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Here we investigate the activation of and a possible role for the hematopoietic Rac1 exchange factor, Vav, in the signaling mechanisms leading to NK cell-mediated cytotoxicity. Our data show that direct contact of NK cells with a panel of sensitive tumor targets leads to a rapid and transient tyrosine phosphorylation of Vav and to its association with tyrosine-phosphorylated Syk. Vav tyrosine phosphorylation is also observed following the activation of NK cells through the low-affinity Fc receptor for IgG (FcyRIII). In addition, we demonstrate that both direct and Ab-mediated NK cell binding to target cells result in the activation of nucleotide exchange on endogenous Rac1. Furthermore, Vav antisense oligodeoxynucleotide treatment leads to an impairment of NK cytotoxicity, with FcyRIII-mediated killing being more sensitive to the abrogation of Vav expression. These results provide new insight into the signaling pathways leading to cytotoxic effector function and define a role for Vav in the activation of NK cell-mediated killing. The Journal of Immunology, 1999, 162: 3148–3152.

The vav protooncogene product encodes a protein of 95-kDa, Vav (1), which has been implicated recently in the reorganization of the actin cytoskeleton following TCR engagement (2, 3). Indeed, vav-/- mice show a defect in TCR-induced actin polymerization and subsequent cap formation. Vav-mediated cytoskeleton regulation mainly depends upon the ability of Vav to catalyze the exchange of bound GDP for GTP on the Rho family GTPase Rac1 (4). Vav exchange activity on Rac1 requires Vav tyrosine phosphorylation, which is mediated by protein tyrosine kinases (PTKs) belonging to the Src and Syk/Zap-70 families (5, 6). Vav undergoes tyrosine phosphorylation after stimulation through a variety of immune receptors, including Ag, Ig, costimulatory, and cytokine receptors (7).

CTL- or NK cell-mediated cytotoxicity largely involves cytoskeleton redistribution, which consists of a polarized concentration of cytoskeleton mediators and repositioning of the microtubule-organizing center (8, 9). However, the signaling pathways leading to cytoskeleton reorganization have not been elucidated yet.

Recently, Syk activation has been shown to be a crucial step in the NK cytoytic response (10). Intriguingly, Syk/Vav association and subsequent Vav tyrosine phosphorylation have been described in B cells in response to B cell receptor engagement (6). Altogether, these data led us to hypothesize a functional involvement of Vav in the regulation of NK cell-mediated cytotoxicity.

We report here that stimulation of human NK cells with a panel of sensitive targets or through the low-affinity Fc receptor for IgG (FcyRIII) (CD16) induces tyrosine phosphorylation of Vav that is associated with Rac1 activation. The down-regulation of Vav protein affects both natural and FcγRIII-mediated cytotoxicity, suggesting that Vav-regulated signals play a central role in the activation events leading to the NK cell-mediated cytotoxic response.

Materials and Methods

Cell lines

The following human cell lines were used as targets: K562 (erythroleukemia), LOVO (colon carcinoma), and Mel 116 (breast cancer) (kindly provided by Dr. M. Maio, Istituto Nazionale Tumori, Centro Europeo, Aviano, Italy). The murine FcγRIIIR P815 (mastocytoma) was used for reverse Ab-dependent cellular cytotoxicity (ADCC).

Preparation of human NK cells

NK cell populations were obtained by a 10-day coculture of nylon nonadherent PBMCs (4 × 10⁵ cells/ml) with irradiated (3000 rad) EBV–RPMI 8866 lymphoblastoid cell line (10⁵ cells/ml) as described previously (11). On day 10, the cell population was routinely 80–95% CD34⁺, CD16⁺, CD3⁻, as assessed by immunofluorescence and cytofluorometric analysis. The experiments were performed on NK cell populations that were >90% pure.

