Brief Definitive Report

Natural Killer (NK) Cell–mediated Cytotoxicity: Differential Use of TRAIL and Fas Ligand by Immature and Mature Primary Human NK Cells

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Summary

Mature natural killer (NK) cells use Ca^{2+}-dependent granule exocytosis and release of cytotoxic proteins, Fas ligand (FasL), and membrane-bound or secreted cytokines (tumor necrosis factor [TNF]-α) to induce target cell death. Fas belongs to the TNF receptor family of molecules, containing a conserved intracytoplasmic "death domain" that indirectly activates the caspase enzymatic cascade and ultimately apoptotic mechanisms in numerous cell types. Two additional members of this family, DR4 and DR5, transduce apoptotic signals upon binding soluble TNF-related apoptosis-inducing ligand (TRAIL) that, like FasL, belongs to the growing TNF family of molecules. Here, we report that TRAIL produced or expressed by different populations of primary human NK cells is functional, and represents a marker of differentiation or activation of these, and possibly other, cytotoxic leukocytes. During differentiation NK cells, sequentially and differentially, use distinct members of the TNF family or granule exocytosis to mediate target cell death. Phenotypically immature $CD161^+/CD56^-$ NK cells mediate TRAIL-dependent but not FasL- or granule release–dependent cytotoxicity, whereas mature $CD56^+$ NK cells mediate the latter two.

Key words: natural killer cells • differentiation • cytotoxicity • TRAIL • Fas ligand

Cytotoxic T lymphocytes (CTLs) and NK cells use a combination of several mechanisms to lyse different target cells. These include (*a*) Ca^{2+}-dependent granule exocytosis and release of cytotoxic proteins (perforin and granzymes) from intracytoplasmic granules; (*b*) FasL, constitutively expressed or induced upon interaction with target cells and inducing Ca^{2+}-independent, Fas (CD95/Apo 1)-mediated apoptosis; and (*c*) membrane-bound or secreted cytokines (TNF-α) (1–5; for a review, see reference 6). Fas and the TNF-R (CD120a, b) belong to a family of molecules containing a conserved intracytoplasmic "death domain" (7, 8) that, through interaction with distinct intermediary adaptor molecules, indirectly results in activation of the caspase enzymatic cascade (9) and ultimately apoptotic mechanisms in numerous cell types (7). Additional members of this family include the death receptors DR4 (R1) and DR5 (R2) (10–12), transducing apoptotic signals upon binding their soluble ligand, the TNF-related apoptosis-inducing ligand (TRAIL [13]). A third receptor (R3), homologous to the other two in its extracellular domain but anchored to the membrane via glycosyl phosphatidylinositol and lacking intracellular domain and apoptosis-inducing capability (14), has been suggested to function as a decoy receptor, protecting normal cells against TRAIL-induced apoptosis.

Engagement of the TCR/CD3 complex on T cells induces increased functional expression of FasL (15). Although most $CD4^+$ T cell–mediated cytotoxicity is Fas/FasL dependent (16), one report indicates Fas/FasL-independent $CD4^+$ cell–mediated lysis of certain melanoma cell lines (17), suggesting that different members in the same families may also be involved in target cell lysis by CTLs.

Upon stimulation with cytokines (18), appropriate target cells (4, 19), or FcγRIIIA ligands (20), functional FasL is induced on NK cells at significant levels and in appropriate configuration to cross-link Fas on positive target cells (e.g., Jurkat) and induce their death. TRAIL expression, and its possible role in cytotoxicity mediated by these cells, has not been analyzed. Here we demonstrate that TRAIL produced and/or expressed by different populations of human

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primary NK cells is functional and represents a marker of differentiation and/or activation of these and possibly other cytotoxic leukocytes.

Materials and Methods

mAbs and Polyclonal Sera. mAbs to CD2 (B67.1, B67.6), CD4 (B66.6), CD5 (B36.1), CD8 (B116.1), CD11b (B43.4), CD14 (B52.1), CD15 (B40.9), CD56 (B159.5 [21, 22]), CD161/NKR-P1A (B199.2) used for cell purification (23), and the TNF-α–neutralizing mAb (B154.2, B154.7 [24]) have been reported previously. mAbs to CD3 (OKT3), CD21 (THB5), CD34, CD32 (IV.3), CD64 (32.2), and the irrelevant P3x63.Ag8.653 Ig were produced from cells from the American Type Culture Collection (Rockville, MD). The Fas-blocking ZB4 mAb and the Fas-triggering CH11 mAb (25) were from Upstate Biotechnology, Inc. (Lake Placid, NY). The goat anti–mouse Ig used for panning was produced in our laboratory, adsorbed on human IgG, and affinity-purified on mouse Ig Sepharose before use (26).

