Mechanism

Apoptosis via an NKp46- and Fas-Dependent Human NK Cells Induce Neutrophil

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J Immunol 2012; 188:1668-1674; Prepublished online 9 January 2012;
doi: 10.4049/jimmunol.1102002
http://www.jimmunol.org/content/188/4/1668

Supplementary Material

http://www.jimmunol.org/content/suppl/2012/01/09/jimmunol.1102002.DC1

References

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Human NK Cells Induce Neutrophil Apoptosis via an NKp46- and Fas-Dependent Mechanism

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Polymorphonuclear neutrophils (PMN) are potent inflammatory effector cells essential to host defense, but at the same time they may cause significant tissue damage. Thus, timely induction of neutrophil apoptosis is crucial to avoid tissue damage and induce resolution of inflammation. NK cells have been reported to influence innate and adaptive immune responses by multiple mechanisms including cytotoxicity against other immune cells. In this study, we analyzed the effect of the interaction between NK cells and neutrophils. Coculture experiments revealed that human NK cells could trigger caspase-dependent neutrophil apoptosis in vitro. This event was dependent on cell–cell contact, and experiments using blocking Abs indicated that the effect was mediated by the activating NK cell receptor NKp46 and the Fas pathway. CDS6-depleted lymphocytes had minimal effects on neutrophil survival, suggesting that the ability to induce neutrophil apoptosis is specific to NK cells. Our findings provide evidence that NK cells may accelerate neutrophil apoptosis, and that this interaction may be involved in the resolution of acute inflammation.


Received for publication July 8, 2011. Accepted for publication December 5, 2011.

This work was supported by grants awarded by Associazione Italiana Ricerca sul Cancro (Investigator grant [IG] project 10643, to A.M.; IG project 4725, to L.M.; and Special Project 5x1000 9962, to A.M. and L.M.), University of Genoa (Progetto Ricerca Ateneo 2010, to E.M.), Swedish Medical Research Council, King Gustaf V’s Memorial Foundation, Gunvor and Ivan Svensson’s Foundation, and the Swedish Research Council. EMBO long-term fellowship to F.B.T.; and by the European Commission (Marie Curie Intra-European Fellowship to F.B.T.).

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The online version of this article contains supplemental material.

Abbreviations used in this article: DC, dendritic cell; FasL, Fas ligand; NCR, natural cytotoxicity receptor; PMN, polymorphonuclear neutrophil; z-IE1TD-fmk, benzoyloxy-carbonyl-Ile-Glu(Ome)Thr-Asp(Ome) fluoromethylketone; z-VAD-fmk, benzoyloxy-carbonyl-Val-Ala-Asp (Ome) fluoromethylketone.

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www.jimmunol.org/cgi/doi/10.4049/jimmunol.1102002

Materials and Methods

mAbs

The following mAbs produced in our laboratory were used in this study: anti-CD56 (A6.220, IgM), anti-HLA class I (A6.136, IgM), anti-2B4
the reaction product was digested with SalI/BamHI restriction enzymes and TTGTCACAAGATTTGGGGTCGACGCGT-3

facturer’s instructions with the addition of To-Pro-3 iodide (0.5 FITC-conjugated Annexin V (BD Biosciences) according to the manu-
m10 Val-Ala-Asp (OMe) fluoromethylketone (z-V AD-fmk; Calbiochem) or

NK cells (defined as CD56+/CD3-). CD56-depleted lymphocytes were obtained using MACS CD56 Micro beads and a MACS LD column. The NK cell content of the depleted lymphocytes was <1%.

Isolation of human leukocytes

Buffy coats were obtained from healthy blood donors and mixed in a 1:1 ratio with 2% Dextran T500 (Pharmacosmos, Holbaek, Denmark). After sedimentation of RBCs, the upper phase was separated into neutrophils and mononuclear cells by density gradient centrifugation. Residual erythrocytes in the pellet were lysed in water to yield a pure population of granulocytes. Lymphocytes were obtained by countercurrent centrifugal elutriation of the mononuclear cells as described previously (19).

