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NKG2D-DAP10 triggers human NK cell–mediated killing via a Syk-independent regulatory pathway

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The immune recognition receptor complex NKG2D-DAP10 on natural killer cells is stimulated by specific ligands carried on virus-infected and malignant cells. Because DAP10 does not have an immunoreceptor tyrosine-based activation motif (ITAM) in its cytoplasmic tail, its ability to trigger killing has been debated. Here we show that a crucial Tyr-Ile-Asn-Met amino acid motif in the cytoplasmic tail of DAP10 couples receptor stimulation to the downstream activation of phosphatidylinositol 3-kinase, Vav1, Rho family GTPases and phospholipase C. Unlike that of ITAM-containing receptors, the activation of NKG2D-DAP10 proceeds independently of Syk family protein tyrosine kinases. Yet the signals initiated by NKG2D-DAP10 are fully capable of inducing killing. Our findings identify a previously unknown mechanism by which receptor complexes that lack ITAM motifs can trigger lymphocyte activation.

Natural killer (NK) cells are a subpopulation of phenotypically identifiable lymphocytes that lack surface immunoglobulin and CD3, express CD16 and mediate the killing of some virus-infected and tumor cells. The mechanistic basis for immune recognition during natural cytotoxicity remained enigmatic for decades, but the molecular identification of activating receptors on NK cells has provided insight into this form of lymphocyte activation[1–3].

One of the activating receptors that has been implicated as a key trigger for some forms of NK cell–mediated cytotoxicity is the C-type lectin–like receptor NKG2D. This receptor specifically recognizes different 'stress-induced', major histocompatibility complex (MHC) class I molecule–like structures, including MICA, MICB and UL16-binding proteins (ULBPs) in humans, and retinoic acid early transcript-1, minor histocompatibility antigen H-60 and murine ULBP-like transcript-1 in mice[4–8]. The upregulation of these molecules during infection or cancer progression has refocused attention on the potentially important role of NK cells in immunosurveillance[9].

So far many investigations have concentrated on the ability of multisubunit immune recognition receptors to use ITAM-containing subunits to initiate immune responses. Molecular characterizations of activating receptor complexes such as NGK2D-DAP10 and 2B4-SAP have also underscored the potential of receptor complexes without ITAM motifs to stimulate granule release and cell-mediated cytotoxicity. For NKG2D-DAP10, the phosphatidylinositol 3-kinase (PI3K)-binding motif Tyr-Ile-Asn-Met (YINM) in the cytoplasmic tail of DAP10 is reminiscent of signaling modules associated with costimulation (such as CD28)[10]. Two reports[11,12] have raised the possibility that DAP10 may not be the only signaling partner of NKG2D in mouse lymphocytes, and that perhaps 'promiscuous' association with an ITAM-containing DAP12 may be required for NKG2D-initiated killing.

Our preliminary observations indicated that some chimeric receptors lacking ITAM-signaling modules could initiate NK cell–mediated killing. We therefore specifically investigated whether NKG2D could trigger NK cell activation without associating with an ITAM-containing subunit. We report here that stimulation of an NKG2D-DAP10 complex is fully sufficient to trigger NKG2D-mediated killing and that its regulatory mechanisms are distinct from those used by ITAM-containing receptor complexes.

RESULTS

Regulation of cytotoxicity versus cytokine production

NKG2D receptors are expressed on all NK cells and on activated $CD8^+$ T cells, activated macrophages and T cells expressing the T cell antigen receptor (TCR) γ- and δ-subunits (γδ T cells)[4]. To assess the role of NKG2D-initiated signaling in NK cell activation, we used homogeneous, cloned populations of nontransformed human NK cells. Selective ligation of the NKG2D receptors expressed on these NK cells with a monoclonal antibody triggered granule release (**Fig. 1a**) and cell-mediated cytotoxicity (**Fig. 1b**). Although ligation of NKG2D receptors alone was sufficient to induce granule release and killing, it was insufficient for inducing the production of interferon-γ.

By contrast, the activation of ITAM-containing receptors such as FcγRIII (hereafter referred to as FcR) was sufficient to induce interferon-γ production from NK cells (**Fig. 1a**) and to trigger granule release (**Fig. 1a**) and cell-mediated cytotoxicity (**Fig. 1b**). Thus, although NKG2D on human NK cells is a 'triggering' receptor for granule-dependent cytotoxicity, it is an insufficient stimulus for the transcription-dependent production of specific cytokines.

NKG2D regulation by proximal tyrosine kinases

Because Src and Syk family protein tyrosine kinases (PTKs) have been implicated in some modes of lymphocyte activation, we examined whether NKG2D stimulation could initiate a similar PTK cascade. NKG2D-triggered cytotoxicity was inhibited in a concentration-

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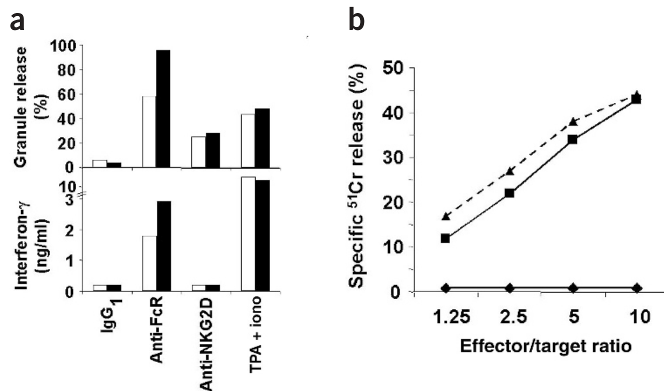

Figure 1 Differential regulation of NK cell–mediated cytotoxicity versus cytokine production. (**a**) Cloned human NK cells were stimulated for 4 h (top) or 20 h (bottom) at 37 °C with 1 g/ml of plate-bound antibody (IgG_1, anti-FcR or anti-NKG2D) or with TPA plus ionomycin. Granule release was measured by a BLT-esterase assay and IFN-γ production by a sandwich ELISA. Two independent experiments (open and filled bars) are shown. (**b**) Cloned human NK cells were incubated at the indicated effector/target ratios with ^{51}Cr-labeled P815 cells in the absence (diamonds) or presence of anti-FcR (triangles) or anti-NKG2D (squares). After 4 h at 37 °C, the amount of ^{51}Cr released into the supernatants was measured.

dependent manner by the Src family PTK inhibitor PP2, with a half-maximal inhibitory concentration (IC_{50}) similar to that for inhibiting killing initiated by the ITAM-containing FcR complex (**Fig. 2a**).