Antibodies

Anti-human Vav and anti-phosphotyrosine (pTyr) (4G10) mAbs were obtained from Upstate Biotechnology (Lake Placid, NY); anti-human Syk (4D10) mAb and affinity-purified rabbit anti-Rac1 Ab were supplied by Santa Cruz Biotechnology (Santa Cruz, CA), B73.1 (anti-FcγRII) and W6/32 (anti-MHC class I) mAbs were kindly provided by Dr. G. Trinchieri (Wistar Institute, Philadelphia, PA).

Cytotoxicity assay

The 51Cr release assay was performed as described previously (12). Maximal and spontaneous release were determined by incubating 51Cr-labeled target cells with 1 M HCl or medium alone, respectively. The percentage of specific lysis was determined as follows: [%mean cpm experimental release – mean cpm spontaneous release]/[mean cpm maximal release – mean cpm spontaneous release] × 100.

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3 Abbreviations used in this paper: PTK, protein tyrosine kinase; ADCC, Ab-dependent cytotoxicity; FcγRIII, low-affinity Fc receptor for IgG; pTyr, phosphotyrosine; ODN, oligodeoxynucleotide; AS, antisense; S, complementary sense; PI-5K, phosphatidylinositol-4 phosphate 5-kinase.
[\textsuperscript{\textit{32P}}]orthophosphate labeling, cell stimulation, and immunoblotting

NK cells were starved for 4 h at 37°C in phosphate-free RPMI 1640 medium (Life Technologies, Gaithersburg, MD) and subsequently labeled (25 × 10\textsuperscript{5} cells/ml) for 4 h with [\textsuperscript{\textit{32P}}]orthophosphate (250 \mu Ci/ml) (Amersham International, Little Chalfont, U.K.) in phosphate-free RPMI 1640 medium. Cells were washed and resuspended (7 × 10\textsuperscript{5} cells/sample) in prewarmed RPMI 1640 medium. Target cells were added (E/T ratio of 2:1), pelleted at 5000 rpm for 5 s, and incubated at 37°C. For anti-Fc\textgamma\textgammaRIII stimulation, cultured NK cells were incubated at 4°C for 15 min with a saturating dose of B73.1 mAb, washed, and used as described above. After stimulation, cells were lysed with lysis buffer (1% v/v Triton X-100, 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 5 mM EGTA, 1.5 mM MgCl\textsubscript{2}, 10% glycerol, plus protease and phosphatase inhibitors) and immunoprecipitated with anti-Vav or W6/32 control mAb bound to protein G-Sepharose beads; the precipitated proteins were separated by SDS-PAGE. Lysates from an equivalent number of target and effector cells were combined before immunoprecipitation and used as time 0 of the kinetics. In the experiments using labeled NK cells, autoradiography was performed on dried gels. The rehydrated gels were then transferred to nitrocellulose membranes and probed with anti-Vav mAb. For experiments with unlabeled NK cells, proteins separated by SDS-PAGE were transferred to nitrocellulose membranes, probed with anti-pTyr mAb, and, after stripping, probed with the anti-Syk mAb. Immunoactivity was detected using an enhanced chemiluminescence kit (Amersham International).

In vivo nucleotide labeling of Rac1

NK cells were labeled for 3 h with [\textsuperscript{\textit{32P}}]orthophosphate and stimulated with target cells as described above. Stimulation was stopped by lysis in 50 mM HEPES (pH 7.4), 1% v/v Triton X-100, 100 mM NaCl, and 5 mM MgCl\textsubscript{2}, plus protease and phosphatase inhibitors. Postnuclear lysates were adjusted to a final concentration of 500 mM NaCl, 0.5% sodium deoxycholate, and 0.05% SDS and immunoprecipitated with anti-Rac1 Ab precoated on protein A-Sepharose beads for 45 min at 4°C. Immunocomplexes were washed and eluted in 5 mM DTT, 5 mM EDTA, O.2% SDS, 0.5 mM GTP, and 0.5 mM GDP at 68°C for 20 min. The nucleotides were separated on polyethyleneimine-cellulose F plates (Merck, Darmstadt, Germany) with 0.75 M KH\textsubscript{2}PO\textsubscript{4} (pH 3.5). TLC plates were analyzed by autoradiography and quantitated by direct scanning for \( \beta \) radiation using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