Induction and quantification of neutrophil apoptosis

Neutrophils were incubated with NK cells or CD56-depleted lymphocytes (37°C, 5% CO2) and assayed for apoptosis after 4 h of coculture. If not otherwise stated, short-term activated allogeneic NK cells (1 ng/ml IL-12, 105 cells) were incubated with 2 μg NKP30Fc, NKP46Fc, or NTB-AFc soluble receptors for 30 min at 4°C, washed, and stained with PE-conjugated F(ab’)2 goat anti-human IgG (Jackson Immunoresearch Laboratories) for 30 min at 4°C. FITC-conjugated Annexin V and To-Pro-3 iodide were added to exclude dead cells from the analysis. Flow cytometry was performed using a FACS Calibur flow cytometer (BD Biosciences), and cells were analyzed with CellQuest Pro software (BD Biosciences).

In vivo model of aseptic inflammation

Skin blisters were induced on the volar side of the forearm of healthy volunteers by a suction chamber coupled to a portable vacuum pump for 2 h (23). At different time points, the blister fluid (∼20 μl/blister) was col-

Results

NK cells trigger apoptosis in neutrophils

Neutrophil apoptosis is a critical step in the termination of acute inflammatory responses. However, incomplete information is available on the proapoptotic signals leading to apoptosis (5, 6). Previous studies have addressed NK cell cytotoxicity against other innate immune cells, such as myeloid DCs and polymched macrophages (14, 16, 18, 24). However, little is known about the NK cell capacity to kill neutrophils. In pilot experiments, freshly isolated neutrophils were incubated with short-term IL-12-activated NK cells and assayed for apoptosis after 4 h of coculture. As shown in Fig. 1A, NK cells induced significant apoptosis in neutrophils (p < 0.0001; n = 18). In contrast with tumor cell lines that are highly susceptible to NK cell-mediated lysis, the majority of dying neutrophils remained intact ( Annexin V- , To-Pro-3 ; Fig. 1B).

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To determine whether the ability to induce neutrophil apoptosis was confined to NK cells or whether it was a general capacity shared by other lymphocytes, we used a CD56-depleted lymphocyte population in apoptosis experiments. As shown in Fig. 1E, CD56-depleted lymphocytes failed to compromise neutrophil survival, suggesting that neutrophil apoptosis is specifically induced by NK cells.

**NK cell-induced neutrophil apoptosis is caspase and contact dependent**

Proinflammatory cytokines, such as GM-CSF, favor prolonged neutrophil survival (6), and supernatants recovered from stimulated NK cells were recently proposed to prolong neutrophil survival (25). To investigate whether the proapoptotic effect of NK cells could override survival signals mediated by cytokines, we exposed neutrophils to short-term activated NK cells in the presence or absence of exogenously added GM-CSF or IL-12, which may trigger cytokine release from NK cells. As expected, exogenously added GM-CSF reduced the rate of spontaneous neutrophil apoptosis ($p < 0.05$; Fig. 2A). However, NK cells induced significant apoptosis also in GM-CSF–protected neutrophils ($p < 0.001$). Presence of IL-12 during NK–neutrophil cocultures slightlyenhanced NK cell-induced neutrophil apoptosis ($p < 0.05, n = 10$, data not shown).

