Association of the X-linked Lymphoproliferative Disease Gene Product SAP/SH2D1A with 2B4, a Natural Killer Cell-activating Molecule, Is Dependent on Phosphoinositide 3-Kinase*

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Natural killer (NK) cells express an activating receptor, 2B4, that enhances cellular cytotoxicity. Upon NK cell activation by ligation of 2B4, the intracellular domain of 2B4 associates with the X-linked lymphoproliferative disease (XLP) gene product, signaling lymphocytic activation molecule-associated protein/SH2D1A (SAP/SH2D1A). Defective intracellular association of 2B4 with mutated SAP/SH2D1A is likely to underlie the defects in cytotoxicity observed in NK cells from patients with XLP. We report here a role for phosphoinositide 3-kinase (PI3K) in the recruitment and association of SAP/SH2D1A to 2B4 in human NK cells. The activation of normal NK cells by ligation of 2B4 leads to the phosphorylation of 2B4, recruitment of SAP/SH2D1A, and association of the p85 regulatory subunit of PI3K. The inhibition of PI3K enzymatic activity with either wortmannin or LY294002 prior to 2B4 ligation does not alter the association of 2B4 with the p85 subunit but prevents the recruitment of SAP/SH2D1A to 2B4. In addition, PI3K inhibitors significantly diminish the cytotoxic function of primary NK cells. This observed inhibition of cytotoxicity, present in normal NK cells, was less apparent or absent in NK cells derived from a patient with XLP. These data indicate that the cytotoxicity of activated NK cells is mediated by the association of 2B4 and SAP/SH2D1A, and that this association is dependent upon the activity of PI3K.

Natural killer (NK) cells are an integral component of the antiviral immune response acting to limit viral replication through both cellular cytotoxic mechanisms and the secretion of cytokines (1, 2). A large number of activating and inhibitory receptors are expressed on the surface of NK cells, and there is a general agreement that the effector function of NK cells is determined by a balance of positive and negative intracellular signals (3, 4). An important activating receptor of NK cells is 2B4, a member of the CD2 subset of the immunoglobulin superfamily (5, 6). Upon 2B4 activation by a natural ligand, CD48, or with a monoclonal antibody, NK cells are induced to augment their cytotoxicity, secrete interferon-γ, and increase calcium flux, phosphoinositol turnover, and perforin degranulation (7–9).

The cytoplasmic tail of 2B4 contains four tyrosine-based motifs that allow for phosphorylation and subsequent binding by molecules containing src-homology 2 (SH2) domains (6, 10, 11). Thus, following stimulation, human 2B4 has been shown to be phosphorylated (12, 13) and associate with a number of SH2 proteins including the SHP-1 and SHP-2 phosphatases (12, 14, 15) and p62dok (Dok1) (16) as well as with the linker for the activation of T-lymphocytes (LAT), an adaptor protein that acts as a substrate for SH2 proteins (17). In addition, 2B4 associates with signaling lymphocytic activation molecule-associated protein/SH2D1A (SAP/SH2D1A), an adaptor molecule containing a single SH2 domain (18).

SAP/SH2D1A is expressed in both NK cells and T-lymphocytes, and the mutations of SAP/SH2D1A are associated with the immunodeficiency syndrome, X-linked lymphoproliferative disease (XLP) (19–21). Consistent with the expression of SAP/SH2D1A in NK cells and its intracellular association with 2B4, XLP patients manifest defects in NK cytotoxic function, particularly following 2B4 activation (18, 22–24). However, the detailed intracellular mechanisms by which SAP/SH2D1A influences 2B4 signaling and NK function are unclear, and in particular, the involvement of phosphoinositide 3-kinase (PI3K) in 2B4-mediated cytotoxicity is unknown.

