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Vav1 Controls DAP10-Mediated Natural Cytotoxicity by Regulating Actin and Microtubule Dynamics[1]

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The NK cell-activating receptor NKG2D recognizes several MHC class I-related molecules expressed on virally infected and tumor cells. Human NKG2D transduces activation signals exclusively via an associated DAP10 adaptor containing a YxNM motif, whereas murine NKG2D can signal through either DAP10 or the DAP12 adaptor, which contains an ITAM sequence. DAP10 signaling is thought to be mediated, at least in part, by PI3K and is independent of Syk/Zap-70 kinases; however, the exact mechanism by which DAP10 induces natural cytotoxicity is incompletely understood. Herein, we identify Vav1, a Rho GTPase guanine nucleotide exchange factor, as a critical signaling mediator downstream of DAP10 in NK cells. Specifically, using mice deficient in Vav1 and DAP12, we demonstrate an essential role for Vav1 in DAP10-induced NK cell cytoskeletal polarization involving both actin and microtubule networks, maturation of the cytolytic synapse, and target cell lysis. Mechanistically, we show that Vav1 interacts with DAP10 YxNM motifs through the adaptor protein Grb2 and is required for activation of PI3K-dependent Akt signaling. Based on these findings, we propose a novel model of ITAM-independent signaling by Vav downstream of DAP10 in NK cells. *The Journal of Immunology,* **2006, 177: 2349–2355.**

Natural cytotoxicity mediated by NK cells is regulated by multiple activating and inhibitory receptors, which confer innate defenses against tumor cells and virus-infected cells. The multistep process leading to target cell lysis involves the formation of a cytolytic synapse and polarized degranulation of the NK cell (1–4). Initial contact between the NK cell and the target cell is mediated by integrins and facilitates engagement of NK-activating receptors by their cognate ligands (2, 4). Postconjugation events are orchestrated by signals emanating from activating receptors and involve F-actin accumulation at the NK-target contact site and microtubule-organizing center (MTOC)[3] polarization toward the target cell. In turn, MTOC polarization leads to the establishment of a microtubule network guiding cytolytic granules to the synapse where they fuse with the plasma membrane and release perforin and granzymes to lyse target cells (2, 4). Cytoskeletal remodeling is critical for NK cytotoxicity because pharmacologic inhibition of F-actin or microtubule dynamics blocks granule polarization and target cell lysis (1). Moreover, NK cells from patients bearing mutations in WASp that disrupt actin dynamics fail to initiate synapse formation and lyse target cells (1, 5).

Activating NK cell receptors primarily signal through ITAM-containing adaptor molecules such as DAP12, CD3ζ, and Fc$R\gamma$, which initiate cellular activation signals by recruiting Syk/Zap-70 family kinases (6–9). Additional NK cell activating receptors, such as NKG2D, trigger cytotoxicity independently of ITAMs by associating with DAP10, a unique adaptor containing a YxNM motif that recruits PI3K (10) and Grb2 (11). Initiation of NKG2D signals occurs upon recognition of specific ligands, stress-induced MHC class I-like molecules such as MICA, MICB, and UL-16 binding protein in humans, as well as Rae-1, H-60, and MULT1 in mice (8, 12). Subsequent to ligand engagement, human NKG2D signals through DAP10, whereas murine NKG2D signals through distinct adaptor molecules. Full-length NKG2D-long (NKG2D-L) signals through DAP10, whereas a shorter splice variant (NKG2D-S) signals through both DAP10 and DAP12 (13–15). In this regard, the relative proportion of NKG2D-L and NKG2D-S in NK cells varies upon in vitro activation with IL-2. Thus, freshly isolated (ex vivo) NK cells predominantly express NKG2D-L, whereas in vitro activation leads to an increase in NKG2D-S expression (13). Nevertheless, experiments with DAP12-deficient murine NK cells demonstrate that DAP10 is sufficient to mediate NKG2D-dependent cytotoxicity (16). Furthermore, human NKG2D promotes NK cytotoxicity despite its inability to interact with DAP12 (17).

Previous studies have implicated the Vav family of Rho guanine nucleotide exchange factors in the regulation of several distinct pathways controlling natural cytotoxicity (18–22). NK cells lacking all three Vav proteins show severely compromised cytotoxicity triggered by both ITAM- and DAP10-associated activating receptors (18). However, while deficiency in Vav1 alone primarily impaired the NKG2D-DAP10 cytolytic pathway, lack of Vav2 and Vav3 reduced cytotoxicity triggered by receptors that signal through ITAM-containing adaptors (18). These observations indicated an unexpected specialization of Vav proteins in regulating distinct cytotoxic pathways and implicated Vav1 in control of signals emanating from DAP10-coupled receptors. However the exact

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[3] Abbreviations used in this paper: MTOC, microtubule-organizing center; NKG2D-L, NKG2D long; NKG2D-S, NKG2D short; SH, Src homology; WT, wild type.

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mechanism of Vav1 coupling to DAP10 remains elusive. In addition, a particular issue is how DAP10 controls cytoskeletal remodeling events during the cytolytic response.

Herein, we sought to elucidate the mechanism of Vav1 function in DAP10-mediated signaling events that control natural cytotoxicity. Using mice deficient in Vav1 and DAP12, we demonstrate a critical function for Vav1 in DAP10-induced PI3K activation, F-actin polymerization, and MTOC polarization and provide evidence that Vav1 is recruited to DAP10 via Grb2.

