus, cytokine production by NK cells influences both innate and adaptive immune responses (12). Parenthetically, the uncontrolled release of TNF and other proinflammatory cytokines in acute and chronic inflammatory disease is a major cause of ongoing tissue damage, pain, and fatality in these conditions (13). Uncontrolled cytokine release by NK cells and other cells together with defective granule release and target cell killing are concomitant features of hemophagocytic lymphohistiocytosis in humans and in mouse models (14–17). Cytokine secretion is thus central to immunity and to immunopathologies.

Despite their importance, little is known about how cytokines are secreted by NK cells. Cytokines made by different cell types can be transported and released by multiple pathways and involve different organelles and carriers (18). In eosinophils and mast cells, cytokines are stored in crystalloid granules or small secretory vesicles and secretory granules, respectively, for release (19–21). In T cells, cytokines can be released by vesicles in polarized and nonpolarized pathways (22). In activated macrophages, we previously described the pathway for trafficking and differential secretion of inflammatory cytokines (23–25). A central organelle for cytokine secretion in macrophages is the recycling endosome (RE), which is the site for regulation and sorting of cytokines, resulting, for instance, in the polarized delivery of TNF to phagocytic cups for release (23). To elucidate cytokine trafficking in NK cells, we studied endogenous and fluorescently tagged IFN-γ and TNF in freshly isolated primary NK cells and in live and fixed NK-92 cells and compared

Abbreviations used in this paper: 3D, three-dimensional; CathD, cathepsin D; EM, electron microscopy; LE, late endosome; MTOC, microtubule-organizing center; PFA, paraformaldehyde; RE, recycling endosome; TACE, TNF converting enzyme; TAPI, TACE inhibitor; Tfn, transferrin; TfnR, transferrin receptor; VAMP3, vesicle-associated membrane protein-3; VAMP3cyto, soluble form of vesicle-associated membrane protein-3; WGA, wheat germ agglutinin.

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these to the trafficking and secretion of perforin in granules. We investigated a role for the RE in these processes. Our findings reveal that trafficking of IFN-γ and TNF is non-directional; their trafficking is distinct from perforin and is dependent on the RE.

Materials and Methods

Abs and reagents

For immunofluorescence staining of primary NK cells the following monoclonal Abs were used: perforin (Alexa Fluor 487-conjugated, clone 4039; Biologend, Australian Bioscience, Karrynun, Western Australia, Australia) and LAMP1/CD107a (FITC-conjugated, clone ID4B; BD Biosciences, San Jose, CA). Rabbit polyclonal Abs against Rab11 (Zymed, Invitrogen, Mulgrave, Victoria, Australia), cathepsin D (CathD) (Chemicon International, Temecula, CA), and Rab27a (gift from Dr. H. Horiiuchi, Kyoto University, Sakyo-ku, Kyoto, Japan) were also used. Secondary Abs were used donkey anti-mouse (Alexa Fluor 488- or Alexa Fluor 647-conjugated) and donkey anti-rabbit (Alexa Fluor 488- or Alexa Fluor 594-conjugated; Molecular Probes, Eugene, OR).

The following primary Abs were used for NK-92 cell staining: goat anti-human TNF (R&D Systems, Minneapolis, MN), mouse anti-human perforin (clone δ9; Pierce, Rockford, IL), mouse anti-human IFN-γ (clone B27; Abcam, Cambridge, MA), goat anti-human IFN-γ (Affinity BioReagents, Golden, CO), mouse anti-GM130, Rab11, and Rab27a (BD Transduits, North Ryde, NSW, Australia), mouse anti-α-tubulin (Molecular Probes), goat anti-γ-tubulin (Santa Cruz Biotechnology, Santa Cruz, CA), rabbit anti-CathD (Chemicon International), and mouse anti-human transferrin receptor (TfnR) (Zymed). Furthermore, Alexa Fluor 647-conjugated wheat germ agglutinin (WGA) (Molecular Probes), which binds to proteins containing sialic acid and N-acetylglucosaminyl residues, localized in high quantities on the plasma membrane and in the Golgi. WGA is used in this paper to visualize the contour of the cell and localization of the Golgi within that cell. Secondary Abs used were donkey anti-rabbit, mouse, and goat conjugated to either Alexa Fluor 488, 594, or 647 (Molecular Probes).

Cell culture, transfection, and activation

Peripheral blood was obtained with informed consent from healthy adult donors at the Karolinska University Hospital, Huddinge, Sweden (after approval by The Regional Ethical Review Board in Stockholm). Primary NK cells were separated from whole blood using density centrifugation (Lymphoprep, Axis Shield, Oslo, Norway) followed by negative selection (Miltenyi Biotec, Auburn, CA), using mouse anti-CD56-PE and anti-CD3-PerCP Abs (BD Biosciences). The cells were verified by flow cytometry to be >98% pure CD56+CD3- cells. Cells were subsequently maintained in RPMI 1640 with 10% FCS and 2 mM L-glutamine (Invitrogen, Carlsbad, CA).

