Functional Dichotomy in Natural Killer Cell Signaling: Vav1-Dependent and -Independent Mechanisms

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pholipase C (PLC), participate to sustain the rise in intracellular calcium, a necessary second messenger during NK cell effector functions (25). In T cells, Vav1 is a critical transducer of TCR signals to the calcium pathway, and in its absence, T cells fail to initiate IL-2 gene transcription and proliferate (26). Vav1 has the ability to bind and be regulated by the lipid substrates and products of PI3K (27). Vav1 has also been proposed to enhance the production of substrates for PLC 2 through activation of phosphatidylinositol 4-phosphate 5 kinase (28). In line with this, the PI3K-specific inhibitor wortmannin abolishes antibody-dependent cell cytotoxicity (ADCC; reference 29), and mice deficient for PLC 1 have decreased natural cytotoxicity (30).

Vav1 is phosphorylated upon contact with tumor targets and upon cross-linking of the sole FcR (Fc RII/III, CD16) expressed by NK cells (31, 32) and therefore may be required for NK cell functions. Virally infected cells produce IFN- / , which are potent activators of NK cells and have been shown to induce phosphorylation of Vav1 in several hematopoietic lineages, including lymphocytes (33).

Binding of NK cells to tumor cells and stimulation of NK cells via CD16 results in activation of the mitogen-acti-//

RMA-S cells. MHC class I Con A blasts (wt) or MHC class I RMA cells were both resistant to lysis (5%), even at the highest E/T ratios. Radioactivity released into the cell-free supernatant was measured, and the percent specific lysis was calculated as following: 100 (experimental release spontaneous release)/ (maximum release spontaneous release).

In Vitro Cytokine Production. Sorted NK cells (2 10^4 cells/ 200 l) were cultured in flat-bottomed microtiter plates in human IL-2 (1,000 U/ml) and stimulated with murine IL-12 (2 ng/ml; PeproTech) or mAbs specific for NK1.1 (clone PK136, 20 g/ml), 2B4 (5 g/ml), CD16 (75 g/ml), or control anti–Gr-1 mAb (10 g/ml). Wells were precoated with mAbs (50 l/well)

tive control, cells were stimulated with 50 ng/ml PMA for 15 min at 37 C. Cells were lysed in 1% NP-40–containing buffer as described (44), and soluble proteins were separated by SDS-PAGE. ERK activation was assessed by immunoblotting with the anti–phospho-ERK mAb E4 (Santa Cruz Biotechnology). The amount of ERK proteins was assessed by reprobing the same membrane with a polyclonal anti-ERK Ab (Santa Cruz Biotechnology). Autoradiographies were quantified by densitometry.

Granule Exocytosis. IL-2–activated NK cells were mixed with YAC-1 cells at a 2:1 ratio. After 4 h, the specific release of esterase was measured in 25 l of cell-free supernatants after addition of 175 l of PBS containing 0.22 mM of DNP (Sigma-Aldrich) and 0.2 mM of N- -benzyloxycarbonyl-

fore, we infected Vav1 / mice and wt mice intravenously with 10^4 L.m., and bacterial burden was measured 48 h later in the liver and spleen (Fig. 2 C). Controls included Rag2/ c / and Rag2 / mice. NK cell–deficient animals displayed an average of ~10-fold more colonies in liver and spleen (data not shown) as compared with wt mice (P 0.002; Fig. 2 C) or Rag2 / mice (data not shown). In contrast to the differences found between wt and Vav1 / mice in tumor clearance, no significant difference was found between wt and Vav1 / mice in terms of bacterial burden. Thus, Vav1 / mice can control the early phases of L.m. infection. As IFN- appears an essential cytokine for early control of L.m. infection, we measured the IFN- levels in the sera of infected mice. Fig. 2 D shows that IFN- production by Vav1 / infected mice was similar to that of wt infected mice. Taken together, these results suggest a functional dichotomy for NK cell functions: normal tumoricidal activity is Vav1 dependent, while IFN- production is Vav1 independent.

Vav1 Regulates Natural Cytotoxicity, ADCC, and Lysis Initiated by Distinct NK Cell Receptors

l.976 Vav1

Figure 4.

B). Therefore, although Vav1 may be required for normal conjugate formation under these conditions, the extent of the lytic defect (up to 80% reduction), strongly suggests that postbinding mechanisms account for defective killer activity of Vav1 / NK cells.