Materials and Methods

Mice and NK cell purification

$Vav1^{-/-}$ and $DAP12^{-/-}$ mice have been described elsewhere and were bred to generate $Vav1^{-/-}DAP12^{-/-}$ double knockout mice (F6 on C57BL/6 background) (23, 24). Splenic NK cells were purified by positive selection with anti-DX-5 microbeads (Miltenyi Biotec), according to the manufacturer's recommendation. Unless otherwise indicated, purified NK cells were cultured in recombinant human IL-2 (1000 U/ml) for 4 days.

Cytotoxicity assays

The NK cells were tested against target cells by standard ^{51}Cr release assay (18).

Pull-down assays

Biotinylated peptides comprising four amino acids flanking the tyrosine motifs of interest were obtained from BioSource International (biotinylated DAP10 peptide sequences were DGRVYINMPGRG, DGRVpY INMPGRG). GST-Src homology (SH)2 fusion proteins were provided by D. Billadeau (Mayo Clinic College of Medicine, Rochester, MN). Peptides were bound to streptavidin-Sepharose and mixed with fusion proteins for 1 h at 4°C. Bound fusion proteins were eluted and detected by Western blot analysis with rabbit anti-GST (Upstate Biotechnology). Alternatively, peptides were bound to streptavidin-Sepharose and used to pull down proteins from NK92 lysates. Where indicated, Grb2 was first depleted from the lysates by six serial immunoprecipitations with rabbit anti-Grb2 (Santa Cruz Biotechnology). After pull down, bound proteins were detected by Western blot with rabbit anti-Vav1 (Santa Cruz Biotechnology) or mouse anti-Grb2 (Santa Cruz Biotechnology).

Biochemistry

Purified splenic NK cells were cultured in IL-2 (1000 U/ml) for 7 days and then starved in serum-free medium for 6 h. NK cells (1.25×10^6/sample) were resuspended in HBSS and incubated on ice with biotinylated anti-NKG2D (Biolegend) at 1 μg/1 $\times$ 10^6 cells. After 15 min, streptavidin (Pierce) was added at 2 μg/1 $\times$ 10^6 cells, and cells were incubated at 37°C for the indicated time points. Cells were then lysed in radioimmunoprecipitation assay buffer and analyzed by Western blotting for phospho-serine 473 Akt (Cell Signaling Technology) or total Akt (Cell Signaling Technology).

Conjugate formation

Target cells were stained with CFSE, and NK cells were stained with hydroethidine. NK cells and targets were pelleted together, gently disrupted, and incubated at 37°C for 15 min. The percentage of cells forming conjugates was determined by FACS.

NK cell staining and imaging

Target cells were stained with CFSE or 7-amino-4-chloromethylcoumarin (Molecular Probes). NK cells and targets were briefly pelleted at a 1:1 ratio and immediately distributed onto poly-L-lysine-coated slides for incubation at 37°C for 30 min. Cells were fixed in paraformaldehyde (2%) and permeabilized in TX-100 (0.1%) before staining with rhodamine-phalloidin (Molecular Probes), rabbit anti-DAP10 (Santa Cruz Biotechnology), or FITC-anti-α-tubulin (Sigma-Aldrich). Cells were visualized on a Zeiss confocal microscope equipped with LSM image analysis software or a Nikon fluorescence microscope. Images were acquired using a $\times$100 objective lens with a $\times$10 ocular lens. Conjugates were scored at random and defined as a NK cell conjugated to a single target cell. Two-dimensional images were captured in an optical slice perpendicular to the NK-target synapse and intersecting the center of the synapse. Quantitations were performed with ImageJ software (National Institutes of Health) to measure the length of the NK membrane at the synapse (in arbitrary units) and the pixel intensity of F-actin staining within a defined area. Statistical analyses were performed using Student's *t* test and the Mann-Whitney *U* test.

Results

Vav1 controls cytotoxicity mediated by NKG2D-DAP10

To specifically examine the requirement of Vav1 in signaling downstream of NKG2D-DAP10, without the confounding effects of NKG2D-DAP12, we generated NK cells lacking both Vav1 and