NK Cell Populations. Immature lineage negative (Lin^-) progenitor cell populations were enriched, as described (23), from umbilical cord blood samples provided by Dr. R. Depp (Department of Obstetrics and Gynecology, Thomas Jefferson University Hospital, Philadelphia, PA). These were >99% $CD3^-/CD161^-/CD16^-/CD56^-$ (indirect immunofluorescence [23]), not cytotoxic against K562 target cells, and did not express CD16 mRNA (reverse transcription [RT]-PCR) or CD161 (23; and data not shown). Their differentiation to NK cells was induced upon coculture with the murine bone marrow stromal cell line Sl/Sl^4 $hSCF^{220}$ (Sl/Sl^4 [27]; provided by Dr. D. Williams, University of Indiana, Indianapolis, IN) and rIL-2 (50 U/ml; Hoffman-LaRoche, Nutley, NJ, through the Biological Response Modifiers Program, National Cancer Institute, Bethesda, MD) or rIL-15 (10 ng/ml, specific activity 2.95×10^8 U/mg; Immunex Corp., Seattle, WA) during a 20–30-d culture period (23, 28). $CD161^+/CD56^-$ immature and $CD56^+$ mature NK cells were purified (23) from $CD3^-/CD161^+$ cells from 25–30-d cultures with IL-2. Homogeneous populations of $CD3^-/CD56^+/CD16^+$ NK cells were also obtained by negative selection from short-term, 10-d cultures of umbilical cord blood lymphocytes with 30 Gy–irradiated RPMI-8866 cells (22). Each population contained >98% cells expressing the appropriate phenotype (direct immunofluorescence).

Soluble TRAIL, Fas, DR4, and DR5. As described (10), Fas, DR4, and DR5 extracellular ligand-binding domain–Fc fusion proteins were produced in human 293 cells transiently transfected with constructs encoding these proteins. Conditioned media were used as source of the soluble proteins. Recombinant His 6–tagged TRAIL was produced in bacteria and purified by affinity chromatography on Ni^{2+} affinity resin.

Cytotoxicity and Cell Death. Cell-mediated cytotoxicity was analyzed in 4- or 6-h ^{51}Cr-release assays using, respectively, the Fas^- K562 and the $Fas^+/DR4^+$ Jurkat cells (J32 clone) as targets (5×10^3/well) and a series of effector cell numbers; LU were calculated at 45% specific ^{51}Cr release (21). When indicated, 1 mM EGTA and 2 mM $MgCl_2$, the Fas-blocking ZB4 or the irrelevant P3x63.Ag8.653 mAb as control (each 1 μg/ml), the Fas-triggering CH11 mAb (0.2 μg/ml), the TNF-α–neutralizing mAb (ascites, 1:500 dilution), His 6-tagged TRAIL (supernatant, 1:400 dilution), and DR4– and DR5–Fc fusion proteins (supernatants, 1:2 dilution) were present throughout the assay. When indicated, effector cells were preincubated (5×10^6 cell/ml, 18 h, 37°C) with rIL-2 (50 U/ml) and rIL-12 (2 ng/ml, specific activity 4.5×10^6 U/mg protein in an IFN-γ–inducing assay; provided by Dr. S. Wolf, Genetics Institute, Andover, MA). J32 cell death was analyzed in a 6-h ^{51}Cr-release assay with the indicated reagents present throughout the assay. rTNF-α (specific activity 10^7 U/mg protein on L929 cells, α subline), was provided by Dr. J.S. Price, Cetus Corp., Emeryville, CA.

RT-PCR Analysis. RT-PCR was performed as described (23) on total RNA extracted from the different cell populations (5×10^4 cells/sample) with RNAzol (Biotecx Laboratories, Houston, TX). FasL and TRAIL primers (produced at the Kimmel Cancer Center Nucleic Acid Facility) used for RT were, respectively, TGGTTGCCTTGGTAGGATTGGGC (5′) and GAGCTTAT-ATAAGCCGAAAAACG (3′) (29), and AACCTCTGAGGAA-ACCAT (5′) and TTAGCCAACTAAAAAGGC (3′). β-actin primers were published previously (23). RT-PCR products were identified on agarose gels after ethidium bromide staining.