Class I PI3K, the isoform most linked to lymphocytes, comprises both a catalytic (p110α, β, γ, or δ) and a regulatory (p85α, p85β, p101, or p55γ) subunit (25, 26). In the case of tyrosine-phosphorylated membrane receptors such as 2B4, PI3K activation begins with the formation of a high affinity interaction between SH2 domains of either p85α or p85β and tyrosine-phosphorylated cytoplasmic domains of the membrane receptor. The association of the p85 subunit recruits the p110 catalytic subunit, which can phosphorylate the D-3 position of the inositol ring of phosphoinositol lipids. The phosphorylated inositol lipids and other metabolites including phosphatidylinositol 3,4-biphosphate are termed 3’-phosphoinositides and act as second messengers in signal transduction. A role for PI3K and 3’-phosphoinositides is suggested because PI3K is known to participate in antibody-dependent cellular cytotoxicity of NK cells (27, 28), and inhibition of PI3K activity has been shown to decrease the cellular cytotoxicity of an NK cell line, NK92 (29). In this study, we investigated the role of PI3K in the downstream signaling events of 2B4 following the activation of primary human NK cells. We show that the p85 regulatory sub-
unit of PI3K associates with 2B4, and that the association of 2B4 with SAP/SH2D1A is abrogated by the inhibition of PI3K enzymatic activity. We also provide evidence that PI3K activity induced by 2B4 stimulation is absent in NK cells expressing mutated SAP/SH2D1A. Finally, we show that PI3K inhibitors diminish the cytotoxicity of NK cells obtained from healthy individuals but have little or no effect on the cytotoxicity of NK cells from an XLP patient.

MATERIALS AND METHODS

Antibodies and Reagents—Polyclonal antiserum to human SAP/SH2D1A was produced by immunizing rabbits with a C-terminal peptide (GGITHREDPVCLKAP) coupled to keyhole limpet hemocyanin (ImmuneChem Pharmaceuticals, Vancouver, British Columbia, Canada). Antibodies used included anti-2B4 (mAb C1.7, Biosign Diagnostics, Saco, ME), anti-p85 (BD Transduction Laboratories, Mississauga, Ontario, Canada), anti-phosphotyrosine (4G10, Upstate Biotechnology, Lake Placid, NY), anti-C3D fluorescent isothiocyanate, and anti-CD56 PE (BD Pharmingen, Mississauga, Ontario, Canada). Chemical reagents used were protease inhibitors mixture, wortmannin, LY294002, and IL-2 (Sigma) and PD98059, protein kinase C inhibitor (19–27), and Lck inhibitor PP2 (Calbiochem).

Natural Killer Cells—The study was approved by the University of British Columbia Research Ethics Board, and informed consent was obtained from all subjects prior to blood collection. NK cells were selected from peripheral blood using RosetteSep (StemCell Technologies, Vancouver, British Columbia, Canada) containing antibodies to CD3, CD3ε, CD4, CD66b, CD19, and glycoporphin A followed by Ficoll density gradient centrifugation. Cells were cultured in complete medium (CM) consisting of RPMI 1640 medium supplemented with 10% human serum, 10 units/ml penicillin, 10 μg/ml streptomycin, 1 mM L-glutamine, 1% nonessential amino acids, 5 × 10⁻⁵ M 2-mercaptoethanol, and 1000 IU/ml IL-2 (Teceleukin, Roche Molecular Biochemicals). These cells were used for all experiments and represent an IL-2-activated NK or lymphokine-activated killer cell phenotype. Prior to experimentation, they were rested for 6 h in AIM-V serum-free medium (Invitrogen). For inhibition experiments, chemical inhibitors (100 nM wortmannin and 10 μg/ml PD98059) were added 5 min before adding 2B4 (data not shown). For activation, NK cells were incubated for 5 min with mAb C1.7 and IL-2 (100 IU/ml).

Cytotoxicity Assays—NK cells were prepared as described above and used in either an inactivated (CM alone) or activated (CM with anti-2B4 and IL-2) state as effector cells. The target cells were either the NK-sensitive chronic myelogenous leukemia cell line, K562 (ATCC, Rockville, MD), or the NK-resistant lymphokine-activated killer cell line, Raji (ATCC, Manassas, VA). Target cells were maintained in RPMI 1640 medium with 10% fetal calf serum and labeled with 51Cr for 1 h. The effector to target ratio was adjusted to 20:1, and/or 10:1 in a total volume of 200 μl. The cells were incubated for 4 h at 37 °C, and 100 μl of supernatant was removed from each well for γ-ray counting. Specific lysis was calculated as described previously (23).