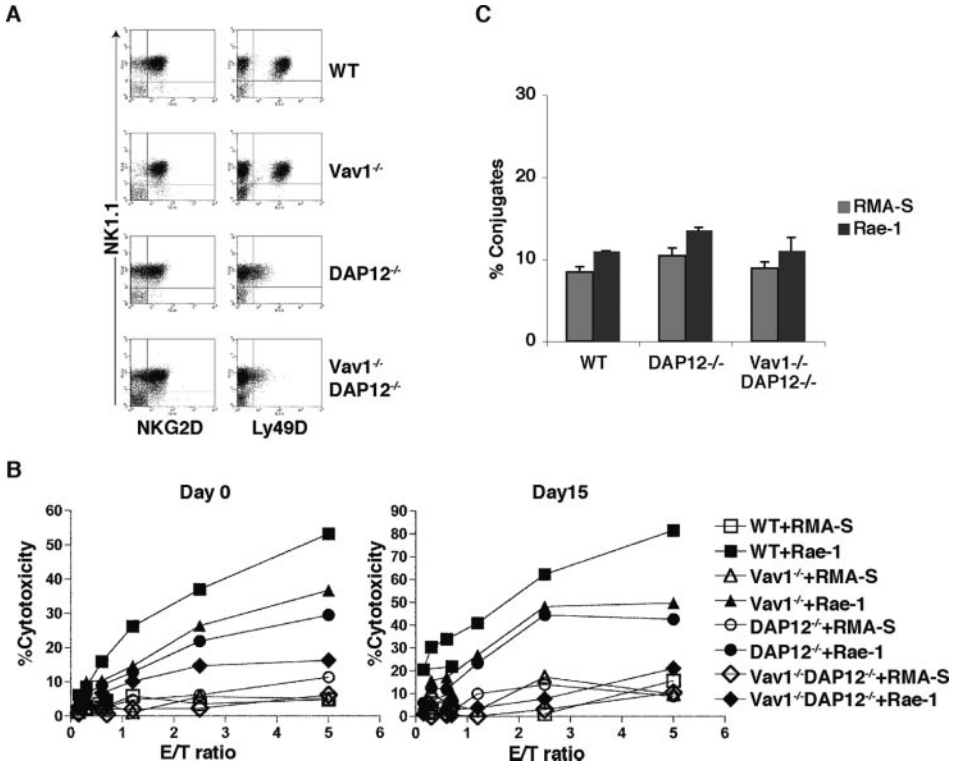

FIGURE 1. Vav1 is required for NKG2D-DAP10-mediated cytotoxicity. *A*, Purified NK cells from WT, $Vav1^{-/-}$, $DAP12^{-/-}$, and $Vav1^{-/-}DAP12^{-/-}$ mice were stained with the indicated Abs and analyzed by FACS for expression of common NK cell surface markers. *B*, Fresh NK cells (*left panel*) or NK cells cultured in IL-2 for 15 days (*right panel*) were tested for cytotoxicity against RMA-S targets or RMA-S targets expressing Rae-1γ in standard chromium release assays. *C*, Purified NK cells were stained with CFSE, and RMA-S or RMA-S Rae-1γ targets were stained with hydroethidine. NK cells and targets were pelleted together and incubated at 37°C for 15 min before fixation and analysis of conjugate formation by FACS.

DAP12. Genetic deletion of Vav1 or DAP12 has no discernible effect on NK cell numbers or expression of NK1.1 (Fig. 1*A* and data not shown). However, both DAP12$^{-/-}$ and Vav1$^{-/-}$DAP12$^{-/-}$ NK cells express slightly lower levels of NKG2D and dramatically reduced levels of Ly49D, as compared with wild-type (WT) cells (Fig. 1*A*), presumably because of a chaperone function conferred by DAP12 (14).

To directly determine the requirement for Vav1 in NKG2D-DAP10 function, we analyzed WT, Vav1$^{-/-}$, DAP12$^{-/-}$, and Vav1$^{-/-}$DAP12$^{-/-}$ NK cells in cytotoxicity assays. In these experiments, fresh or in vitro-activated NK cells were tested for cytotoxicity against RMA-S cells or RMA-S transfectants expressing the NKG2D ligand Rae-1γ. In comparison to WT NK cells, Vav1$^{-/-}$ NK cells are less efficient at lysing RMA-S targets expressing Rae-1γ (Fig. 1*B* and Ref. 18), suggesting that Vav1 plays a role in NKG2D-mediated killing. Similarly, DAP12$^{-/-}$ NK cells exhibit reduced lysis of Rae-1γ targets compared with WT NK cells, which is consistent with the notion that murine NKG2D signals through both DAP10 and DAP12 and that NKG2D expression is reduced in DAP12$^{-/-}$ NK cells (Fig. 1*B*). Strikingly, both fresh (day 0) and IL-2-activated (day 15) Vav1$^{-/-}$DAP12$^{-/-}$ NK cells demonstrate an essentially complete block in cytotoxicity against targets expressing Rae-1γ, thus revealing a critical requirement for Vav1 in NKG2D-DAP10-mediated cytotoxicity. Despite exhibiting defects in cytotoxicity, Vav1$^{-/-}$DAP12$^{-/-}$ NK cells are indistinguishable from WT and DAP12$^{-/-}$ NK cells in their ability to form conjugates with RMA-S and Rae-1γ targets (Fig. 1*C*). These results indicate that defects observed in Vav1$^{-/-}$DAP12$^{-/-}$ NK cytotoxicity are not due to impaired conjugate formation but rather to specific defects in NKG2D-DAP10 signaling and post-conjugation events.

Vav1 interacts with DAP10 via the adaptor Grb2

Having established the requirement for Vav1 in cytotoxicity mediated by NKG2D-DAP10, we pursued potential mechanisms of Vav1 recruitment to DAP10. Based on previously published findings (11, 25), we hypothesized that Vav1 could interact with DAP10 directly via Grb2. To test this hypothesis, we performed pull-down assays and found that DAP10 peptides containing tyrosine phosphorylated, but not unphosphorylated, YxNM motifs can directly interact with the Grb2 SH2 domain (Fig. 2*A*). As a positive control in this assay, the Grb2 SH2 domain interacts with CD22 phospho-peptides containing a YxN motif (Fig. 2*A*). However, peptides comprising Y174 of Vav1, which lack the YxN motif, fail to interact with Grb2 SH2 fusion proteins, indicating specificity in the interaction of DAP10 tyrosine motifs with Grb2 (Fig. 2*A*).