The requirement for a proximal Src family PTK in NKG2D-initiated regulation was further confirmed using a somatic genetic approach. Flag-tagged CD4-DAP10 chimeric receptors were expressed in either wild-type or Lck-deficient Jurkat (Jurkat versus JCaM1)[13] cells to assess the role of Src family PTKs in coupling the activation of DAP10 to downstream signaling events. In this genetic model, activation of NKG2D was coupled to the tyrosine phosphorylation of phospholipase C-γ (PLC-γ) in Lck^+ Jurkat but not in Lck^- JCaM1 cells (**Fig. 2b**). These experiments verify the requirement for Src family PTKs in coupling the stimulation of DAP10 to the downstream activation of PLC-γ.

After Src family PTK–dependent phosphorylation of ITAM-containing receptors, Syk family PTKs are required for signal propagation. Thus, if NKG2D stimulation proceeds via the DAP12 signal-transducing subunit expressed on NK cells, then tyrosine phosphorylation of ZAP70 and Syk should occur. For NKG2D-initiated lymphocyte activation, however, we found no requirement for activation of Syk family PTKs (**Fig. 2c,d**). Selective ligation of endogenous NKG2D receptors did not induce the tyrosine phosphorylation of either Syk (**Fig. 2c**) or ZAP70 (**Fig. 2d**).

In additional experiments, we tested the ability of the Flag-tagged CD4-DAP10 chimeric receptor to function in the ZAP70-deficient, Syk-deficient p116 subline of Jurkat[14]. In the absence of ZAP70 and Syk, activation of CD4-DAP10 coupled to downstream signals such as PLC-γ activation (data not shown). Taken together, these data suggest that NKG2D can trigger NK cell–mediated cytotoxicity by a mechanism dependent on Src family PTKs but not Syk family PTKs.

Coupling of NKG2D stimulation to downstream signals

Protein-protein interactions dictated by the subdomain structures of adaptor molecules are crucial in lymphocyte activation. Although both SLP76 (ref. 15) and LAT[16] have been proposed to regulate ITAM-containing receptors in human NK cells, their role in NK cell activation initiated by NKG2D ligation remains unexplored. Because SLP76 and LAT become tyrosine phosphorylated after stimulation by ITAM-containing receptors complexes, we assessed whether similar biochemical modifications occurred after NKG2D-initiated signaling (**Fig. 3**). These analyses indicated that selective crosslinking of endogenous NKG2D induced the tyrosine phosphorylation of SLP76 (**Fig. 3a**) but not LAT (**Fig. 3b**).

We next evaluated whether the Syk-independent, LAT-independent proximal signaling mechanism could couple to the downstream guanine nucleotide exchange factor (GEF) Vav1 (**Fig. 3c**) and to PLC (**Fig. 3d**). NKG2D stimulation resulted in the tyrosine phosphorylation of Vav1 (**Fig. 3c**). The functional significance of the biochemical modification of Vav1 activation was underscored by the observation

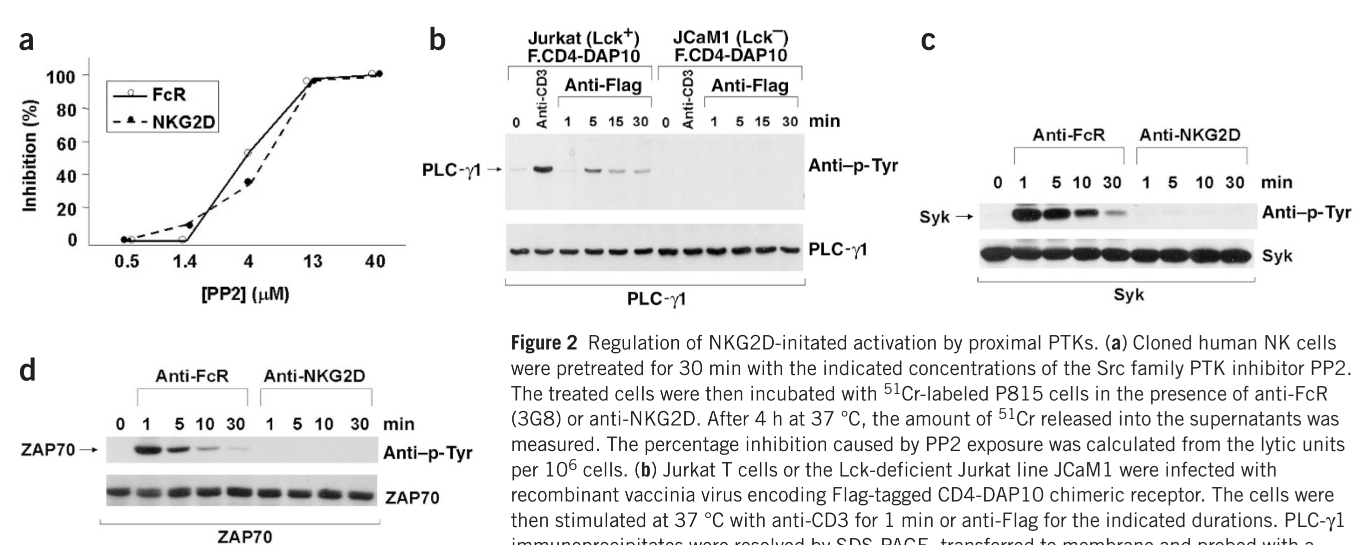

Figure 2 Regulation of NKG2D-initated activation by proximal PTKs. (**a**) Cloned human NK cells were pretreated for 30 min with the indicated concentrations of the Src family PTK inhibitor PP2. The treated cells were then incubated with ^{51}Cr-labeled P815 cells in the presence of anti-FcR (3G8) or anti-NKG2D. After 4 h at 37 °C, the amount of ^{51}Cr released into the supernatants was measured. The percentage inhibition caused by PP2 exposure was calculated from the lytic units per 10^6 cells. (**b**) Jurkat T cells or the Lck-deficient Jurkat line JCaM1 were infected with recombinant vaccinia virus encoding Flag-tagged CD4-DAP10 chimeric receptor. The cells were then stimulated at 37 °C with anti-CD3 for 1 min or anti-Flag for the indicated durations. PLC-γ1 immunoprecipitates were resolved by SDS-PAGE, transferred to membrane and probed with a monoclonal antibody to phosphotyrosine (top) or polyclonal rabbit antisera to PLC-γ1 (bottom). (**c**,**d**) Human NK cells were stimulated at 37 °C with anti-FcR or anti-NKG2D for the indicated durations. Syk (**c**) or ZAP70 (**d**) immunoprecipitates from each sample were treated as in **b** and probed with a monoclonal antibody to phosphotyrosine (top) or the indicated polyclonal rabbit antisera (bottom). In **b–d**, results are representative of three separate experiments.

