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

By Francesco Colucci,* Eleftheria Rosmaraki,⁎ Søren Bregenholt,⁎ Sandrine I. Samson,* Vincenzo Di Bartolo,‡ Martin Turner,§ Lesley Vanes,‖ Victor Tybulewicz,‖ and James P. Di Santo*

From the *Laboratory for Cytokines and Lymphoid Development and the ‡Laboratory for Molecular Immunology, Pasteur Institute, 75015 Paris, France; †The Babraham Institute, Cambridge CB2 4AT, United Kingdom; and †The National Institute for Medical Research, London NW7 1AA, United Kingdom

Abstract
The product of the protooncogene Vav1 participates in multiple signaling pathways and is a critical regulator of antigen–receptor signaling in B and T lymphocytes, but its role during in vivo natural killer (NK) cell differentiation is not known. Here we have studied NK cell development in Vav1−/− mice and found that, in contrast to T and NK-T cells, the absolute numbers of phenotypically mature NK cells were not reduced. Vav1−/− mice produced normal amounts of interferon (IFN)-γ in response to Listeria monocytogenes and controlled early infection but showed reduced tumor clearance in vivo. In vitro stimulation of surface receptors in Vav1−/− NK cells resulted in normal IFN-γ production but reduced tumor cell lysis. Vav1 was found to control activation of extracellular signal-regulated kinases and exocytosis of cytotoxic granules. In contrast, conjugate formation appeared to be only mildly affected, and calcium mobilization was normal in Vav1−/− NK cells. These results highlight fundamental differences between proximal signaling events in T and NK cells and suggest a functional dichotomy for Vav1 in NK cells: a role in cytotoxicity but not for IFN-γ production.

Key words: tumor clearance • Listeria infection • exocytosis • lymphoid development • cytokines

Introduction
The protooncogene Vav1 is one of three members of the Vav family of guanine nucleotide exchange factors (GEFs) for the small GTPases of the Rho family (1). Its product, p95vav1 (or Vav1) contains an array of structural motifs including a Dbl homology domain, a single Src homology (SH)2 and two SH3 domains, a pleckstrin homology domain, and a calponin homology domain, enabling it to interact with different proteins involved in multiple signal transduction pathways (1). Vav1 is expressed preferentially in hematopoietic cells (2) and is rapidly phosphorylated after stimulation of growth factor receptors and Ag receptors on B and T lymphocytes (3–5). Upon tyrosine phosphorylation, Vav1 promotes Rac1 and other Rho family proteins to their active GTP-bound state (6–10), thereby mediating vital functions such as survival, differentiation, motility, and cell division (1, 11). In lymphocytes, activation of the small Rho family proteins by Vav1 promotes Ag–receptor capture, actin polymerization, and Ag-induced proliferation of B and T cells in vitro and effective T cell selection in vivo (5, 12–17). Therefore, it is clear that Vav1 is essential for T cell development and for Ag–receptor-induced responses of B and T lymphocytes. In contrast, signaling requirements for NK cell development are not fully appreciated, although soluble growth factors (including Flt3L/Flk2L, stem cell factor [SCF], IL-7, and IL-15, which appear to be the dominant cytokines) and stromal elements in the bone marrow are essential (18–22). Because Vav1 has been reported to be part of the signaling pathways activated by the receptors for Flt3L/Flk2L and SCF (23, 24), it may be crucial in early NK cell development.

Vav1 may participate in signaling pathways during the activation of NK cells. Proximal events, including phosphorylation of phosphatidylinositol 3 kinase (PI3K) and phos-
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 PLCγ2 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 PLCγ1 have decreased natural cytotoxicity (30).

Vav1 is phosphorylated upon contact with tumor targets and upon cross-linking of the sole FcR, (FcγRII/III, CD16) expressed by NK cells (31, 32) and therefore may be required for NK cell functions. Virally infected cells produce IFN-α/β, 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-activated protein kinase (MAPK) cascades. One of this cascades, the extracellular signal-regulated kinase (ERK) is part of the intracellular signaling leading to IFN-γ production, granule exocytosis, and cytotoxicity by NK cells (34–36). In T cells, Vav1 is required to transduce TCR signals to the ERK pathway (26), thereby mediating gene transcription. By analogy, Vav1 may be involved in the process of IFN-γ production in NK cells. On the other hand, p38 MAPK is also activated upon binding of NK cells to tumor targets and leads to cytokine production and cytotoxicity (37). However, Vav1 does not seem to be an essential regulator either of the p38 MAPKs or of the c-Jun NH2-terminal kinase (JNK, or stress-activated kinase, SAPK) in T cells (15, 16, 26).

Inactivation of the Vav1/Rac1 pathway in human NK cell lines has helped address the role of Vav1 in NK cell functions. As such, Vav1 has been shown to be critical for signaling through CD16 and through the receptor(s) mediating natural cytotoxicity (32, 38). In these studies, it was reported that natural cytotoxicity and ADCC were both decreased in human NK cell lines in the presence of a dominant-negative form of Rac1 (32), or antisense oligonucleotides for Vav1 (38). However, whether deficiency in Vav1 will affect NK cell ontogeny or cytokine production has not yet been investigated. Here, we characterize NK cell differentiation in Vav1−/− mice.