To test the possibility that Vav1 can interact with DAP10 by means of the adaptor function of Grb2, we used DAP10 YxNM peptides to pull down Vav1 from lysates of NK92 cells containing endogenous levels of Grb2 or from NK92 lysates depleted of Grb2 by serial immunoprecipitation (Fig. 2*B*). Strikingly, phosphorylated DAP10 YxNM peptides readily pull down Vav1 from NK92 lysates, while depletion of Grb2 from the lysate before pull-down abrogates the interaction of YxNM peptides with Vav1 (Fig. 2*B*). Importantly, depletion of Grb2 has no discernible effect on total levels of Vav1 present in the lysates (Fig. 2*B*). The observation that DAP10 peptides pull down Vav1 in a Grb2-dependent manner is consistent with previous findings that Vav1 constitutively associates with Grb2 via a SH3-SH3 interaction (25). In this context, our results suggest that Grb2 may simultaneously bind Vav1 through its SH3 domain and DAP10 YxNM motifs through its SH2 domain, although we cannot rule out the possibility that Vav1 may also be recruited through additional mechanisms. Nevertheless, these data implicate Grb2-Vav1 as a potential downstream signaling module used by NKG2D-DAP10. To address such a potential role for Vav1 as a downstream signaling effector of DAP10, we examined the induction of PI3K activity in Vav1$^{-/-}$DAP12$^{-/-}$ NK cells stimulated through NKG2D. To this end, WT, DAP12$^{-/-}$, or Vav1$^{-/-}$DAP12$^{-/-}$ NK cells were stimulated by cross-linking

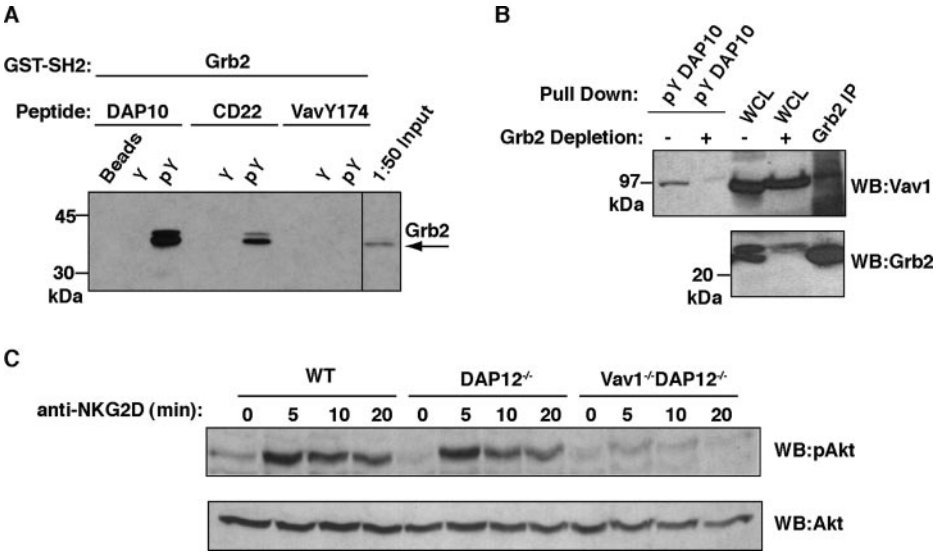

FIGURE 2. Vav1 interacts with DAP10 through Grb2. *A*, Biotin-conjugated peptides containing tyrosine motifs derived from the indicated proteins were used in pull-down assays to test direct interactions with the Grb2 SH2 domain. Phosphorylated (pY) or unphosphorylated (Y) peptides were bound to streptavidin-Sepharose and incubated with GST-Grb2-SH2. Bound protein complexes were detected by Western blotting with anti-GST. *B*, Biotinylated DAP10 YxNM peptides bound to streptavidin-Sepharose were used to pull down Vav1 from NK92 lysates that contained endogenous levels of Grb2 or were depleted of Grb2 by serial immunoprecipitation (IP). Bound protein complexes were analyzed by Western blotting with anti-Vav1. Additionally, whole cell lysates (WCL) from NK92 cells or Grb2-depleted lysates were analyzed by Western blotting to detect Grb2 and Vav1. *C*, Purified splenic NK cells were stimulated with anti-NKG2D and lysed at the indicated time points. Lysates were analyzed for phosphorylated Akt and total Akt by Western blot (WB).

NKG2D with specific Abs, and phosphorylation of Akt kinase was measured at various time points as a surrogate assay of PI3K activation. Strikingly, while stimulation of WT and DAP12$^{-/-}$ NK cells with anti-NKG2D leads to a strong induction of Akt phosphorylation by 5 min (Fig. 2*C*), Vav1$^{-/-}$DAP12$^{-/-}$ NK cells show essentially no Akt phosphorylation in response to NKG2D cross-linking at any of the time points tested (Fig. 2*C*). These results indicate that Vav1 is essential for normal regulation of PI3K activity in response to NKG2D-DAP10 signals.

Vav1 is essential for postconjugation events induced by NKG2D-DAP10 in NK cells

Given that Vav1$^{-/-}$DAP12$^{-/-}$ NK cells efficiently conjugate with RMA-S cells but fail to kill these targets, we examined the role of Vav1 in postconjugation events. Analyses of NK cell-target conjugates using DAP10 Ab staining revealed that WT, DAP12$^{-/-}$, and Vav1$^{-/-}$DAP12$^{-/-}$ NK cells all display a spherical morphology when conjugated to RMA-S targets in the absence of NKG2D ligands (Fig. 3, *A* and *B*). In contrast, WT and DAP12$^{-/-}$ NK cells conjugated with RMA-S targets expressing Rae-1γ adopt a compressed morphology marked by spreading of the plasma membrane along the contour of the target cell and expansion of the cell body at the target interface (Fig. 3, *A* and *B*). Strikingly, Vav1$^{-/-}$DAP12$^{-/-}$ NK cells completely fail to undergo this cellular compression and maintain a spherical morphology when conjugated to targets expressing Rae-1γ (Fig. 3, *A* and *B*).

