Published in final edited form as:

Sci Signal. ; 5(232): . doi:10.1126/scisignal.2002754.

Complementary Phosphorylation Sites in the Adaptor Protein SLP-76 Promote Synergistic Activation of Natural Killer Cells

Hun Sik Kim^{1,2,3,*} and Eric O. Long^{1,*}

¹Laboratory of Immunogenetics, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Rockville, MD 20852, USA

²Department of Medicine, Graduate School, University of Ulsan, Seoul 138-736, Korea

³Department of Microbiology, Cellular Dysfunction Research Center and BMIT, University of Ulsan College of Medicine, Seoul 138-736, Korea

Abstract

The cytotoxic effects of natural killer (NK) cells and their ability to secrete cytokines require the induction of synergistic signals from co-activation receptors, such as CD314 (NKG2D) and CD244 (2B4), which bind to ligands expressed on target cells. Synergy is required to overcome inhibition of the guanine nucleotide exchange factor (GEF) Vav1, a central regulator of NK cell activation, by the E3 ubiquitin ligase c-Cbl. However, the molecular basis for this synergy is unknown. Here, we showed that the adaptor protein Src homology 2 (SH2) domain-containing leukocyte phosphoprotein of 76 kD (SLP-76) was required for this synergy, and that distinct tyrosine residues in SLP-76 were phosphorylated by each receptor of a synergistic pair. Selective phosphorylation of tyrosine 113 or tyrosine 128 in SLP-76, each of which enables binding of SLP-76 to Vav1, was unique to receptors that stimulate ligand-dependent target cell killing, because antibody-dependent stimulation by Fc receptor CD16 promoted phosphorylation at both sites. Knockdown and reconstitution experiments with SLP-76 showed the distinct role of each tyrosine in the synergistic mobilization of Ca²⁺, revealing an unexpected degree of selectivity in the phosphorylation of SLP-76 by NK cell co-activation receptors. Together, these data suggest that complementation of separate phospho-tyrosine targets in SLP-76 forms the basis of synergistic NK cell activation.

INTRODUCTION

Natural killer (NK) cells play a key role in the first line of defense against infection by providing rapid responses through cytokine production and direct lysis of transformed or virus-infected cells without prior immunization (1–3). NK cells rely on an array of germ line-encoded receptors, each of which has unique ligand specificity and signaling properties, to distinguish normal healthy cells from diseased target cells (4, 5). Activation of NK cells is tightly regulated by the requirement for the engagement by target cells of multiple co-activating receptors on NK cells, which are not activating on their own (6, 7). Thus, the cytotoxicity of NK cells towards sensitive target cells is triggered by combined signals, which can operate in synergy (6, 8, 9). In addition, signals from activating receptors are kept in check by inhibitory receptors specific for major histocompatibility complex (MHC) class

*To whom correspondence should be addressed: eLong@nih.gov (E.O.L.); hunkim@amc.seoul.kr (H.S.K.).

Author contributions: H.S.K. performed experiments, and H.S.K. and E.O.L. designed experiments, analyzed the data, and wrote the paper.

Competing interests: The authors declare that they have no competing interests.

I molecules on target cells, which protect healthy cells from lysis by NK cells (10). Inhibitory receptors such as killer cell immunoglobulin (Ig)-like receptors (KIRs) and the lectin-like CD94-NKG2A heterodimer are dominant over activation signals, even though NK cells can be triggered through multiple activating receptors that use discrete signaling pathways. The intersection of signals from different activating receptors by a single class of inhibitory receptors that contain immunoreceptor tyrosine-based inhibition motifs (ITIMs) suggests that inhibition would target a central common point in the activation of NK cells.

Because of the lack of central control by a single activating receptor, signaling pathways for the activation of NK cells require the integration of distinct signals delivered by co-activation receptors (11). In contrast, activation of T and B cells is dominated by signals from a single antigen-specific receptor that are augmented by costimulatory receptors. It is still unclear how signals from different receptors on NK cells are integrated to achieve proper functional responses. Among the receptor combinations that provide synergistic activation in resting NK cells are the lectin-like receptor NKG2D (CD314) and the signaling lymphocyte-activation molecule (SLAM) family member 2B4 (CD244), as well as 2B4 and the Ig-like DNAM-1 (CD226). NKG2D and DNAM-1 do not synergize and signaling through this combination of receptors is unable to mount a productive response (6). The natural ligands of NKG2D are the stress-inducible MHC class I chain-related molecules A (MICA) and MICB and the small family of UL16-binding protein (ULBP) molecules (12, 13). NKG2D is associated through its transmembrane region with DNAX-activating protein of 10 kD (DAP10). Upon stimulation by NKG2D ligands, DAP10 becomes tyrosine phosphorylated and recruits either phosphatidylinositol-3-kinase (PI3K) or a complex of the small adaptor protein Grb2 bound to the guanine nucleotide exchange factor (GEF) Vav1 (14, 15). The receptor 2B4 recognizes CD48 (16), an Ig-like molecule found predominantly on hematopoietic cells, and recruits through its own cytoplasmic tyrosine-based motifs the small adaptor protein SLAM-associated protein (SAP) and the SAP-associated tyrosine kinase Fyn (17). The signaling properties of DNAM-1 are still largely unknown. DNAM-1 binds to cellular adhesion molecules called nectins, including CD112 and the poliovirus receptor CD155 (18), and its cytoplasmic tail is phosphorylated by protein kinase C (PKC) (19). A difficulty in the study of synergy between activating receptors on NK cells is that each co-activation receptor uses distinct signaling modules. Because synergy requires the integration of such diverse signals, uncovering the basis for synergy would help us to understand how disparate signals converge to a certain point at which synergy occurs.

Engagement of NKG2D, 2B4, and DNAM-1 on NK cells results in the phosphorylation of Vav1 (20). Vav1 is a multifunctional protein, which acts as a GEF for the Rho family of guanosine triphosphatases (GTPases) and as an adaptor protein through multiple regions including typical C-terminal Src homology 2 (SH2) and SH3 domains (21–23). It has a central role in the regulation of actin cytoskeleton dynamics and lymphocyte receptor signaling, thereby coordinating various effector functions such as adhesion, degranulation, and cytokine production (23, 24). Deficiency in Vav diminishes the recruitment of phospholipase C (PLC- β) to receptor signaling complexes, inhibiting subsequent Ca^{2+} responses (21, 25, 26). Among the three Vav isoforms, synergistic activation of NK cells by NKG2D and 2B4 is controlled selectively by Vav1, given that knockdown of Vav1, but not Vav2 or Vav3, leads to decreased synergistic PLC- β phosphorylation, Ca^{2+} mobilization, and degranulation (20). The central role of Vav1 in NK cell activation is underscored by its identification as a primary substrate for dephosphorylation by the SH2-containing tyrosine phosphatase 1 (SHP-1) during the engagement of inhibitory receptors by MHC class I on target cells (27, 28). Vav1 is also an essential component in the synergy among combinations of NKG2D, 2B4, and DNAM-1 (20). Thus, Vav1 might represent a central common point for multiple activation pathways in NK cells. Synergistic NK cell activation by two co-activation receptors is accompanied by enhanced phosphorylation of Vav1, which

is equivalent to the sum of the extent of phosphorylation induced by each receptor alone, and it is required to overcome inhibition by the E3 ubiquitin ligase c-Cbl (20). At present, there is no information about how distinct signals from synergizing receptors converge to regulate Vav1 and its downstream signaling.

Based on the additive, rather than the synergistic, phosphorylation of Vav1 in NK cells during the simultaneous stimulation of pairs of co-activating receptors, we hypothesized that different pools of Vav1 might complement each other to achieve synergistic activation of NK cells. As a first step, we tested the roles of adaptor proteins known to contribute to signaling by Vav1 for lymphocyte activation. We investigated whether adaptor proteins such as SH2 domain-containing leukocyte phosphoprotein of 76 kD (SLP-76) and linker of activated T cells (LAT) were required for the synergistic activation of NK cells, and, if so, whether they regulated NK cell responses to co-activation receptors through modulation of Vav1. The adaptor proteins SLP-76 and LAT form the backbone of critical signaling complexes in T cells (29, 30). The absence of these proteins results in profound defects in lymphocyte activation and development (31). Moreover, activation of PLC- γ , a signaling molecule that is required for the synergistic activation of NK cells (20), depends on both SLP-76 and LAT in T cells (32–34).

We sought to assess the changes in early activation signals in NK cells that were stimulated by receptor ligation with specific antibodies or by direct contact with target cells. To avoid the complexity of interactions between NK cells and mammalian target cells that have multiple, and as yet unidentified, ligands, we used *Drosophila* S2 cells as target cells. S2 cells could be manipulated to express ligands of human NK cell receptors either alone or in combination. This system enabled us to dissect the contribution of individual receptors to the signaling pathways required for synergy in the context of defined and physiological receptor-ligand interactions (20). The results revealed that SLP-76 was required for the optimal phosphorylation of Vav1 and effector function of NK cells during synergy between NKG2D and 2B4. The phosphorylation of SLP-76 by NKG2D and 2B4 was regulated by distinct and complementary signals. Moreover, we found that complementary phosphorylation of SLP-76 tyrosine residues contributed to the synergistic activation of NK cells. Together, these data suggest that the complementary phosphorylation of SLP-76 during synergy between receptors that stimulate natural cytotoxicity represents a checkpoint in NK cell activation and forms the basis of synergy.