**Materials and Methods**

**Mice.** Vav1−/− mice on the B10.BR background were derived as described (14). Vav1−/−, B10.BR (wt), Rag2−/−/pc−/− (39), and Rag2−/−/mice (40) were housed at the animal facility of the Pasteur Institute.

**FACS® Analysis and Cell Sorting.** Cell suspensions were prepared from spleen, thymus, liver, and bone marrow. They were depleted of red cells and stained for flow cytometry as described (39). mAbs directly conjugated to FITC, PE, Tricolor (TR1), alkaline phosphocyanin, or biotin included mAbs specific for CD3, CD4, CD8, CD11a (LFA-1), CD11b (Mac-1), CD19, CD45R (B220), CD90 (Thy-1.2), CD117 (c-kit), CD122 (IL-2Rβ), CD161 (NK1.1), DX5, 2B4, Ly49A, Ly49C/I, Ly49D, Ly49G2, TCR-α/β, IgM (all from PharMingen), and H-2Kb (Caltag Laboratories). Biotin-conjugated mAbs were revealed by streptavidin–TR1 (Caltag). Cells (106) were first incubated with anti–FcγRII/III (hybridoma 2.4G2) for 20 min on ice to avoid specific binding to low-affinity FcRs. To purify cells, splenocyte suspensions were stained with mAbs specific for NK1.1–PE and CD3–FITC; CD3–NK1.1+ NK cells and CD3–NK1.1− T cells were sorted using a FACSStar® (Becton Dickinson). The purity of the sorted populations was reproducibly >95%. Alternatively, splenocytes were passed over nylon wool columns to deplete myeloid and B cells and thereafter depleted of T cells by panning on anti–TCR-β (clone H57) coated OptiLys® Petri dishes (Falcon). Cells were then expanded in IL-2 (1,000 U/ml) for 7–10 d.

**In Vivo NK Cell Functions.** A standard lung clearance assay was used to test the ability of mice to reject tumors in vivo as described (41). Briefly, RMA-S cells (106) were labeled in 100 μCi of 51Cr for 1 h at 37°C, and after several washings, 105 cells per mouse were injected intravenously. 4 h later, mice were killed, and the residual radioactivity was measured in the explanted lungs.

In a typical experiment, total radioactivity of the injected inoculum (105 cells/200 μl) would be ∼25–50,000 cpm. In a second tumor model, modified from that described in reference 43, RMA-S cells (2.5 × 106) were injected intraperitoneally, and 48 h later the peritoneal exudate cells recovered, counted, and stained with mAbs specific for CD3, NK1.1, and H-2Kb. Residual tumor cells could readily be distinguished by their larger size. CD3 staining, and absence of H-2Kb staining. In the lymphoma metastases model, mice were injected intravenously with H-2–compatible (H-2b) but class I–mRD4 cells (106), and survival was scored for up to 14 d. Those mice that survived were killed at day 14 and their livers fixed in Bouin’s medium for 48 h. Macroscopic metastases were readily detected by visual inspection. For the infection model, 104 Listeria monocytogenes (L.m.) bacteria (strain LO28) were injected intravenously, and 48 h later the serum was collected from each mouse and the IFN-γ content was quantified by ELISA (Genzyme). Bacterial burden in livers and spleens was determined 48 h after injection as described previously (42).

**In Vitro NK Cell Cytotoxicity.** A standard 51Cr-release assay was used to measure NK activity in vitro as described (43). Target cells (H-2b murine YAC-1 thymoma, H-2b murine macrophage tumor line 1C-21, H-2b murine EL-4 thymoma, H-2b murine P815 mastocytoma, Con A–activated B10.BR, and β2m−/−/blasts, and H-2b murine lymphomas RMA and transporter associated with antigen processing [TAP]-deficient RMA-S) were labeled with 100 μCi 51Cr (ICN Pharmaceuticals), and 2.5–5 × 105 targets were incubated with graded numbers of effector cells in 200 μl of medium for 4 h. For natural cytotoxicity, effector cells were NK-enriched splenocytes (by passage on nylon wool columns) from untreated mice or from mice primed 40 h earlier with 0.2 mg of poly(I:C) (Sigma-Aldrich). NK-enriched splenocytes and FACS®-purified NK cells were expanded in IL-2 (1,000 U/ml) for 7–10 d and thereafter used as effectors for (a) ADCC using EL-4 cells coated with anti–Thy-1.1 mAb (lysis of EL4 cells without the Ab never exceeded 10% at the highest E/T ratios); (b) “reverse” ADCC (R-ADCC) using FeRγ+ P815 cells in presence or absence of anti-NK1.1, anti-2B4, or anti-CD16 mAbs; (c) MHC inhibition and recognition of “missing self” using Con A–activated B10.BR and β2m−/−/blasts or RMA and 1414 Natural Killer Cell Functions in Vav1−/− Mice
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 × 10⁵ cells/200 µL) were cultured in flat-bottomed microtiter plates in human IL-2 (1,000 U/ml; PeproTech) or mAbs specific for NK1.1 (clone PK136, 20 µg/ml), 2B4 (5 µg/ml), CD16 (75 µg/ml), or control anti–Gr-1 mAb (10 µg/ml). Wells were precoated with mAbs (50 µL/well) for 4 h before adding NK cells. After 24 h, the cell-free supernatants were collected and the amount of IFN-γ was quantitated by ELISA (Genzyme). In parallel experiments, FACs⁺-purified NK and T cells (2 × 10⁶) were incubated in the presence of brefeldin A (10 µg/ml) for 4 h at 37°C on flat-bottomed microtiter plates precoated with anti-NK1.1 or anti-CD3. Cells were then washed, fixed in 2% paraformaldehyde for 30 min on ice, thereafter stained with anti–IFN-γ-FITC (PharMingen) in 0.5% saponin. As controls, cells were incubated in medium alone or with PMA (50 ng/ml) and ionomycin (1 µg/ml).