To quantitate this postconjugation plasticity in NK cells, we used a morphometric assay that measures the relative proportion of the NK cell membrane that is directly apposed to the target cell at the synapse. Accordingly, two-dimensional images of conjugates were acquired in a plane perpendicular to the plane of the synapse and intersecting the center of the synapse. Within this optical slice, the length of the NK cell membrane contacting the target cell was measured using ImageJ software, and this value was divided by the NK cell's circumference as measured in the same plane. NK cells derived from WT mice and DAP12$^{-/-}$ mice allocate a larger proportion of their membrane to the contact site with Rae-1γ targets as compared with Vav1$^{-/-}$DAP12$^{-/-}$ NK cells (Fig. 3*C*). Thus, Vav1 controls cellular plasticity in response to NKG2D-DAP10 engagement.

Because NK cells vary significantly in their size, we also devised an alternative measure of NK cell plasticity, which is based on measurement of the angle θ defined by the apex of a triangle bound within the confines of the NK cell membrane (Fig. 3*D*). The first of the three points defining the triangle is located at the center of the NK cell membrane region in contact with the target. The remaining two points of the triangle are located at the widest points of the NK cell such that the line connecting them is parallel to the NK-target contact site. An angle θ is defined as the angle formed by the triangle's apex located at the NK-target contact site. In this assay, NK cells maintaining a spherical morphology upon conjugation with target cells have a corresponding θ value of ~90 degrees. However, morphological changes associated with compression and widening of the NK cell base at the target cell surface are associated with θ values > 90 degrees. Because baseline in this assay is 90 degrees rather than 0 degrees, we expressed the data as