Results and Discussion

NK cells induced to differentiate from Lin^- umbilical cord blood cells in cultures with IL-15 mediated lower levels of cytotoxicity against K562 target cells than those mediated by cells from cultures with IL-2 (Fig. 1 *a*), but cells from either culture condition, such as mature adult peripheral or umbilical cord blood NK cells (26, 30), mediated similar levels of cytotoxicity after stimulation with IL-2 and IL-12. In both cases cytotoxicity was restricted to the $CD3^-/CD161^+/CD56^+$ NK cell population, the only one detected in cultures with IL-15 (our unpublished data; Fig. 1, *b* and *c*). An additional immature $CD161^+/CD56^-$ NK cell population generated in cultures with IL-2 neither

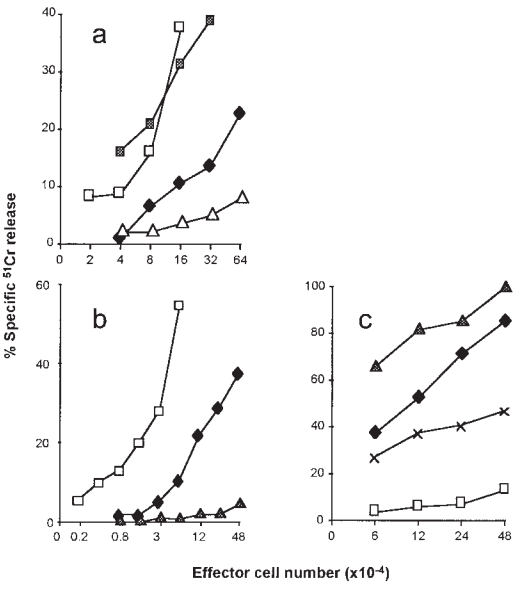

Figure 1. Development of granule exocytosis–dependent cytotoxicity during NK cell differentiation. (*a*) Lin^- umbilical cord blood cells were cultured with the Sl/Sl^4 cell line and IL-2 (◆, ■) or IL-15 (△, □) for 30 d, incubated (18 h, 37°C) without (◆, △) or with IL-2 and IL-12 (■, □), and tested for cytotoxicity on K562 target cells. (*b*) Cytotoxicity of total (◆), $CD56^+$ (□), and $CD56^-$ (▲) cells from cultures with IL-15, K562 target cells. (*c*) Cytotoxicity of $CD56^+$ (◆, ▲) and $CD56^-$ (△, X) NK cells from cultures with IL-2, K562 (◆, □), or Jurkat (▲, X) target cells. Experiment representative of at least three performed with similar results.

produces IFN-γ (23) nor mediates cytotoxicity against K562 cells (Fig. 1 *c*) although, like mature NK cells, cells in this population contain perforin and enzymatically active granzyme B (23) and form conjugates with K562 cells (not shown). The inability of this cell population to kill K562 cells likely reflects a defect in either expression or granule exocytosis–triggering function of one or more NK cell receptors activating cytotoxicity or of other accessory molecules involved in it. Interestingly, however, cells in the same population reproducibly mediated significant levels of cytotoxicity against the Jurkat cell line (Fig. 1 *c*).

Killing of K562 cells by umbilical cord (Fig. 2 *a*) and adult peripheral blood NK cells (31; data not shown) is completely Ca^{2+} dependent, granule exocytosis mediated; that of the Fas^+ Jurkat cell line (25) is only partially so (Fig. 2 *b*, using umbilical cord blood as effector cells; not shown with adult PBLs). In this case, activation of the Ca^{2+}- and granule release–independent apoptotic death pathway(s) depends on engagement of Fas by FasL, expressed or induced at significant levels on the effector cells upon target cell interaction (18, 31). Thus, the effector cells function primarily to present target cells with the appropriate configuration of the ligand(s) (FasL) capable of binding and cross-linking membrane receptor(s) (Fas) that initiate the target cell apoptotic signals. The Ca^{2+}-independent cytotoxicity mediated against Jurkat cells by NK cells from cultures with IL-15 or IL-2 (Fig. 2, *c* and *d*, respectively) was inhibited, as expected, in the presence of Fas-blocking mAb, and no greater inhibition was observed upon further addition of TNF-α–neutralizing mAb. Thus, analogous to the observations with freshly purified mature umbilical cord blood and adult peripheral blood NK cells (31), Ca^{2+}-dependent granule exocytosis and Fas/FasL-dependent cytotoxic mechanisms account for most cytotoxic activity of mature NK cells from Lin^- cells under the culture conditions analyzed.