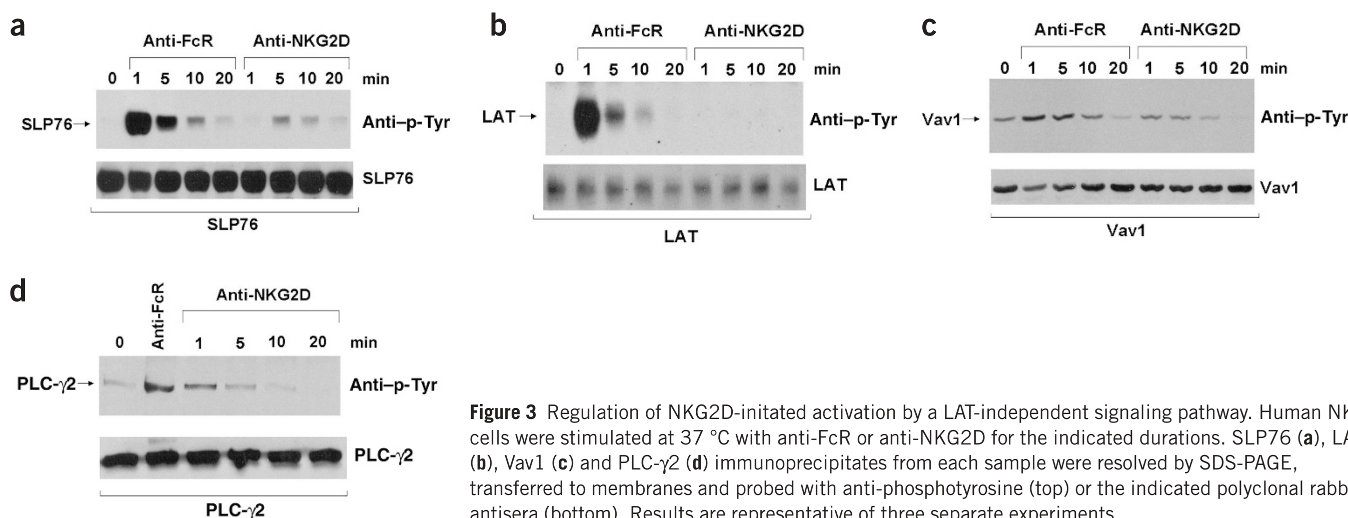

Figure 3 Regulation of NKG2D-initated activation by a LAT-independent signaling pathway. Human NK cells were stimulated at 37 °C with anti-FcR or anti-NKG2D for the indicated durations. SLP76 (**a**), LAT (**b**), Vav1 (**c**) and PLC-γ2 (**d**) immunoprecipitates from each sample were resolved by SDS-PAGE, transferred to membranes and probed with anti-phosphotyrosine (top) or the indicated polyclonal rabbit antisera (bottom). Results are representative of three separate experiments.

that overexpression of Vav1 in NK cells enhanced NKG2D-initiated killing, whereas the expression of mutant Vav1 constructs lacking GEF activity (Vav-dbl$^-$) or containing only the carboxy-terminal region (Vav-trunc) did not (**Fig. 4a**).

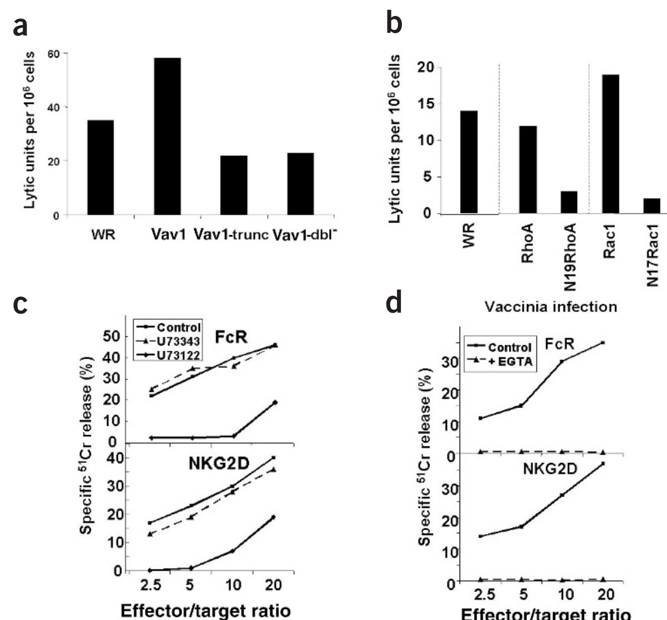

Figure 4 Regulation of NKG2D-initiated killing by Vav1, RhoA, Rac1 and PLC-γ2. Human NK cells were infected for 5 h with recombinant vaccinia virus encoding the indicated variants of Vav1 (**a**) or RhoA and Rac1 (**b**), or with control virus (WR). The cells were then incubated with ^{51}Cr-labeled P815 cells in the presence of anti-NKG2D. After 4 h at 37 °C, the amount of ^{51}Cr released into the supernatants was measured. Data are expressed as lytic units per 10^6 cells. Results are representative of four separate experiments. The NK cells did not kill ^{51}Cr-labeled P815 cells in the absence of anti-NKG2D (not shown). (**c**) Human NK cells were incubated for 5 min at 37 °C with the PLC-γ inhibitor U73122, the inactive analog U73343 or a dimethylsulfoxide control, and assayed as cytotoxic effectors against ^{51}Cr-labeled P815 cells in the presence of anti-FcR or anti-NKG2D. (**d**) Human NK cells were assayed as cytotoxic effectors against ^{51}Cr-labeled P815 cells with the indicated monoclonal antibodies and in the presence or absence of EGTA.

Because activated Vav family members act as potential exchange factors for the low molecular weight Rho-Rac family GTPases, we thought that RhoA or Rac1 might influence NKG2D-initiated NK cell activation. Consistent with this hypothesis, expression of dominant-negative versions of RhoA (N19RhoA) or Rac1 (N17Rac1) in NK cells considerably inhibited NKG2D-initiated killing as compared with the expression of wild-type RhoA or wild-type Rac1 or after infection with the control vaccinia virus WR (**Fig. 4b**). These data suggest that NKG2D does use Vav family GEFs and Rho-Rac family GTPases during the development of NK cell–mediated cytotoxicity. It has been also shown that Vav1 knockout mice have impaired killing of cells expressing NKG2D ligands (F. Colucci and S. Zompi, personal communication).