Conjugate Formation. IL-2–activated NK cells (2 × 10⁵) were labeled with 1 µg/ml of 2'-7'-biscarboxyethyl-5(6)-carboxyfluorescein (BCECF; Calbiochem) for 30 min at room temperature, washed, and mixed with a 1:1 ratio of YAC-1 targets that had been labeled with 40 µg/ml of hydroethydine (HE; Polysciences, Inc.) for 10 min at room temperature. Cell mix was spun at 1,000 rpm for 5 min and incubated for 1, 2, 5, 10, or 20 min at 37°C. After gentle resuspension, cells were analyzed by flow cytometry. BCECF emits in FL1 and HE emits in FL3, therefore conjugates can be readily detected as double positive (FL1/FL3). Controls were sham conjugates obtained by centrifuging and incubating under the same conditions differentially labeled (HE and BCECF) YAC-1 cells. Background double-positive staining was reproducibly <1%.

Calcium Flux. Purified NK and T cells were labeled with 5 µg/ml of Indo-1 (Molecular Probes) for 45 min at 37°C. Washed cells were resuspended in 10% RPMI and divided into aliquots (5 × 10⁶ cells each) that were incubated for 30 min at 4°C with 20 µg/ml of one of the following mAbs: anti-2B4, anti-NK1.1, anti-CD16, anti-Ly49D, or anti-CD3. Cells were then washed and kept at room temperature until analysis, which was done using an LSR flow cytometer (Becton Dickinson). Immediately before acquisition, cells were resuspended in 500 µl of prewarmed medium. Acquisition was performed for 45 s to set the baseline and then briefly interrupted to add 25 µg/ml of goat F(ab̂)2 anti–mouse IgG to cross-link 2B4 and anti-NK1.1 mAbs or goat F(ab̂)2 anti–rat IgG to cross-link anti-CD16, anti-Ly49D, or anti-CD3 mAbs, and finally interrupted after 4–5 min to stimulate cells with 1 µg/ml of ionomycin. Cross-linking Abs failed to generate calcium flux in Indo-1–labeled cells not preincubated with primary mAbs.

ERK Activation. NK and T sorted cells were first expanded in IL-2. Before analysis, cells were starved in IL-2–free medium for 4 h at 37°C and thereafter incubated on ice for 30 min with 20 µg/ml of anti-NK1.1 or anti-CD3 mAbs, followed by cross-linking with the relevant F(ab̂)2 Ab for 3 min at 37°C. As a posi-

Figure 1. Vav1 is dispensable for NK cell development. (A) Cells from spleen, marrow, and liver were stained with α-CD3FITC and α-NK1.1PE mAbs. Thymocytes were stained with α-CD3FITC, α-NK1.1PE, and α-HSAADEO mAb, and a gate was set to exclude HSA⁺ immature cells. Figures in the dot plots indicate percentages of NK, NK-T, and T cells. Data are from one representative of eight independent experiments. (B) Spleen cells were stained with α-NK1.1PE, α-TCR-αβFITC, and α-CD19PE and one of the indicated FITC-conjugated mAbs. Biotinylated Abs were revealed with streptavidin–TR1, and only NK1.1⁺ TCR-αβ⁺ CD19⁺ NK cells were included in the analysis. Figures show percentage of NK cells staining positive for FITC. No significant differences were detected between wt and Vav1⁻/⁻ NK cells. Results are from one representative of five independent experiments.
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 assayed 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 μl of cell-free supernatants after addition of 175 μl of PBS containing 0.22 mM of DNP (Sigma-Aldrich) and 0.2 mM of N-α-benzoylcarbonyl-L-lysine thioethyl ester (BLT; Sigma-Aldrich) as described (44). Maximal release was obtained by measuring the esterase content in the supernatant of NK cells after three freeze–thaw cycles.

Statistics. Statistical significance of data was evaluated by applying Student’s t test (Microsoft Excel® software).

Results

Vav1 Is Not Required for NK Cell Development but Is Essential for T and NK-T Cell Development. We enumerated lymphocyte subsets in the spleen, bone marrow, liver, and thymus of Vav1−/− mice and littermate controls (Vav1+/−, hereafter referred to as wt). While T and NK-T cells were reduced in Vav1−/− mice, NK cell percentages and absolute numbers in the spleen and bone marrow of Vav1−/− mice were found to be greater than in controls (Fig. 1 A and Table I). Mature NK cells express a constellation of surface markers, including members of the Ly49 family of MHC receptors, whose upregulation starts at 10 d of age and reaches levels comparable to those of mature NK cells at 2–3 wk of age (45). We found that the expression pattern of 13 different markers on splenic and marrow NK cells (including Ly49A, Ly49C/I, Ly49D, Ly49G2, 2B4, DX5, CD2, CD11b, CD16, CD69, CD90, CD117, and CD122; Fig. 1 B and data not shown) was comparable between Vav1−/− and wt NK cells. Therefore, Vav1 is not required for NK cell development, unlike T and NK-T cell development.