RESULTS

SLP-76 is required for the synergistic activation of NK cells

Stimulation of the receptor NKG2D induces the tyrosine phosphorylation of SLP-76 but not LAT (35), whereas engagement of 2B4 results in the phosphorylation of LAT in human NK cells (36). Because their roles in the synergistic activation of NK cells was unexplored, we tested whether SLP-76 and LAT were required for the effector functions of NK cells when triggered by both NKG2D and 2B4. We performed small interfering RNA (siRNA)-mediated knockdown of SLP-76 and LAT in NKL cells, a human NK cell line. After 48 hours, we detected a noticeable reduction in the amount of SLP-76 and LAT protein in the cells (Fig. 1A). The synergistic increase in Ca^{2+} mobilization and cytotoxicity was markedly diminished by knockdown of SLP-76, but not LAT, in NKL cells (Fig. 1A and 1B). Synergistic signals that stimulated the secretion of interferon- γ (IFN- γ) and the chemokine macrophage inflammatory protein (MIP)-1 by NKL cells that were activated with beads coated with monoclonal antibodies (mAbs) against NKG2D and 2B4 were also abolished by knockdown of SLP-76 (Fig. 1C), consistent with a previous report that showed the dependence of NKG2D on SLP-76 for IFN- γ production in mouse NK cells (37). Knockdown of LAT caused a slight reduction in the production of cytokines (Fig. 1C). The

central role of SLP-76, but apparently not LAT, during the synergy between NKG2D and 2B4 suggested a distinct requirement for adaptor proteins in signaling by co-activation receptors, given that both adaptor proteins regulate antibody-dependent cellular cytotoxicity (ADCC) against antibody-coated target cells by NK cells through engagement of Fc γ RIIIA (CD16) (35, 38).

To determine whether SLP-76 was required for the synergistic activation of NK cells by a different combination of co-activation receptors, we examined NKL cells after the simultaneous crosslinking of the receptors DNAM-1 and 2B4. DNAM-1 synergizes with 2B4, but not NKG2D, to stimulate Ca^{2+} mobilization and cytotoxicity (6, 20). We found that synergy between DNAM-1 and 2B4 to stimulate Ca^{2+} mobilization was markedly impaired by knockdown of SLP-76 in NKL cells compared to that in control cells (fig. S1), underscoring the pivotal role of SLP-76 in synergistic activation through different combinations of co-activation receptors.

SLP-76 controls Vav1 phosphorylation during synergy between NKG2D and 2B4

The notable dependence of the synergy between NKG2D and 2B4 on SLP-76 (Fig. 1) and Vav1 (20) prompted us to investigate whether SLP-76 regulated this synergy by modulating the activation of Vav1. Vav1 associates with SLP-76 upon TCR ligation (39, 40) and regulates the association between SLP-76 and PLC- γ 1 (41), thereby contributing to the proper activation of PLC- γ 1. We found that activation of NKG2D or 2B4 or the combined activation of NKG2D and 2B4 all induced the phosphorylation of Vav1 at Tyr¹⁶⁰ (Y160), as expected (20), and at Tyr¹⁷⁴ (Y174) (fig. S2), which are two of the three tyrosines that contribute to Vav1 activation when phosphorylated (42).

To examine the association between SLP-76 and Vav1 in the context of physiological receptor-ligand interactions, we mixed NKL cells with S2 cells that expressed ULBP1 (a ligand for NKG2D) and CD48 (a ligand for 2B4), either alone or in combination. We then immunoprecipitated SLP-76 from NKL cell lysates and performed Western blotting analysis to detect Vav1 phosphorylated at Y160 (pY160-Vav1). After mixing with S2-ULBP1 and S2-CD48 cells, NKG2D and 2B4 each induced the association of SLP-76 with pY160-Vav1 in NKL cells (Fig. 2A). The association between SLP-76 and pY160-Vav1 was not seen in NKL cells that were mixed with control S2 cells that did not express NK cell ligands. Mixing of NKL cells with S2 cells expressing both ligands resulted in an enhanced association of SLP-76 with pY160-Vav1, which corresponded to the sum of the associations induced by engagement of single receptors. Therefore, it is likely that SLP-76 links NKG2D- and 2B4-proximal signaling to the synergistic activation of NK cells through its association with, and modulation of, Vav1. Supporting this notion, knockdown of SLP-76 or Vav1 caused comparable reductions in the extent of Ca^{2+} mobilization induced by the combined stimulation of NKL cells through NKG2D and 2B4 (fig. S3A). The defect in Ca^{2+} mobilization that occurred as a result of Vav1 knockdown was further decreased by the additional knockdown of SLP-76 (fig. S3B), which suggested that SLP-76 and Vav1 cooperated to induce optimal Ca^{2+} mobilization. The same experiment performed in NKL cells stimulated through both 2B4 and DNAM-1 revealed a lesser dependence on Vav1 than on SLP-76 for Ca^{2+} mobilization (fig. S3C and D), when compared to stimulation through 2B4 and NKG2D. This suggested that discrete pathways existed for SLP-76 and Vav1 to trigger Ca^{2+} mobilization depending on the combinations of receptors stimulated.

We next tested the requirement of SLP-76 for Vav1 phosphorylation during combined stimulation of NKL cells through NKG2D and 2B4 by performing experiments in which we knocked down SLP-76. NKG2D and 2B4 on NKL cells were stimulated, either individually or together, by preincubating the cells with specific mAbs which were then crosslinked with goat F(ab)₂ anti-mouse IgG. The extent of phosphorylation of Vav1 that was induced by

NKG2D stimulation was slightly reduced by SLP-76 knockdown compared to that in control cells (Fig. 2B). The weak phosphorylation of Vav1 induced by 2B4 was also reduced in the SLP-76 knockdown cells compared to that in control cells (Fig. 2B). The small difference in the amount of pY160-Vav1 induced by NKG2D or 2B4 alone in the presence or absence of SLP-76 was not statistically significant. In contrast, SLP-76 knockdown significantly diminished the extent of Vav1 phosphorylation in response to combined stimulation of NKG2D and 2B4 compared to that in stimulated control cells (Fig. 2B, $P < 0.05$), which suggested that SLP-76 was required for the optimal phosphorylation of Vav1 during synergistic activation of cells.

To validate this finding in the physiological context of contacts between NK cells and target cells, we examined Vav1 phosphorylation in NKL cells that were mixed with S2 cells expressing ULBP1, CD48, or both. We obtained similar results to those from the earlier experiments with antibody-based stimulation. In particular, we saw a substantial reduction in the extent of Vav1 phosphorylation stimulated by NKG2D and 2B4 co-engagement in SLP-76-knockdown cells, and slight inhibition by engagement of the single receptor (Fig. 2C). These results, obtained in the context of receptors interacting with their physiological ligands, confirmed that the activation of Vav1 in cells stimulated through both NKG2D and 2B4 was largely dependent on SLP-76. Moreover, the prominent loss of Vav1 phosphorylation through NKG2D and 2B4 co-engagement that we observed in SLP-76-knockdown cells suggested that SLP-76 might serve to integrate signals for Vav1 activation through NKG2D and 2B4 synergy.

Synergy between NKG2D and 2B4 induces complementary signals for SLP-76 phosphorylation

Tyrosine phosphorylation of SLP-76 is required for its association with Vav1 (39, 43). Upon TCR stimulation, the SH2 domain of Vav1 binds to two phosphorylated tyrosine residues in the N-terminal, acidic region of SLP-76 (44). Given the inducible association of SLP-76 with pY160-Vav1 during costimulation of cells through NKG2D and 2B4 (Fig. 2A), we examined whether NKG2D and 2B4 could induce tyrosine phosphorylation of SLP-76. After crosslinking receptors with specific mAbs, we immunoprecipitated tyrosine-phosphorylated proteins from cell lysates and then performed Western blotting analysis to detect SLP-76. We observed tyrosine phosphorylation of SLP-76 in NKL cells stimulated through NKG2D, as expected (35), and in cells stimulated through 2B4 alone, and this was further enhanced in cells stimulated through both NKG2D and 2B4 (Fig. 3A). Thus, engagement of 2B4 as well as of NKG2D led to the tyrosine phosphorylation of SLP-76.

The N-terminal region of human SLP-76 contains three tyrosine residues (Tyr¹¹³, Tyr¹²⁸, and Tyr¹⁴⁵) that become phosphorylated upon TCR ligation (29). Phosphorylation of SLP-76 at Tyr¹¹³ was clearly detected in NKL cells after crosslinking of 2B4, but not NKG2D, and was further increased in extent by crosslinking both receptors (Fig. 3B). In contrast, SLP-76 phosphorylation at Tyr¹²⁸ was observed after crosslinking of NKG2D, but not 2B4 (Fig. 3B). The basal extent of SLP-76 phosphorylation at Tyr¹⁴⁵ was not increased by engagement of NKG2D, 2B4, or both. Therefore, NKG2D and 2B4 induced distinct signals for the tyrosine phosphorylation of SLP-76. An important question was whether complementary phosphorylation of SLP-76 also occurred during the synergistic activation of primary, resting NK cells. Thus, we stimulated NKG2D and 2B4 on resting NK cells by ligating the receptors with specific mAbs, either individually or together. Similar to the experiments with NKL cells, we found selective phosphorylation of Tyr¹¹³ by crosslinking 2B4 and of Tyr¹²⁸ by crosslinking NKG2D in primary, resting NK cells (Fig. 3C). To confirm this finding in the context of physiological receptor-ligand interactions, we incubated S2 cells that stably expressed either ULBP1, CD48, or both with NKL cells that had been rested in the absence of IL-2 for 24 h (Fig. 3D) or primary, resting NK cells (Fig.