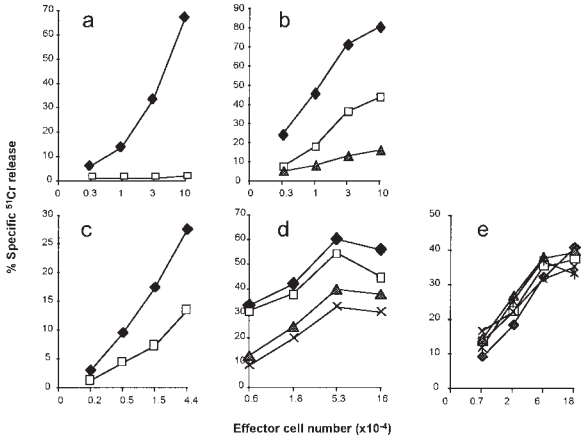

Figure 2. Granule exocytosis–dependent and independent cytotoxicity of umbilical cord blood NK cell subsets. Mature $CD3^-/CD56^+/CD16^+$ NK cells from short-term cultures were tested against K562 (*a*) or Jurkat target cells (*b*): cytotoxicity assay without (◆) or with EGTA (□), or with both EGTA and Fas-neutralizing ZB4 mAb (▲). Cytotoxicity of total cells from cultures of Lin^- cells with IL-15 (*c*) or IL-2 (*d*) was tested against Jurkat cells in the presence of EGTA alone (◆) or both EGTA and: (*c*) Fas-neutralizing mAb (□), (*d*) TNF-α– (□), or Fas-neutralizing mAb (▲), or a mixture of TNF-α– and Fas-neutralizing mAb (X). (*e*) Cytotoxicity of $CD3^-/CD161^+/CD56^-$ cells from cultures with IL-2 against Jurkat cells without (◆) or with added EGTA alone (*), or with both EGTA and: anti–TNF-α (□), Fas-neutralizing mAb (▲), or a mixture of TNF-α– and Fas-neutralizing mAb (X). Each experiment is representative of at least three performed with similar results.

Interestingly, the immature $CD161^+/CD56^-$ NK cells from cultures with IL-2 (Fig. 2 *e*) also lysed Jurkat cells, although less efficiently than their mature $CD56^+$ cell counterparts from the same culture. As expected based on lack of detectable granule exocytosis upon target cell recognition (23), Jurkat cell lysis by these cells, unlike that by mature NK cells, was essentially Ca^{2+} independent (Fig. 2 *e*). Immunofluorescence analysis revealed no detectable FasL, whereas RT-PCR detected constitutive FasL mRNA expression in all of the NK cell populations (not shown), in agreement with findings in mature adult human NK cells (19) and murine NK cell clones (18). However, Fas-blocking mAb did not inhibit killing of Jurkat cells by the immature cells (Fig. 2 *e*), suggesting that these use soluble or membrane-bound molecules distinct from FasL to mediate this effect.

$CD161^+/CD56^-$ immature NK cells express TNF-α mRNA constitutively (23), and most produce TNF-α upon stimulation (our unpublished observations). Two lines of evidence exclude a significant role of TNF-α, either secreted (32) or in membrane-bound form (3), in Jurkat cell death: (*a*) no significant ^{51}Cr release above background was detected from Jurkat cells after a 6-h incubation with up to 10^3 U/ml rTNF-α (not shown); and (*b*) no inhibition of immature NK cell–mediated Jurkat cell killing was detected in the presence of a TNF-α–neutralizing mAb, alone or with added Fas-blocking ZB4 mAb (Fig. 2 *e*). Similar results with cells from cultures with IL-2 (containing both mature and immature NK cells) confirmed that the TNF-α/TNF-R pathway does not participate significantly in NK cell–mediated Jurkat cell lysis under the experimental conditions used, and ligands other than TNF-α on the immature NK cells bind target cell receptors distinct from TNF-R and Fas but sharing their apoptosis-inducing activity.