The human NK cell line, NK-92 (26), was maintained in Myelocult (H100 medium (ThermoTrace, Thermo Fisher Scientific, Sorensby, Victoria, Australia) supplemented with HL-2 (200 U/ml; eBioscience, San Diego, CA) and hydrocortisone (final concentration 10 μM). In several experiments, NK-92 cells were additionally activated using PMA (final concentration 10 ng/ml) in combination with ionomycin (final concentration 1 μg/ml) for 10–120 min. This activation served to switch on cytokine production similarly in NK-92 cells and primary NK cells.

Conducts of TNF, Rab11 (GDP-bound Rab11S25N), vesicle-associated membrane protein-3 (VAMP3) (soluble form [VAMP3cyto]), Rab27a, and Rab7b were cloned into pEGFP-C2 vector (Clontech, BD Biosciences, North Ryde, New South Wales, Australia) to produce a N-terminal GFP-tagged protein as previously described (23).

NK-92 cells were transfected for the transient expression of TNF, Rab11 (GDP-bound Rab11S25N), vesicle-associated membrane protein-3 (VAMP3) (soluble form [VAMP3cyto]), Rab27a, and Rab7b were cloned into pEGFP-C2 vector (Clontech, BD Biosciences, North Ryde, New South Wales, Australia) to produce a N-terminal GFP-tagged protein as previously described (23).

Intracellular cytokine staining of primary NK cells by FACS

Freshly isolated PBMCs were incubated alone or with the myelogenous leukemia cell line K562 at a 1:1 E:T ratio for 6 h. After 1 h, 1 μg/ml brefeldin A was added for the remainder of the incubation. Cells were then labeled with anti-CD3 and anti-CD56 for 45 min at 4 °C in PBS with 2% FCS and 2 mM EDTA. Subsequently, cells were washed, fixed in 100% paraformaldehyde (PFA), and permeabilized with 0.5% saponin, followed by labeling with allophycocyanin XL-TNF and allophycocyanin XL-IFN-γ for 1 h at 4 °C. FACS was performed on a FACS Calibur (BD Biosciences).

ELISA

To determine the levels of secreted IFN-γ, TNF (BD OptEIA; BD Biosciences), and perforin (Mabtech, Nacka, Sweden), commercial ELISA kits were used according to the manufacturer’s instructions.

Preparation of cell lysates and Western blotting

NK-92 cells were washed three times with PBS and resuspended in lysis buffer (20 mM Tris [pH 7.4], containing 1 mM EDTA, 1% Triton X-100, 150 mM NaCl, and Complete Protease Inhibitors [Roche, Basel, Switzerland]). Cells were subsequently disrupted by passing them through a series of successively smaller needles, and lysates were centrifuged for 10 min at 17,000 × g. Protein concentration was determined using the Bio-Rad protein Assay, and 30–100 μg per lane was loaded and analyzed using SDS-PAGE separation and Western blotting.

Indirect immunofluorescence

Indirect immunofluorescence of primary NK cells was performed on either unstimulated or PMA- (100 ng/ml) and ionomycin-stimulated (0.5 μM; both Calbiochem, San Diego, CA) cells, which were fixed in 4% PFA (Sigma-Aldrich), permeabilized with 0.5% saponin (Sigma-Aldrich), and blocked in PBS containing 5% FCS, 0.1% BSA-acetylated (Aurion, Wageningen, Netherlands) or normal donkey serum (Jackson ImmunoResearch Laboratories, West Grove, PA). Slides were mounted using ProLong Gold with DAPI (both from Invitrogen), and images were acquired on a DMI2020 confocal microscope (Leica Microsystems, Deerfield, IL).

NK-92 cells were allowed to adhere to poly-γ-lysine–coated slides before fixation in 4% PFA for 60 min or ice-cold methanol for 5 min. After fixation, cells were washed, permeabilized, and incubated with Abs as previously described (25). Immunofluorescence still images were captured on an inverted microscope (IX71; Olympus, Melville, NY) with a ×100 oil objective and a 12-bit 1280 × 1024-pixel charge-coupled device camera (IMAGO SuperVGA; TILL Photonics, Victor, NY). Confocal images were captured on a confocal microscope (LSM510 META; Zeiss, Oberkochen, Germany) using a ×100 oil objective and optical spectral separation or digital emission fingerprinting. Single images were captured with an optical thickness of 3–7 μm, and for a z-series, a 0.25–0.36-μm step interval was used. Analysis was performed using LSM510 META software (Zeiss) and Photoshop CS2 (Adobe, Chatswood, New South Wales, Australia). For three-dimensional (3D) reconstructions, Velocity 3.7 (Improvison, Wallingford, MA) software was used. The thresholds for the fluorescence intensity of each channel were carefully adjusted to most closely represent the signal strength of the original two-dimensional images collected. Colocalization was quantified by determining Pearson’s coefficient on confocal cell images, using ImageJ 1.37p (National Institutes of Health, Bethesda, MD); a coefficient of >0.4 was read as significant colocalization. This analysis was performed on five confocal images from 50 cells per experiment in images showing channels for stained proteins of interest but excluding DAPI and other fiducary markers.