3E). Similar to the results observed after crosslinking the receptors with mAbs, we saw that SLP-76 phosphorylation at Tyr¹¹³ and Tyr¹²⁸ was obtained by mixing NK cells with S2 cells expressing both ligands but not by mixing NK cells with S2 cells expressing either ligand alone (Fig. 3D and E). Therefore, co-engagement of NKG2D and 2B4 is required to induce tyrosine phosphorylation of SLP-76 at both tyrosine residues.

To test whether complementary phosphorylation of SLP-76 was induced by a different combination of co-activation receptors, we examined primary, resting NK cells after co-engagement of DNAM-1 with 2B4 or with NKG2D. Ligation of DNAM-1 induced selective phosphorylation of SLP-76 at Tyr¹²⁸, as did the crosslinking of NKG2D (Fig. 4A). Because SLP-76 phosphorylation at Tyr¹¹³ was induced by crosslinking 2B4, co-engagement of DNAM-1 with 2B4, but not with NKG2D, was required for the phosphorylation of SLP-76 at both Tyr¹¹³ and Tyr¹²⁸ (Fig. 4A). In contrast, crosslinking CD16 alone, which mediates ADCC and not natural cytotoxicity (i.e. ligand-dependent target cell killing), induced robust activation of resting NK cells and was sufficient to induce SLP-76 phosphorylation at both Tyr¹¹³ and Tyr¹²⁸ (Fig. 4A). Similarly, TCR ligation on Jurkat cells resulted in SLP-76 phosphorylation at both tyrosines (Fig. 4B), indicating distinct regulation of SLP-76 phosphorylation by co-activation receptors for natural cytotoxicity in human NK cells.

In T cells, SLP-76 phosphorylation induced by TCR engagement is dependent on the zeta-chain-associated protein kinase 70 (ZAP70) (45). We tested the sensitivity of SLP-76 phosphorylation at Tyr¹¹³ and Tyr¹²⁸ to different inhibitors after simulation of NK cells through NKG2D and 2B4 or after crosslinking of CD16. Phosphorylation of SLP-76 at Tyr¹¹³ and Tyr¹²⁸ induced by CD16 was sensitive to inhibitors of the Syk and ZAP70 family of kinases and to inhibition of the Src family kinases (fig. S4). In contrast, phosphorylation of Tyr¹¹³ and Tyr¹²⁸ induced by the combined stimulation of NKG2D and 2B4 was not sensitive to a Syk inhibitor (Syk inhibitor II) (fig. S4) or an inhibitor of Syk and ZAP70 (piceatannol) (fig. S5), but was abolished by inhibition of the Src family kinases by PP2 (fig. S4). These results show that tyrosine phosphorylation of SLP-76 during synergistic signaling by the combination of NKG2D and 2B4 does not depend on the same tyrosine kinases as those required during signaling by CD16.

Given that 2B4 recruits the kinase Fyn through the adaptor SAP, and that phosphorylation of SLP-76 was dependent on Src-family kinases, we next assessed the phosphorylation of SLP-76 after knockdown of Fyn. We found that SLP-76 phosphorylation at Tyr¹¹³ induced by 2B4 was Fyn-dependent, whereas phosphorylation of Tyr¹²⁸ induced by NKG2D was not (fig. S6), further demonstrating the selective phosphorylation of SLP-76 tyrosine residues by NK cell co-activation receptors. Together, our data suggest that even after co-engagement of synergistic NK cell co-activation receptors, the dual phosphorylation of SLP-76 at Tyr¹¹³ and Tyr¹²⁸ follows a different signaling pathway than that triggered by the immunoreceptor tyrosine-based activation motif (ITAM)-dependent receptor CD16 in NK cells and that stimulated by the TCR in T cells.

SLP-76 is required for independent signaling by NKG2D, 2B4, and DNAM-1

Crosslinking of NKG2D, 2B4, or DNAM-1 alone resulted in the phosphorylation of SLP-76 at one of the residues Tyr¹¹³ or Tyr¹²⁸. This raised the question of whether SLP-76 regulated signaling by each one of the co-activation receptors, as it does their synergistic combinations. An alternative would be that SLP-76 is required only for synergistic signaling, downstream of signals propagated by each co-activation receptor. Activation of NK cells through the engagement of a single co-activation receptor can be unmasked by knockdown of c-Cbl (20). We found that knockdown of c-Cbl rendered otherwise unresponsive NKL cells sensitive to the engagement of NKG2D, DNAM-1, or 2B4 alone, as determined by measurement of Ca²⁺ mobilization (Fig. 5). Ca²⁺ mobilization induced by

single co-activation receptors after knockdown of c-Cbl was markedly diminished by the additional knockdown of SLP-76 (Fig. 5A). In contrast, Ca^{2+} mobilization induced by individual co-activation receptors in the context of c-Cbl knockdown was not notably affected by the additional knockdown of LAT (Fig. 5B), consistent with the lack of a role for LAT in mediating synergy between NKG2D and 2B4. Therefore, these data suggest that the requirement for SLP-76 in the synergy among NKG2D, 2B4, and DNAM-1 may be attributed to the dependence of signaling through each one of these co-activation receptors on SLP-76.

Tyrosine phosphorylation of SLP-76 is required for the synergistic activation of NK cells

Next, we examined the role of SLP-76 tyrosine phosphorylation in the synergistic activation of NK cells. To test how an increased abundance of SLP-76 might affect synergistic activation signals, we transfected NKL cells with a plasmid encoding yellow fluorescent protein (YFP)-tagged SLP-76 and then measured Ca^{2+} mobilization in YFP-containing (YFP⁺) cells after crosslinking both NKG2D and 2B4. Despite the higher abundance of SLP-76-YFP compared to that of endogenous SLP-76, Ca^{2+} mobilization was not enhanced in YFP⁺ cells (fig. S7). To test the function of SLP-76 phosphorylation without interference by endogenous protein, we used a “suppression and re-expression” strategy in which we first depleted cells of endogenous SLP-76 with specific siRNA and then reconstituted cells with various siRNA-resistant mutants of SLP-76 (Fig. 6A). Wild-type SLP-76-YFP in those cells restored Ca^{2+} mobilization induced by the synergy between NKG2D and 2B4 (Fig. 6B), but YFP alone did not, thus validating this strategy for the study of SLP-76 function.

To assess the role of SLP-76 tyrosine phosphorylation in the effector functions of NK cells, we individually mutated the SLP-76 tyrosines at positions 113, 128, and 145 to phenylalanines (Y113F, Y128F, and Y145F). We also generated SLP-76 with mutations at both Tyr¹¹³ and Tyr¹²⁸ (Y113+128F). After depletion of SLP-76 by siRNA, NKL cells were transfected with plasmids encoding YFP-tagged wild-type SLP-76 or SLP-76 tyrosine mutants. The amounts of YFP-tagged proteins after transfection of NKL cells were similar for wild-type SLP-76 and its tyrosine mutants (Fig. 7A). We then measured Ca^{2+} mobilization in YFP⁺ NKL cells. SLP-76 tyrosines are required for Ca^{2+} mobilization in T cells (46) and thymocytes (47) after TCR stimulation, and in neutrophils after Fc R crosslinking (48). The Ca^{2+} mobilization induced by combined stimulation of NKG2D and 2B4 was restored by wild-type SLP-76-YFP but not by any of the tyrosine mutants tested (Fig. 7B), indicating that each tyrosine of SLP-76 was required for optimal activation of NK cells. As expected, mutation of Tyr¹⁴⁵, which is required for the binding of IL-2-inducible T-cell kinase (ITK) to SLP-76 in T cells (29), resulted in defective Ca^{2+} mobilization. Therefore, signals through Tyr¹¹³ and Tyr¹²⁸ of SLP-76 are not independent, but complement each other to trigger synergistic Ca^{2+} mobilization. Ca^{2+} mobilization was similar in extent in all cells after stimulation with the Ca^{2+} ionophore ionomycin (fig. S8). To examine the effects of SLP-76 tyrosine mutations on the effector functions of NK cells, we determined the ability of the SLP-76 tyrosine mutant proteins to support cytotoxicity and cytokine production by combined stimulation of NKG2D and 2B4. A similar defect was obtained in functional studies of cells expressing each SLP-76 tyrosine mutant, as shown by reduced cytotoxicity (Fig. 7C) and impaired release of IFN- and MIP-1 (Fig. 7D). We conclude that complementary phosphorylation of SLP-76 at Tyr¹¹³ and Tyr¹²⁸ is the basis of the synergistic activation of NK cell effector functions.