Unlike K562 cells (13; not shown), Jurkat cells are sensitive both to Fas-mediated (25; not shown) and to soluble TRAIL-mediated cytotoxicity (13). Engagement of the TRAIL receptors DR4 and DR5 (10, 11) by soluble TRAIL results in apoptosis (33, 34). The J32 Jurkat cell clone used, like most clones of this cell line, undergoes apoptotic cell death upon binding either soluble TRAIL or Fas-triggering mAb (Fig. 3). TRAIL-induced ^{51}Cr release was inhibited to background levels by a mixture of soluble TRAIL-Rs (DR4 and DR5), but not by soluble Fas or Fas-blocking mAb. Conversely, Fas-induced ^{51}Cr release using Fas-triggering mAb was inhibited by the same Fas-blocking mAb, but not by the mixture of TRAIL-R–Fc fusion proteins. The lack of inhibition of the anti-Fas mAb–induced cell death by soluble Fas likely reflects inefficient competition of this molecule for the anti-Fas binding site on the mAb.

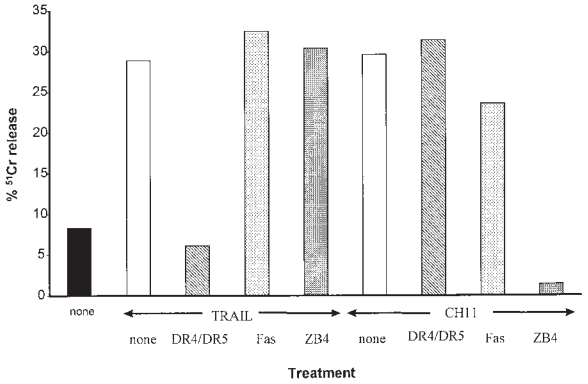

Figure 3. Fas and TRAIL sensitivity of Jurkat T cells. ^{51}Cr-labeled J32 Jurkat cells were incubated for 6 h with the indicated reagents; released radioactivity was measured in the supernatants from triplicate wells. His 6–tagged TRAIL (1:400 dilution), DR4– and DR5–, and Fas–Fc fusion molecules (each 1:2 dilution), Fas-blocking ZB4 and Fas-stimulating CH11 mAb (1 and 0.2 μg/ml, respectively) were present throughout the assay as indicated. The experiment is representative of two performed with similar results.

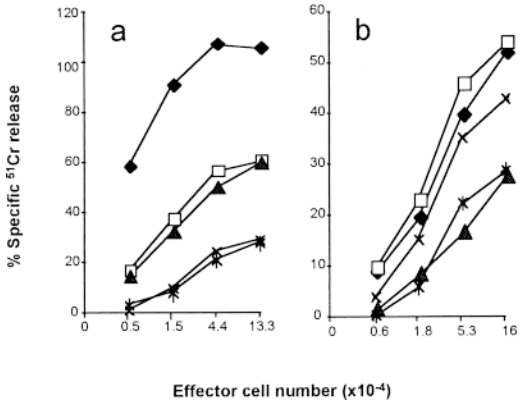

Figure 4. Differential use of TRAIL and FasL by immature and mature NK cells. Cytotoxicity of mature CD161$^+$/CD56$^+$ (*a*) and CD161$^+$/CD56$^-$ NK cells (*b*) purified from 30-d cultures of Lin$^-$ umbilical cord blood cells with IL-2 and the Sl/Sl^4 cell line (reference 23) was measured in a 6-h assay against Jurkat target cells without (◆) or with both EGTA and: control P3x63.Ag8.653 Ig (□), DR4– and DR5–Fc chimeric proteins (▲), anti–Fas-blocking mAb ZB4 (X), or a mixture of DR4/DR5–Fc fusion proteins and ZB4 mAb (*) (concentrations as in the legend to Fig. 3) throughout the assay. Experiment representative of four performed with similar results.

These data establish that functional Fas and TRAIL-R1 and 2 (DR4 and/or DR5) are expressed on this Jurkat cell clone with their expected and exclusive specificity.