Electron microscopy

Electron microscopy (EM) of resin-embedded cells was performed using standard methods. Immunofluorescence cryosections was performed as previously described (28). In brief, before fixation in 4% PFA (EM grade; ProSciTech, Thuringowa, Queensland, Australia), primary NK and NK-92 cells were embedded in warm gelatin and frozen onto cryotubs. Ultrathin cryosections were collected onto copper grids and immunolabeled according to Slot et al. (29). Abs were detected with either differently sized protein A–gold conjugates (provided by J. Slot, University of Utrecht, Utrecht, The Netherlands) or species-specific gold probes (British BioCell from Australian Laboratory Services, East Brisbane, Queensland, Australia). Sections were viewed on a JEOL 1011 electron microscope (JEOL Australasia, Brookwater, Australia) at 80 kV, and images were captured using the iTEM image analysis program (Soft Imaging System, Olympus, Berlin, Germany).

Live cell epifluorescence imaging

Live cell epifluorescence imaging was performed as described previously (25). Briefly, NK-92 cells were cultured in glass-bottom 35-mm dishes (MatTek, Ashland, MA) coated with poly-γ-lysine. Live cell epifluorescence imaging was performed and anti-CD3–conjugated protein A–gold was incubated with ×100 oil objective (IX81 OBS real time; Olympus). Frame capture rates were between 500 and 2000 ms, with total capture periods ranging from 2 to 20 min. Image control and postacquire image analysis performance were performed using CellIR 2.5 software.

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Conjugate formation

K562 cells were used to investigate conjugate and synapse formation. K562 cells and NK-92 cells were incubated at ratios ranging from 5:1 to 1:1 for 10–60 min. Images shown were all taken at a 30 min time point. NK-92 cells and K562 cells were pelleted at low speed, washed twice, and taken up in PBS. Next, they were allowed to adhere to poly-L-lysine–coated slides for 10–20 min and fixed in either 4% PFA or methanol as described above.

HRP-transferrin inactivation of RE

A HRP inactivation assay of the RE was modified from Ang et al. (30). Briefly, NK-92 cells were incubated with HRP-labeled transferrin (Tfn) (10 mg/ml) in media for 1 h in the dark at 37°C. Following a 5 min chase, cells were washed with ice-cold PBS, and surface-bound Tfn was removed by washing with ice-cold 0.15 M NaCl and 20 mM citric acid (pH 5). Following another wash in ice-cold PBS, cells were incubated on ice in the dark for 1 h with PBS containing 0.1 mg/ml diaminobenzidine and 0.025% H2O2. The control sample contained PBS alone. Cells were then washed with PBS containing 1% BSA to stop the reaction and incubated in prewarmed media. Afterwards, the sample contained PBS alone. Cells were then washed with PBS containing 0.1 mg/ml diaminobenzidine and 0.025% H2O2. The control sample contained PBS alone. Cells were then washed with PBS containing 1% BSA to stop the reaction and incubated in prewarmed media. Afterwards, cells were activated with PMA/ionomycin as described above, and supernatant was collected for TNF, IFN-γ, and perforin detection. Cells were washed, fixed, and immunostained for perforin and TNF for analysis by confocal microscopy or FACS.

Analysis of TNF cell-surface delivery by FACS

For analysis by FACS, NK-92 cells were transiently transfected with constructs containing either GFP alone, GDP-bound GFP-RAB11S25N, or GFP-VAMP3syt5 (a soluble form), as described above. Subsequently, cells were activated, stained for extracellular and intracellular TNF, and fixed as described above. Cells were analyzed with an Amnis ImageStream System (Amnis, Seattle, WA) equipped with 488, 350, and 658 nm lasers. Analysis of data was performed with IDEAS 3.0 (Amnis).

Statistical analysis

All quantifications are given as mean ± SEM from at least three representative experiments. Differences between two groups were compared using a non-parametric t test (Mann-Whitney U test); p < 0.05 were considered significant.