The dependence of NK cell activation on synergistic signals from co-activation receptors was partially lifted after knockdown of c-Cbl (20). To test whether Ca^{2+} mobilization induced by NKG2D alone, in the absence of inhibition by c-Cbl, was still dependent on phosphorylation of Tyr¹²⁸ but not Tyr¹¹³, we expressed the Y113F or Y128F SLP-76 mutants in NKL cells in which both c-Cbl and SLP-76 had been knocked down. Ca^{2+}

mobilization induced by NKG2D alone was rescued by the Y113F SLP-76 mutant, but not the Y128F mutant (fig. S9). Conversely, Ca^{2+} mobilization triggered by 2B4 alone required Tyr¹¹³ but not Tyr¹²⁸ of SLP-76 (fig. S9). Therefore, signaling by NKG2D through Tyr¹²⁸, independently of Tyr¹¹³, and by 2B4 through Tyr¹¹³, independently of Tyr¹²⁸, occurred also in the absence of inhibition by c-Cbl, further demonstrating the selectivity in SLP-76 tyrosine phosphorylation induced by the NK cell co-activation receptors NKG2D and 2B4.

DISCUSSION

An interesting aspect of the regulation of natural cytotoxicity by NK cells is that several pairwise combinations of receptors, which have very distinct signaling properties, results in potent synergistic activation of intracellular Ca^{2+} release, cytokine production, and cytotoxicity. The main question addressed here was how distinct signals from co-activation receptors were integrated to regulate such synergy, and which molecular checkpoint(s) controlled this process. Here, we offer a new perspective on the synergistic signaling pathway for natural cytotoxicity, which we suggest relies on the integration of distinct and complementary signals at the level of tyrosine phosphorylation of the adaptor protein SLP-76. Using synergistic activation by receptors NKG2D and 2B4 as a model, we showed that they synergized through a pathway that was largely dependent on complementary phosphorylation at N-terminal tyrosine residues in SLP-76. Signaling by NKG2D induced SLP-76 phosphorylation preferentially at Tyr¹²⁸, but not Tyr¹¹³. Conversely, 2B4 engagement induced the preferential phosphorylation of SLP-76 at Tyr¹¹³ rather than at Tyr¹²⁸. Phosphorylation of SLP-76 at both Tyr¹¹³ and Tyr¹²⁸, which was required for synergy between NKG2D and 2B4, was achieved by the joint contribution of both co-activation receptors. Thus, synergistic stimulation of NK cells by two co-activation receptors is not achieved by simple mutual enhancement of signaling by the two receptors.

The control of SLP-76 phosphorylation in NK cells is clearly different from that of the well-known example of TCR signaling: Engagement of TCR alone is sufficient to induce SLP-76 phosphorylation at both N-terminal tyrosines, which is followed by downstream signaling events and the activation of T cells (29). Likewise, stimulation of NK cells by the Fc receptor CD16 resulted in the phosphorylation of SLP-76 at both Tyr¹¹³ and Tyr¹²⁸. Therefore, selective phosphorylation of each tyrosine is a property of co-activation receptors for natural cytotoxicity, and is not a result of a fundamental difference between T cells and NK cells. In T cells, phosphorylation of SLP-76 after TCR stimulation is mediated by the tyrosine kinase ZAP-70 (45). As expected, we found that SLP-76 phosphorylation in NK cells induced by CD16 was blocked by inhibitors of the related tyrosine kinase Syk. In contrast, phosphorylation of SLP-76 at Tyr¹¹³ induced by 2B4 and at Tyr¹²⁸ by NKG2D, and phosphorylation at both sites induced by the synergistic actions of 2B4 and NKG2D were not sensitive to inhibitors of the Syk and ZAP70 family of kinases. It is therefore possible that the selective phosphorylation of both tyrosines is mediated by specific kinases through coupling to different NK cell receptors. Supporting this, we observed that the kinase Fyn was required for the phosphorylation of SLP-76 at Tyr¹¹³ by 2B4 but not at Tyr¹²⁸ by NKG2D.

ITAM-dependent pathways, such as those triggered by antigen-specific receptors of adaptive immune cells (29) and by α -containing integrins in neutrophils and macrophages (49) signal through SLP-76. Although LAT is usually required for ITAM-dependent signaling, a LAT-independent function of SLP-76 contributes to Fc RI-mediated activation of mast cells (50). We showed that SLP-76, but not LAT, was indispensable for NK cell activation triggered by the synergistic action of NKG2D or DNAM-1 with 2B4, thus demonstrating a pivotal role for SLP-76 in mediating NK cell activation downstream of non-ITAM receptors.

We previously showed that the E3 ubiquitin ligase c-Cbl acts as a gatekeeper for NK cell activation by preventing activation of NK cells in response to a single activating receptor, and that a combination of synergizing receptors is required to provide sufficient stimulation (20). Knockdown of c-Cbl enables the co-activation receptors NKG2D and 2B4 to induce natural cytotoxicity individually and therefore bypasses the requirement for synergy. However, this raises the question of why synergistic co-activation requires unique combinations of receptors. For example, NKG2D can synergize with 2B4 but not with DNAM-1 to activate NK cells, but 2B4 can synergize with both NKG2D and DNAM-1. Despite the use of different signaling modules by each co-activation receptor, signals stimulated through the crosslinking of NKG2D, 2B4, or DNAM-1 in each case led to the tyrosine phosphorylation of SLP-76. However, NKG2D and DNAM-1 induced the selective phosphorylation of Tyr¹²⁸, whereas 2B4 induced the phosphorylation of Tyr¹¹³. Accordingly, the combination of NKG2D and 2B4, and of 2B4 and DNAM-1, but not of NKG2D and DNAM-1, resulted in the phosphorylation of SLP-76 at both sites.

We have previously shown that overexpression of Vav1 results in permissive NK cell activation and degranulation after stimulation with NKG2D or 2B4 alone (20). These results were more consistent with the requirement for strong Vav1 signals to trigger activation, and excluded the possibility of an obligate complement to Vav1 signals. In support of this, we observed that engagement of either NKG2D or 2B4 alone induced Vav1 phosphorylation at both Tyr¹⁶⁰ and Tyr¹⁷⁴ independently, which was further enhanced by co-engagement of NKG2D with 2B4. This was distinct from the complementary phosphorylation of SLP-76 selectively at Tyr¹²⁸ by NKG2D and at Tyr¹¹³ by 2B4. Our study provides evidence that signals from synergizing receptors converge just upstream of Vav1 at the level of SLP-76 through complementary phosphorylation of SLP-76 tyrosine residues.

SLP-76 participates in T cell development and in the activation of diverse cells, including T cells, mast cells, neutrophils, and even platelets (29). SLP-76 serves to nucleate the formation of multiprotein signaling complexes and thereby supports signaling downstream of integrins and of multiple immunoreceptors. Here, we showed that SLP-76 has an indispensable role in synergistic signaling by NKG2D and 2B4 for Ca²⁺ mobilization, cytokine secretion, and cytotoxicity by NK cells. These data were obtained from experiments with primary, resting NK cells or an NK cell line after a period of rest. Stimulation was triggered by crosslinking of receptors with mAbs against NKG2D and 2B4, and was also evaluated in the more physiological context of receptor-ligand interactions between NK cells and *Drosophila* S2 cells expressing ligands for NK cell receptors. Synergistic signaling by NKG2D and 2B4 induced degranulation but not granule polarization, which is controlled by the α_2 integrin lymphocyte function-associated antigen 1 (LFA-1) (51). Thus, our experimental approach in the absence of a ligand to stimulate LFA-1 avoided potential complications as a result of the contribution of SLP-76 to “outside-in” signaling by LFA-1 (52, 53). We conclude that SLP-76 has an indispensable role in regulating synergy by NK cell stimulatory receptors independently of its role in outside-in signaling by LFA-1.

In response to TCR ligation, Vav1 is selectively recruited into SLP-76 microclusters to increase their stability and function (22), and thereby cooperates with SLP-76 to activate Ca²⁺-dependent T cell responses (39, 40, 54). An interaction between SLP-76 and Vav1 can occur through the C-terminal SH2 domain of Vav1, which binds to phosphorylated Tyr¹¹³ and Tyr¹²⁸ in the N-terminal, acidic domain of human SLP-76 (39). Consistent with this, stimulation of NK cells through either NKG2D or 2B4 alone induced the association of pY160-Vav1 with SLP-76. Association of Vav1 with SLP-76 was further enhanced by co-engagement of NKG2D and 2B4, which corresponded approximately to the sum of the associations induced by each receptor individually. Thus, it is likely that SLP-76 serves to

integrate Vav1-dependent activation signals by recruiting Vav1 to the phosphorylated tyrosines 113 and 128 of SLP-76. A quantitative biochemical analysis has shown that SLP-76 phosphopeptides containing the N-terminal tyrosines enable simultaneous binding of Vav1 molecules to both tyrosines, forming a 2:1 stoichiometric complex of Vav1 and SLP-76 (44).