Vav1 Regulates NK Cell–mediated Tumor Clearance. NK cells recognize cells with disparate class I MHC expression and absolute numbers in the spleen and bone marrow of mice retained by measuring the esterase content in the supernatant of NK cells after three freeze–thaw cycles.

| Table I. Lymphoid Subsets in Vav1−/− Mice |
|-------------------------------------------|
| n  | wt | Vav1−/− |
|----|----|---------|
| Spleen Total lymphocytes | 11  | 653 ± 140 | 671 ± 110 |
| NK1.1+CD3− NK cells | 11  | 17 ± 5 | 25 ± 7 |
| NK1.1+CD3+ NK-T cells | 8  | 9.4 ± 3 | 5.2 ± 2 |
| TCR-αβ+ T cells | 9  | 174 ± 19 | 81 ± 22 |
| IgM+ B cells | 10  | 415 ± 130 | 533 ± 92 |
| Bone marrow Total lymphocytes | 5  | 23.4 ± 5.4 | 25.8 ± 6.3 |
| NK1.1+CD3+ NK cells | 5  | 0.19 ± 0.02 | 0.37 ± 0.17 |
| Liver Total lymphocytes | 7  | 27.4 ± 9.6 | 10.4 ± 1.7 |
| NK1.1+CD3+ NK cells | 7  | 1.75 ± 0.5 | 1.41 ± 0.5 |
| NK1.1+CD3+ NK-T cells | 7  | 6.2 ± 0.6 | 1.1 ± 1 |
| Thymus Total lymphocytes | 9  | 587 ± 126 | 100 ± 63 |
| NK1.1+CD3− NK cells | 9  | 0.54 ± 0.4 | 0.8 ± 0.4 |
| NK1.1+CD3+ NK-T cells | 9  | 5.0 ± 0.24 | 0.42 ± 0.14 |

Cell suspensions were enumerated and stained with a mixture of mAbs specific for TCR-αβ, IgM, and NK1.1. Absolute cell numbers were calculated based on the percentages of positive cells out of the total number of lymphoid cells. Statistically significant differences (P < 0.05, Student’s t test) are indicated in bold type. Splenocyte numbers are expressed in 106, and for the other organs, cell numbers are expressed in 105.

In a second model of NK cell–dependent tumor rejection (47), we tested the capacity of Vav1−/− mice to elimi-
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 $Rag2^{-/-}$ 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-$\gamma$ appears an essential cytokine for early control of $L.m.$ infection, we measured the IFN-$\gamma$ levels in the sera of infected mice. Fig. 2 D shows that IFN-$\gamma$ 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-$\gamma$ production is $Vav1$ independent.

$Vav1$ Regulates Natural Cytotoxicity, ADCC, and Lysis Initiated by Distinct NK Cell Receptors but Is Dispensable for IFN-$\gamma$ Production In Vitro. To dissect the cellular mechanisms responsible for defects in $Vav1^{-/-}$ mice in vivo, we compared the lytic activity of freshly isolated and IL-2–activated NK cells from $Vav1^{-/-}$ and $wt$ mice. As shown in Fig. 3, A–E, target cell lysis by $Vav1^{-/-}$ NK cells was defective, ranging from 20 to 50% of control values. The assays included: (a) natural cytotoxicity versus murine thymoma cells (YAC-1), mastocytoma (P815), or macrophage cell line (IC-21); (b) ADCC using EL4 cells precoated with $\alpha$–CD90; (c) killing of class I–deficient cells ($B2m^{-/-}$ Con A–activated blasts and TAP–deficient RMA-S thymomas); and (d) “reverse” ADCC (R-ADCC) versus FeR$^+$ P815 cells in the presence of $\alpha$–CD16, $\alpha$–2B4, or $\alpha$–NK1.1 mAbs. These results strongly suggest that all forms of NK cell–mediated cytolyis depend on $Vav1$, irrespective of which cellular receptor is activated. Importantly, prior activation of NK cells with poly(I:C) in vivo or with IL-2 in vitro did not rescue the lytic defect in $Vav1^{-/-}$ NK cells (Fig. 3, B and D), and no significant difference was observed in the proliferation induced by IL-2 (data not shown).

In contrast, $Vav1^{-/-}$ NK cells responded with normal IFN-$\gamma$ production (Fig. 4 A), after stimulation of 2B4, CD16, and NK1.1 activating receptors, and with IL-12. It should be noted that stimulation of $Vav1^{-/-}$ NK cells via the same receptors (2B4, CD16, and NK1.1) did not result in normal cytotoxicity of FeR$^+$ P815 cells (Fig. 3 E). To better evaluate the dichotomy between cytokine production and cytotoxicity observed in $Vav1^{-/-}$ NK cells, we measured the levels of intracellular IFN-$\gamma$ produced by $wt$ and $Vav1^{-/-}$ NK cells 4 h after activation. This can be compared with the 4-h Cr-release assay used to measure cytotoxicity. No significant defect in IFN-$\gamma$ production was detected, as similar percentages of $wt$ and mutant NK cells stained positively for intracellular IFN-$\gamma$ upon NK1.1 stimulation (Fig. 4 B). In contrast, CD3 stimulation did not result in IFN-$\gamma$ production by $Vav1^{-/-}$ T cells (Fig. 4 B).