TRAIL mRNA expression was detectable (by RT-PCR) in the immature NK cells, although TRAIL protein was undetectable in DR4/DR5 binding analysis (data not shown). To determine whether engagement of functional TRAIL-R by TRAIL, possibly expressed or induced to significant levels on these cells upon target cell interaction, elicits Ca^{2+}-independent target cell death, we analyzed the effect of DR4/DR5–Fc fusion proteins on cytotoxicity of mature and immature NK cells against Jurkat cells. Like the Ca^{2+}-independent lysis mediated by mature NK cells from short-term (10-d) cultures of primary PBLs (Fig. 4 *a*), that mediated by CD161$^+$/CD56$^+$ mature NK cells from cultures with IL-15 (not shown) was reproducibly inhibited by the Fas-blocking mAb, but not by the DR4/DR5–Fc fusion proteins. Addition of these to the anti-Fas mAb in the presence of EGTA did not result in inhibition greater than that mediated by the mAb alone. However, at the same concentrations, these reagents significantly inhibited J32 cell lysis by immature NK cells (Fig. 4 *b*; >75%, as quantitated based on LU in the cell populations). This indicates that the molecules transducing apoptotic signals in this case are either or both DR4 and DR5, thus implicating TRAIL in the cytotoxicity mediated by this cell subset. No additional inhibition was observed under the same conditions with ZB4 mAb added, excluding a major role, if any, for the Fas/FasL-dependent cytotoxic pathway by immature NK cells. The possibility that FasL on the immature NK cells is induced, and used, at later times after interaction with the appropriate target cells is unlikely, given the relatively fast kinetics of FasL expression both in T and NK cells. However, formal proof awaits results of experiments using additional target cells in longer assays. The possibility that other (yet to be identified) molecule(s) with ligand binding specificity identical to TRAIL and in addition to it are involved in Jurkat cell killing by the immature NK cells is unlikely, based on a recent report showing anti-TRAIL Ab–mediated inhibition of Jurkat and melanoma cell lysis by cytotoxic human CD4$^+$ T cell clones (35).

The data reported here provide the first direct evidence for the use of functional TRAIL, or a molecule with identical DR4/DR5 binding specificity, by primary human NK cells, and point to this as a marker of differentiation of these and possibly other cytotoxic cell types. Our results also indicate a switch in the surface molecules used for target cell recognition and/or lysis during NK cell differentiation. TRAIL–TRAIL-R interactions represent the first major (but possibly not exclusive) mechanism of target cell lysis used by NK cells to mediate their functions as innate effectors of defense, and this ability is acquired by NK cells at a specific, phenotypically identifiable stage of differentiation (exclusively CD161$^+$) that precedes acquisition of the ability to mediate granule exocytosis– or FasL-dependent cytotoxicity (23; and this paper) and to produce IFN-γ (23).

Interesting and testable questions are raised by these data related to whether (*a*) the same molecules are used by NK cells at different stages of differentiation to kill other target cells; (*b*) TRAIL and FasL are also markers of differentiation of other cytotoxic cells, or of specific functional capabilities acquired by activated mature cytotoxic effector cells or lymphocyte subsets, phenotypically similar to the immature CD161$^+$/CD56$^-$ NK cells and present in peripheral blood (23). This possibility is suggested by preliminary data (RT-PCR, not shown) indicating TRAIL mRNA expression in mature NK cells; and (*c*) like granule exocytosis (for a review, see reference 36), both FasL- and TRAIL-dependent cytotoxicity are negatively regulated by MHC-bind-

ing receptors inhibiting cytotoxicity. TRAIL-Rs, expressed on numerous cell types, have been shown to be functional on transformed, but not normal cell-derived cell lines (37). Definition of the expression and functionality of these receptors on primary tumor cells and hematopoietic cells at distinct stages of differentiation or activation/proliferation is essential to establish whether the use of TRAIL rather than FasL by immature NK cells, or even (a) phenotypically identical subset among mature NK cells (23), may endow these cells with (relative) specificity in the control of tumor and possibly immature hematopoietic cell growth.

