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Ala Aoukaty and Rusung Tan

NK cells from individuals with X-linked lymphoproliferative (XLP) disease exhibit functional defects when stimulated through the NK receptor, 2B4 (CD244). These defects are likely a consequence of aberrant intracellular signaling initiated by mutations of the adaptor molecule SLAM-associated protein. In this report, we show that NK cells from individuals with XLP but not healthy individuals fail to phosphorylate and thereby inactivate glycogen synthase kinase-3 (GSK-3) following 2B4 stimulation. Lack of GSK-3 phosphorylation prevented the accumulation of the transcriptional coactivator β-catenin in the cytoplasm and its subsequent translocation to the nucleus. Potential signaling pathways leading from 2B4 stimulation to GSK-3 phosphorylation were also investigated. Ligation of 2B4 resulted in the phosphorylation of the guanine nucleotide exchange factor, Vav-1, and subsequent activation of the GTP-binding protein Rac-1 (but not Ras) and the serine-threonine kinase Raf-1 in healthy but not XLP-derived NK cells. In addition, the activity of MEK-2 (but not MEK-1) was up-regulated, and Erk1/2 was phosphorylated in normal NK cells but not those from an individual with XLP suggesting that these proteins relay SLAM-associated protein-dependent signals from 2B4. Finally, inactivation of GSK-3 using a specific inhibitor of GSK-3α/β increased the cytotoxicity and cytokine secretion of both healthy and XLP NK cells. These data indicate that the signaling of 2B4 in NK cells is mediated by GSK-3 and β-catenin, possibly through a signal transduction pathway that involves Vav-1, Rac-1, Raf-1, MEK-2, and Erk1/2 and that this pathway is aberrant in individuals with XLP. The Journal of Immunology, 2005, 174: 4551–4558.

N atural killer cells are vital components of the antiviral immune response (1) and important regulators of lymphocyte homeostasis (2). These immunological functions are accomplished through a combination of cell migration, cellular cytotoxicity and cytokine secretion that in turn are dependent on the sequential signaling of tyrosine kinase and G protein-coupled receptors (3, 4). The NK cell surface receptor 2B4 (CD244) transduces extracellular signals that lead to augmentation of both cellular cytotoxicity and IFN-γ secretion (5–9). Ligation-induced phosphorylation of 2B4 recruits intracellular proteins that include PI3K and SLAM-associated protein (SAP)3 to its cytoplasmic domain, thus initiating a signaling cascade, substantial elements of which are unknown (6, 10–14). Mutations of SAP, an intracellular adaptor protein containing a single Src homology SH2 domain, are associated with the fatal disorder X-linked lymphoproliferative (XLP) disease (15–17) and NK cells from XLP patients lack normal function when stimulated through 2B4 (10, 12, 13, 18, 19). Although the precise physiologic functions of SAP in NK cells remain unknown, it is thought that this protein competes with SH2 protein tyrosine phosphatases for binding to tyrosine-phosphorylated 2B4 thus preventing the delivery of negative signals through 2B4 (16). We have shown previously that PI3K is necessary both for the association of SAP with 2B4 and for the optimal function of NK cells (10). PI3K phosphorylates the myo-inositol ring of phosphatidylinositol resulting in the production of 3′ phosphoinositides, including phosphatidylinositol 4,5-bisphosphate and phosphatidylinositol 3,4,5-trisphosphate, and these lipid products act to localize and initiate additional downstream signal transduction molecules. However, the total extent of signaling from surface ligation of 2B4 to cellular changes is still unclear. Both phosphatidylinositol 4,5-bisphosphate and phosphatidylinositol 3,4,5-trisphosphate are known to mediate activation of a diverse family of proteins including protein kinase B/Akt, phospholipase Cγ1, protein kinase C, Vav, Tec, and Itk (20–24) and in addition, PI3K interacts directly, independent of its lipid products, with small G proteins such as Ras and Rac (25, 26).

In investigating the downstream effects of PI3K following 2B4 stimulation of NK cells, we have identified glycogen synthase kinase-3 (GSK-3) and the transcriptional coactivator, β-catenin as potential regulators of NK cell cytotoxicity and IFN-γ secretion. In addition, we have investigated the role of likely signal transduction proteins that might mediate signals between 2B4 and GSK-3/β-catenin, and have identified a potential pathway that involves Vav-1, Rac-1, Raf-1, MEK-2, and Erk1/2. Elements of this pathway are aberrant in XLP NK cells and may underlie the deficiencies seen in NK function following 2B4 stimulation.

Materials and Methods

NK cells

The use of human cells was approved by the University of British Columbia Clinical Research Ethics Board and informed consent was obtained from all subjects. Blood samples were obtained from healthy volunteers and from two subjects with XLP due to a mutation of Arg55 in SAP. NK cells were negatively selected using RosetteSep (StemCell Technologies) containing Abs to CD3, CD36, CD4, CD66b, CD19, and glycoporphin A.