Receptor-initiated activation of PLC is also a feature of several modes of lymphocyte activation. Activated PLC hydrolyzes membrane phosphatidylinositol(4,5)bisphosphate into inositol(1,4,5)trisphosphate and *sn*-1,2-diacylglycerol, which in turn mediate the mobilization of intracellular calcium and the activation of protein kinase C, respectively. To assess whether PLC has a role in NKG2D-initated activation of NK cells, we first examined whether receptor ligation induced the biochemical modification of PLC-γ2. NKG2D crosslinking triggered the tyrosine phosphorylation of PLC-γ2, which was detectable within 1 min and returned to baseline within 10–20 min (**Fig. 3d**). We assessed the functional significance of these changes to PLC-γ with the PLC inhibitor U73122. This inhibitor potently inhibited NKG2D-initiated killing, whereas the pharmacologically inactive analog U73343 did not (**Fig. 4c**).

Consistent with a role for PLC-γ in NKG2D-dependent regulation, the calcium signal generated by PLC-γ was required for NKG2D-initiated killing (**Fig. 4d**). Taken together, these data suggest that NKG2D controls NK cell activation via a previously unknown regulatory pathway—that is, a Syk-independent, LAT-independent coupling of the receptor to the downstream effectors Vav1, Rho family GTPases and PLC-γ2.

DAP10-dependent triggering of NKG2D-initiated killing

Reports suggest that NKG2D on murine NK cells can associate with the ITAM-containing DAP12 subunit[11,12]. As noted above, however, the Syk family PTK–independent regulation of our homogeneous, clonal population of human NK cells was not consistent with DAP12-associated activation. We therefore directly evaluated whether DAP12 is used during NKG2D activation in human NK cells.

First, we assessed whether DAP12 is biochemically modified after NKG2D ligation. The tyrosine phosphorylation of DAP12 is a characteristic of DAP12-associated receptor complexes[17]. Stimulation of

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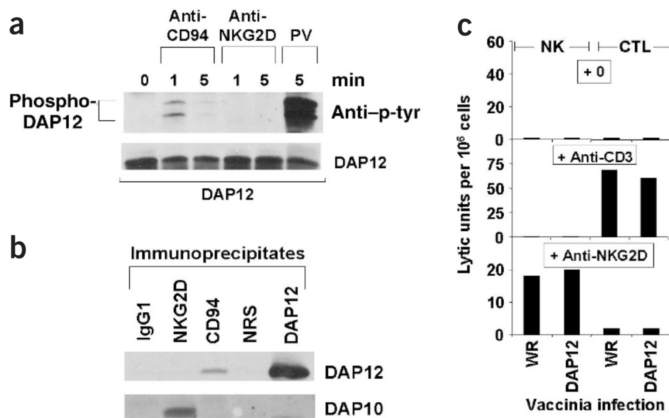

Figure 5 Lack of association of NKG2D with DAP12 in clonal, nontransformed human NK cell lines. Human NK cell lines were functionally screened in ^{51}Cr-release assays for clonal populations expressing the activating form of the CD94-DAP12 complex. (**a**) The NK cells were stimulated at 37 °C for the indicated durations with anti-CD94, anti-NKG2D or pervanadate (PV). DAP12 immunoprecipitates from each sample were resolved by SDS-PAGE, transferred to membrane, and probed with a monoclonal antibody to phosphotyrosine (top) or polyclonal rabbit antisera to DAP12 (bottom). (**b**) Lysates from human NK cells expressing the activating form of CD94 were subjected to immunoprecipitation as indicated, treated as in **a**, and probed with polyclonal rabbit antisera to DAP12 (top) or a monoclonal antibody to DAP10 (bottom). In **a**,**b**, results are representative of three separate experiments. (**c**) NK cells or CD8+ T cells were infected for 5 h at 37 °C with either control virus (WR) or recombinant vaccinia virus encoding full-length DAP12. The effector cells were tested for their ability to kill ^{51}Cr-labeled P815 cells in the absence of antibody (top) or in the presence of anti-CD3 (middle) or anti-NKG2D (bottom). Results are representative of experiments using five different NK clones and six different CTL clones.

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clonal populations of NK cells bearing the activating form of the CD94-DAP12 complex with a CD94-specific monoclonal antibody resulted in tyrosine phosphorylation of DAP12 (**Fig. 5a**). By contrast, ligation of NKG2D, which induced the tyrosine phosphorylation of DAP10 (data not shown), did not result in detectable tyrosine phosphorylation of DAP12 in clonal populations of human NK cells (**Fig. 5a**). We also determined whether there was a physical association between NKG2D and DAP12 in human NK cells. Although the activating form of CD94 associated with DAP12, and NKG2D associated with DAP10, no association was detected between NKG2D and DAP12 in cell lysates prepared using Triton X-100 (**Fig. 5b**), digitonin (data not shown) or Nonidet P-40 (data not shown).

Because a study[11] has suggested that DAP12 may allow NKG2D to function as a triggering receptor on murine CD8+ T cells, we examined NKG2D-initiated functions in clonal populations of human CD8+ T cell lines. Consistent with previous analyses[4], all of the human CD8+ T cell lines expressed NKG2D, but not DAP12, on their surface (data not shown). Although the CD8+ T cell lines could be triggered to kill after ligation of CD3, no killing was triggered after NKG2D ligation (**Fig. 5c**).

To assess whether the absence of DAP12 was responsible for the inability of the CD8+ T cells to mediate NKG2D-dependent killing, we expressed full-length DAP12 in the CD8+ T cells using recombinant vaccinia virus (**Fig. 5c**). Immunoblot analysis showed that there was no DAP12 in the T cells infected with the control WR vaccinia virus, but high expression of DAP12 in T cells infected with recombinant vaccinia encoding full-length DAP12 (data not shown). Expression of DAP12 did not enable the CD8+ T cells to mediate NKG2D-initiated killing (**Fig. 5c**). Thus, factors other than DAP12 are responsible for the inability of human CD8+ T cells to mediate killing after NKG2D ligation.