Table II. $Vav1^{-/-}$ Mice Have a Reduced Capacity to Clear Tumor Cells

|        | $wt$ | $Vav1^{-/-}$ | $Rag2^{-/-}$ |
|--------|------|-------------|-------------|
| Mortality | 0/4 | 1/6 | 3/5 |
| Liver metastases in surviving mice | 0/4 | 2/5 | 2/2 |

Mice were injected intravenously with $10^4$ lymphoma cells (RDM4, H-2$,^b$, class I$^{+}$), and survival was followed for 14 d. Those mice that survived were killed and examined for the presence of liver metastases.
Vav1 has only a minor role in NK cell conjugate formation. It has been shown that conjugate formation is decreased in human NK cell lines expressing a dominant-negative form of the Vav1 substrate Rac1 (32). We therefore tested the ability of Vav1−/− NK cells to form stable conjugates using a flow cytometric approach. As shown in Fig. 5A, conjugate formation by Vav1−/− NK cells was readily detectable, ruling out an essential role for Vav1 in this process. A detailed kinetic study revealed that Vav1−/− NK cells formed somewhat less conjugates, although the observed decrease never exceeded 30%, and was not statistically significant in four of five time points analyzed (Fig. 5B).
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-γ 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...
1420 Natural Killer Cell Functions in Vav1−/− Mice

was detected (Fig. 7 A). However, stimulation of Vav1−/− NK cells via Ly49D resulted in reduced activation of both ERK1 (threefold) and ERK2 (twofold) kinases (data not shown). Collectively, these results suggest that Vav1 controls the ERK pathway in NK cells.

Vav1 Controls Exocytosis of Cytotoxic Granules upon Target Cell Binding. Intracellular polarization of NK cell cytotoxic granules toward sensitive target cells precedes granule exocytosis. The Vav1/Rac1 pathway has been suggested to be a critical regulator of this process (32). To directly measure granule exocytosis upon contact with targets, we quantitated the granzyme A esterase content in the supernatants of NK cell cultures stimulated with targets. Although total esterase content was similar in wt and mutant NK cells (data not shown), Vav1−/− NK cells showed a significant reduction in esterase exocytosis compared with wt NK cells (7.2 ± 4.5 versus 17.3 ± 5%, P = 0.03; Fig. 7 B). Thus, the absence of Vav1 results in defective granule exocytosis and reduced ability to lyse tumor targets.

Discussion

The data presented here show that Vav1 is an essential regulator of NK cell-mediated cytolysis of tumor cells. On the other hand, Vav1 is dispensable for NK cell development and cytokine production. Thus, Vav1−/− mice generate normal numbers of phenotypically mature NK cells but show a dichotomy in NK cell-mediated effector functions. They are competent for IFN-γ-dependent control of early bacterial infections yet have a reduced capacity to reject tumors. We could define the cellular mechanisms that are Vav1 dependent. The absence of Vav1 had only minor effects on conjugate formation, and calcium flux was normal. In contrast, the absence of Vav1 reduced ERK activation and exocytosis of cytotoxic granules that allow NK cells to lyse their targets.

The phenotype of NK cell functions in Vav1−/− mice is reminiscent of that of Beige mice, the murine model for Chediak-Higashi syndrome, a monogenic disease caused by mutations in the lysosomal trafficking regulator (Lyst) gene (50). Although the function of the Lyst protein has not yet been fully understood, it has been suggested to interact with microtubules to regulate protein sorting and late endosomal organization (51). Whatever the mechanisms underlying the Chediak-Higashi syndrome, the end result is a severely impaired granule exocytosis, much like what we have observed in Vav1−/− NK cells. Interactions between}

Figure 5. Vav1 plays a minor role in NK cell conjugate formation. (A) BCECF-labeled NK cells were allowed to form conjugates with HE-labeled YAC-1 targets for 20 min at 37°C and immediately analyzed by flow cytometry. Double-positive cells are conjugates, and the percentages (NK cells bound to targets out of the total NK cells) for the two genotypes are indicated for one representative of six independent experiments. Non-specific aggregates were reproducibly <1%. (B) Kinetics of conjugate formation. Values at each time point were analyzed statistically, and significant differences (P < 0.05 by Student’s t test) are indicated by an asterisk.

Figure 6. T cells but not NK cells require Vav1 to initiate calcium flux. Purified NK cells and T cells were stimulated with the primary mAbs (anti-NK1.1, anti-CD16, or anti-CD3), and analysis was done for 45 s to set the baseline levels of intracellular calcium. Acquisition was interrupted once to add cross-linking goat F(ab’)2 Abs and again to add 1 μg/ml of ionomycin. Cells were analyzed by LSR-FACS®. Intracellular calcium is proportional to the FL5/FL4 ratio.
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 cytotoxicity, 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 FasL-dependent mechanisms, which would not depend on lytic activity of targets. How can we explain the quantitative defect in the toxicity of Vav1−/− cells.