Oligodeoxynucleotides (ODNs) and cell treatment

Antisense (AS) and complementary sense (S) ODNs that targeted the translation initiation region of the protooncogene vav were synthesized in fully phosphorothioated form. The sequence was as follows: AS-vav (5'-CATT GGC GCC CAC AGCTT CAT-3'), S-vav (5'-ATG GAC CGT GGG CCC AATG-3'). Cultured NK cells (2.5 × 10\textsuperscript{5} cells/ml) were exposed to ODNs (100 \mu g/ml) in heat-inactivated (65°C for 20 min) conditioned medium, derived from primary culture (see preparation of human NK cells), for 56 h. To analyze Vav expression, equivalent amounts of cell lysates from ODN-treated or untreated cells were separated by SDS-PAGE, transferred to a nitrocellulose membrane, and probed with anti-Vav mAb. The same membrane was probed with anti-Syk mAb.

Results

Natural and Fc\gammaRIII-mediated killing induce tyrosine phosphorylation of Vav in human NK cells

As PTK activity has been demonstrated to provide early and requisite signals for the development of NK cell-mediated cytotoxic functions (13), we investigated whether Vav could be one of the substrates of PTK(s) triggered by the interaction of NK cells with the cell line K562, the prototype NK-sensitive target.

To analyze NK cell- but not target cell-derived Vav, cultured NK cells were labeled with [\textsuperscript{\textit{32P}}]orthophosphate. In target-stimulated NK cells, Vav immunoprecipitates show increased [\textsuperscript{\textit{32P}}]incorporation in a 95-kDa band corresponding to Vav, as revealed by anti-Vav immunoblot (Fig. 1A). The increase in Vav phosphorylation peaks at 5 min and almost declines to baseline levels by 20 min.

We subsequently analyzed whether the triggering of Fc\gammaRIII-mediated cytotoxicity would also result in the stimulation of Vav phosphorylation. [\textsuperscript{\textit{32P}}]-labeled NK cells were challenged with NK-resistant Fc\gammaRIIIB73.1 mAb in the presence of anti-Fc\gammaRIII B73.1 mAb (reverse ADCC). A rapid phosphorylation of Vav was observed when NK cells were allowed to contact Ab-coated P815 cells; however, unlike natural killing, Fc\gammaRIII-induced Vav phosphorylation was more persistent (still present at 20 min of stimulation). Low levels of Vav phosphorylation were also induced by direct contact with P815 cells, which are barely lysed by cultured NK cells (Fig. 1B). No bands corresponding to Vav were observed in samples immunoprecipitated with isotype-matched control mAb (data not shown).

Recent reports have shown that the nucleotide exchange activity of Vav is regulated by PTK-dependent tyrosine phosphorylation (4). Thus, we further determined whether NK cell Vav was undergoing phosphorylation on tyrosine residues upon target cell binding. For this purpose, NK cells were stimulated with LOVO or Mel 116 Vav-negative tumor cell lines, and Vav was subsequently immunoprecipitated and immunoblotted with anti-pTyr mAb. Upon target stimulation, NK cells show a marked tyrosine phosphorylation of Vav (Fig. 2, upper panels). Interestingly, a 70-kDa tyrosine-phosphorylated protein was consistently coprecipitated with Vav from target-stimulated but not unstimulated NK cell lysates (Fig. 2, upper panels). On the basis of the described Vav/Syk

![FIGURE 1.](https://example.com/figure1.png)
association (6) and the involvement of Syk in the NK cytotoxicity signaling pathway (10), the same blot was reprobed with anti-Syk mAb after stripping of the bound Abs. Fig. 2 (middle panels) shows that Syk was present in anti-Vav immunoprecipitates from target-stimulated NK cells. The anti-Vav blot (Fig. 2, lower panels) shows the absence of Vav in the LOVO and Mel 116 cell lines and an equivalent amount of Vav immunoprecipitated from unstimulated and target-stimulated NK cells.