**FIGURE 1.** NK cells trigger apoptosis in neutrophils. A, Freshly isolated human neutrophils were incubated alone or together with short-term activated NK cells (1 ng/ml IL-12, 20 h) and assayed for apoptosis after 4 h. NK cells induced significant apoptosis in neutrophils ($***p < 0.0001, n = 18$; paired samples t test). B, A representative experiment shows that NK cells predominantly induced apoptosis (Annexin V+ To-Pro-3−) rather than lysis (Annexin V− To-Pro-3−). C and D, Resting autologous (C) and allogeneic (D) NK cells triggered significant apoptosis in coincubated neutrophils. Values are given as percentage of total Annexin V+ neutrophils (mean ± SEM, $n = 5$ and $n = 4$, respectively). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

E, Neutrophils were incubated with NK cells or CD56-depleted lymphocytes, either resting or incubated overnight (20 h) with IL-12 (1 ng/ml). Resting NK cells induced significantly more neutrophil apoptosis than CD56-depleted lymphocytes ($***p < 0.001$). Pretreatment of lymphocytes with IL-12 enhanced NK cell cytotoxicity ($***p < 0.01$) but did not make CD56-depleted lymphocytes capable of triggering neutrophil apoptosis ($***p < 0.001$). Data are mean ± SEM, $n = 7$.

To determine whether the ability to induce neutrophil apoptosis was confined to NK cells or whether it was a general capacity shared by other lymphocytes, we used a CD56-depleted lymphocyte population in apoptosis experiments. As shown in Fig. 1E, CD56-depleted lymphocytes failed to compromise neutrophil survival, suggesting that neutrophil apoptosis is specifically induced by NK cells.

**FIGURE 2.** NK cell-induced neutrophil apoptosis is caspase dependent and surmounts the antiapoptotic effect of GM-CSF. A, NK cells induced neutrophil apoptosis both in the absence or presence of 100 U/ml GM-CSF (mean ± SEM, $n = 6$; ***$p < 0.001$). GM-CSF reduced the spontaneous apoptosis rate in neutrophils (*$p < 0.05$; $n = 6$). B, Neutrophils preincubated with the pan-caspase inhibitor z-VAD-fmk (10 or 100 μM) were exposed to NK cells. z-VAD-fmk–treated neutrophils were significantly protected against NK-induced apoptosis (*$p < 0.05$ and ***$p < 0.001$ for 10 and 100 μM z-VAD-fmk, respectively). Values are given as percentage of Annexin V+ neutrophils (mean ± SEM, $n = 5$).

C, Confocal photomicrographs of neutrophils in the presence or absence of short-term activated NK cells as indicated (original magnification ×220). Nuclei were made visible by DAPI staining (blue), and green staining of the cytosol indicated caspase activation and ongoing apoptosis. D, Conditioned media obtained from cell cultures were collected and stored at $-80^\circ$C until use. Neutrophils were incubated in indicated conditioned media for 4 h and stained with Annexin V and To-Pro-3. No increase in neutrophil apoptosis was detected when comparing control medium with conditioned media recovered from NK cell or NK–neutrophil cocultures (mean ± SEM, $n = 3$).
In the next series of experiments, neutrophils were preincubated with the pan-caspase inhibitor z-VAD-fmk before coincubation with NK cells. As seen in Fig. 2B, apoptosis was reduced in the presence of z-VAD-fmk in a dose-dependent manner. Next, we used confocal microscopy to visualize the activation of caspases in neutrophils. Activation of caspases was visualized using a fluorescent inhibitor of caspases (FLICA) that was added to the cell suspension (21). This cell-permeable, carboxyfluorescein-labeled reagent binds to activated caspases and can be detected inside apoptotic cells. As shown in Fig. 2C, in the presence of NK cells, neutrophils displayed green cytosolic staining, indicating caspase activation and ongoing apoptosis.

In a series of experiments, we examined whether the NK cell-mediated apoptosis induction was dependent on soluble factors or whether cell–cell contact was needed. Conditioned media, obtained from NK cell or NK–neutrophil cocultures, were collected and added to freshly isolated neutrophils. As shown in Fig. 2D, none of these conditioned media contained soluble mediators capable of inducing apoptosis as determined after 4 h of incubation. These results suggest that NK cell-induced neutrophil apoptosis is not mediated through soluble factors, and that cell–cell contact is important for apoptosis induction.