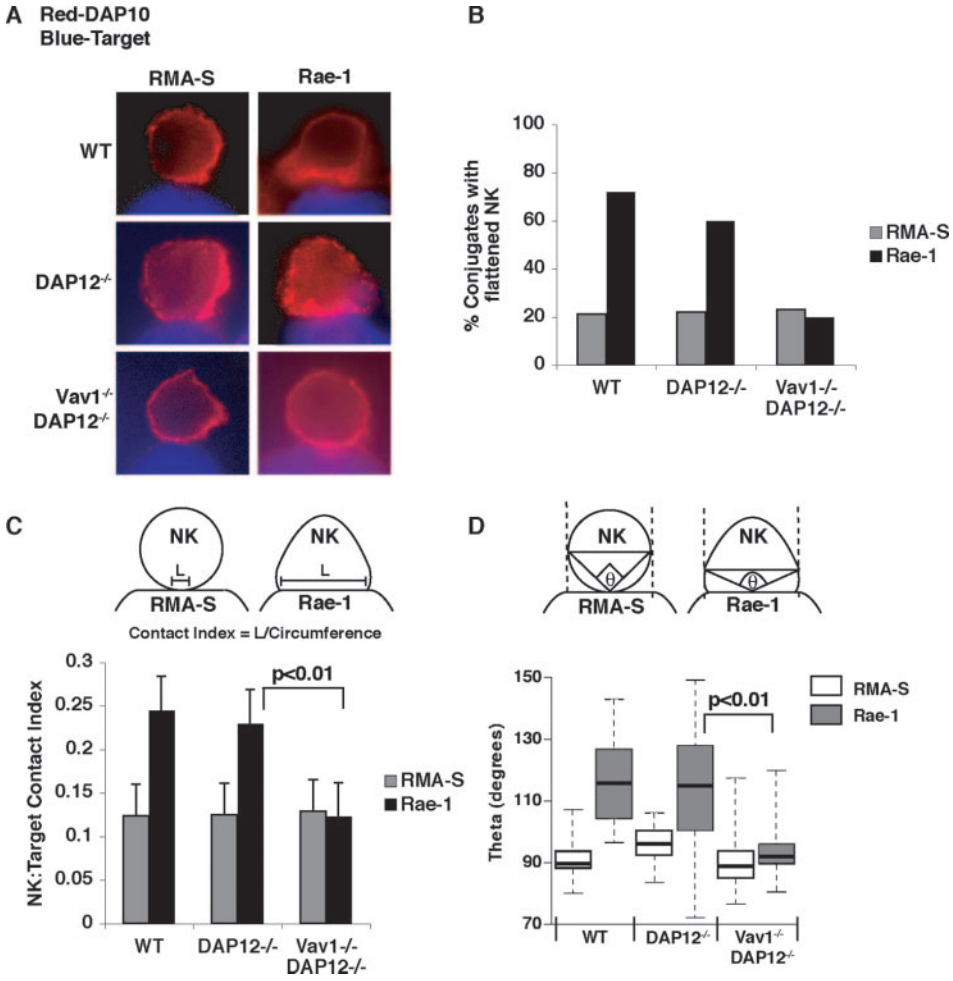

FIGURE 3. Vav1 is required for postconjugation events induced by NKG2D-DAP10. *A*, RMA-S or RMA-S Rae-1γ targets were stained with CMAC (blue) and conjugated with NK cells. Conjugates were fixed and stained with anti-DAP10 (red) to mark the NK cell membrane. *B*, Percentages of conjugates containing flattened NK cells were scored and tabulated. Conjugates were scored positive for flattening if the NK cell diameter at the synapse was wider than the diameter at the center of the cell. A total of 97 conjugates was scored. *C*, Quantitation of NK cell morphological changes was performed by measuring the length of the NK cell membrane in contact with the target and dividing this number by the circumference of the NK cell (as described in *Results* and *Materials and Methods*). Data represent the NK:target contact index or mean proportion of NK cell membrane contained within the synapse + SD of $n > 15$ conjugates for each different conjugate pair. *D*, Quantitation of NK cell morphological changes was performed by constructing a triangle within the confines of the NK cell membrane (as described in *Materials and Methods*) and measuring the angle θ. The box plot represents $n \geq 15$ conjugates for each different conjugate pair. Medians are represented as thick horizontal lines, 25th and 75th percentiles as boxes, and 10th and 90th percentiles as whiskers.

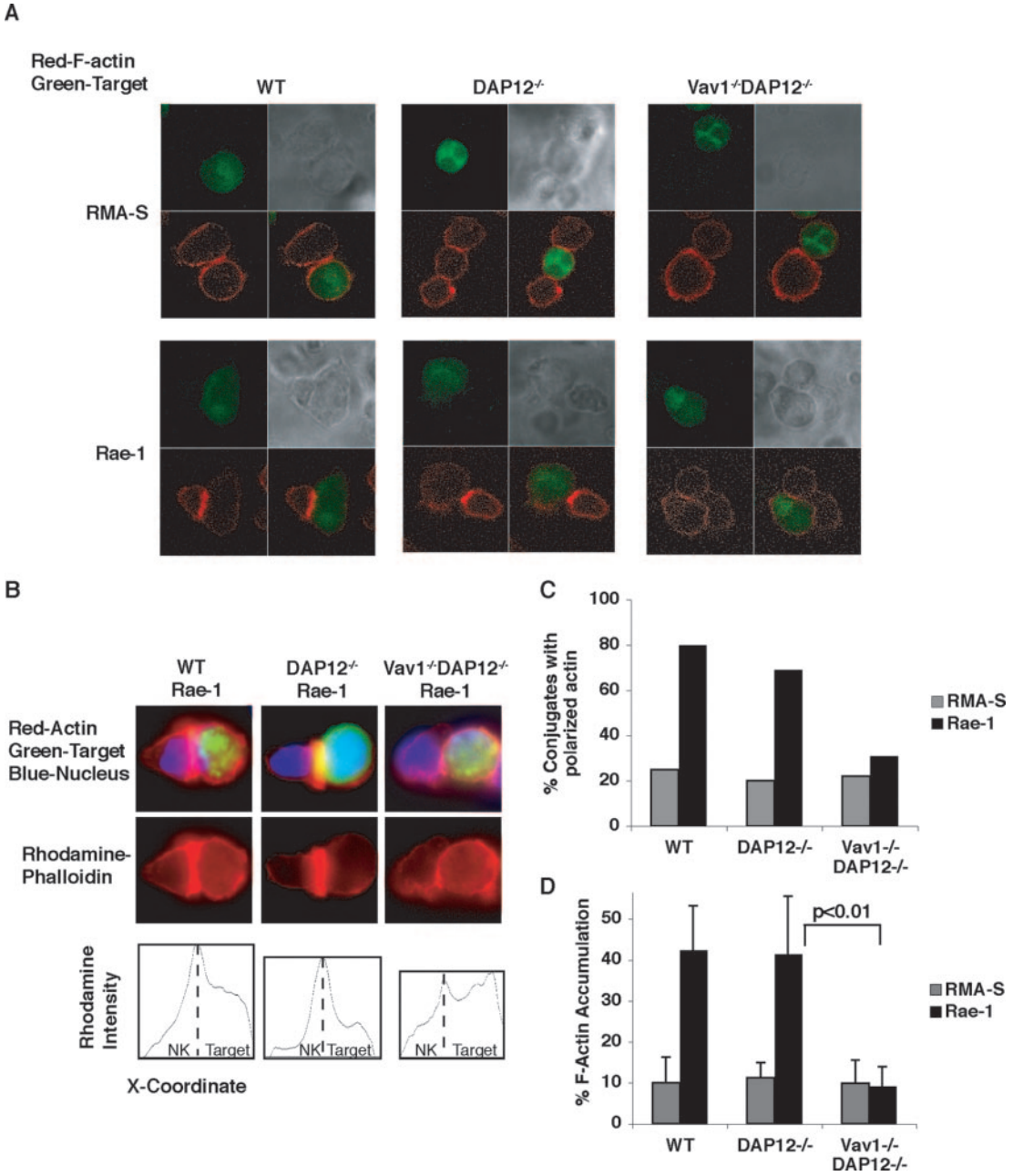

FIGURE 4. Vav1 controls actin polymerization induced by NKG2D-DAP10. *A*, RMA-S or RMA-S Rae-1γ targets were stained with CFSE (green) and conjugated with NK cells. Conjugates were fixed and stained with phalloidin (red) to mark F-actin. *B*, Images of conjugates stained as in Fig. 3*A* were converted into histograms representing phalloidin fluorescence intensity along the *x*-axis. *C*, Percentages of conjugates containing NK cells with F-actin accumulation at the synapse were scored and tabulated. NK cells were scored positive for actin accumulation if phalloidin staining was more intense in the half of the cell (based on volume) facing the target as compared with the distal half of the cell. A total of 49 conjugates was scored. *D*, Quantitation of actin polymerization was performed in optical slices perpendicular to the synapse and intersecting the center of the synapse. Accumulation of F-actin within the NK cell at the site of contact with the target cell was determined by fluorescence intensity of phalloidin staining. Data represent the mean percentage of total phalloidin fluorescence localized at the contact site $\pm$ SD of $n \geq 15$ conjugates for each different conjugate pair.