Results

TNF and IFN-γ production and secretion in NK cells

In response to contact with K562 target cells, primary NK cells produce the cytokines IFN-γ and TNF (Fig. 1A). Activation of NK-92 cells with CpG DNA results in the secretion of TNF but not IFN-γ, whereas activation with the protein kinase C activator PMA in combination with CpG DNA results in the secretion of TNF but not IFN-γ (Fig. 1B). TNF is initially produced as a transmembrane precursor (26 kDa), which is cleaved upon release by the ADAM-17 protease (TNF converting enzyme [TACE]) to a 17 kDa soluble form (Fig. 1C). Incubation of cells with TACE inhibitor (TAPI) results in accumulation of uncleaved 26 kDa TNF precursors in cells (31), often also seen as increased cell-surface staining (32). De novo synthesis of IFN-γ and TNF can be detected here perinuclear staining of each cytokine in activated cells (Fig. 1D). TACE inhibition with TAPI accumulates TNF on the surface of NK-92 cells (Fig. 1D) and blocks the secretion of soluble TNF into the culture medium, without affecting the concomitant release of IFN-γ (Fig. 1E). Thus, the abundant and rapid secretion of these cytokines can be measured as one of the key responses of activated NK cells.

Perforin granules in NK-92 and primary NK cells

NK cells also have granules for secretion of cytolytic agents, such as perforin. Preformed perforin granules can be stained in unstimulated primary NK cells and NK-92 cells (Fig. 2A, 2C, respectively). Upon stimulation, the perforin granules can be seen clustering around the MTOC (Fig. 2B, 2D and Supplemental Movie 1), which can be stained by γ-tubulin (Fig. 2E). In both the NK cell line (Fig. 2F–H) and primary cells (Fig. 2J, 2K), many of the perforin granules are demarked by overlapping staining with late endosome (LE) markers, such as Rab7 and CathD, and by Rab27a. This was confirmed by Pearson’s coefficients for Rab7 (0.71 ± 0.07), Rab27a (0.61 ± 0.06), and CathD (0.66 ± 0.09) that reflect significant colocalization of these markers with perforin (Fig. 2I). Cryo-EM and immunogold labeling also show these granules colabeled with CathD in both the NK cell line (Fig. 2L, 2M) and primary cells (Fig. 2N). Thus, perforin granules and their secretory pathway are seen to have typical and well-established characteristics in NK cells, and their labeling with LE markers is consistent with LE involvement in granule maturation. Having established the morphology and labeling of perforin granules, we were now able to compare these to cytokine-carrying structures.

Relative localization of cytokines and perforin in secretory organelles

TNF and perforin are stained in separate structures after cell activation (Fig. 3A). Preformed perforin granules predominate in unstimulated cells, which are not producing cytokines. By 30 min after activation with PMA/ionomycin, staining of newly synthesized TNF appears but is separate from perforin labeling. By 2 h, TNF is still being produced and can be seen in the perinuclear area and trapped on the surface of 50% (46.7 ± 7.1) of TAPI-treated cells (Fig. 3A, 3B). By this time, the distinct perforin granules have been released in ~70% (70.7 ± 7.0) of cells (Fig. 3A, 3C). Also, IFN-γ–positive secretory vesicles appear distinct from perforin-containing granules in activated cells (Fig. 3D, 3E and Supplemental Movie 2). Punctate structures labeled for TNF do not colabel with CathD, further distinguishing them from perforin granules (Fig. 3F).

Conjoint path for TNF and IFN-γ secretion

Newly synthesized TNF and IFN-γ were examined in detail in activated NK-92 cells (Fig. 4A–H). One hour after stimulation ~30% (28.0 ± 1.4) of cells are producing both IFN-γ and TNF, whereas other cells express only one of the cytokines (Fig. 4A). The perinuclear accumulation of TNF precursors largely overlaps with the Golgi marker GM130 in these cells (Figs. 1D, 4B). IFN-γ and TNF localize with one another and are coproduced in the same Golgi complexes (Fig. 4C). GFP-TNF can be expressed to study the secretory pathway of endogenous cytokines (23). Here, transient expression of GFP-TNF reflects the staining pattern of endogenous TNF, and it can also be seen colocalizing with endogenous IFN-γ in fixed NK-92 cells (Fig. 4D). GFP-TNF is clustered around the MTOC (here demarked by a concentration of α-tubulin), and several TNF carriers can be seen in the cell periphery (Fig. 4E). In cells expressing both IFN-γ and GFP-TNF over half (57.9 ± 1.9%) of these carriers contained both cytokines (Fig. 4F, 4G). GFP-TNF was expressed and imaged in live NK cells. Again, GFP-TNF appeared in perinuclear and peripheral vesicular structures, some of which could be tracked moving from the cell interior toward the cell surface (Fig. 4H and Supplemental Movie 3). Notably these vesicles moved multidirectionally and were delivered to multiple points around the cell circumference; some were captured fusing with the surface at these points delivering GFP-TNF to the surface prior to its release (Fig. 4H, arrows). This multivesicle delivery at the surface was confirmed in fixed cells by immunostaining endogenous TNF at early times (30 min) after cell activation in the presence of TAPI (Fig. 4I). Similarly, incubation of live, stimulated cells with TAPI and TNF Abs was performed to capture endogenous TNF at its initial delivery sites on the surface (Fig. 4J). This also showed that TNF is delivered to multiple sites at 30 min and is completely circumferential by 1 h. Therefore, the cytokines, TNF and IFN-γ, share post-Golgi carriers that are distinct from perforin, and they are delivered to multiple sites on the cell surface for release.
FIGURE 1. TNF and IFN-γ synthesis and secretion in NK cells. A. Freshly isolated PBMCs were incubated alone or with K562 target cells (E:T ratio of 1:1) for 6 h, with brefeldin A (1 mg/ml) added after 1 h. CD56+CD3− NK cells were gated by FACS for analysis of intracellular cytokine levels. Left panels show IFN-γ production in unstimulated cells and cells incubated with K562 cells, respectively. TNF production in cells incubated with K562 cells is shown in the right panel. B, NK-92 cells were activated with either CpG DNA or PMA in combination with ionomycin for 2–4 h, and levels of TNF and IFN-γ secretion are shown as measured by ELISA. C, Western blotting detected the nonprocessed (26 kDa) and processed (17 kDa) forms of TNF after 30–180 min of stimulation with PMA and ionomycin. D, Epifluorescence images of IFN-γ (red) and TNF (green) staining in NK-92 cells showing mainly perinuclear localization and plasma membrane staining of TNF in the presence of TAPI. Results are representative of 50 cells. Bars represent 5 μm. E, Graphs showing the secretion of TNF and IFN-γ, determined by ELISA, after 2 h of PMA/ionomycin stimulation, in the presence or absence of TAPI. Data are represented as mean ± SEM.
Cytokines pass through the RE