We expressed chimeric receptors on human NK cells to determine directly whether the DAP10 cytoplasmic tail can generate NK cell–mediated cytotoxicity and whether the DAP10-initiated signals are the same as those seen after crosslinking endogenous NKG2D receptors. Recombinant vaccinia viruses were generated expressing Flag-tagged CD4 chimeric receptors with no cytoplasmic tail (Flag–CD4-TM), ITAM-containing cytoplasmic tails (Flag–CD4-ζ, Flag–CD4-γ, Flag–CD4-DAP12) or the DAP10 cytoplasmic tail (Flag–CD4-DAP10). As expected, the chimeric receptors with ITAM-containing cytoplasmic tails triggered killing, whereas the tailless chimeric receptor did not (**Fig. 6**). Notably, the chimeric receptor with the DAP10 cytoplasmic tail also triggered effective killing in all of the 30 human NK clones in which it was expressed (**Fig. 6**). Although the chimeric receptors were expressed equivalently on the surface of the human NK clones, the CD4-γ chimeric receptor consistently triggered the greatest degree of killing, whereas the CD4-DAP10 receptor triggered quantitatively less killing than did the ITAM-containing chimeric receptors.

If the endogenous NKG2D receptor on human NK cells selectively uses DAP10 to regulate the initiation of killing, then the proximal signals generated by crosslinking of endogenous NKG2D receptors should be similar to those initiated by selective ligation of the Flag–CD4-DAP10 chimeric receptor. Consistent with this hypothesis, crosslinking of endogenous NKG2D (**Figs. 2c**,**d** and **3a-d**) or the DAP10-containing chimeric receptor (**Fig. 7a–f**) resulted in coupling to activation of Vav1, SLP-76 and PLC-γ2 without detectable tyrosine phosphorylation of Syk, ZAP70 or LAT.

Taken together, these analyses based on both biochemical and genetic approaches indicate that NKG2D stimulation on human NK cells couples to the signal-transducing subunit DAP10 to generate selective proximal signals that are fully capable of initiating NK cell–mediated cytotoxicity.

The YINM motif of DAP10

Previous analyses have shown that binding of the p85 regulatory subunit of PI3K to the tyrosine-phosphorylated YINM motif of DAP10

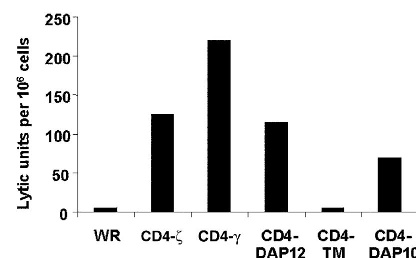

Figure 6 DAP10-dependent induction of NK cell–mediated cytotoxicity. Separate aliquots of human NK cells were infected with recombinant vaccinia viruses encoding Flag-tagged CD4 chimeric receptors either lacking a cytoplasmic tail (Flag–CD4-TM) or containing cytoplasmic tails from ITAM-containing signal transducing subunits (Flag–CD4-ζ, Flag–CD4-γ, Flag–CD4-DAP12) or from DAP10 (Flag–CD4-DAP10), as indicated. The cells were then incubated with ^{51}Cr-labeled P815 cells in the presence of anti-CD4. Results are representative of ten separate experiments.

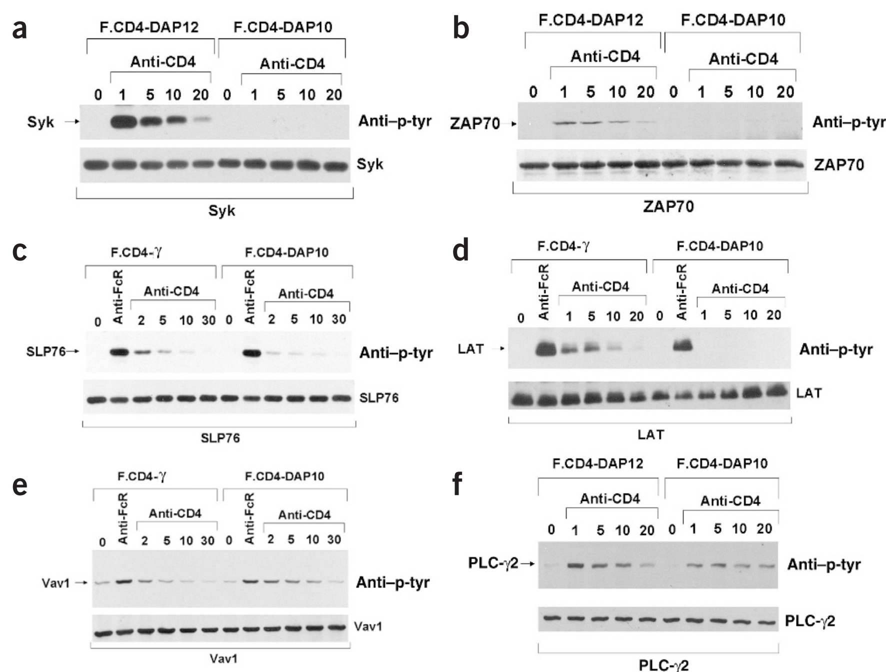

Figure 7 DAP10-dependent regulation of NK cell activation by Syk PTK–independent and LAT-independent pathways. Human NK cells were infected with recombinant vaccinia virus encoding the indicated Flag-tagged chimeric receptors. The cells were then stimulated at 37 °C with anti-FcR for 1 min or with anti-CD4 for the indicated durations. Syk (**a**), ZAP70 (**b**), SLP76 (**c**), LAT (**d**), Vav1 (**e**) and PLC-γ2 (**f**) immunoprecipitates were resolved by SDS-PAGE, transferred to membranes and then probed with either monoclonal antibodies to phosphotyrosine (top) or the indicated polyclonal rabbit antisera (bottom). Results are representative of three separate experiments.

couples NKG2D stimulation to activation of PI3K[10]. The importance of this mechanism is underscored by functional studies showing that NKG2D-initiated activation of NK cell–mediated killing is inhibited in a concentration-dependent manner by the PI3K inhibitor wortmannin (data not shown). The relationship of PI3K activation to the other NKG2D-initiated signaling events (such as the tyrosine phosphorylation of Vav1 and PLC-γ) remains, however, unknown.