a "box-and-whisker" plot wherein the boxes represent the 25th and 75th percentiles and the whiskers represent the 10th and 90th percentiles (Fig. 3*D*). Measurements in WT and DAP12$^{-/-}$ NK cells conjugated with Rae-1γ targets reveal values of θ that are 20–40 degrees greater than a right angle (Fig. 3*D*). In stark contrast, Vav1$^{-/-}$DAP12$^{-/-}$ NK cells maintain a spherical morphology and a θ value near 90 degrees when conjugated to RMA-S targets with or without Rae-1γ (Fig. 3*D*). Thus, we conclude from these experiments that Vav1 is critical for compression and flattening of the NK cell at the interface with target cells expressing NKG2D ligands.

Vav1 is required for actin accumulation at the NK-target contact site in response to NKG2D-DAP10 signals

Given that Vav proteins have the capability to regulate actin remodeling in T cells (26), we hypothesized that the morphological defects observed in Vav1$^{-/-}$DAP12$^{-/-}$ NK cells subsequent to conjugation are due to impaired cytoskeletal remodeling. Actin polymerization is absolutely required for NK cell function, as disruption of actin dynamics due to WASp mutations or pharmacologic inhibition with cytochalasin-D abolishes cytotoxicity (1, 5). Thus, we examined the requirement of Vav1

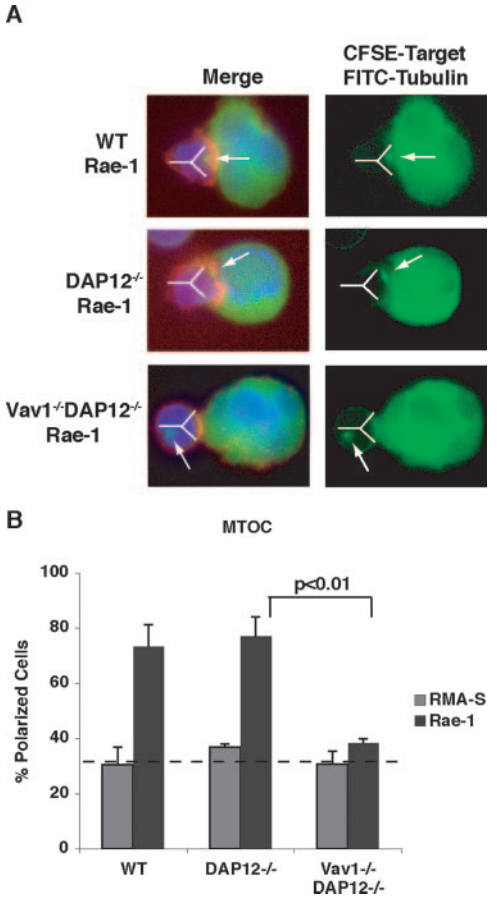

FIGURE 5. Vav1 controls MTOC polarization induced by NKG2D-DAP10. RMA-S or RMA-S Rae-1γ targets were stained with CFSE (green) and conjugated with NK cells. Conjugates were fixed and stained with anti-tubulin for MTOCs (green spots marked by white arrows), phalloidin to mark cortical actin at the cell boundary (red), and 4′,6′-diamidino-2-phenylindole to mark the nucleus (blue). *B*, Quantitation was performed by dividing the NK cell into three equal regions, with one region facing the target cell (see Fig. 4*A*). MTOC polarization was defined by the presence of MTOCs in the region of the NK cell facing the target. The percentage of cells containing polarized MTOCs in $n > 15$ conjugates for each different conjugate pair was determined in each experiment. Data represent the mean percentage of polarized cells + SD in three separate experiments. Random distribution of MTOCs predicts that 33% of NK cells will possess polarized MTOCs.

for actin polymerization during NK cell activation. We found that WT and DAP12$^{-/-}$ NK cells exhibit robust F-actin accumulation at sites of contact with target cells expressing Rae-1γ but not parental RMA-S cells lacking NKG2D ligands (Fig. 4, *A–C*). In contrast, Vav1$^{-/-}$DAP12$^{-/-}$ NK cells fail to accumulate F-actin at the contact sites with RMA-S target cells expressing Rae-1γ (Fig. 4, *A–C*).

Quantitation of actin polymerization was performed in optical slices perpendicular to the synapse and intersecting the center of the synapse (Fig. 4*B*). In Fig. 4*D*, actin polymerization is reflected as the percentage of total F-actin within the NK cell that localizes at the target cell interface, and background actin polymerization is defined as the percentage of actin accumulated at the site of contact between NK cells and RMA-S targets. Using this assay, we find a 4-fold increase over background actin polymerization in both WT or DAP12$^{-/-}$ NK cells conjugated with Rae-1γ targets, as compared with conjugates with RMA-S targets (Fig. 4*D*). In contrast, no increase over background actin polymerization is observed in Vav1$^{-/-}$DAP12$^{-/-}$ NK cells conjugated with RMA-S targets expressing Rae-1γ, indicating a strict requirement for Vav1 in actin polymerization downstream of NKG2D-DAP10 (Fig. 4*D*).