**Neutrophil expression of ligands for NK cell receptors and role for NKp46 in neutrophil apoptosis induction**

NK cell cytotoxicity is regulated by the balance between inhibitory and activating signals emanating from interactions between target cell ligands and NK cell receptors. In a series of experiments, we characterized the expression of NK cell receptor ligands on the neutrophil cell surface. As shown in Fig. 3A, neutrophils displayed very low levels of ligands for the activating receptors 2B4 (CD48) and NKG2D (MICA, ULBP 1-3). DNAM-1 ligands, Nectin-2 and poliovirus receptor, were expressed at higher levels, although some variation was observed among different individuals (Fig. 3A).

To assess the cell surface expression of ligands to the natural cytotoxicity receptors (NCRs), NKp46 and NKp30, on neutrophils, we determined the binding of NCRFc fusion proteins by flow cytometry. As shown in Fig. 3B, neutrophils displayed weak but significant binding of a soluble NKp46 receptor. Also, NKp30Fc stained neutrophils but to a lower extent, whereas no binding of NTB-AFc was observed, confirming previous reports stating that NTB-A ligands are specifically expressed in eosinophilic granulocytes (26). These data provide, to our knowledge, the first evidence that neutrophils constitutively express cell surface ligands for the NCRs NKp30 and NKp46.

Next, we investigated the role of different activating NK cell receptors for NK cell-mediated apoptosis induction in human neutrophils. As shown in Fig. 3C, masking of NKp46 with a specific mAb significantly protected neutrophils from NK cell-induced apoptosis, whereas Abs directed to other activating NK cell receptors failed to significantly protect neutrophils. The absence of a suitable IgM Ab against NKG2D precluded analysis of the role of NKG2D in NK cell-induced neutrophil apoptosis. These data suggest that a hitherto unidentified structure on neu-
crophils interacts with the activating NK cell receptor NKp46 to trigger NK cell activation and subsequently neutrophil apoptosis. Alternatively, NKp46 binding to neutrophil-encoded ligand may generate an apoptosis-promoting signal in neutrophils.

To address this possibility, we determined NK cell degranulation after coincubation with neutrophils. When NK cells become activated upon interaction with a target cell, lytic granules fuse with the plasma membrane to release perforin and granzymes to kill the encountered cell (27, 28). Because these granules are specialized secretory lysosomes, they contain lysosomal membrane proteins (e.g., CD107a) that can be detected on the cell surface after granule fusion (20, 29). As shown in Fig. 4A, short-term activated NK cells incubated with neutrophils displayed significant degranulation, albeit not of the same magnitude observed with the classical NK-susceptible target cells K562.

NK cells also use FasL to kill target cells; upon binding of FasL, the cytoplasmic part of Fas recruits death domain-containing adapter proteins that interact with caspase-8 to form the death-inducing signaling complex (30). Within this complex, caspase-8 becomes active and promotes a series of events leading to apoptosis. In a next series of experiments, we examined whether caspase-8 was involved in NK cell-induced neutrophil apoptosis. As shown in Fig. 4B, preincubation with the caspase-8 inhibitor, z-IETD-fmk, significantly protected neutrophils from NK-induced apoptosis, suggesting a role for caspase-8 in NK cell-mediated induction of neutrophil apoptosis. However, granzymes released from NK cells share the substrate specificity with caspase-8. Thus, to discriminate between the granzyme and Fas pathways, we performed experiments with neutralizing Abs to Fas and FasL. As shown in Fig. 4C, blocking of FasL or Fas resulted in significantly decreased apoptosis of neutrophils (p < 0.001; n = 4), strongly suggesting a key role for the Fas pathway in NK cell-induced neutrophil apoptosis.