Pharmacological inhibition of PI3K using wortmannin concentrations that fully inhibited both the catalytic activity of PI3K and NK cell–mediated cytotoxicity did not affect the capacity of endogenous NKG2D receptors (**Fig. 8a**) or the DAP10-containing chimeric receptors (**Fig. 8b**) to stimulate the tyrosine phosphorylation of Vav1 and PLC-γ2. By contrast, a tyrosine-to-phenylalanine mutation in the YINM motif of DAP10 abolished the activation of Vav1 (**Fig. 8c**) and PLC-γ2 (**Fig. 8d**). These results indicate that, although the catalytic activity of PI3K does not influence NKG2D coupling to Vav1 and PLC-γ2, the YINM motif has an essential role in this process. This is consistent with a mechanism whereby unknown signaling molecules

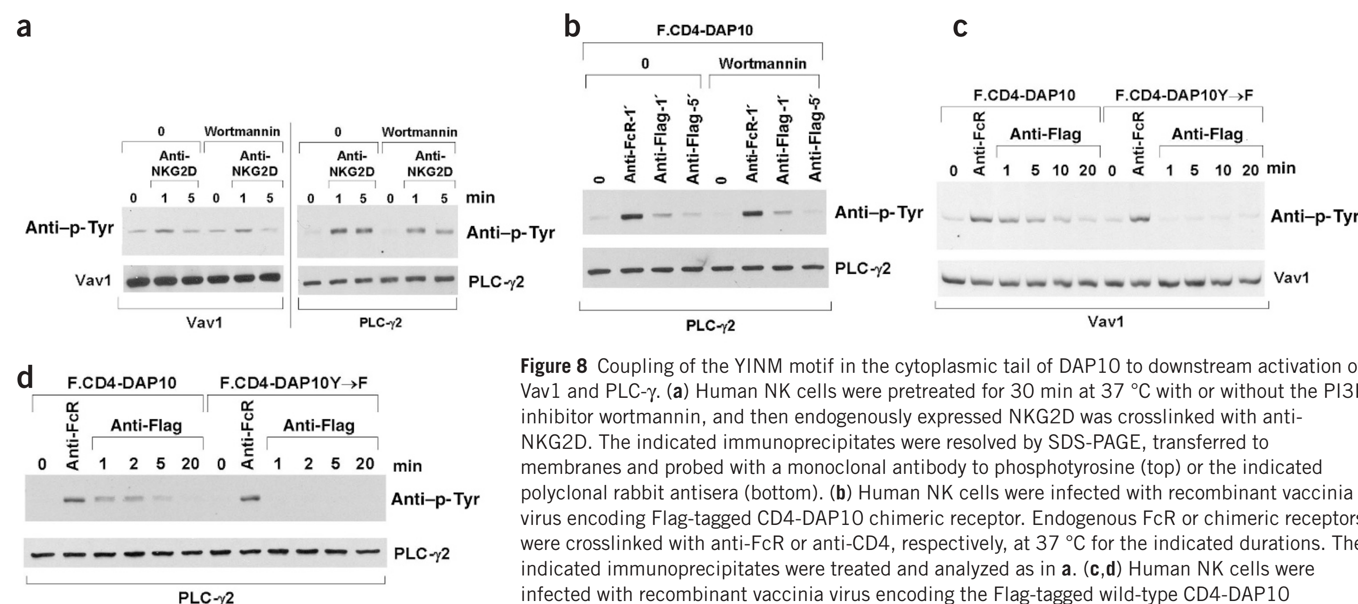

Figure 8 Coupling of the YINM motif in the cytoplasmic tail of DAP10 to downstream activation of Vav1 and PLC-γ. (**a**) Human NK cells were pretreated for 30 min at 37 °C with or without the PI3K inhibitor wortmannin, and then endogenously expressed NKG2D was crosslinked with anti-NKG2D. The indicated immunoprecipitates were resolved by SDS-PAGE, transferred to membranes and probed with a monoclonal antibody to phosphotyrosine (top) or the indicated polyclonal rabbit antisera (bottom). (**b**) Human NK cells were infected with recombinant vaccinia virus encoding Flag-tagged CD4-DAP10 chimeric receptor. Endogenous FcR or chimeric receptors were crosslinked with anti-FcR or anti-CD4, respectively, at 37 °C for the indicated durations. The indicated immunoprecipitates were treated and analyzed as in **a**. (**c**,**d**) Human NK cells were infected with recombinant vaccinia virus encoding the Flag-tagged wild-type CD4-DAP10 chimeric receptor or a CD4-DAP10 chimeric receptor with a tyrosine to phenylalanine (Y→F) mutation in the YINM cytoplasmic motif. Endogenous FcR or chimeric receptors were then crosslinked with anti-FcR or anti-CD4, respectively, at 37 °C for the indicated durations, and the indicated immunoprecipitates were treated and analyzed as in **a**. Results are representative of three separate experiments.

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either bind directly to the phosphorylated YINM motif or do so in association with p85. In either case, unlike ITAM-containing receptor complexes, the YINM motif in DAP10 in NK cells can couple to Vav1 and PLC-γ2 independently of the activity of Syk family PTKs.

DISCUSSION

Our understanding of the regulation of diverse immune effector cells has been aided by the recognition of conserved signaling motifs. In particular, many immune recognition receptors use ITAMs in their cytoplasmic tails to initiate cellular activation[18]. There are also several costimulatory receptors that contain different signaling modules, such as the Tyr-x-x-Met (YxxM) motif of CD28 and inducible costimulator (ICOS), to amplify and to modulate cellular responses. The prevailing model suggests that unlike ITAM-containing receptor complexes, costimulatory receptors by themselves cannot drive specific effector responses. Two reports[11,12] provide a variation on this theme. Specifically, it has been shown that the immune recognition receptor NKG2D can interact with either an ITAM-containing subunit (DAP12) or a YxxM-containing subunit (DAP10) and, depending on the expression pattern of these subunits in different immune cells (such as the presence of DAP12 in NK cells and its absence in resting T cells), there are differential cellular responses.

Our results suggests another level of complexity—that is, although signaling modules containing a YxxM motif are costimulatory only in specific lymphocytes such as T cells, these non-ITAM-containing signaling modules can be fully activating in other types of cell such as NK cells. Our data using chimeric receptors are consistent with this hypothesis. The capacity of the YxxM-containing module to fully activate killing in NK cells is also consistent with reports that CD28 (refs. 19,20) or ICOS[21]—two receptors that are traditionally considered to be costimulators in T cells—can trigger cell-mediated cytotoxicity in NK or 'NK-like' cells. Because NKG2D crosslinking by itself on human NK cells is insufficient to trigger cytokine production (ref. 22 and our results), it will be interesting to assess the differential signaling requirements for NKG2D-initiated transcription versus NKG2D-initiated granule release.

Although DAP12 has been shown to couple with NKG2D in murine NK cells[11,12], similar findings in human NK cells have not been reported. In our human NK clones, which contain both DAP12 and DAP10, we did not detect any physical association between NKG2D and DAP12. This is consistent with previous studies that found no evidence for an association between human NKG2D and human DAP12 (refs. 10,22). NKG2D crosslinking also resulted in no detectable tyrosine phosphorylation of DAP12 in our human NK clones. An NKG2D-initiated Syk-independent regulatory pathway is also not consistent with the involvement of DAP12.