In other cells, the RE is adjacent to the trans-Golgi network clustered around the MTOC. In NK cells, the RE, labeled with the marker Rab11 or with the RE cargo TfnR, is also localized in a patch adjacent to the trans-Golgi network that is stained by γ-tubulin. F–H. Confocal immunofluorescence images showing colocalization of perforin (red) with LE/granule markers Rab7 (F, green) and Rab27a (G, green) translocated into NK-92 cells and partial colocalization with lysosome marker CathD (H, green). Results are representative of 50 cells. I. Graph showing levels of colocalization between perforin and the various LE/granule/lysosome markers using Pearson’s coefficient (n = 5). Data are represented as mean ± SEM. J and K. Costaining of perforin (red) with Rab27a (J, green) and CathD (K, green) in primary NK cells. Results are representative of 50 cells. L–N. EM showing localization of perforin (10 nm gold) and CathD (15 or 5 nm gold) in granules from NK-92 (L, M) and primary NK cells (N). Scale bars in immunofluorescence images represent 5 μm, and EM scale bars represent 100 nm.

Cytokines are secreted in a nonpolarized fashion

When NK cells are activated by interaction with appropriate target cells, release of cytolytic agents, such as perforin, occurs in a highly polarized fashion. Accordingly, perforin granules are directed to the synapse formed between NK-92 cells and K562 target cells (Fig. 6A). High-magnification images revealed that some granules docked beneath the cell surface at the site of contact, as demarked by WGA staining of sialoglycoproteins on the surface of NK cells (Fig. 6B). Cytokine staining was examined in cells forming synapses. Both IFN-γ and TNF can be seen in the Golgi complex (and possibly in the surrounding RE), located near the MTOC and aligned with the synapse (Fig. 6C). Despite this orientation and proximity to the synapse, the cytokines are
not delivered in a polarized fashion only toward the synapse. TNF and IFN-γ are detected and partially colocalized in carriers in the periphery (Fig. 6D). In the presence of TAPI, TNF was delivered and trapped over the entire cell surface, as shown by confocal imaging and in a 3D reconstruction (Fig. 6E, 6F and Supplemental Movie 5). Observation of synapse formation over a time course showed no overlap between perforin-positive granules and IFN-γ or TNF (data not shown). The 3D reconstruction of staining at such a synapse (Fig. 6F) revealed the separate staining of TNF carriers and perforin granules at the synapse membrane, suggesting that some cytokine can also be released within the synapse. Thus, although cells are undergoing polarized delivery of granules to the synapse for perforin release, cytokines are delivered to the cell surface at both the synapse and elsewhere in a truly non-polarized fashion (Supplemental Fig. 1).

**RE function in secretion from NK cells**

Different approaches were used to test whether the RE is functionally involved in cytokine secretion, perforin release, or both using the NK cell line. First, the RE was inactivated by allowing cells to take up HRP-Tfn, followed by peroxidase-induced deposition of substrate in the RE (30). Inactivated (HRP-Tfn loaded followed by substrate) and unreacted (HRP-Tfn loaded without substrate) REs were examined in cells stimulated with PMA/ionomycin, after which TNF, IFN-γ, and perforin release were monitored. Although unreacted REs showed no significant effect on cytokine release, secretion of TNF and IFN-γ was radically and significantly reduced (a 3- to 6-fold decrease) in cells with functionally inactivated REs. In contrast, the secretion of perforin from preformed granules was not significantly affected by RE inactivation (Fig. 7A). This shows that transition through the RE is essential for cytokine release but not perforin release from preformed granules.