Vav1 is critical for MTOC polarization at the NK-target contact site in response to NKG2D-DAP10 signals

Downstream of actin polymerization, microtubule dynamics are required for MTOC polarization and subsequent degranulation of NK cells (1). Given the profound defects in actin dynamics and cytotoxicity in Vav1$^{-/-}$DAP12$^{-/-}$ NK cells, we speculated that microtubule dynamics may be disrupted as well. To examine this process, we conducted a quantitation of MTOC polarization by dividing the NK cell into three equal sections with one section facing the target (Fig. 5*A*). Results from these quantitations show that ~70% of WT and DAP12$^{-/-}$ NK cells polarize MTOCs toward target cells expressing Rae-1γ (Fig. 5*B*). In contrast, MTOCs are randomly distributed in Vav1$^{-/-}$DAP12$^{-/-}$ NK cells (~33% of cells polarized), indicating a failure of polarization toward Rae-1γ targets. These findings reveal an absolute requirement for Vav1 in regulating microtubule dynamics in the context of NKG2D-DAP10 signaling.

Discussion

Previous studies demonstrated the requirement for Vav proteins in NK cytotoxicity mediated by ITAM-containing adaptors such as DAP12 and FcRγ; however, evidence for the requirement for Vav proteins downstream of the DAP10-associated receptor NKG2D has been complicated by the fact that murine NKG2D associates with both DAP10 and DAP12 (13–15). In this report, we provide genetic evidence for the critical role of Vav1 in natural cytotoxicity induced by NKG2D-DAP10 in the absence of DAP12-generated ITAM signals. Moreover, we provide biochemical evidence that Vav1 can interact with DAP10 through the linker Grb2, although it is possible that Vav1 and Grb2 may also interact indirectly within a DAP10-signalosome.

DAP10 has been shown to recruit PI3K to its YxNM motif (10), and our data suggest a model, not mutually exclusive with this notion, in which DAP10 YxNM motifs recruit Grb2. Vav1 may be recruited directly to Grb2 and/or activated by phosphatidylinositol 3,4,5-trisphosphate generated by PI3K (27). Indeed, a recent report (28) suggested that Vav1 can be activated by PI3K, although these studies did not distinguish between multiple individual NK-activating receptors that could have been engaged. In a separate report (17), Vav was proposed to act upstream of PI3K based on the observation that inhibition of PI3K with wortmannin failed to block phosphorylation of Vav in human NK cells stimulated with anti-NKG2D. Despite these apparently conflicting models, Vav1 may act both upstream and downstream of PI3K in the context of NKG2D-DAP10 signaling. Of note, evidence in T cells suggests that Vav and PI3K participate in a self-reinforcing, positive feedback loop downstream of the TCR (29). In the context of DAP10 signaling, our data provide an explanation for how Grb2-Vav may act cooperatively with PI3K.

In addition to demonstrating the requirement of Vav1 in NKG2D-DAP10 signaling, we identify a previously unknown role for Vav1 in the regulation of postconjugation events at the NK-target interface. Specifically, we show that Vav1 is essential for F-actin polymerization at the NK-target contact site and for polarization of MTOCs toward the target cell. The qualitative nature of the cytoskeletal defects observed in Vav1$^{-/-}$DAP12$^{-/-}$ NK cells is underscored by the fact that postconjugation events are blocked completely. Based on these findings, we propose a novel model for Vav1 function in DAP10-mediated cytotoxicity, implicating a critical and receptor-proximal role for Vav1 as a regulator of cytoskeletal dynamics.

In vivo, NKG2D-DAP10 signaling through Vav1 occurs in concert with many additional signaling pathways. NK cells possess a

diverse repertoire of activating and inhibitory receptors, which interact with specific ligands on potential target cells and transduce opposing signals. NK cells must integrate and interpret these signals to discriminate between infected or transformed cells and healthy cells. The importance of Vav proteins for propagating activation signals downstream of receptors containing ITAMs is well documented (18); however, we report an additional role for Vav1 in NK cell activation initiated by DAP10 YxNM motifs. Given the widespread requirement for Vav proteins in NK cell activation, it follows that Vav would be a target for antagonism by NK inhibitory receptors. Indeed, recruitment of SHP-1 to ITIMs in inhibitory receptors appears to specifically target Vav1 and lead to its dephosphorylation (21). In this context, our findings suggest a possibility that dephosphorylation of Vav (30, 31) may be functionally linked to inhibition of postconjugation events such as actin polymerization and MTOC polarization. Of note, the NK inhibitory synapse lacks robust F-actin accumulation and MTOC polarity, which is consistent with a mechanism involving Vav dephosphorylation as a result of Src homology region 2 domain-containing phosphatase-1 recruitment to ITIM-containing NK inhibitory receptors (32, 33). Thus, Vav may represent a critical point of convergence between opposing activation and inhibitory signals in NK cells.

While Vav1 is absolutely required for DAP10-mediated natural cytotoxicity, it is dispensable for several ITAM-mediated signaling events, including DAP12-mediated natural cytotoxicity (18), generation of Ag-specific T cell-APC immune synapses (34), and TCR-induced formation of signaling microclusters and F-actin polymerization (our unpublished observations). In this context, we note that the strict dependence of DAP10 signaling on Vav1 illuminates a highly specialized signal transduction module in NK cells.

Note added in proof. During review of this manuscript, another group reported that DAP10 signals through a GRB2/Vav1 complex in human NK cells (35).

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Disclosures

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