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References

1. Montel, A.H., M.R. Bochan, W.S. Goebel, and Z. Bramhi. 1995. Fas-mediated cytotoxicity remains intact in perforin and granzyme B antisense transfectants of a human NK-like cell line. *Cell. Immunol.* 165:312–317.
2. Lee, P.K., J. Spielman, D.Y. Zhao, K.J. Olsen, and E.R. Podack. 1996. Perforin, Fas ligand, and tumor necrosis factor are the major cytotoxic molecules used by lymphokine-activated killer cells. *J. Immunol.* 157:1919–1925.
3. Perez, C., I. Albert, K. DeFay, N. Zacharides, L. Gooding, and M. Kriegler. 1990. A nonsecretable cell surface mutant of tumor necrosis factor (TNF) kills by cell-to-cell contact. *Cell.* 63:251–258.
4. Oshimi, Y., S. Oda, Y. Honda, S. Nagata, and S. Miyazaki. 1996. Involvement of Fas ligand and Fas-mediated pathway in the cytotoxicity of human natural killer cells. *J. Immunol.* 157:2909–2915.
5. Lowin, B., M.C. Peitsch, and J. Tschopp. 1995. Perforin and granzymes: crucial effector molecules in cytolytic T lymphocyte and natural killer cell-mediated cytotoxicity. *Curr. Top. Microbiol. Immunol.* 198:1–24.
6. Berke, G. 1994. The binding and lysis of target cells by cytotoxic lymphocytes: molecular and cellular aspects. *Annu. Rev. Immunol.* 12:735–773.
7. Nagata, S., and P. Golstein. 1995. The Fas death factor. *Science.* 267:1449–1456.
8. Itoh, N., and S. Nagata. 1993. A novel protein domain required for apoptosis. Mutational analysis of human Fas antigen. *J. Biol. Chem.* 268:10932–10937.
9. Cohen, G.M. 1997. Caspases: the executioners of apoptosis. *Biochem. J.* 326:1–16.
10. MacFarlane, M., A. Manzoor, S.M. Srinivasula, T. Fernandes-Alnemri, G.M. Cohen, and E.S. Alnemri. 1997. Identification and molecular cloning of two novel receptors for the cytotoxic ligand TRAIL. *J. Biol. Chem.* 41:25417–25420.
11. Chaudhary, P.M., M. Eby, A. Jasmin, A. Bookwalter, J. Murray, and L. Hood. 1997. Death receptor 5, a new member of the TNFR family, and DR4 induce FADD-dependent apoptosis and activate NF-κB. *Immunity.* 7:821–830.
12. Walczak, H., M.A. Degli-Esposti, R.S. Johnson, P.J. Smolak, J.Y. Waugh, N. Boiani, M.S. Timour, M.J. Gerhart, K.A. Schooley, C.A. Smith, et al. 1997. TRAIL-R2: a novel apoptosis-mediating receptor for TRAIL. *EMBO (Eur. Mol. Biol. Organ.) J.* 16:5386–5397.
13. Wiley, S.R., K. Schooley, P.J. Smolak, W.S. Din, C.P. Huang, J.K. Nicholl, G.R. Sutherland, T.D. Smith, C. Rauch, and C.A. Smith. 1995. Identification and characterization of a new member of the TNF family that induces apoptosis. *Immunity.* 3:673–682.
14. Degli-Esposti, M.A., P.J. Smolak, J. Walczak, J. Waugh, C.-P. Huang, R.F. DuBose, R.G. Goodwin, and C.A. Smith. 1997. Cloning and characterization of TRAIL-R3, a novel member of the emerging TRAIL receptor family. *J. Exp. Med.* 186:1165–1170.
15. Vignaux, F., E. Vivier, B. Malissen, V. Depraetere, S. Nagata, and P. Gotstein. 1995. TCR/CD3 coupling to Fas-based cytotoxicity. *J. Exp. Med.* 181:781–786.
16. Stalder, T., S. Hahn, and P. Erb. 1994. Fas antigen is the major target molecule for CD4+ T cell-mediated cytotoxicity. *J. Immunol.* 152:1127–1133.
17. Thomas, W.D., and P. Hersey. 1998. CD4 T cells kill melanoma cells by mechanisms that are independent of Fas (CD95). *Int. J. Cancer.* 75:384–390.
18. Tsutsui, H., K. Nakanishi, K. Matsui, K. Higashino, H. Okamura, Y. Miyazawa, and K. Kaneda. 1996. IFN-γ-inducing