How could the interaction between SLP-76 and Vav1 result in synergistic co-activation by NK cell receptors? The simultaneous binding of two Vav1 molecules to adjacent phosphotyrosines on SLP-76 may result in greater than additive Vav1 activity, either through reciprocal transactivation, or by protecting Vav1 from inhibition and degradation. In the context of c-Cbl knockdown, the ability of NKG2D and 2B4 to activate NK cells individually (20) still occurs selectively through the residues Tyr¹²⁸ and Tyr¹¹³, respectively, which suggests that SLP-76-dependent synergy may protect Vav1 from c-Cbl-mediated degradation. Once structural information on the complete Vav1 molecule becomes available, it will be possible to model and test some of these predictions. Alternatively, NK cell responses may be regulated by a finely tuned activation threshold. Phosphorylation of Vav1 could represent a critical threshold, given that signals from diverse activating and inhibitory receptors converge to compete for phosphorylation and dephosphorylation of Vav1 (11, 55).

MATERIALS AND METHODS

Cell lines and reagents

Human NK cell populations were isolated from peripheral blood by negative selection with an NK cell isolation kit (StemCell Technologies) as previously described (56). Human blood samples from healthy donors were drawn for research purposes at the NIH Department of Transfusion Medicine under a protocol approved by the NIH IRB with informed consent. Resting NK cells were resuspended in Iscove's modified Dulbecco medium (IMDM) (Invitrogen) supplemented with 10% human AB serum (Valley Biomedical) and were used within 2 days of isolation. These cells were 97% to 99% CD3⁻CD56⁺ as assessed by flow cytometry. The human NK cell line NKL (which was a gift of M. Robertson, Indiana University Medical Center, Indianapolis, IN) was cultured in RPMI 1640 supplemented with 10% fetal bovine serum (FBS), 1 mM sodium pyruvate, and rIL-2 (200 U/ml, Roche). NKL cells were rested in RPMI 1640 supplemented with 5% FBS, 0.5 mM sodium pyruvate without rIL-2 for 24 hours. P815 cells (American Type Culture Collection) were maintained in IMDM supplemented with 10% FBS and 2 mM L-glutamine. *Drosophila* Schneider line 2 (S2) cells expressing ULBP1 and CD48, either singly or in combination, have been described previously (20). Culture, maintenance, and transfection of S2 cells were performed as described (57).

Antibodies

Antibodies used in this study are listed according to their targets and indicate their source, as follows: NKG2D (149810) was from R&D Systems; CD244/2B4 (C1.7) was obtained from Beckman Coulter; CD226/DNAM-1 (DX11), CD16 (3G8), pY113-SLP-76 (J80-373), pY128-SLP-76 (J141-668.36.58), pY145-SLP-76 (J81-1214.48), and actin (C4) were purchased from BD Biosciences; CD3 (OKT3) was obtained from eBioscience; SLP-76 (AS55-P) was from Antibody Solutions; LAT (06-807) and phosphotyrosine (4G10) were from Millipore; isotype control mouse IgG1 (MOPC-21) was from Sigma; Vav1 (H211) was from Santa Cruz; pY160-Vav1 was from Biosource; pY174-Vav1 was obtained from Abcam; and SLP-76 (4958) was obtained from Cell Signaling. Goat F(ab)₂ antibody against mouse IgG was obtained from Jackson Immunoresearch. Horseradish peroxidase (HRP)-conjugated antibodies against mouse and rabbit antibodies were from Santa Cruz.

Flow cytometric analysis of Ca²⁺ mobilization

Intracellular Ca²⁺ mobilization was measured by flow cytometric analysis of cells that were labeled with Fluo-4 AM (Invitrogen). For experiments involving siRNA-mediated knockdown, experiments were performed as described previously (20). Briefly, NK cells were labeled for 30 min at 30°C with dye-loading buffer [Hanks' balanced salt solution (HBSS) with 1% FBS, Fluo-4 AM (4 µg/ml), and 4 mM probenecid]. For experiments involving transfection with plasmids encoding YFP-tagged SLP-76 constructs, NK cells were labeled for 30 min at 30°C in HBSS, 1% FBS with Asante Calcium Red-AM (6 µg/ml, Teflabs) and 4 mM probenecid. Cells were then washed twice, resuspended in HBSS, 1% FBS, and incubated with the indicated stimulating mAbs (10 µg/ml) for 30 min on ice. Cells were washed twice and resuspended in HBSS containing 1% FBS, and transferred to flow cytometry analysis tubes. Cells were warmed for 5 min at 37° in a water bath, and placed on the flow cytometer. After 30 s of data acquisition, tubes were removed, and 4 µg of cross-linking goat anti-mouse F(ab)₂ was added. Cells were mixed by vortexing, placed back on the flow cytometer, and events were acquired for a further 5 min. Data were analyzed with FlowJo software (Tree Star).

Cytotoxicity assays

For Europium-based cytotoxicity assays, P815 cells were labeled with 40 µM BATDA (Perkin Elmer) for 30 min at 37°C. Cells were then washed in medium containing 1 mM sulfinpyrazone (Sigma), resuspended at 1×10^6 cells/ml in the medium, and incubated for 30 min at room temperature with mAbs (10 µg/ml) specific for NK receptors. Cells were washed and incubated with effector cells in the presence of sulfinpyrazone for 2 hours at 37°C. Plates were mixed briefly and centrifuged at 400× *g* for 3 min. Supernatant (20 µl) was incubated with 200 µl of 20% Europium solution (Perkin Elmer) in 0.3 M acetic acid for 5 min and analyzed with a Wallac plate reader (Perkin Elmer).

Measurement of cytokine secretion

The amounts of IFN- (Pierce) and MIP-1 (R&D Systems) released after stimulation of cells with beads coated with mAbs against NK receptors were determined by enzyme-linked immunosorbent assay (ELISA) as described previously (6).

RNA interference

NKL cells were transfected with 300 pmoles of siRNAs with the Amaxa Nucleofector II system. A total of 2×10^6 cells were resuspended in 100 µl of Amaxa kit solution V (Lonza), mixed with siRNA, and immediately transfected with program O-17. Cells were then incubated for a total of 48 hours at 37°C, for the last 24 hours of which the cells were rested, and then the cells were assayed as indicated. The siRNAs specific for SLP-76 and LAT were part of TriFECTa Dicer-substrate kit obtained from Integrated DNA Technologies (IDT). The following siRNA sequences were used: SLP-76: 5' - CCAGACAGAAGAGAGAAUGAUGAAG-3' (sense) and 5' - CUUCAUCAUUCUCUCUCUGUCUGGUC-3' (antisense); LAT: 5' - GCACAUCCUCAGAUAGUUUGUAUCC-3' (sense) and 5' - GGAUACAAACUAUCUCUGAGGAUGUGCUG-3' (antisense). A second set of siRNA sequences for SLP-76 was used, which was as follows: 5' - CGAAGAGAGGAGGAGCAUCUU-3' (sense) and 5' - GAUGCUCUCCUCUCUCUGCUU-3' (antisense). Similar results were obtained with either one of the SLP-76 siRNAs. The results shown in this paper are those obtained with the former set of siRNA oligonucleotides. The siRNAs used for Fyn knockdown were obtained from IDT and the sequences are as follows: 5' - CACGGACAGAAGAUGACCUGAGUTT-3' (sense) and 5' -

AAACUCAGGUCAUCUUCUGUCCGUGUC-3 (antisense). The siRNA oligos used to knockdown Vav1 and c-Cbl were described previously (20) and their sequences are as follows: Vav1: 5'-CGUCGAGGUCAAGCACAUdTdT-3 (sense) and 5'-AAUGUGCUUGACCUCGACGdTdT-3 (antisense); c-Cbl: 5'-CCUCUCUCCAAGCACUGAdTdT-3 (sense) and 5'-UCAGUGCUUGGAAGAGAGGdTdT-3 (antisense). The negative siRNA controls were obtained from IDT and Dharmacon.

DNA constructs and cell transfections

The plasmids encoding SLP-76-YFP and LAT-YFP were provided by L. Samelson (National Cancer Institute, Bethesda, MD). The SLP-76-YFP encoded by the SLP-76-YFP plasmid was made resistant to the two separate SLP-76-specific siRNAs by independent mutagenesis with the Quik Change kit (Stratagene) and were used for the suppression and re-expression experiments. Four silent mutations were introduced in each one, using the oligonucleotides 5'-GCATGGATGGGGACCAGAtAGgAGAGAAaAcGATGAAGATGATGTGCATCAG-3 and 5'-GGAAATCAACAAGAACGAAGAAaAGaAGaAGtATCTTCACACGCAAACCCC-3, respectively. Point mutations introduced into wild-type SLP-76 are indicated by lower-case letters. Each one of the two mutated SLP-76-YFP plasmids was further mutated to generate the Y113F, Y128F, Y145F, and Y113+128F SLP-76 mutants. All final constructs were verified by sequencing. Similar results were obtained with either one of the siRNA-resistant SLP-76 mutants. The data shown in this paper are those obtained with the former. NKL cells (5×10^6 cells) that had been transfected with SLP-76-specific siRNA for 24 hours were then transfected with 5 to 8 μ g of plasmid DNA by Amaxa Nucleofector II, with solution V, and program O-17. Transfected cells were assayed 24 hours after transfection after a period of rest.