**Activation of the small GTP-binding protein Rac1 upon NK/target cell interaction**

Vav tyrosine phosphorylation results in a dramatic enhancement of its guanine nucleotide exchange activity toward Rac1 (4). Therefore, we analyzed whether Rac1 was activated upon NK/target cell contact. The high intrinsic GTPase activity of Rac1 has prevented the detection of its GTP-bound form in living cells. Thus for these experiments, we took advantage of a recently described technique that evaluates Rac1 exchange activity by determining the levels of 32P-labeled GDP bound to Rac1 after a brief exposure to [32P]orthophosphate-containing medium (4, 14). Stimulating NK cells by direct contact with K562 target cells or through FcγRIII consistently increased the levels of radioactive GDP bound to Rac1 (three- to eightfold). Under these experimental conditions, low levels of labeled nucleotides were observed in unstimulated NK cells (Fig. 3).

Vav is functionally involved in NK cell-mediated cytotoxic activity

The above data indicate that Vav undergoes tyrosine phosphorylation upon NK cell stimulation by sensitive targets or through FcγRIII; the activation of Rac1 is also observed under the same conditions.

To provide direct evidence of the functional requirement for Vav in the generation of NK cytotoxicity, we attempted to perturb Vav expression using AS-ODNs. NK cells were incubated with AS-ODNs, S-ODNs, or control medium for 56 h, and Vav expression was analyzed by immunoblotting. The viability of cells cultured in the presence of AS-ODNs, S-ODNs, or control medium was >90% as assessed by trypan blue exclusion (data not shown). Fig. 4g shows that Vav was almost undetectable in cells exposed to AS-ODNs compared with cells exposed to S-ODNs or to untreated

**FIGURE 2.** Vav tyrosine phosphorylation and Vav/Syk association upon stimulation of NK cells with target cells. For each sample, 5 × 10^6 cultured NK cells were mixed with 2.5 × 10^7 of the indicated target cells, pelleted, and incubated at 37°C for 10 min. Vav or control mAb (W6/32, Ctr) immunoprecipitates were resolved by SDS-PAGE, transferred to a nitrocellulose membrane, and probed with anti-pTyr mAb (upper panels). After stripping of bound Abs, the same blot was reprobed with anti-Syk (middle panels) or anti-Vav mAbs (lower panels). Sizes are indicated in kilodaltons. Data are representative of three independent experiments.

**FIGURE 3.** Rac1 activation after stimulation of NK cells with target cells. TLC of the nucleotides eluted from Rac1 immunoprecipitates of 32P-labeled NK cells is shown. A total of 5 × 10^7 32P-labeled cultured NK cells were mixed with the indicated target cells (K562 or P815) (2.5 × 10^7), pelleted, and incubated at 37°C for the indicated times before lysis and immunoprecipitation of endogenous Rac1. For anti-FcγRIII stimulation, 32P-labeled cultured NK cells were pretreated with B73.1 mAb, washed, and mixed with P815 target cells. Data derived from quantitation of the same experiment by direct scanning for β radiation are shown. Data are representative of three independent experiments.
controls. The specificity of the AS-ODN effect is shown by blotting the same membrane with anti-Syk mAb (Fig. 4b). Vav AS-ODN treatment markedly reduced FcγRIII-mediated cytotoxic activity (Fig. 4, a, c, and e), whereas natural killing was less sensitive to the effect of Vav down-regulation (Fig. 4, b, d, and f). S-ODN-treated cells exhibit a level of cytotoxicity that is comparable with that of untreated NK cells.