Our data underscore fundamentally divergent roles for Vav1 in the biology of T and NK lymphocytes. First, T and NK-T cell development and T cell functions are impaired in Vav1−/− mice, a consequence of the fact that T cells must signal through the same (Ag) receptor to complete differentiation and to exert their effector functions. Instead, requirement for Vav1 in NK cell functions (at least for cell-mediated cytotoxicity) dissociates from requirement for development. Several cytokine receptors are thought to drive NK cell differentiation (i.e., Flt3, c-kit, IL-7R, IL-15R), and we can suggest that Vav1 is not essential for transducing signals through any of these receptors, although it is phosphorylated upon ligation of some of them (23, 24). Moreover, it is likely that NK cell cytotoxicity is triggered by ligand–receptor systems different from those required for development, otherwise developing NK cells might be constitutively stimulated in the absence of inhibitory signals (which develop only later in life) and potentially dangerous.

Second, proximal signaling (measured by rise in intracellular calcium) in Vav1−/− NK cells is not impaired as opposed to defective capping and calcium flux upon cross-linking of TCRs in Vav1−/− T cells (14–16). Therefore, the abnormal biological responses of Vav1−/− T cells seem to be due to defective cellular activation. Along these lines, cytotoxic CD8+ T cells of Vav1−/− mice fail to generate a primary response to viral infections, while they are able to mount a normal CTL secondary response, suggesting that Vav1 is required for T cell–mediated cytotoxicity only when the activation threshold is high, during a primary response (54). But this is unlikely to occur in Vav1−/− NK cells, as we show here that IL-2 activation in vitro and stimulation with poly(I:C) in vivo does not rescue the NK cell lytic defect. Although several receptors have been reported to be able to activate NK cell effector functions (55), the ones that activate natural cytotoxicity remain unknown (56). This incomplete knowledge has obviously been an obstacle for advancing our understanding of the molecular mechanisms transducing NK cell activating signals into ef-
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 Var1−/− mice analyzed in this study. However, two independent groups, using mice carrying different Vav1 mutations, did not find an essential role for Vav1 in ERK activation (15, 16). We confirm here the results by Costello et al. (26) showing that Var1−/− T cells have a reduced ERK activation. Moreover, we show that Vav1 controls the ERK pathway in NK cells. Although both cytotoxicity and IFN-γ production have been shown to depend on ERK (34–36), we found that only cytotoxicity was affected in Var1−/− NK cells. It is possible that different thresholds of ERK phosphorylation control cytokine productions and granule exocytosis, explaining the divergent effect seen in Var1−/− NK cells.

Finally, receptor ligation in Var1−/− NK cells resulted in divergent effects on tumor cell killing and on cytokine production, showing that Vav1 differentially controls NK cell effector functions. This situation is not paralleled in T cells, where TCR-initiated events tested so far (calcium flux, proliferation, apoptosis, IL-2 gene transcription, cytotoxicity) are all defective in the absence of Vav1 (14–16, 26). Our results are consistent with a functional dichotomy in NK cell signaling with respect to Vav1, where Vav1-dependent cytotoxicity contrasts with Vav1-independent cytokine production.

We would like to thank Fabienne Mazerolles (Necker Hospital, Paris) for helping with the detection of conjugate formation, Petter Hoglund (Karolinska Institute, Stockholm) for suggestions on the lung clearance assay, Claude Leclerc (Pasteur Institute, Paris) and Thomas Ranson (Pasteur Institute, Paris) for help with providing the RDM4 cell line, Olivier Gaillot (Necker Hospital, Paris) and Odile Richard for discussions and for critical reading of the manuscript. The skillful technical help of Erwan Corcuff and Odile Richard is much appreciated.

This work was supported by grants from the Pasteur Institute (Paris, France), Fondation pour la Recherche Medica (France), Ligue Nationale contre le Cancer (France), Association contre le Cancer (France), Medical Research Council (UK), and Biotechnology and Biological Sciences Research Council (BBSRC). F. Colucci is a recipient of a Poste Vert position sponsored by the Institute National pour la Santé et la Recherche Médicale (France). E. Rosmaraki is a recipient of a Marie Curie Training and Mobility of Researchers grant from the European Commission. S. Bregenholst was supported by a postdoctoral fellowship from the Danish Research Agency.

Submitted: 11 October 2000
Revised: 4 April 2001
Accepted: 15 May 2001

References

1. Collins, T.L., M. Deckert, and A. Altman. 1997. Views on Vav. Immunol. Today. 18:221–225.

2. Katzav, S., D. Martin-Zanca, and M. Barbacid. 1989. vav, a novel human oncogene derived from a locus ubiquitously expressed in hematopoietic cells. EMBO J. 8:2283–2290.

3. Bustelo, X.R., J.A. Ledbetter, and M. Barbacid. 1992. Product of vav proto-oncogene defines a new class of tyrosine protein kinase substrates. Nature. 356:68–71.

4. Margolis, B., P. Hu, S. Katzav, W. Li, R.J.M. Olive, A. Ullrich, A. Weiss, and J. Schlessinger. 1992. Tyrosine phosphorylation of vav proto-oncogene product containing SH2 domain and transcription factor motifs. Nature. 356:71–74.

5. Tarakhovsky, A., M. Turner, S. Schaal, P.J. Mee, L.P. Duddy, K. Rajewsky, and V.L. Tybulewicz. 1995. Defective antigen receptor-mediated proliferation of B and T cells in the absence of Vav. Nature. 374:467–470.