Receptor crosslinking and cell mixing experiments

For antibody-mediated crosslinking of NK receptors, NK cells were preincubated with isotype control mAb or mAbs specific for NK receptors (all at 10 μ g/ml) for 30 min on ice. After washing with medium, NK cells were stimulated by crosslinking with goat anti-mouse F(ab)₂ Ab (30 μ g/ml) at 37°C for the indicated times. For cell mixing experiments, NK cells and S2 target cells separately chilled on ice were mixed at an effector (NK cell) to target (S2 cell) ratio of 1:1. Cells were initially incubated for 20 min on ice before being incubated at 37°C for the indicated times. Cells were then returned to ice and lysed for further analysis.

Western blotting and immunoprecipitations

Stimulated NK cells were washed with ice-cold phosphate-buffered saline (PBS) and lysed in lysis buffer [50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% Triton X-100, 5 mM EDTA, 1 mM NaVO₃, 50 mM NaF, 1 mM PMSF, and protease inhibitor cocktail (Roche)] for 30 min on ice. Cell debris, including nuclei, was removed by centrifugation, and supernatants were recovered. Protein concentrations in the cell lysates were determined with the Micro BCA protein assay kit (Pierce). For immunoprecipitations, lysates were precleared with protein G-conjugated agarose beads (Invitrogen) followed by incubation with 2 μ g Ab coupled to protein G agarose beads overnight at 4°C. Beads were washed three times in 20 volumes of ice-cold lysis buffer, resuspended in 1 $\times$ NuPAGE LDS sample buffer (Invitrogen) containing 50 mM DTT, and boiled for 5 min at 95°C. Immunoprecipitation of tyrosine-phosphorylated proteins was performed with 30 μ l of agarose-conjugated 4G10 for 2 hours at 4°C. Immunoprecipitated samples were washed in lysis buffer, and bound proteins were eluted with 30 μ l of 100 mM sodium phenylphosphate in PBS. Eluates were diluted with 4 $\times$ NuPAGE LDS sample buffer containing 50 mM DTT. Equal amounts of protein for each

sample were resolved on NuPAGE 4 to 12% Bis-Tris gels (Invitrogen) and subsequently transferred to PVDF membranes (Millipore) in 1 × NuPAGE transfer buffer (Invitrogen). After blocking with 5% bovine serum albumin (BSA) or skim milk for 1 hour in tris-buffered saline (TBS) containing 0.1% Tween 20 (TBS-T), membranes were sequentially incubated with primary antibodies and then with the appropriate HRP-conjugated secondary antibodies (Santa Cruz). Blots were developed with either SuperSignal West Pico or Dura (Pierce) and exposed to Biomax MR film (Kodak).

Statistical analysis

Each graph was generated from at least three independent experiments. Individual data points between two groups were analyzed by two-tailed Student's *t* test. Differences between multiple groups were statistically analyzed with one-way analysis of variance (ANOVA). Repeated-measures ANOVA with Bonferroni correction was used to compare multiple interrelated measurements between groups.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank M. March for useful discussions, M. Robertson for the NKL cell line, and L. Samelson for plasmids encoding LAT and SLP-76. **Funding:** This work was supported by the Intramural Research Program of the National Institute of Allergy and Infectious Diseases, National Institutes of Health, and, in part, by a National Research Foundation of Korea Grant from the Korean Government to H.S.K. (NRF-2011-0014138) and by the Korea Healthcare Technology R&D Project (A110893) and National R&D Program for Cancer Control (1220030), Ministry for Health, Welfare & Family Affairs, Korea to H.S.K..

REFERENCES AND NOTES

1. Vivier E, Tomasello E, Baratin M, Walzer T, Ugolini S. Functions of natural killer cells. *Nat Immunol.* 2008; 9:503–510. [PubMed: 18425107]
2. Moretta L, Bottino C, Pende D, Castriconi R, Mingari MC, Moretta A. Surface NK receptors and their ligands on tumor cells. *Semin Immunol.* 2006; 18:151–158. [PubMed: 16730454]
3. Lodoen MB, Lanier LL. Natural killer cells as an initial defense against pathogens. *Curr Opin Immunol.* 2006; 18:391–398. [PubMed: 16765573]
4. Lanier LL. NK cell recognition. *Annu Rev Immunol.* 2005; 23:225–274. [PubMed: 15771571]
5. Bryceson YT, March ME, Ljunggren HG, Long EO. Activation, coactivation, and costimulation of resting human natural killer cells. *Immunol Rev.* 2006; 214:73–91. [PubMed: 17100877]
6. Bryceson YT, March ME, Ljunggren HG, Long EO. Synergy among receptors on resting NK cells for the activation of natural cytotoxicity and cytokine secretion. *Blood.* 2006; 107:159–166. [PubMed: 16150947]
7. Bryceson YT, Ljunggren HG, Long EO. Minimal requirement for induction of natural cytotoxicity and intersection of activation signals by inhibitory receptors. *Blood.* 2009; 114:2657–2666. [PubMed: 19628705]
8. Lanier LL, Corliss B, Phillips JH. Arousal and inhibition of human NK cells. *Immunol Rev.* 1997; 155:145–154. [PubMed: 9059890]
9. Pende D, Cantoni C, Rivera P, Vitale M, Castriconi R, Marcenaro S, Nanni M, Biassoni R, Bottino C, Moretta A, Moretta L. Role of NKG2D in tumor cell lysis mediated by human NK cells: cooperation with natural cytotoxicity receptors and capability of recognizing tumors of nonepithelial origin. *Eur J Immunol.* 2001; 31:1076–1086. [PubMed: 11298332]
10. Long EO. Negative signaling by inhibitory receptors: the NK cell paradigm. *Immunol Rev.* 2008; 224:70–84. [PubMed: 18759921]

11. Bryceson YT, Long EO. Line of attack: NK cell specificity and integration of signals. *Curr Opin Immunol.* 2008; 20:344–352. [PubMed: 18439809]
12. Bauer S, Groh V, Wu J, Steinle A, Phillips JH, Lanier LL, Spies T. Activation of NK cells and T cells by NKG2D, a receptor for stress-inducible MICA. *Science.* 1999; 285:727–729. [PubMed: 10426993]
13. Cosman D, Mullberg J, Sutherland CL, Chin W, Armitage R, Fanslow W, Kubin M, Chalupny NJ. ULBPs, novel MHC class I-related molecules, bind to CMV glycoprotein UL16 and stimulate NK cytotoxicity through the NKG2D receptor. *Immunity.* 2001; 14:123–133. [PubMed: 11239445]
14. Upshaw JL, Arneson LN, Schoon RA, Dick CJ, Billadeau DD, Leibson PJ. NKG2D-mediated signaling requires a DAP10-bound Grb2-Vav1 intermediate and phosphatidylinositol-3-kinase in human natural killer cells. *Nat Immunol.* 2006; 7:524–532. [PubMed: 16582911]
15. Graham DB, Cella M, Giurisato E, Fujikawa K, Miletic AV, Kloepfel T, Brim K, Takai T, Shaw AS, Colonna M, Swat W. Vav1 controls DAP10-mediated natural cytotoxicity by regulating actin and microtubule dynamics. *J Immunol.* 2006; 177:2349–2355. [PubMed: 16887996]
16. Brown MH, Boles K, van der Merwe PA, Kumar V, Mathew PA, Barclay AN. 2B4, the natural killer and T cell immunoglobulin superfamily surface protein, is a ligand for CD48. *J Exp Med.* 1998; 188:2083–2090. [PubMed: 9841922]
17. Chen R, Relouzat F, Roncagalli R, Aoukaty A, Tan R, Latour S, Veillette A. Molecular dissection of 2B4 signaling: implications for signal transduction by SLAM-related receptors. *Mol Cell Biol.* 2004; 24:5144–5156. [PubMed: 15169881]
18. Bottino C, Castriconi R, Pende D, Rivera P, Nanni M, Carnemolla B, Cantoni C, Grassi J, Marcenaro S, Reymond N, Vitale M, Moretta L, Lopez M, Moretta A. Identification of PVR (CD155) and Nectin-2 (CD112) as cell surface ligands for the human DNAM-1 (CD226) activating molecule. *J Exp Med.* 2003; 198:557–567. [PubMed: 12913096]
19. Shibuya A, Lanier LL, Phillips JH. Protein kinase C is involved in the regulation of both signaling and adhesion mediated by DNAX accessory molecule-1 receptor. *J Immunol.* 1998; 161:1671–1676. [PubMed: 9712030]
20. Kim HS, Das A, Gross CC, Bryceson YT, Long EO. Synergistic signals for natural cytotoxicity are required to overcome inhibition by c-Cbl ubiquitin ligase. *Immunity.* 2010; 32:175–186. [PubMed: 20189481]
21. Turner M, Billadeau DD. VAV proteins as signal integrators for multi-subunit immune-recognition receptors. *Nat Rev Immunol.* 2002; 2:476–486. [PubMed: 12094222]
22. Sylvain NR, Nguyen K, Bunnell SC. Vav1-Mediated Scaffolding Interactions Stabilize SLP-76 Microclusters and Contribute to Antigen-Dependent T Cell Responses. *Sci Signal.* 2011; 4:ra14. [PubMed: 21386095]
23. Saveliev A, Vanes L, Ksionda O, Rapley J, Smerdon SJ, Rittinger K, Tybulewicz VL. Function of the nucleotide exchange activity of vav1 in T cell development and activation. *Sci Signal.* 2009; 2:ra83. [PubMed: 20009105]
24. Tybulewicz VL. Vav-family proteins in T-cell signalling. *Curr Opin Immunol.* 2005; 17:267–274. [PubMed: 15886116]
25. Weber M, Treanor B, Depoil D, Shinohara H, Harwood NE, Hikida M, Kurosaki T, Batista FD. Phospholipase C-gamma2 and Vav cooperate within signaling microclusters to propagate B cell spreading in response to membrane-bound antigen. *J Exp Med.* 2008; 205:853–868. [PubMed: 18362175]
26. Manetz TS, Gonzalez-Espinosa C, Arudchandran R, Xirasagar S, Tybulewicz V, Rivera J. Vav1 regulates phospholipase cgamma activation and calcium responses in mast cells. *Mol Cell Biol.* 2001; 21:3763–3774. [PubMed: 11340169]
27. Stebbins CC, Watzl C, Billadeau DD, Leibson PJ, Burshtyn DN, Long EO. Vav1 dephosphorylation by the tyrosine phosphatase SHP-1 as a mechanism for inhibition of cellular cytotoxicity. *Mol Cell Biol.* 2003; 23:6291–6299. [PubMed: 12917349]
28. Peterson ME, Long EO. Inhibitory receptor signaling via tyrosine phosphorylation of the adaptor Crk. *Immunity.* 2008; 29:578–588. [PubMed: 18835194]
29. Koretzky GA, Abtahian F, Silverman MA. SLP76 and SLP65: complex regulation of signalling in lymphocytes and beyond. *Nat Rev Immunol.* 2006; 6:67–78. [PubMed: 16493428]