Vav1 Is Required to Initiate Calcium Flux in T Cells but Not in NK Cells. Vav1 is required for normal calcium flux in response to Ag–receptor signaling in T cells (26), and by analogy, reduced cytolysis by Vav1 / NK cells could result from a similar defect in proximal signaling. To test this, we compared the rise in intracellular calcium in NK cells upon cross-linking membrane receptors. Normal calcium flux was induced after stimulation of the NK1.1 and CD16 receptors (Fig. 6) as well as the 2B4 and Ly49D receptors (data not shown) in both wt and Vav1 / NK cells. In contrast, and as expected from previous reports (26), CD3 cross-linking did not induce calcium flux in Vav1 / T cells (Fig. 6). Thus, Vav1 is not essential to transduce signals to the calcium pathway in NK cells and acts downstream of the rise in intracellular calcium to control the NK cell cytotoxicity machinery.

Vav1 Controls ERK Activation in NK and T Cells. Vav1 appears essential in transducing TCR signals to the ERK pathway in T cells (26), although studies using T cells from two other Vav1 mutant mice did not support an essential role for Vav1 in ERK activation (15, 16). ERKs have been implicated in the control of both cytotoxicity and IFN- production by NK cells (34–36). We therefore analyzed ERK1/2 phosphorylation in NK cells after stimulation of NK1.1 and compared it to CD3-initiated ERK1/2 phosphorylation in T cells. We found a reduced activation of ERK1 (eightfold) and ERK2 (2.5-fold) in Vav1 / T cells (Fig. 7 A), confirming the essential role of Vav1 in TCR-mediated ERK activation (26). NK1.1-mediated ERK1 phosphorylation was 4.4-fold reduced in Vav1 / NK cells, while no significant reduction in activation of ERK2

Vav1 and Lyst have never been documented, but it is likely that they both regulate components of the cytoskeleton (actin filaments and microtubules, respectively) required for successful exocytosis. However, NK cells of Beige mice have a complete defect in cellular cytotoxity, whereas NK cells of Vav1$^{-/-}$ mice have reduced capacity to kill tumor targets. How can we explain the quantitative defect in the lytic activity of Vav1$^{-/-}$ NK cells?

Cell-mediated cytotoxicity can be induced by calcium-dependent, perforin-mediated mechanisms and calcium-independent, FasL-mediated pathways (52). The residual cytotoxicity of Vav1$^{-/-}$ NK cells could be accounted for FasL-dependent mechanisms, which would not depend on exocytosis. In fact, we found that target cell lysis was totally abrogated in calcium-free medium in both wt and Vav1$^{-/-}$ NK cells (data not shown), ruling out calcium-independent mechanisms as responsible for the residual cytotoxic activity seen in Vav1$^{-/-}$ NK cells.

Activation of small GTPases can be induced by several GEFs, and functional redundancy among members of this family may account for some degree of compensation in the absence of Vav1. Human NK cells have been recently shown to express Vav2, which regulates the development of cell-mediated cytotoxicity (53). We have also detected Vav2 proteins in murine NK cells by intracellular staining (our unpublished observation), suggesting that suboptimal granule exocytosis and residual cytolysis by Vav1$^{-/-}$ NK cells may be accounted for by compensatory mechanisms (activation of Rac1 or Rho?) initiated by Vav2. The analysis of NK cell functions in mice deficient for Vav1 and Vav2 will help answer this question.

Vav1

Inactivation of the Vav1 substrate Rac1 reduces the capacity of human NK cells to form stable 38dsVygtes twithTj 0.0678 Tw T* (garget cell (d3). Tow vera, ur uata nnduicte Rony m

pav21in mhaepbiolog of hTand rK clymphcytoe . Firs, wTTj 0.0864Tw 0 -1.122 TD (fnd rK -Tcell levelopment)and rTcell functions
apaird in cTj /T1_1 1 Tf 0 Tw 13.92 0 Td (Vav1)Tj /T1_2 1 Tf 0.664 0 1.4165 6.664 2372.84 3471664 2m ()Tj /T1_1 1 Tf 6.66

fector functions (57). The NK cell receptors we have used to elicit calcium flux (NK1.1, CD16, 2B4) are known to enhance cytotoxicity, but it would be interesting to measure rise in intracellular calcium upon target cell binding.

Vav1 has been shown to control the ERK pathway in T cells (26), using the same Vav1$^{-/-}$ mice analyzed in this study. However, two independent groups, using mice carrying different Vav1 mutations, did not find an essential

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