Discussion

NK cell-mediated cytotoxicity can be initiated by different receptor systems. ADCC is triggered by the engagement of FcγRIII by the Fc portion of IgG bound to cell-associated Ags. “Natural cytotoxicity” refers to the capacity of NK cells to directly interact with tumor or virus infected cells by means of incompletely defined receptors (15). Thus far, the signaling events leading to NK cytotoxic functions have been only partially characterized.

In this report we have addressed the role of the specific Rac1 activator, Vav, in the generation of NK cell cytotoxic activity. Our findings show that the activation of NK cells through FcγRIII or by interaction with sensitive target cells results in the rapid stimulation of Vav tyrosine phosphorylation. The Vav phosphorylation stimulated by FcγRIII triggering exhibited a more sustained kinetic (still persisting at 20 min) with respect to that observed upon direct NK/target cell contact. This difference can be explained by the ability of the D3 phosphoinositide products of phosphatidylinositol-3 kinase to regulate Vav phosphorylation (16). In this regard, recent evidence indicates a requirement for phosphatidylinositol-3 kinase in FcγRIII-mediated NK cytotoxicity but not in natural killing (17).

Vav/Syk association leading to Vav tyrosine phosphorylation has been described in B lymphocytes upon Ag receptor stimulation (6). A recent study has identified Syk as a tyrosine kinase that is essential for NK cell cytotoxic activity (10). Our results show that NK/target cell interaction induces the formation of Vav/Syk complexes, suggesting that Syk could be the PTK responsible for Vav phosphorylation and control the recruitment of activated Vav in the area of NK/target cell contact. However, we cannot rule out the possibility that PTKs other than Syk may be involved in Vav tyrosine phosphorylation in response to FcγRIII or target stimulation.

Our study also provides evidence that Vav is required for NK cell-mediated cytotoxic function. Vav AS-ODN-treated NK cells exhibited a marked decrease in FcγRIII-mediated killing, whereas natural killing was affected to a much lesser extent. This observation could be explained by discrete signaling pathways regulating these different modalities of cytotoxicity (15); in this context, Rac1 exchange factors other than Vav (Dbl, Ost, and Tiam1) may be involved (18).

The dynamic organization of the actin cytoskeleton is an essential requirement for cytotoxic activity (8, 9); recent reports demonstrate that Vav functions by regulating the actin cytoskeleton (2, 3). Vav-mediated regulation of the cytoskeleton may be achieved via its guanine nucleotide exchange activity on the small G protein Rac1 (4). Rac1-mediated control of macrophage-dependent phagocytosis, actin polymerization in platelets, and mast cell degranulation has been demonstrated recently (19–22). We show here that both FcγRIII-mediated and direct NK/target cell contacts induce GDP/GTP exchange activity on endogenous Rac1.

In agreement with our data, a recent report describes the induction of Vav phosphorylation upon NK/target cell contact. Moreover, it provides genetic evidence that the Vav/Rac1 pathway regulates effector/target cell binding and cytotoxic granule polarization during cellular cytotoxicity (23). The downstream effectors of Rac1 that are involved in the Vav-mediated control of cytotoxic activity still need to be defined. Rho family GTPases may control actin polymerization by binding and activating the enzyme phosphatidylinositol-4-phosphate 5-kinase (PI-5K), thus leading to phosphatidylinositol 4,5-bisphosphate generation (21, 24). Interestingly, Vav-mediated PI-5K activation upon CD19 coreceptor engagement on B lymphocytes (25) has been described recently. Moreover, the action of phospholipase C on phosphatidylinositol 4,5-bisphosphate generates phosphatidylinositol 3,4,5 trisphosphate and diacylglycerol, which lead to protein kinase C activation and intracellular calcium mobilization, two signaling intermediates that play a central role in the development of NK cytotoxicity (15). The involvement of PI-5K in the Vav-mediated regulation of cytotoxic activity is under investigation.

Our results provide insight into the molecular events that regulate cytotoxic function and identify a critical role for Vav in NK-mediated killing.

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