REs could, however, be contributing to earlier stages of perforin granule maturation or trafficking. To examine this, we first depleted the pre-existing perforin granules using a 3 h stimulation with PMA/ionomycin. Subsequently, during a 24 h period, recovery of perforin-containing granules was assessed, with or without RE inactivation during the recovery phase. In control cells, fully formed perforin-containing granules can be detected again after 8 h, and by 24 h these cells fully restore their pool of granules localized around the MTOC (Fig. 7B, 7C). In contrast, in RE-inactivated cells, perforin was detected after 8 h, but it was still localized in the Golgi complex, and only by 24 h could perforin granules again be seen, although in smaller numbers (Fig. 7D, 7E). Thus, RE inactivation appeared to delay exit of newly synthesized perforin from the Golgi or retarded granule formation, or both, suggesting that RE function is required at some stage in the granule-mediated secretory pathway. Although this concept has been previously suggested in CTLs (34), a role for REs in granule formation in NK cells needs to be more fully explored.

To confirm a role for REs in cytokine secretion, we next employed dominant-negative forms of the RE proteins Rab11 and VAMP3. VAMP3 on REs has been shown to be involved in TNF secretion in mast cells (21) and in macrophages (23), where in the latter case expression of GFP-VAMP3cyto, a soluble mutant, abrogated TNF surface delivery. Rab11 mutants, such as GDP-bound GFP-RAB11S25N, were previously used in epithelial cells to block the exocytosis of other proteins trafficking to the cell surface through the RE (33, 35). Here, these constructs were transiently transfected into cells. The transfection efficiency of this cell line is known to be exceptionally low,
and FACS experiments were supplemented by analysis of multiple, individual transfected cells (Fig. 7F). After stimulation in the presence of TAPI, cells were analyzed for the relative levels of cell-surface and intracellular TNF staining, as a measure of TNF surface delivery. We compared control cells expressing GFP to cells transfected with GFP-RAB11S25N or GFP-VAMP3cyto mutants. All of the cells expressing the GFP-RAB11S25N were able to produce TNF (internal staining) but lacked cell-surface TNF (Fig. 7F). Transfection of GFP-VAMP3cyto resulted in similar findings. Thus, a predominance of nonfunctional Rab11 or VAMP3 disrupted surface delivery of newly synthesized TNF.

**FIGURE 4.** Conjoint path for TNF and IFN-γ secretion. A, Graph showing the percentages of cells expressing either TNF or IFN-γ, or both (n = 5). B, Epifluorescence image showing colocalization of endogenous TNF (red) and Golgi marker, GM130 (green), in stimulated NK-92 cells. C, D, Colocalization of newly synthesized TNF (red) and GFP-TNF (green), respectively, with IFN-γ (green in C, red in D) in the perinuclear Golgi area. E, Confocal immunofluorescence image showing secretory vesicles containing GFP-TNF are mainly clustered around the MTOC (here visualized as a concentration of α-tubulin, red) but are also detected in the periphery. F, Secretory vesicles in the periphery containing GFP-TNF also contain IFN-γ (red), as shown by confocal immunofluorescence. G, Graph showing the percentages of peripheral vesicles containing either TNF or IFN-γ, or both (n = 5). H, A series of frames from a movie of live imaging of an NK-92 cell expressing GFP-TNF and stimulated for 30 min with PMA/ionomycin in the presence of TAPI. Arrows indicate GFP-TNF–containing vesicles fusing with the cell surface at multiple points around the cell for delivery of GFP-TNF (Supplemental Movie 3). I, Immunostaining for endogenous TNF (green) on cells fixed 30 min after incubation with PMA/ionomycin in the presence of TAPI. J, Live cells incubated with PMA/ionomycin with TAPI (control) or with TNFAb added to the medium were fixed at 30 and 60 min and stained without permeabilization with secondary Abs. Endogenous TNF captured at points of surface delivery is shown. Pearson’s coefficients are indicated on representative panels (n = 5). Bars represent 5 μm. Data are represented as mean ± SEM. *p < 0.01; ***p < 0.001.