30. Balagopalan L, Coussens NP, Sherman E, Samelson LE, Sommers CL. The LAT story: a tale of cooperativity, coordination, and choreography. *Cold Spring Harb Perspect Biol.* 2010; 2:a005512. [PubMed: 20610546]
31. Jordan MS, Singer AL, Koretzky GA. Adaptors as central mediators of signal transduction in immune cells. *Nat Immunol.* 2003; 4:110–116. [PubMed: 12555096]
32. Beach D, Gonen R, Bogin Y, Reischl IG, Yablonski D. Dual role of SLP-76 in mediating T cell receptor-induced activation of phospholipase C-gamma1. *J Biol Chem.* 2007; 282:2937–2946. [PubMed: 17148460]
33. Zhang W, Sloan-Lancaster J, Kitchen J, Triple RP, Samelson LE. LAT: the ZAP-70 tyrosine kinase substrate that links T cell receptor to cellular activation. *Cell.* 1998; 92:83–92. [PubMed: 9489702]
34. Finco TS, Kadlecsek T, Zhang W, Samelson LE, Weiss A. LAT is required for TCR-mediated activation of PLCgamma1 and the Ras pathway. *Immunity.* 1998; 9:617–626. [PubMed: 9846483]
35. Billadeau DD, Upshaw JL, Schoon RA, Dick CJ, Leibson PJ. NKG2D-DAP10 triggers human NK cell-mediated killing via a Syk-independent regulatory pathway. *Nat Immunol.* 2003; 4:557–564. [PubMed: 12740575]
36. Bottino C, Augugliaro R, Castriconi R, Nanni M, Biassoni R, Moretta L, Moretta A. Analysis of the molecular mechanism involved in 2B4-mediated NK cell activation: evidence that human 2B4 is physically and functionally associated with the linker for activation of T cells. *Eur J Immunol.* 2000; 30:3718–3722. [PubMed: 11169415]
37. Hidano S, Sasanuma H, Ohshima K, Seino K, Kumar L, Hayashi K, Hikida M, Kurosaki T, Taniguchi M, Geha RS, Kitamura D, Goitsuka R. Distinct regulatory functions of SLP-76 and MIST in NK cell cytotoxicity and IFN-gamma production. *Int Immunol.* 2008; 20:345–352. [PubMed: 18203684]
38. Jevremovic D, Billadeau DD, Schoon RA, Dick CJ, Irvin BJ, Zhang W, Samelson LE, Abraham RT, Leibson PJ. Cutting edge: a role for the adaptor protein LAT in human NK cell-mediated cytotoxicity. *J Immunol.* 1999; 162:2453–2456. [PubMed: 10072481]
39. Raab M, da Silva AJ, Findell PR, Rudd CE. Regulation of Vav-SLP-76 binding by ZAP-70 and its relevance to TCR zeta/CD3 induction of interleukin-2. *Immunity.* 1997; 6:155–164. [PubMed: 9047237]
40. Wu J, Motto DG, Koretzky GA, Weiss A. Vav and SLP-76 interact and functionally cooperate in IL-2 gene activation. *Immunity.* 1996; 4:593–602. [PubMed: 8673706]
41. Reynolds LF, Smyth LA, Norton T, Freshney N, Downward J, Kioussis D, Tybulewicz VL. Vav1 transduces T cell receptor signals to the activation of phospholipase C-gamma1 via phosphoinositide 3-kinase-dependent and -independent pathways. *J Exp Med.* 2002; 195:1103–1114. [PubMed: 11994416]
42. Bustelo XR. Vav proteins, adaptors and cell signaling. *Oncogene.* 2001; 20:6372–6381. [PubMed: 11607839]
43. Tuosto L, Michel F, Acuto O. p95vav associates with tyrosine-phosphorylated SLP-76 in antigen-stimulated T cells. *J Exp Med.* 1996; 184:1161–1166. [PubMed: 9064333]
44. Barda-Saad M, Shirasu N, Pauker MH, Hassan N, Perl O, Balbo A, Yamaguchi H, Houtman JC, Appella E, Schuck P, Samelson LE. Cooperative interactions at the SLP-76 complex are critical for actin polymerization. *EMBO J.* 2010; 29:2315–2328. [PubMed: 20562827]
45. Bubeck Wardenburg J, Fu C, Jackman JK, Flotow H, Wilkinson SE, Williams DH, Johnson R, Kong G, Chan AC, Findell PR. Phosphorylation of SLP-76 by the ZAP-70 protein-tyrosine kinase is required for T-cell receptor function. *J Biol Chem.* 1996; 271:19641–19644. [PubMed: 8702662]
46. Jordan MS, Sadler J, Austin JE, Finkelstein LD, Singer AL, Schwartzberg PL, Koretzky GA. Functional hierarchy of the N-terminal tyrosines of SLP-76. *J Immunol.* 2006; 176:2430–2438. [PubMed: 16456002]
47. Jordan MS, Smith JE, Burns JC, Austin JE, Nichols KE, Aschenbrenner AC, Koretzky GA. Complementation in trans of altered thymocyte development in mice expressing mutant forms of the adaptor molecule SLP76. *Immunity.* 2008; 28:359–369. [PubMed: 18342008]

48. Lenox LE, Kambayashi T, Okumura M, Prieto C, Sauer K, Bunte RM, Jordan MS, Koretzky GA, Nichols KE. Mutation of tyrosine 145 of lymphocyte cytosolic protein 2 protects mice from anaphylaxis and arthritis. *J Allergy Clin Immunol*. 2009; 124:1088–1098. [PubMed: 19895996]
49. Mocsai A, Abram CL, Jakus Z, Hu Y, Lanier LL, Lowell CA. Integrin signaling in neutrophils and macrophages uses adaptors containing immunoreceptor tyrosine-based activation motifs. *Nat Immunol*. 2006; 7:1326–1333. [PubMed: 17086186]
50. Kambayashi T, Okumura M, Baker RG, Hsu CJ, Baumgart T, Zhang W, Koretzky GA. Independent and cooperative roles of adaptor molecules in proximal signaling during FcεRI-mediated mast cell activation. *Mol Cell Biol*. 2010; 30:4188–4196. [PubMed: 20606011]
51. Bryceon YT, March ME, Barber DF, Ljunggren HG, Long EO. Cytolytic granule polarization and degranulation controlled by different receptors in resting NK cells. *J Exp Med*. 2005; 202:1001–1012. [PubMed: 16203869]
52. Wang H, Wei B, Bismuth G, Rudd CE. SLP-76-ADAP adaptor module regulates LFA-1 mediated costimulation and T cell motility. *Proc Natl Acad Sci U S A*. 2009; 106:12436–12441. [PubMed: 19617540]
53. Baker RG, Hsu CJ, Lee D, Jordan MS, Maltzman JS, Hammer DA, Baumgart T, Koretzky GA. The adapter protein SLP-76 mediates “outside-in” integrin signaling and function in T cells. *Mol Cell Biol*. 2009; 29:5578–5589. [PubMed: 19667077]
54. Fang N, Motto DG, Ross SE, Koretzky GA. Tyrosines 113, 128, and 145 of SLP-76 are required for optimal augmentation of NFAT promoter activity. *J Immunol*. 1996; 157:3769–3773. [PubMed: 8892604]
55. Mesecke S, Urlaub D, Busch H, Eils R, Watzl C. Integration of activating and inhibitory receptor signaling by regulated phosphorylation of Vav1 in immune cells. *Sci Signal*. 2011; 4:ra36. [PubMed: 21632469]
56. March ME, Long EO. β_2 Integrin Induces TCR ζ -Syk-Phospholipase C- γ Phosphorylation and Paxillin-Dependent Granule Polarization in Human NK Cells. *J Immunol*. 2011; 186:2998–3005. [PubMed: 21270398]
57. Barber DF, Long EO. Coexpression of CD58 or CD48 with intercellular adhesion molecule 1 on target cells enhances adhesion of resting NK cells. *J Immunol*. 2003; 170:294–299. [PubMed: 12496412]

Fig. 1.