6. Adams, J.M., H. Houston, J. Allen, T. Lints, and R. Harvey. 1992. The hematopoietically expressed vav proto-oncogene shows homology with the dbl GDP-GTP exchange factor, the bcr gene and a yeast gene (CDC24) involved in cytoskeletal organization. Oncogene. 7:611–618.

7. Boguski, M.S., A. Bairoch, T.K. Attwood, and G.S. Michaels. 1992. Proto-vav and gene expression. Nature. 358:113.

8. Crespo, P., X.R. Bustelo, D.S. Aaronson, O.A. Coso, M. Lopez-Barahona, M. Barbacid, and J.S. Gutkind. 1996. Rac-1 dependent stimulation of the JNK/SAPK signaling pathway by Vav. Oncogene. 13:455–460.

9. Olson, M.F., N.G. Pasteris, J.L. Gorski, and A. Hall. 1996. Fas-cigenital dysplasia protein (FGD1) and Vav, two related proteins required for normal embryonic development, are upstream regulators of Rho GTPases. Curr. Biol. 6:1628–1633.

10. Crespo, P., K.E. Schuebel, A.A. Ostrom, J.S. Gutkind, and X.R. Bustelo. 1997. Phosphotyrosine-dependent activation of Rac-1 GDP/GTP exchange by the vav proto-oncogene product. Nature. 385:169–172.

11. Cantrell, D. 1998. Lymphocyte signalling: a coordinating role for Vav? Curr. Biol. 8:R535–R538.

12. Fischer, K.D., A. Zmuldzinas, S. Gardner, M. Barbacid, A. Bernstein, and C. Guido. 1995. Defective T-cell receptor signalling and positive selection of Vav-deficient CD4+CD8+ thymocytes. Nature. 374:474–477.

13. Zhang, R., F.W. Alt, L. Davidson, S.H. Orkin, and W. Swat. 1995. Defective signalling through the T- and B-cell antigen receptors in lymphoid cells lacking the vav proto-oncogene. Nature. 374:470–473.

14. Turner, M., P.J. Mee, A.E. Walters, M.E. Quinn, A.L. Mellor, R. Zamoyska, and V.L. Tybulewicz. 1997. A requirement for the Rho-family GTP exchange factor Vav in positive and negative selection of thymocytes. Immunity. 7:451–460.

15. Fischer, K.D., Y.Y. Kong, H. Nishina, K. Tedford, I.E. Marengere, I. Koziardzki, T. Sasaki, M. Starr, G. Chan, S. Gardener, et al. 1998. Vav is a regulator of cytoskeletal reorganization mediated by the T-cell receptor. Curr. Biol. 8:554–562.

16. Holsinger, L.J., I.A. Graef, W. Swat, T. Chi, D.M. Bautista, L. Davidson, R.S. Lewis, F.W. Alt, and G.R. Crabtree. 1998. Defects in actin-cap formation in Vav-deficient mice implicate an actin requirement for lymphocyte signal transduction. Curr. Biol. 8:563–572.