**FIGURE 5.** Cytokines pass through the RE. A, B, Epifluorescence images showing localization of RE mainly in the perinuclear region using TfnR (A, green) and Rab11 (B, green) as markers. C, Confocal immunofluorescence image showing localization of WGA (binding to proteins in the trans-Golgi network, green) at the MTOC as demarked by a concentration of α-tubulin (red). D, Western blotting detected expression of Rab11 and VAMP3 before and after stimulation with PMA and ionomycin. E, F, Partial colocalization of endogenous TNF (red) with RE markers Rab11 and TfnR (both green), respectively, as shown by epifluorescence images. G, Graph showing quantification of colocalization between TNF and both Rab11 and TfnR, using Pearson’s coefficient (n = 5). Data are represented as mean ± SEM. H, High-resolution 3D confocal reconstruction of a cross section through a dual GFP-TNF and TfnR-positive compartment (red) in an NK-92 cell (Supplemental Movie 4). I, J, Epifluorescence images showing marginal overlap of RE marker Rab11 (green) and perforin (red) in primary NK and NK-92 cells, respectively. Fluorescent images are representative of 50 cells. Bars represent 5 μm.
FIGURE 6. Cytokines are secreted in a nonpolarized fashion. Confocal fluorescence imaging of conjugates formed between NK-92 and K562 cells incubated for 30 min. A, Conjugate between an NK-92 cell and its K562 target shows directional delivery of perforin granules (green) toward the synapse. B, High-magnification image shows granules (red) lining up beneath the synapse cell surface, as demarked by WGA staining (green). Bars represent 5 μm. C, TNF (green) and IFN-γ (red) colocalization in the perinuclear Golgi region of a polarized NK-92 cell conjugated to a target cell. Dotted line indicates the location of the synapse membrane. D, IFN-γ (red) and TNF (green) single- and double-positive (arrow) peripheral vesicles are detected peripherally all around the conjugated NK cells. Fluorescent images are representative of 50 cells. E, Confocal 3D images showing cell-surface–trapped TNF (green), in the presence of TAPI, after contact with a K562 target (Supplemental Video 5). F, A 3D reconstruction of a synapse region showing perforin granules (red) and TNF staining (green) in the presence of TAPI. TNF (arrows) can be seen at the NK cell surface (white) labeled with WGA at the synapse and nonsynapse regions.

This provides another line of evidence implicating the RE in a requisite role in cytokine trafficking between the Golgi and cell surface.

Discussion
In this paper, we describe for the first time how cytokines, IFN-γ and TNF, are transported and secreted by NK cells. We reveal that the pathway for cytokine secretion is distinct from that for perforin secretion via cytotoxic granules. We show in particular that REs are essential for the surface delivery and release of newly synthesized IFN-γ and TNF. Moreover, we find that despite the alignment of the RE with the synapse and the polarity established to ensure directional delivery of granules to synapses, the cytokines are delivered to other sites on the cell surface for largely nonpolarized secretion. The separation of these pathways is an important mechanism allowing NK cells to simultaneously kill target cells and to recruit other immune cells, with different sets of secreted mediators, and thus to fulfill their role in immunity (36).

Primary blood NK cells produced IFN-γ and TNF after activation, a process that was faithfully reproduced by the release of endogenous cytokines in activated NK-92 cells. Complementary use of this cell line enabled expression and tracking of GFP-labeled TNF in fixed and live cells and manipulation of RE proteins for functional studies. IFN-γ and TNF—both endogenous and recombinant forms—were colocalized in NK cells, appearing together in the Golgi complex of activated cells, colocalizing with RE markers, and being found together in carriers in the cell periphery. We thus show that although IFN-γ is secreted in greater abundance by NK cells, it is trafficked and released simultaneously with TNF. This finding is relevant to the different immunologic roles of IFN-γ and TNF, for instance, in fighting viral infections (9), and their different pathological consequences when secreted in an uncontrolled fashion in disease (17).

The conjoint, nondirectional delivery of IFN-γ and TNF to the NK cell surface differs from the handling and secretion of cytokines in other cell types. In Th cells, IFN-γ is delivered in a polarized fashion to synapses with APCs, whereas TNF is secreted—separately—in a nonpolarized fashion (22). In activated macrophages, TNF is delivered in a polarized fashion to phagocytic cups or filopodia for release (23). This differs also from the localization of TNF and other cytokines in preformed granules in mast cells (37). Thus, in NK cells, as in other cell types, the trafficking and secretion of cytokines are customized for their cell-specific roles in immunity.