Western Blots and Immunoprecipitations—NK cells (5 × 10⁶ cells) were incubated for 30 min at 4 °C with gentle agitation in lysis buffer (10 mM Tris, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 0.2 mM sodium vanadate, 0.2 mM phenylmethylsulfonyl fluoride, 1% Triton X-100, 0.5% Nonidet P-40, and protease inhibitors mixture). The preparation was centrifuged at 14,000 × g for 15 min, and the lysate was precleared by incubation with protein A/G-agarose at 4 °C for 15 min to reduce the nonspecific binding of proteins to agarose beads. The protein concentration of the lysates was measured by the Bradford method, and equal amounts were used in Western blots or immunoprecipitates. For immunoprecipitates, the lysates were incubated overnight at 4 °C with a 1:10 dilution of the relevant antibody. The immune complexes were collected by the addition of protein A/G-agarose, and the pellet was washed three times in immunoprecipitated buffer before suspension in SDS sample buffer. Proteins were separated by SDS-PAGE, transferred onto polyvinylidene difluoride membranes, and blocked with 5% skim milk in Tris-buffered saline containing 0.05% Tween 20 with the exception of tyrosine phosphorylation experiments where 1% bovine serum albumin was added to the Tris-buffered saline containing 0.05% Tween 20. The membranes were incubated with primary antibody in blocking buffer overnight at 4 °C and then with secondary antibody for 2 h at room temperature before detection using either a color development method (Bio-Rad) or ECL using SuperSignal WestPico substrate (Pierce). For all assays, wide range molecular weight standards were used (Bio-Rad). Phosphatidylinositol Kinase Assay—PI3K assays were performed as described previously (30). NK cells were either left intact or pretreated with 100 nM wortmannin as described earlier and then were incubated in CM alone or with mAb C1.7 and IL-2 (100 IU/ml) for 5 min. Cell lysates were immunoprecipitated with mAb C1.7, and immunocomplexes were washed first with buffer containing 137 mM NaCl, 20 mM Tris base, pH 7.5, 1 mM CaCl₂, 1 mM MgCl₂, 0.1 mM sodium orthovanadate, 1% Nonidet P-40 and then with buffer containing 0.5 mM LiCl, 100 mM Tris-HCl, pH 7.5, and 1 mM sodium orthovanadate and finally with Tris/NaCl/EDTA buffer containing 0.1 mM sodium orthovanadate. The protein complexes were mixed with 50 μl of Tris/NaCl/EDTA buffer, 10 μg (20 μg) of phosphatidylinositol 4,5-bisphosphate (Sigma), and 10 μl of 100 mM MgCl₂. The reaction was started by the addition of 30 μC/sample of [γ-³²P]ATP (Amersham Biosciences) and incubated for 10 min at 25 °C. MgCl₂ (20 mM) was then added to this mixture for an additional 15 min. The reaction was stopped by the addition of 20 μl of 6 N HCl and 160 μl of CHCl₃/MeOH (1:1). The organic phase was separated from the chloroform layer, and 50 μl was spotted on thin layer chromatography aluminum Silica Gel 60 F₂₅₄ precoated sheets (Sigma). Thin layer chromatography plates were developed and revealed with [³²P]ATP, methanol, H₂O, NaOH (60:47:11:2), left to dry, and phosphatidylinositol 1,4,5-trisphosphate products were visualized by autoradiography.

RESULTS

NK Cell Activation by Anti-2B4 Results in 2B4 Phosphorylation and the Recruitment of SAP/SH2D1A in Normal but Not XLP NK Cells—Previous reports indicate that the activation of NK cell lines by the ligation of 2B4 is followed by 2B4 phosphorylation and the recruitment of SAP/SH2D1A (12, 17). Using NK cells from healthy individuals, we found that activation of primary NK cells with an antibody to 2B4 (31) results in 2B4 phosphorylation within 2 min of stimulation (Fig. 1A) and the recruitment of SAP/SH2D1A (Fig. 1B). The association of 2B4 and SAP/SH2D1A is present following ligation with anti-2B4 alone but is significantly enhanced when IL-2 is present, suggesting a synergistic interaction between 2B4 and IL-2 and also implying that downstream pathways of IL-2 signaling are important for the recruitment of SAP/SH2D1A (Fig. 1B). The association between 2B4 and SAP/SH2D1A begins at 5 min following NK activation and persists at least 20 min (Fig. 1C).