The similarity in signals after crosslinking endogenous NKG2D receptors and after stimulation of the CD4-DAP10 chimeric receptor was striking. It is possible that there is a considerable difference between human and murine NK cells in the use of signal-transducing adaptor subunits. There is a precedent for this in the use of ζ- and γ-subunits by the human FcR but only γ-subunits by the murine FcR[23–27]. Alternatively, we cannot rule out the possibility that, even though NKG2D, DAP12 and DAP10 are present in our NK clones, coupling between NKG2D and DAP12 is dependent on a particular state of activation not found in these NK clones. Additional analyses will be needed to determine the mechanistic basis of these differences.

Despite these issues concerning the conditions required for NKG2D to couple with DAP12, it is increasingly clear that DAP12 is not required to initiate killing. In addition to our own observation that the CD4-DAP10 chimeric receptor could trigger killing, published observations support this hypothesis. First, NK cells from mice expressing a DAP12 mutant lacking a functional ITAM can preferentially kill H-60–bearing targets after NKG2D recognition (see Supplementary Fig. 2b in ref. 11). Second, NK cells from mice deficient for DAP10 show reduced killing of target cells bearing NKG2D ligands[12]. Last, activated NK cells from mice with global functional defects for ITAM-containing receptors (owing to the lack of both Syk and ZAP70) can preferentially kill targets bearing NKG2D ligands[28]. Taken together, these findings suggest that NKG2D receptors on NK cells do not require an association with DAP12 to generate NK cell–mediated cytotoxicity against target cells bearing NKG2D ligands.

Previous work has suggested that the tyrosine-phosphorylated YxxM motif of the cytoplasmic tail of DAP10 recruits the p85 regulatory subunit of PI3K[10]. This is consistent with the hypothesis that PI3K catalytic activity is required for NKG2D-initiated killing. An additional feature of the YxxM motif, however, is its requirement for coupling NKG2D stimulation to the activation of PLC-γ2 and Vav1. The catalytic activity of PI3K is not required for this coupling mechanism. These observations suggest two alternative mechanisms. First, the phosphorylated form of the consensus motif might bind to a signaling component that is separate from PI3K. So far, however, no such binding partner has been identified. Second, after recruitment of the p85 regulatory subunit of PI3K to NKG2D, p85 might associate both physically and functionally with other signaling components. Precedents for a p85-Vav1 association have been described in models of B cell activation[29] and cytokine-induced differentiation[30]. Thus, a macromolecular complex might form in which p85-associated Vav family members could interact with SLP76 and PLC-γ and also act as GEFs for required Rho-Rac family GTPases (RhoA and Rac1). Ultimately, these potential interactions allow NKG2D-initiated activation to proceed with a previously unidentified Syk-independent and LAT-independent regulatory mechanism for cellular activation initiated by multisubunit immune recognition receptors.

METHODS

Reagents, cells and antibodies. Unless otherwise stated, all chemicals were from Sigma. The human Jurkat cell line, the JCaM1 subline and the murine mastocytoma cell line P815 were from the American Type Culture Collection. Human NK cells and $CD8^+$ cytotoxic T lymphocytes were cloned and passaged as described[31]. We purified the 3G8 monoclonal antibody to FcγRIIIA by affinity chromatography over protein A–agarose. We also used monoclonal antibodies to NKG2D (R&D Systems), Flag (M2, Sigma-Aldrich), CD4 (Becton Dickinson), Syk (Santa Cruz Biotechnology) and phosphotyrosine (4G10, Upstate Cell Signaling Solutions). Rabbit polyclonal antiserum to DAP12 was obtained after immunizing rabbits with the keyhole limpet hemocyanin (KLH)-conjugated DAP12 peptide 87–113 (Cocalico Biologicals). The rabbit polyclonal antibodies to PLC-γ1, PLC-γ2, Vav1, ZAP70, SLP76 and LAT were prepared as described[15,16,32–34]. We purchased the Src family PTK inhibitor PP2 from Biomol Research Labs.

Generation of DNA constructs and recombinant vaccinia. The recombinant Flag-tagged proteins RhoA, N19RhoA, Rac1 and N17Rac1 have been described[34,35]. The Flag-tagged CD4 chimeric receptors were generated by polymerase chain reaction and standard molecular biology techniques. In brief, the sequence encoding the CD8 leader and Flag epitope were amplified from pSP11-CD8L-Flag-2B4 using the sense-strand oligonucleotide 5′-CTC<u>AAGCTT</u>ACCGAGTCGGCGACACAGTGTGGG-3′ and the antisense-strand oligonucleotide 5′-TCCCCGCGG<u>CTGCAG</u>CTTGTCGTCATCGTCCTTGTCAT-3′. The underlined nucleotides identify engineered restrictions sites or incorporated mutations for all the oligonucleotides listed below. We subcloned the resulting polymerase chain reaction (PCR) product into the vaccinia recombination substrate vector pSP11 as a *Hin*dIII-*Pst*I fragment.

The sequence encoding the CD4 extracellular and transmembrane domain (amino acids (aa) 24–418)[36] was amplified from a vector containing the CD4

complementary DNA (a gift of C.V. Paya, Mayo Clinic, Rochester, Minnesota) using the oligonucleotides 5′-GCCCTGCAGGGAAACAAAGTGGTGCTGGGC-3′ and 5′-CTTGCGGCCGCCGGTGCCGGCACCTGGTACCGAAGAAGATGCC-3′. We subcloned the resulting PCR product into the pSP11 vector containing the CD8L-Flag as a *Pst*I-*Not*I fragment. This vector, Flag-CD4-TM, was used as the basis for generating chimeric receptors with different intracellular signaling domains. Nucleotides encoding cytoplasmic amino acids from the indicated signaling components were amplified by PCR with reverse transcription (RT-PCR) from NK cell messenger RNA and subcloned into pSP11-CD8L-Flag-CD4-TM as *Kpn*I-*Not*I fragments using the following oligonucleotides: DAP10, 5′-CTGGGTACCCGCCCACGCCGCAGC-3′ and 5′-GTCGCGGCCGCAGGAGGGTCAGCCCCTGCC-3′; DAP12, 5′TTCGGTACCCGGCTGGTCCCTCGG-3′ and 5′-GGTATCATGCGGCCGCCTGTCATGATTCGGG-3′; TCR ζ-chain, 5′-TTGGGTACCAGAGTGAAGTTCAGC-3′ and 5′-CTGGGCGGCCGCAGGTCTGGCCTTTGAGTGG-3′; and FcRε γ-chain, 5′-CTCGGTACCCGACTGAAGATCCAA-3′ and 5′-ATGGCGGCCGCTATTCTAAAGCTACTG-3′. We sequenced all constructs in full to ensure fidelity.