Notably, the lack of any demonstrable colocalization of IFN-γ or TNF with perforin showed that the secretory pathways for cytokines and cytolytic agents diverge at the level of the Golgi complex. Perforin exiting the Golgi complex was localized here in NK cell granules where it overlapped with the LE markers Rab7, CathD, and Rab27a, all of which are associated with the maturation and release of secretory granules in a number of cell types (34, 38, 39). There was no colocalization of cytokines with LE markers and no evidence of cytokines inside lysosome/granule structures. Because cytokines in NK cells are excluded from granule maturation pathways and from the granules themselves, alternative carriers are implicated for cytokine transport. The appearance and behavior of the carriers in NK cells labeled for both the soluble IFN-γ and the membrane-attached TNF precursor are similar to the pleomorphic tubules and vesicular structures that have been shown to mediate cytokine transport between organelles and the cell surface in other cells (23, 40, 41).
Partial colocalization with RE marker proteins first pointed to this organelle as a transitory post-Golgi destination for cytokines in NK cells. To show functionally that the RE is part of the cytokine secretory pathway, we used HRP-labeled Tfn to first populate and then inactivate REs in NK cells. Previous studies have established that by filling the REs with HRP substrate the traffic of cargo proteins through this compartment is blocked (30, 42). This manipulation reduced both IFN-γ and TNF secretion to ~17% of levels secreted by control cells. The similar effect on secretion of both cytokines further confirms that both cytokines are trafficked together but that, unlike

FIGURE 7. RE function in secretion from NK cells. A, A graph showing TNF, IFN-γ, and perforin secretion, as determined by ELISA, following HRP-Tfn inactivation of the RE. B–E, Epifluorescence images showing the recovery of perforin-containing granules (green) in control cells (B, C) and cells where RE was inactivated during recovery (D, E). F, NK-92 cells were transfected with either Rab11 mutant (GFP-Rab11S25N), VAMP3 mutant (GFP-VAMP3cyto), or GFP alone and subsequently stimulated in the presence of TAPI and stained for intracellular TNF (white) and cell-surface TNF (red). Bars represent 5 μm. Data are represented as mean ± SEM. **p < 0.01.
TNF and IL-6 in macrophages (25), they are not sorted at the RE for separate release. Additional functional evidence for RE involvement in cytokine secretion was obtained by overexpressing mutants of VAMP3 and the RE-resident GTPase, Rab11, both of which, in several assays, reduced cytokine surface delivery without disrupting de novo synthesis. Thus, several lines of evidence confirm the RE as a common destination and indeed as an obligate presurface compartment for both IFN-γ and TNF.

Interestingly, a small amount of perforin was localized at REs, and functional studies also showed that inactivating REs retarded or impaired granule maturation and reffiling with perforin. This likely signifies contact or transport between cytotoxic granules and REs during granule maturation in NK cells. Ménager et al. (34) suggested a role for REs in granule secretion in CTLs. In their activated T cells, vesicles representing Rab11-positive REs fused, via Munc13-4, with Rab27a-positive LEs, and the fused compartment was delivered to the immunological synapse. The granules and RE–LE fused compartment then came together, possibly, to enact the final stage of granule fusion with the synapse membrane (34). This exact scenario seems not to exist in NK cells, because we did not find tight association of Rab11 or other RE markers with the NK target cell synapses (data not shown) and RE inactivation did not greatly impair the release of preformed, mature NK cell granules. Instead, our data may be consistent with transient fusion between REs and NK cell granules—possibly early in maturation—which may preprime the NK cell granules for release. Future studies in CTLs and NK cells will need to address specific, temporal roles for any of these molecules or REs in granule maturation and release. Thus, the RE functions, but in quite different capacities, in secretory pathways for perforin granules and for cytokines. The RE may serve to separate the terminal stages of secretory pathways to regulate and segregate cell killing and cytokine release. The separate trafficking of cytokines and perforin granules is consistent with findings showing that separate signaling pathways govern the segregated release of cytokines and cytotoxic granules in NK cells (43, 44).

Increasingly, the RE is emerging as an exocytic compartment as well as a sorting and regulatory site for polarized cell-surface delivery in multiple cell types (25, 35). In macrophages, subcompartments of REs help to sort proinflammatory cytokines, IL-6 and TNF, for separate release (25). In Th cells, IFN-γ and TNF are trafficked separately (22), and this is also likely regulated by the RE given the roles of syntaxin 6 and Vti1b (24). Other cargo moving through the MTOC-clustered REs in Th cells are TCRs, which are recycled and delivered to the Th cell–APC synapse (45). In CTLs, granules cluster at the MTOC and from there are directed to the synapse for release (4, 46). Our images in NK cells confirm that REs are mostly clustered at the MTOC—demarked by tubulin staining—and that they align with target cell synapses during granule release. Thus, the RE, at the MTOC, in NK cells and T lymphocytes potentially sorts multiple proteins and elicits multiple vesicles, directing them either to the synapse or to other points on the cell surface. Given their sorting capacity, it is interesting to speculate that REs may be able to reroute cytokines according to different stimuli for immunity or pathology. For instance, in NK cells, IFN-γ and TNF are not wholly directed to lytic synapses, but sorting and redirection at the level of the RE could possibly occur to deliver them to regulatory synapses formed with dendritic cells (47). Our findings provide a basis for understanding key secretory pathways in NK cells, which ultimately regulate the separate lytic and regulatory capacity of these cells at the frontline of antiviral and antitumor responses.

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**Disclosures**

The authors have no financial conflicts of interest.

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