SLP-76 is required for the synergistic activation of NK cells by NKG2D and 2B4. (A) NKL cells were transfected with control siRNA (siControl) or siRNA specific for (top) SLP-76 (siSLP76) or (bottom) LAT (siLAT). After 24 hours, the cells were rested for another 24 hours, and then lysates were prepared and analyzed by Western blotting for SLP-76, LAT, and actin, as indicated. Ca²⁺ mobilization was also analyzed in the same NKL cells, which were stimulated through NKG2D and 2B4 (indicated by the arrows) after the measurement of baseline Ca²⁺ concentrations for 30 s. Changes in fluorescence are shown as a function of time. (B) Lysis of FcR⁺ P815 cells by rested NKL cells transfected with the indicated siRNAs, in the presence of Abs and at the indicated effector to target cell ratios (E:T). Cytotoxicity against P815 cells in the presence of mAbs specific for NKG2D and 2B4 was determined after 2 hours with the Europium assay. Error bars represent the SD. (C) Cytokine release assays were performed with rested NKL cells that had been transfected with control siRNA or siRNAs specific for (left) SLP-76 or (right) LAT, and stimulated with beads coated with isotype control mAb (cIgG1) or mAbs specific for NKG2D and 2B4 (NKG2D+2B4). After incubation for 16 hours, the amounts of IFN- and MIP-1 in the culture media were measured by ELISA. Values represent mean $\pm$ SD. Data are representative of at least three independent experiments. n.s., not significant; * P < 0.05; ** P < 0.01; *** P < 0.001.

Fig. 2.

Enhanced Vav1 phosphorylation during synergistic activation of NK cells is SLP-76-dependent. (A) Rested NKL cells were mixed with S2, S2-ULBP1, S2-CD48, or S2-ULBP1+CD48 cells, as indicated. After incubation for 20 or 40 min, SLP-76 was immunoprecipitated from NKL cell lysates and samples were analyzed by Western blotting with an antibody against pY160-Vav1 (left). The blot was then stripped and analyzed with an antibody against SLP-76. Band intensities of pY160-Vav1 relative to those of total SLP-76 were quantified with ImageJ software and are presented for each condition (right). (B) Rested NKL cells transfected with control siRNA or SLP-76-specific siRNA were stimulated with isotype control mAb (cIgG1) or with mAbs against NKG2D and 2B4, individually or in combination. After receptor crosslinking for 2 min, cells were lysed and analyzed by Western blotting to detect pY160-Vav1, SLP-76, and actin (left). Blots were then stripped and analyzed with antibody against total Vav1. Band intensities of pY160-Vav1 relative to those of total Vav1 were quantified (right). (C) Rested NKL cells transfected with control siRNA or SLP-76-specific siRNA were stimulated with control S2 cells or with S2 cells expressing ULBP1, CD48, or both ligands for 20 min. NKL cell lysates were analyzed by Western blotting for pY160-Vav1, SLP-76, and actin (left). Blots were then stripped and analyzed for total Vav1. Band intensities of pY160-Vav1 relative to those of total Vav1 were quantified (right). Western blots are representative of at least three

experiments, whereas graphs of densitometric data show the average of at least three independent experiments. n.s., not significant; * $P < 0.05$.

Fig. 3. NKG2D and 2B4 induce complementary signals for SLP-76 phosphorylation at Tyr¹¹³ and Tyr¹²⁸. **(A)** Rested NKL cells were preincubated on ice for 30 min with mAbs specific for NKG2D and 2B4, either individually or in combination, and then were stimulated by crosslinking with secondary goat F(ab)₂ anti-mouse IgG at 37°C for the indicated times. NKL cell lysates were subjected to immunoprecipitation with mAb against pTyr and analyzed by Western blotting with an antibody against SLP-76. **(B)** Rested NKL cells were treated as described for (A) to stimulate NKG2D and 2B4, either individually or in combination, at 37°C for the indicated times. NKL cell lysates were analyzed by Western blotting with antibodies against SLP-76 phosphorylated at Tyr¹¹³ (pY¹¹³), Tyr¹²⁸ (pY¹²⁸), or Tyr¹⁴⁵ (pY¹⁴⁵), and with antibody against SLP-76. **(C)** Freshly isolated, resting human NK cells were stimulated with isotype control mAb or with mAbs specific for NKG2D and 2B4, either individually or in combination. After receptor crosslinking for 2 min, cells were lysed and analyzed by Western blotting with antibodies specific for pY¹¹³-SLP-76, pY¹²⁸-SLP-76, pY¹⁴⁵-SLP-76, and total SLP-76. **(D)** Rested NKL cells were incubated with S2 cells or with S2 cells expressing ULBP1, CD48, or both, as described for Fig. 2A. NKL cell lysates were then analyzed by Western blotting with antibodies specific for pY¹¹³-SLP-76, pY¹²⁸-SLP-76, and total SLP-76. **(E)** Resting NK cells were incubated with S2 cells or with S2 cells expressing ULBP1, CD48, or both for 20 min. NK cells were then lysed and analyzed by Western blotting with antibodies against pY¹¹³-SLP-76, pY¹²⁸-SLP-76, and total SLP-76. All Western blots are representative of at least three independent experiments.

Fig. 4. Complementary phosphorylation of SLP-76 by stimulation of NKG2D, 2B4, and DNAM-1. **(A)** Primary, resting human NK cells were preincubated with mAbs to the indicated receptors either singly or in combination and then were stimulated by receptor crosslinking for 2 min as described for Fig. 3A. NKL cell lysates were then analyzed by Western blotting with antibodies against pY113-SLP-76, pY128-SLP-76, and total SLP-76. **(B)** Jurkat cells were preincubated with mAb specific for CD3 and stimulated for the indicated times as described for Fig. 3A. Jurkat cell lysates were then analyzed by Western blotting with antibodies specific for pY113-SLP-76, pY128-SLP-76, and total SLP-76. All Western blots are representative of three experiments.

Fig. 5. SLP-76 is required for the Ca^{2+} mobilization triggered by individual signaling by NKG2D, 2B4, or DNAM-1 in the context of c-Cbl knockdown. (**A** and **B**). Ca^{2+} mobilization in rested NKL cells transfected with control siRNA or siRNAs specific for (A) c-Cbl and SLP-76 or (B) c-Cbl and LAT. NKL cells were stimulated with mAbs against NKG2D, 2B4, or DNAM-1 as described for Fig. 1A. Data are representative of at least three independent experiments.

Fig. 6.

Reconstitution of cells lacking endogenous SLP-76 with SLP-76 restores Ca²⁺ mobilization in response to combined stimulation of NKG2D and 2B4. To express SLP-76 in cells treated with SLP-76-specific siRNA, we introduced silent mutations into YFP-tagged SLP-76 to render it resistant to SLP-76-specific siRNA. After siRNA-mediated knockdown of SLP-76, NKL cells were transfected with plasmids encoding the indicated proteins. **(A)** Rested NKL cells depleted of SLP-76 and then transfected with the indicated plasmids were analyzed by Western blotting for SLP-76 and actin. **(B)** Ca²⁺ mobilization in YFP⁺ rested NKL cells depleted of SLP-76 that were transfected with plasmids encoding the indicated proteins. NKL cells were stimulated with mAbs to NKG2D and 2B4 as described for Fig. 1A. Data are representative of at least three independent experiments.

Fig. 7.

The N-terminal tyrosines of SLP-76 are required to mediate the synergistic signaling of NKG2D and 2B4. After knockdown of SLP-76 by siRNA, NKL cells were transfected with plasmids encoding the indicated siRNA-resistant, YFP-tagged SLP-76 proteins. **(A)** Rested NKL cells depleted of SLP-76 and then transfected with plasmids encoding the indicated proteins were analyzed by Western blotting for SLP-76 and actin. **(B)** Ca²⁺ mobilization in rested YFP⁺ NKL cells that were depleted of endogenous SLP-76 and then transfected with plasmids encoding the indicated proteins. NKL cells were stimulated with mAbs against NKG2D and 2B4 as described for Fig. 1A. **(C)** Redirected lysis of P815 cells by rested NKL cells depleted of SLP-76 and then transfected with plasmids encoding the indicated proteins at the indicated effector to target cell ratios (E:T). NKL cells were stimulated with mAbs against NKG2D and 2B4 as described for Fig. 1B. Error bars represent the SD. **(D)** Cytokine release assays with rested NKL cells after depletion of SLP-76 and then transfection with plasmids encoding the indicated proteins and stimulation with beads coated with mAbs specific for NKG2D and 2B4, as described for Fig. 1C. Values represent mean $\pm$ SD. Data are representative of at least three independent experiments. n.s., not significant; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.