**FIGURE 4.** NK cell degranulation and involvement of granzymes and the Fas pathway. A, Short-term activated NK cells displayed significant upregulation of CD107a on the cell surface after 4 h of coincubation with neutrophils (p = 0.02; n = 4). The MHC class I+ cell line K562 was used as positive control. B, Neutrophils preincubated with the granzyme/caspase-8 inhibitor z-IETD-fmk (10 μM) were exposed to allogeneic short-term activated NK cells. z-IETD-fmk-treated neutrophils were significantly protected against NK-induced apoptosis (mean ± SEM, n = 5; *p = 0.01). C, Blocking Abs to FasL or Fas protected neutrophils from NK cell-induced apoptosis (mean ± SEM, n = 4; ***p < 0.001).

**FIGURE 5.** Neutrophils and NK cells appear in skin blisters. Blisters were induced on the forearm of healthy volunteers by a suction chamber coupled to a portable vacuum pump. A, At indicated time points, the blister fluid was collected and the presence of immune cells in the blister fluid was determined by flow cytometry. As shown in Fig. 5, neutrophils started to accumulate already after 2 h. At a later time point, NK cells (CD3− NKp46+), together with other lymphocytes and monocytes, were detected in the blister fluid (Fig. 5A, 5B). Interestingly, the appearance of NK cells coincided with an increased percentage of apoptotic neutrophils in the blister fluid (Fig. 5C).

**Discussion**

Neutrophils are the most abundant leukocyte in human peripheral blood, and a vast number of mature neutrophils are released from the bone marrow every day (31, 32). However, they are not long-lived, and in the absence of an inflammatory insult, they enter an apoptotic program. In contrast, when neutrophils are recruited to a site of inflammation, their life span is extended. At these sites, neutrophils recognize and phagocytose microbes and participate in the inflammatory response. As long as the threat remains, proinflammatory mediators, such as GM-CSF, IFN-γ, IL-1β, IL-3, IL-6, TNF-α, and LPS enhance neutrophil survival (6). Activated neutrophils produce chemokines that attract more neutrophils, as well as other immune cells, to the lesion (33). For example, NK cells express receptors for many of the mediators produced or generated by activated neutrophils, such as CXCL8 (IL-8), CCL3 (MIP-1α), and chemerin (33–36). The recruitment of more neutrophils is an important amplification loop in the immediate response to infection. Ingested pathogens are killed using oxygen...
cytokine GM-CSF. In contrast, neutrophils were fully protected from NK cell cytotoxic response, respectively. In this study, an Ab to the NKp46 receptor was found to significantly protect neutrophils from NK cell-mediated lysis (27, 28). Whether FasL is present only in lytic granules or is also stored in distinct intracellular structures is still a matter of debate (48, 49). Our data suggest that degranulation occurs when NK cells interact with neutrophils even though only a small fraction of NK cells upregulated CD107a expression after coinoculation with neutrophils as compared with coinoculation with K562 cells. However, K562 cells represent an exceptional target cell line in their ability to induce NK cell activation and degranulation. In this context, it should be noted that several target cell lines that are susceptible to NK cells in chromium release assays, such as several EBV-infected cells, also fail to induce substantial degranulation as determined in a CD107a assay (A. Moretta, unpublished observations). Pretreatment with the caspase-8 inhibitor, z-IETD-fmk, or neutralizing Abs to FasL or Fas significantly protected neutrophils from NK cell-induced apoptosis, strongly suggesting that NK cells trigger PMN apoptosis by use of the Fas pathway.

In conclusion, in this study, we show that human NK cells are capable of triggering apoptosis in neutrophils. NK cell-induced apoptosis was contact and Fas dependent, and mediated by the activating NK cell receptor, NKp46. These data expand the role for NK cells as modulators of immune responses and suggest that NK cells could contribute to the safe removal of neutrophils, and thus play an important part in the resolution of inflammation.

Acknowledgments
We thank Michela Falco (Istituto Giannina Gaslini, Genova) for supplying the NTB-AFc fusion protein.

Disclosures
The authors have no financial conflicts of interest.

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