In contrast, cells from an XLP patient who carries an inactivating arginine to leucine (R55L) mutation of SAP/SH2D1A (32) failed to recruit SAP/SH2D1A to 2B4 following activation (Fig. 1D). The lack of association between 2B4 and SAP/SH2D1A was not because of an absolute lack of SAP/SH2D1A expression in mutated cells, although there was a decreased level of expression seen (Fig. 1E).

2B4 Associates with the p85 Subunit of PI3K and Activated PI3K Recruited SAP/SH2D1A to 2B4—Because PI3K has been previously implicated in both NK cytotoxicity (29) and chemotaxis (30, 33), we investigated whether PI3K associates with 2B4 in activated NK cells. The PI3K isoform in lymphocytes consists of a regulatory subunit p85, which contains two SH2 domains, and a catalytic subunit p110, which functions as a lipid kinase. The presence of SH2 domains within the regulatory subunit suggests a potential association between phosphorylated 2B4 and PI3K. NK cells cultured from healthy individuals were either left inactivated or activated briefly with anti-2B4 and IL-2. Cell lysates were immunoprecipitated with anti-2B4 and immunoblotted with anti-p85. Figure 1E indicates that the p85 subunit of PI3K associates with 2B4 in 3 min following NK activation and dissociates sometime between 10 and 20 min.

The observed association between 2B4 and the p85 subunit of PI3K implies a role for 2B4 in the induction of PI3K activity and the subsequent generation of phosphorylated membrane lipids. To verify that the 2B4-p85 complex has as-
Fig. 1. NK cell activation by anti-2B4 results in 2B4 phosphorylation and the recruitment of SAP/SH2D1A in normal but not XLP NK cells. A, phosphorylation of 2B4 following activation. NK cells were incubated at 37 °C in CM alone (lane 1), CM with mouse IgG (lane 2), or CM with mAb C1.7 and IL-2 (lane 3). Cell lysates were collected after 2 min, immunoprecipitated (IP) with anti-2B4, and immunoblotted (IB) with antiphosphotyrosine. B, SAP/SH2D1A associates maximally with 2B4 following stimulation with anti-2B4 and IL-2. NK cells were incubated at 37 °C for 5 min under the indicated conditions. Cell lysates were immunoprecipitated with anti-SAP/SH2D1A and immunoblotted with mAb C1.7. C, time course of SAP/SH2D1A-2B4 association. NK cells were incubated at 37 °C in CM alone (lane 1), or in CM with mAb C1.7 and IL-2 (lanes 2–6, respectively) for indicated times before cell lysis. Cell lysates were immunoprecipitated with anti-2B4 and immunoblotted with anti-SAP/SH2D1A. D, mutated SAP/SH2D1A does not associate with 2B4. NK cell lysates from normal controls or an XLP patient were immunoprecipitated with anti-2B4 and immunoblotted with anti-SAP/SH2D1A. E, expression of SAP/SH2D1A in normal and XLP NK cells. Cell lysates from were resolved by SDS-PAGE and immunoblotted with anti-SAP/SH2D1A. WB, Western blot. The molecular masses are indicated by the markers on the left.

Fig. 2. 2B4 associates with the p85 subunit of PI3K and activated PI3K recruits SAP/SH2D1A to 2B4. A, PI3K associates with 2B4 in activated NK cells. NK cells were incubated in CM alone (lane 1) or with mAb C1.7 and IL-2 for the indicated times (lanes 2–5, respectively) before cell lysis. Lysates were immunoprecipitated (IP) with mAb C1.7 and immunoblotted (IB) with anti-p85 mAb. B, PI3K activity is absent in NK cells carrying a mutation in SAP/SH2D1A. NK cells were isolated and prepared as described above. The cells were treated as indicated, and cell lysates were immunoprecipitated with anti-2B4 and co-immunoblotted with phosphatidylinositol 4,5-bisphosphate in the presence of [γ-32P]ATP. The arrows indicate either the PI3K products, phosphatidylinositol 1,4,5-trisphosphate (PIP3), or the original spot (Orig.). C, Activated 2B4 recruits PI3K in NK cells of XLP patient. NK cells were incubated as indicated, and cell lysates were immunoprecipitated with anti-2B4 and immunoblotted with anti-p85 mAb. D, the inhibition of PI3K does not prevent association of 2B4 with PI3K. NK cells were treated as indicated. The cells were washed and further incubated for 5 min at 37 °C in CM alone (lane 1) or with mAb C1.7 and IL-2 (lanes 2–5). Cell lysates were immunoprecipitated with mAb C1.7 and immunoblotted with anti-p85 mAb.