17. Kong, Y.Y., K.D. Fischer, M.F. Bachmann, S. Mariathasan, I. Koziardzki, M.P. Nghiem, D. Bouchard, A. Bernstein, P.S. Ohashi, and J.M. Penninger. 1998. Vav regulates pep
tide-specific apoptosis in thymocytes. J. Exp. Med. 188:2099–2111.
18. Williams, N.S., J. Klem, I.J. Puzanov, P.V. Sivakumar, J.D. Schatzle, M. Bennett, and V. Kumar. 1998. Natural killer cell differentiation: insights from knockout and transgenic mouse models and in vitro systems. Immunol. Rev. 165:47–61.
19. McKenna, H.J., K.L. Stocking, R.E. Miller, K. Brasel, T. De Smedt, E. Maraskovsky, C.R. Maliszewski, D.H. Lynch, J. Smith, B. Pulendran, et al. 2000. Mice lacking flt3 ligand have deficient hematopoiesis affecting hematopoietic progenitor cells, dendritic cells, and natural killer cells. Blood. 95:3489–3497.
20. Colucci, F., and J.P. Di Santo. 2000. The receptor tyrosine c-kit provides a critical signal for survival, expansion, and maturation of mouse natural killer cells. Blood. 95:984–991.
21. Kennedy, M.K., M. Glaccum, S.N. Brown, E.A. Butz, J.L. Viney, M. Embers, M. Matsuki, K. Charrier, L. Sedger, C.R. Willis, et al. 2000. Reversible defects in natural killer and memory CD8 T cell lineages in interleukin 15-deficient mice. J. Exp. Med. 191:771–780.
22. Moore, T.A., U. van Freeden Jeffry, R. Murray, and A. Zlotnik. 1996. Inhibition of gamma delta T cell development and early thymocyte maturation in IL-7−/− mice. J. Immunol. 157:2366–2373.
23. Alai, M., A.L. Mui, R.L. Cutler, X.R. Bustelo, M. Barbacid, and G. Krystal. 1992. Steel factor stimulates the tyrosine phosphorylation of the proto-oncogene product, p95vav, in human hemopoietic cells. J. Biol. Chem. 267:18021–18025.
24. Dosil, M., S. Wang, and I.R. Lemischka. 1993. Mitogenic signalling and substrate specificity of the Flk2/Flt3 receptor tyrosine kinase in fibroblasts and interleukin 3-dependent hematopoietic cells. Mol. Cell. Biol. 13:6572–6585.
25. Trinchieri, G. 1989. Biology of natural killer cells. Adv. Immunol. 47:187–376.
26. Costello, P.S., A.E. Walters, P.J. Mee, M. Turner, L.F. Reynolds, A. Prisco, N. Sarner, R. Zamoyska, and V.L. Tybulewicz. 1999. The Rho-family GTP exchange factor Vav is a critical transducer of T cell receptor signals to the calcium, ERK, and NF-kappaB pathways. Proc. Natl. Acad. Sci. USA. 96:3035–3040.
27. Han, J., K. Luby-Phelps, B. Das, X. Shu, Y. Xia, R.D. Mosteller, U.M. Krishna, J.R. Falck, M.A. White, and D. Broek. 1998. Role of substrates and products of PI 3-kinase in regulating activation of Rac-related guanosine triphosphatases by Vav. Science. 279:558–560.
28. O’Rourke, L.M., R. Tooze, M. Turner, D.M. Sandoval, R.H. Carter, V.I. Tybulewicz, and D.T. Fearon. 1998. CD19 as a membrane-anchored adaptor protein of B lymphocytes: costimulation of lipid and protein kinases by recruitment of Vav. Immunity. 8:635–645.
29. Bonnema, J.D., J.M. Karmitz, R.A. Schoon, R.T. Abraham, and P.J. Leibson. 1994. Fc receptor stimulation of phosphatidylinositol 3-kinase in natural killer cells is associated with protein kinase C-independent granule release and cell-mediated cytotoxicity. J. Exp. Med. 180:1427–1435.
30. Wang, D., J. Feng, R. Wen, J.C. Marine, M.Y. Sangster, E. Pargas, A. Hoffmeyer, C.W. Jackson, J.L. Cleveland, P.J. Murray, et al. 2000. Phospholipase Cgamma2 is essential in the functions of B cell and several Fc receptors. Immunity. 13:35–35.
31. Xu, X., and A.S. Chong. 1996. Vav in natural killer cells is tyrosine phosphorylated upon cross-linking of Fc gamma RI/IIA and is constitutively associated with a serine/threonine kinase. Biochem. J. 318:527–532.
receptor expression by developing natural killer cells. J. Exp. Med. 187:609–618.
46. Karre, K., H.G. Ljunggren, G. Piontek, and R. Kiessling. 1986. Selective rejection of H-2-deficient lymphoma variants suggests alternative immune defence strategy. Nature. 319:675–678.
47. Smyth, M.J., K.Y.T. Thia, E.A. Street, E. Cretney, J. Trapani, M. Tamiguchi, T. Kawano, S.B. Pelikan, N. Crowe, and D.I. Godfrey. 2000. Differential tumor surveillance by natural killer (NK) and NKT cells. J. Exp. Med. 191:661–668.
48. Biron, C.A., K.B. Nguyen, G.C. Pien, L.P. Cousens, and T.P. Salazar-Mather. 1999. Natural killer cells in antiviral defense: function and regulation by innate cytokines. Annu. Rev. Immunol. 17:189–220.
49. Wherry, J.C., R.D. Schreiber, and E.R. Unanue. 1991. Regulation of gamma interferon production by natural killer cells in scid mice: roles of tumor necrosis factor and bacterial stimuli. Infect. Immun. 59:1709–1715.
50. Barbosa, M.D., Q.A. Nguyen, V.T. Tchernev, J.A. Ashley, J.C. Detter, S.M. Blaydes, S.J. Brandt, D. Chotai, C. Hodgman, R.C. Solari, et al. 1996. Identification of the homologous beige and Chediak-Higashi syndrome genes. Nature. 382:262–265.
51. Faigle, W., G. Raposo, D. Tenza, V. Pinet, A.B. Vogt, H. Kroghofer, A. Fischer, G. de Saint-Basile, and S. Amigorena. 1998. Deficient peptide loading and MHC class II endosomal sorting in a human genetic immunodeficiency disease: the Chediak-Higashi syndrome. J. Cell Biol. 141:1121–1134.
52. Shresta, S., C.T. Pham, D.A. Thomas, T.A. Graubert, and T.J. Ley. 1998. How do cytotoxic lymphocytes kill their targets? Curr. Opin. Immunol. 10:581–587.
53. Billadeau, D.D., S.M. Mackie, R.A. Schoon, and P.J. Leibson. 2000. The rho family guanine nucleotide exchange factor vav-2 regulates the development of cell-mediated cytotoxicity. J. Exp. Med. 192:381–392.
54. Penninger, J.M., K.D. Fischer, T. Sasaki, I. Kozieradzki, J. Le, K. Tedford, K. Bachmaier, P.S. Ohashi, and M.F. Bachmann. 1999. The oncogene product Vav is a crucial regulator of primary cytotoxic T cell responses but has no apparent role in CD28-mediated co-stimulation. Eur. J. Immunol. 29:1709–1718.
55. Lanier, L.L. 1998. NK cell receptors. Annu. Rev. Immunol. 16:359–393.
56. Moretta, A., R. Biassoni, C. Bottino, M.C. Mingari, and L. Moretta. 2000. Natural cytotoxicity receptors that trigger human NK-cell-mediated cytolysis. Immunol. Today. 21:228–234.
57. Leibson, P.J. 1997. Signal transduction during natural killer cell activation: inside the mind of a killer. Immunity. 6:655–661.