factor up-regulates Fas ligand-mediated cytotoxic activity of murine natural killer cell clones. *J. Immunol.* 157:3967–3973.
19. Mori, S., A. Jewett, K. Murakami-Mori, M. Cavalcanti, and B. Bonavida. 1997. The participation of the Fas-mediated cytotoxic pathway by natural killer cells is tumor-cell-dependent. *Cancer Immunol. Immunother.* 44:282–290.
20. Eischen, C.M., J.D. Schilling, D.H. Lynch, P.H. Krammer, and P.J. Leibson. 1996. Fc receptor-induced expression of Fas ligand on activated NK cells facilitates cell-mediated cytotoxicity and subsequent autocrine NK cell apoptosis. *J. Immunol.* 156:2693–2699.
21. Perussia, B., S. Starr, S. Abraham, V. Fanning, and G. Trinchieri. 1983. Human natural killer cells analyzed by B73.1, a monoclonal antibody blocking Fc receptor functions. I. Characterization of the lymphocyte subset reactive with B73.1. *J. Immunol.* 130:2133–2141.
22. Perussia, B., C. Ramoni, I. Anegon, M.C. Cuturi, J. Faust, and G. Trinchieri. 1987. Preferential proliferation of natural killer cells among peripheral blood mononuclear cells co-cultured with B lymphoblastoid cell lines. *Nat. Immun. Cell Growth Regul.* 6:171–188.
23. Bennett, I.M., O. Zatsepina, L. Zamai, L. Azzoni, T. Mikheeva, and B. Perussia. 1996. Definition of an NKR-P1A$^+$/CD56$^-$/CD16$^-$ functionally immature human NK cell subset that differentiates in vitro in the presence of IL-12. *J. Exp. Med.* 184:1845–1856.
24. Cuturi, M.C., M. Murphy, M.P. Costa-Giomi, R. Weinmann, B. Perussia, and G. Trinchieri. 1987. Independent regulation of tumor necrosis factor and lymphotoxin production by human peripheral blood lymphocytes. *J. Exp. Med.* 165:1581–1594.
25. Alderson, M., T.W. Tough, S. Braddy, T. Davis-Smith, E. Roux, K. Schooley, R.E. Miller, and D.H. Lynch. 1994. Regulation of apoptosis and T cell activation by Fas-specific mAb. *Int. Immunol.* 6:1799–1806.
26. Trinchieri, G., M. Matsumoto-Kobayashi, S.C. Clark, J. Seehra, L. London, and B. Perussia. 1984. Response of resting human peripheral blood natural killer cells to interleukin 2. *J. Exp. Med.* 160:1147–1169.
27. Cuturi, M.C., I. Anegon, F. Sherman, R. Loudon, S.C. Clark, B. Perussia, and G. Trinchieri. 1989. Production of hematopoietic colony-stimulating factors by human natural killer cells. *J. Exp. Med.* 169:569–583.
28. Suda, T., T. Takahashi, P. Golstein, and S. Nagata. 1993. Molecular cloning and expression of the Fas ligand, a novel member of the tumor necrosis factor family. *Cell.* 75:1169–1178.
29. Toksoz, D., K. Zsebo, K. Smith, S. Hu, D. Brankow, S.V. Suggs, F. Martin, and D.A. Williams. 1992. Support of human hematopoiesis in long-term bone marrow cultures by murine stromal cells selectively expressing the membrane-bound and secreted forms of the human homolog of the steel gene factor, stem cell factor. *Proc. Natl. Acad. Sci. USA.* 89: 7350–7354.
30. Gaddy, J., G. Risdon, and H.E. Broxmeyer. 1995. Cord blood natural killer cells are functionally and phenotypically immature but readily respond to interleukin-2 and interleukin-12. *J. Interferon Cytokine Res.* 15:527–536.
31. Montel, A.H., M.R. Bochan, J.A. Hobbs, D.H. Lynch, and Z. Brahmi. 1995. Fas involvement in cytotoxicity mediated by human NK cells. *Cell. Immunol.* 166:236–246.
32. Chong, A.S., P. Scuderi, W.J. Grimes, and E.M. Hersh. 1989. Tumor targets stimulate IL-2 activated killer cells to produce interferon-γ and tumor necrosis factor. *J. Immunol.* 142:2133–2139.
33. Marsters, S.A., R.M. Pitti, C.J. Donahue, S. Ruppert, K.D. Bauer, and A. Ashkenazi. 1996. Activation of apoptosis by Apo-2 ligand is independent of FADD but blocked by CrmA. *Curr. Biol.* 6:750–752.
34. Pitti, R.M., S.A. Marsters, S. Ruppert, C.J. Donhaue, A. Moore, and A. Ashkenazi. 1996. Induction of apoptosis by Apo-2 ligand, a new member of the tumor necrosis factor cytokine family. *J. Biol. Chem.* 271:12687–12690.
35. Thomas, W.D., and P. Hersey. 1998. TNF-related apoptosis-inducing ligand (TRAIL) induces apoptosis in Fas ligand-resistant melanoma cells and mediates CD4 T cell killing of target cells. *J. Immunol.* 161:2195–2200.
36. Renard, V., A. Cambiaggi, F. Vely, M. Blery, L. Olcese, S. Olivero, M. Bouchet, and E. Vivier. 1997. Transduction of cytotoxic signals in natural killer cells: a general model of fine tuning between activatory and inhibitory pathways in lymphocytes. *Immunol. Rev.* 155:205–221.
37. Gura, T. 1997. How TRAIL kills cancer cells, but not normal cells. *Science.* 277:768.