Although PI3K activity was not because of a lack of association between 2B4 and PI3K, though this association was weaker in XLP-derived NK cells (Fig. 2C). We also investigated the effect of PI3K inhibitors, wortmannin or LY294002, on the association of 2B4 with PI3K p85 itself. The pretreatment of NK cells with either inhibitor did not prevent the association of the p85 subunit with 2B4 (Fig. 2D). Therefore, the expression of mutated SAP/SH2D1A alters the association and/or function of PI3K in NK cells, and PI3K activity is absent following 2B4-mediated activation of XLP-derived NK cells.

Wortmannin Prevents the Association of 2B4 and SAP/SH2D1A—To investigate further the role of PI3K following 2B4 activation, we studied the effect of wortmannin on the association between 2B4 and SAP/SH2D1A (Fig. 3A). Lysates obtained from healthy individuals were left unstimulated or briefly stimulated with anti-2B4 and IL-2, and the lysates were harvested at 5 min. As expected, because 2B4 is phosphorylated following activation (Fig. 1A), 2B4 and SAP/SH2D1A were associated in stimulated NK cells, however, the pretreatment of cells with PI3K inhibitors but not with other kinase inhibi-
tors resulted in a loss of this association. Because SAP/SH2D1A has previously been shown to associate only with phosphorylated 2B4 (34), we next determined the effect of wortmannin on 2B4 phosphorylation. NK cells from healthy individuals were pretreated with wortmannin and then activated with anti-2B4 to determine whether 2B4 phosphorylation was altered. In normal NK cells, wortmannin did not alter the phosphorylation of 2B4 at 3 min or the dephosphorylation of 2B4 that occurred within 5 min (Fig. 2A). However, in XLP-NK cells, the presence of wortmannin resulted in significantly less phosphorylated 2B4 at both 3 and 5 min, suggesting that dephosphorylation of 2B4 was occurring faster in XLP cells. These observed differences between healthy and XLP NK cells were not because of differences in the phenotype of the cells nor the levels of 2B4 expression (Fig. 3C). Together, these data demonstrate a critical and functional role for PI3K in the recruitment of SAP/SH2D1A to 2B4.

NK Cells with a Mutation of SAP/SH2D1A Have Defects in Activation and Cytotoxicity—In the experiments described above, we determined that the inhibition of PI3K activity prevents an association of SAP/SH2D1A with 2B4, and we showed that NK cells with a mutation of SAP/SH2D1A lack PI3K enzymatic activity. To assess the functional significance of abnormal PI3K signaling, we established NK cytotoxicity assays using cells derived from normal controls and an XLP patient. We showed previously that NK cells carrying the R55L mutation in SAP/SH2D1A have defective cytotoxicity when tested against K562 target cells (23). In those experiments, the reduction of killing by freshly isolated (uncultured) NK cells was a consequence of fewer (CD3-CD56+) NK cells present in XLP males. By using a stringent purification method for selecting NK cells and by culturing them in high dose IL-2, we have since been able to reliably generate CD3-CD56+ NK populations of >90% purity from both healthy volunteers and an XLP patient (Fig. 4A). We compared the cytotoxic function of these effector cells, both normal and XLP-derived, against K562 and Raji targets with or without 2B4 activation. Surprisingly, NK cytotoxicity against K562 cells was not significantly different between the two groups (Fig. 4B), suggesting that IL-2 cultured NK cells from XLP patients are fully capable of killing MHC class I-deficient/low target cells. However, when tested against Raji targets that express surface MHC class I, NK cells from normal controls exhibited more cytotoxicity than XLP NK cells (Fig. 4C, *p* < 0.002). Moreover, when NK cells were activated with anti-2B4, the differences in cytotoxicity were significantly more apparent against both K562 (Fig. 4D, *p* < 0.001) and Raji (Fig. 4E, *p* < 0.0001) cells. These data indicate that XLP-derived NK cells kill MHC class I-deficient target cells in normal fashion but display diminished cytotoxicity against cells expressing MHC class I. In addition, as previously reported, XLP-derived NK cells manifest a significant defect in 2B4-activated cytotoxicity.