Recombinant vaccinia viruses were generated using the various pSP11 CD4 chimeric constructs as described[35]. The DAP10 Y→F mutant was generated using PCR site-directed mutagenesis as described[34] with the oligonucleotides 5′-GCCCAAGATGGCAAAGTCTTCATCAACATGCCAGGCAGG-3′ and 5′-CCTGCCTGGCATGTTGATGAAGACTTTGCCATCTTGGGC-3′.

The recombinant Vav1 vaccinia virus has been described[35]. The GEF-deficient vaccinia virus (Vav-dbl^{-}) contains point mutations (Y209F and L213Q) at two highly conserved residues in the Dbl homology domain that inactivate GEF activity. To make this construct, we mutated the wild-type Vav1 cDNA using the oligonucleotides 5′-CAGACGGAGGAGAAGTCCACTGACACGCAGGGCTCCATCCAGCAG-3′ and 5′-CTGCTGGATGGAGCCCTGCGTGTCAGTGGACTTCTCCTCCGTCTG-3′ and the Quick-Change Site-Directed mutagenesis kit (Stratagene) in accordance with the manufacturer's instructions. To generate the C-terminal portion (aa 598–845) of Vav1 (Vav-trunc), the wild-type Vav1 cDNA was amplified by PCR using the oligonucleotides 5′-CTGGGTCTGCCCAAGCTTGAGGTGTTTCAGGAATAC-3′ and 5′-TGCCGCGGCCGCAGGGCTCAGCAGTATTCAGAATAATC-3′. The resulting PCR product was subcloned into the pSHN11 vector and recombinant vaccinia virus was generated.

The DAP12 cDNA was amplified by RT-PCR from NK cell mRNA using the following oligonucleotide pairs: 5′-GCAGCATCAAGCTTCATGGGGGGACTTGAACCCTGC-3′ and 5′-TGCCAGCACGCCCATGGTCACCGTAG-3′ (aa 1–40) and 5′- CTACGGTGACCATGGGCGTGCTGGCA-3′ and 5′-GGTATCATGCGGCCGCCTGTCATGATTCGGGCTCA-3′ (aa 41–113). We subcloned the resulting PCR products into pSHN11 and inserted a Flag epitope sequence[35] proximal to the putative transmembrane region at the *Nco*I site between aa 39 and 41. The Flag-DAP12 virus was generated as described[35].

Measurements of granule release and cytokine production. We incubated 96-well plates overnight at 4 °C with 1 g/ml of monoclonal antibody to FcR or NKG2D in 0.05 M carbonate buffer, pH 9.6 (100 l per well). After washing the plates, we added NK cell or T cell suspensions (3 10^6 cells/ml) to each well (250 l/well) in RPMI medium containing 10% calf serum and 100 U/ml of interleukin-2. After incubation at 37 °C for 4 h (for granule release) or 20 h (for cytokine production), supernatants were evaluated either for secretion using a standard *N*-benzyloxycarbonyl lysine thiobenzyl ester (BLT) esterase assay[37] or for production of interferon-γ using a sandwich enzyme-linked immunosorbent assay (ELISA) and interferon-γ-specific antibodies[38]. As positive controls, supernatants from NK cells treated with 100 g/ml of *O*-tetradecanoylphorbol 13-acetate (TPA) plus 1 M ionomycin were evaluated using the BLT esterase assay and the interferon-γ-specific ELISA.

Cytotoxicity assays. The ^{51}Cr-release assays were done as described[31]. In some experiments, we pretreated NK cells with the PTK inhibitor PP2 (various concentrations, 30 min), the PLC-γ inhibitor U73122 (3.3 M, 5 min), the pharmacologically inactive analog U73343 (3.3 M, 5 min) or the calcium chelator EGTA (1 mM, 1 min). In all experiments, spontaneous release did not exceed 10% of maximum release. In redirected cytotoxicity assays, NK clones killed the FcR$^+$ P815 target cells only in the presence of monoclonal antibodies to FcR, NKG2D or CD4. We calculated lytic units on the basis of 20% cytotoxicity[39].

Cell stimulation and immunoblot analysis. In experiments involving vaccinia infection, NK clones or Jurkat T cells (5–10 10^6 cells per sample) were infected with control (WR) or recombinant vaccinia virus at a multiplicity of infection (MOI) of 20 for 5 h (NK clones) or 10 for 2 h (Jurkat lines). In experiments evaluating the effects of the PI3K inhibitor wortmannin, cells were preincubated for 30 min at 37 °C with 100 nM wortmannin. In experiments involving specific cell surface receptor crosslinking, cells were incubated for 3 min on ice with the indicated monoclonal antibodies. We then incubated the washed cells with goat anti–mouse IgG F(ab′)$_2$ at 37 °C for the indicated durations. After stimulation, the cells were lysed on ice for 10 min in 1 ml of buffer containing 20 mM Tris-HCl, 40 mM NaCl, 5 mM EDTA, 50 mM NaF, 30 mM $Na_4P_2O_7$, 0.1% bovine serum albumin, 1 mM Na_3VO_4 1 mM PMSF, 5 g/ml of aprotinin, 10 g/ml of leupeptin and 1% Triton X-100, pH 7.4. Cellular debris was removed by centrifugation at 18,000*g* for 5 min at 4 °C.

Immunoprecipitations were done on cell lysates for 1–2 h at 4 °C using either the indicated polyclonal rabbit antisera bound to protein A–Sepharose beads or the indicated mouse monoclonal antibody bound to goat anti–mouse IgG conjugated to agarose beads. Protein complexes were then eluted in 40 l of SDS sample buffer, resolved by SDS-PAGE and transferred to Immobilon-P membranes (Millipore). Tyrosine-phosphorylated proteins were detected using 4G10 monoclonal antibody to phosphotyrosine, followed by sheep anti–mouse IgG coupled to horseradish peroxidase (Amersham Pharmacia Biotech) and the enhanced chemiluminescence (ECL) detection system from Amersham. We used protein A coupled to horseradish peroxidase and the ECL detection system to detect proteins recognized with specific rabbit polyclonal sera.

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COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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