Inhibition of PI3K Diminishes the Cytotoxicity of Normal but Not XLP-derived NK Cells—Using the system noted above, we investigated the effect of PI3K inhibitors on the cytotoxicity of normal and XLP-derived NK cells. NK cells were incubated in the presence or absence of PI3K inhibitors prior to cytotoxicity assays performed with or without 2B4 ligation. We found that
Target cells used were K562 (B) CM alone (in CM with 1000 IU/ml IL-2 for 14 days. Cells were washed and incubated in AIM-V medium for 6 h prior to assays. NK cells were incubated in data point is the mean value of the repeated experiments, and the error bars refer to the mean of XLP-derived NK cytotoxicity was insignificant (Fig. 5, isothiocyanate and anti-CD56 and analyzed on a FACSCalibur flow cytometer (BD PharMingen) using Cellquest™ software. (Figs. 5, were also significantly inhibited by wortmannin or LY294002 (D) and E), and the NK cytotoxicity was measured in chromium release assays. Target cells used were K562 (B and D) or Raji (C and E). All assays were performed in triplicate and on at least three separate occasions. Each data point is the mean value of the repeated experiments, and the error bars refer to the mean ± S.D. generated from the three independent assays. Student’s t test was used to calculate p values. *, p = 0.002; **, p = 0.0006; and †††, p < 0.0001.

both wortmannin and LY294002 significantly reduced the cytotoxicity of normal NK cells against K562 target cells with or without 2B4 activation (Fig. 5, A and C, *p < 0.0001 in all cases comparing CM alone with wortmannin or LY294002). However, the reduction in cytotoxicity was far less significant in NK cells that carry the XLP mutation (Fig. 5A, **, p = 0.018, and C, ***, p = 0.036). Against Raji target cells, normal NK cells were also significantly inhibited by wortmannin or LY294002 (Figs. 5, B and D, *p < 0.0001 in all cases), but the inhibition of XLP-derived NK cytotoxicity was insignificant (Fig. 5, B, ***, p = 0.18, and D, ***, p = 0.17). These findings suggest that a mutation of SAP/SH2D1A partially conceals the effect that PI3K inhibitors have on NK cytotoxicity.

**DISCUSSION**

We have investigated the role of PI3K in 2B4-mediated signal transduction following NK cell activation. In agreement with previous reports (12, 13), we found that 2B4 is phosphorylated ~2 min after 2B4 stimulation, and we showed that phosphorylation of 2B4 is followed first by the association of PI3K at ~3 min and then by the association of SAP/SH2D1A at 5 min. These kinetics of association are consistent with a previous report (34) that SAP/SH2D1A interacts only with the phosphorylated form of 2B4. Interestingly, we found that by inhibiting PI3K activity with either wortmannin or with LY294002, the association of SAP/SH2D1A to 2B4 was prevented, implying that PI3K either directly or indirectly facilitates the binding of SAP/SH2D1A to 2B4. In an XLP patient with an inactivating mutation of SAP/SH2D1A, 2B4 and PI3K continued to be associated (Fig. 2C), but PI3K activity was not present (Fig. 2B), suggesting that conversely PI3K activity is dependent on functioning SAP/SH2D1A. These findings taken together imply a complex sequence of events following 2B4 stimulation where SAP/SH2D1A-2B4 association is dependent on PI3K activity and where PI3K activity is dependent on functional SAP/SH2D1A. These events suggest that SAP/SH2D1A has multiple functions beyond simply binding to phosphorylated 2B4 and may also bind to one or more other phosphotyrosine motifs that facilitate PI3K activity. For instance, it was shown previously that LAT constitutively associates with 2B4, and that the ligation of 2B4 leads to the phosphorylation of LAT and the recruitment of other intracellular proteins to the LAT-2B4 complex (17). It has also been suggested in T cells that the p85 subunit of PI3K associates with LAT directly or with the tyrosine-phosphorylated immunoreceptor tyrosine-based motif of the FcR γ-chain via the SH2 domains of p85 (35). Thus, SAP/SH2D1A may facilitate PI3K activation through its association with LAT rather than 2B4 (17). Nevertheless, the above findings argue that PI3K plays an important role in mediating the cytotoxicity of 2B4-activated NK cells.

PI3K and its downstream products have been implicated in basic cell processes that influence NK cytotoxicity including chemotaxis (33), trafficking of perforin/granzyme B (29), and plasma membrane reorganization (36). It is unclear as to which of these activities is most important in the context of 2B4-mediated NK cytotoxicity, because all three mechanisms may play critical roles. For example, the recruitment and activation of PI3K may affect NK plasma membrane reorganization. In support of this hypothesis, previous work (37, 38) indicates that CD2, which is closely related to 2B4, assists in adhesion between T cells and antigen-presenting cells, and that the adhesion-promoting properties of CD2 are mediated by the forma-
Association of XLP Product

**FIG. 5.** Inhibition of PI3K diminishes the cytotoxicity of normal but not XLP-derived NK cells. The effect of the PI3K inhibition on NK cytotoxicity. NK cells were isolated and prepared as described above. The cells were left untreated (CM) or treated with Lck inhibitor (PP2), wortmannin (Wort), or LY294002 (LY) for 30 min at 37°C. NK cells were washed and incubated for 5 min in CM (A and B) or CM with C1.7 plus IL-2 (C and D) before NK cytotoxicity was measured in chromium release assays at a 20:1 effector to target ratio. Target cells used were K562 (A and C) or Raji (B and D). All assays were performed in triplicate and on at least three separate occasions. Each data point is the mean value of the repeated experiments, and the error bars refer to the mean ± S.D. generated from the three independent assays. Student's t test was used to calculate p values. *p < 0.0001 (A–D); **p = 0.018 (A); ***p < 0.18 (B); **p = 0.036 (C); **p = 0.17 (D).

of lipid rafts. Raft formation occurred in both activated T cells, a subset of which also express 2B4, (38, 39) and in NK cells (40, 41), and interestingly, it was dependent on PI3K activity (42). Thus, 2B4 stimulation and the downstream functions of both SAP/SH2D1A and PI3K may assist in lipid raft formation, which is critical for signaling and cytotoxicity. In addition, defects of the perforin/granzyme B pathway would certainly affect NK cell cytotoxicity. In this regard, Djeu and colleagues (29, 43) have shown that intracellular redistribution of perforin granules is dependent on PI3K and its downstream effectors, MEK and extracellular signal-regulated kinase-2 (ERK2).

In addition to preventing the association of 2B4 with SAP/SH2D1A, wortmannin and LY294002 were able to inhibit significantly the cytotoxicity of normal NK cells (Fig. 5). This inhibition was much less apparent or absent in NK cells from a patient with XLP, suggesting that a block at the stage of PI3K was made redundant by a block at the stage of SAP/SH2D1A association. The implication of this redundancy is that PI3K and SAP/SH2D1A participate serially in the same signaling pathway. Therefore, we propose a model whereby the activation of PI3K allows SAP/SH2D1A to associate with 2B4, and we suggest that this association is critical for NK cell effector function. In XLP patients, SAP/SH2D1A is unable to bind to 2B4. Thus, any inhibition of PI3K has no further impact on cytotoxicity, and we conclude that the major effect of wortmannin and LY294002 on blocking cytotoxicity occurs via preventing the association of SAP with 2B4. This conclusion is also consistent with our finding that NK cells carrying mutated SAP/SH2D1A cannot induce PI3K activity following 2B4 stimulation (Fig. 3), although the precise mechanism is unknown.

During investigation of the role of PI3K in NK cytotoxicity, we found as others have (18, 24) that NK cells expressing mutated SAP/SH2D1A lyse K562 target cells as efficiently as normal NK cells. The differences in cytotoxicity between normal and XLP cells only appeared when lymphokine-activated killer-sensitive (NK-resistant) Raji target cells were used or when NK cells were activated with anti-2B4. One difference between K562 cells and Raji cells is that the K562 cells lack or have very low MHC class I surface expression, whereas Raji cells express HLA A, B, C (44). Taken together, one interpretation of these observations is that NK cells from XLP patients are fully capable of killing MHC class I-deficient targets, but because they lack functional SAP/SH2D1A, they are unable to achieve the broader killing repertoire of 2B4-activated NK cells. Because MHC class I-deficient target cells are likely to generate fewer NK inhibitory signals, unstimulated NK cells are still able to efficiently lyse them. We speculate that different viral infections may generate different expression levels of MHC class I on infected cells and that clinically apparent XLP develops only when containment of viral replication requires 2B4-dependent elimination of infected targets.

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