Department of Pathology and Laboratory Medicine, British Columbia’s Children’s Hospital and University of British Columbia, Vancouver, British Columbia, Canada

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2 Address correspondence and reprint requests to Dr. Rusung Tan, Department of Pathology and Laboratory Medicine, British Columbia Children’s Hospital, 4480 Oak Street, Vancouver, British Columbia V6H 3V4, Canada. E-mail address: roo@interchange.ubc.ca

3 Abbreviations used in this paper: SAP, SLAM-associated protein; XLP, X-linked lymphoproliferative; GSK, glycogen synthase kinase; SH, Src homology; LAK, lymphokine-activated killer; PAK, p21-activated protein kinase; PBD, PAK-1 binding domain; PVDF, polyvinylidene difluoride; PDK, phosphoinositide-dependent protein kinase; MBP, myelin basic protein; SGK, serum- and glucocorticoid-induced protein kinase.
Cells were cultured in complete medium consisting of RPMI 1640 supplemented with 10% human serum, 10 U/ml penicillin, 100 mg/ml streptomycin, 1 mM l-glutamine, 1% nonessential amino acids, 5 × 10⁻⁴ M 2-ME, and 1000 U/ml IL-2 (Tecelulekin; Hoffmann-LaRoche). These cells, which were >95% CD3⁺, CD16⁺, and CD56⁺ (data not shown), were used for all experiments and represent an IL-2-activated NK cell or lymphokine-activated killer (LAK) cell phenotype. Before each experiment, NK cells were rested for 6 h in AIM V serum-free medium (Invitrogen Life Technologies). For experiments that involved 2B4 cross-linking LAK cells were stimulated with 0.1 µg/ml C1.7 mAb and 10 U/ml IL-2 for 10 min at 37°C.

Abs and reagents

The following Abs were used: anti-2B4 mAb C1.7 (Biodesig Internacional); anti-phosphotyrosine 4G10, anti-DSG-3, anti-DSG-3β, anti-β1 integrin, anti-Y Rho kinase, anti-phospho-serine/threonine (Upstate Biotechnology); and anti-CD3 FITC, anti-CD16 PE, and anti-D56 allophycocyanin (BD Biosciences). Chemical reagents used were a protease inhibitor mix and GSK-3β inhibitor (Calbiochem). All other chemicals used in this study were obtained from Sigma-Aldrich.

Preparation of cytoplasmic and nuclear fractions

Separations of cytoplasmic and nuclear cell fractions were performed according to previously described method (27). Cell fractions were prepared by suspending 5 × 10⁵ cells in 200 µl of buffer A (10 mM HEPES, pH 7.5, 10 mM KCl, 1.5 mM MgCl₂, 0.5 mM DTT, 0.2 mM EDTA, 25% (v/v) glycerol, 1 mM PMSF, 1 mM Na₃VO₄, 1 µg/ml aprotinin, 1 µg/ml leupeptin, 25 mM β-glycerophosphate). Cell suspensions were incubated on ice for 15 min and 0.5% Igepal was added. The samples were centrifuged for 1 min at 1100 × g at 4°C, and the supernatant was isolated and used as cytoplasmic fraction. The remaining pellet was washed with buffer A and extracted with 100 µl of buffer B (20 mM HEPES, pH 7.5, 0.4 mM NaCl, 1.5 mM MgCl₂, 0.5 mM DTT, 0.2 mM EDTA, 25% (v/v) glycerol, 1 mM PMSF, 1 mM Na₃VO₄, 1 µg/ml aprotinin, 1 µg/ml leupeptin, 25 mM β-glycerophosphate, 0.5% Igepal) for 20 min on ice. The sample was centrifuged for 3 min at full speed in a cold Eppendorf microfuge and the supernatant was collected and used as nuclear fraction.

Cytosotopy assays

NK cells were used in either an inactivated (complete medium alone) or activated (complete medium with anti-2B4 and IL-2) state as effector cells. The target cells were either the NK-sensitive chronic myelogenous leuke-

ELISPOT assay

ELISPOT assays were performed in 96-well BD ELISPOT plates (BD Biosciences) using IFN-γ and granzyme B ELISPOT kits (BD Biosciences) according to the manufacturer’s instructions. Briefly, between 1 × 10⁵ and 1 × 10⁶ NK cells were incubated for 24 h in the presence of 250 µl of buffer A or Raji target cells at 10:1 and 5:1 E:T ratio, with or without stimulation of the 2B4 receptor. Spots were counted using ELISPOT counter (Immunobinsys).

Phosphotyrosine arrays

NK cells were harvested, incubated with anti-2B4, washed with PBS and centrifuged. The cell pellet was homogenized in ice-cold lysis buffer containing: 20 mM MOPS, pH 7.0, 2 mM EGTA, 5 mM EDTA, 30 mM sodium fluoride, 40 mM β-glycerophosphate, pH 7.2, 10 mM sodium pyrophosphate, 2 mM sodium orthovanadate, 1 mM PMSF, 3 mM benamanidine, 5 mM pepstatin A, 10 µg leupeptin, and 0.5% Nonidet P-40. The final concentration for the homogenizing buffer was adjusted to 7.0. Protein concentration was measured using Bradford method, and lysates were suspended in SDS-PAGE sample buffer containing: 30 mM Tris-HCl (pH 6.8), 1% SDS (w/v), 12.5% glycerol (v/v), 0.02% bromophenol blue, and 1.25% 2-ME. The samples were boiled for 5 min at 100°C in the SDS-PAGE sample buffer before protein screening. Controls and experimental samples were loaded in lanes for gel electrophoresis. Proteins were transferred to polyvinylidene difluoride (PVDF) membranes and probed with phosphotyrosine-specific Abs. The phosphoprotein arrays were performed by Kinexus Bioinformatics using proprietary Abs specific for the following phosphoproteins: NR1, Adducin-α, Adducin-γ, Src, cyclin-dependent kinase 1, p38 MAPK, protein kinase C-α, β, and γ, stress-activated protein kinase, MEK1/2 MEK3, MEK6, p70 S6K, Erk, SMAD1, CREB, mitogen-stimulated kinase 1/2, kinase protein R, protein kinase Ca, and GSK-3β.

Western blots, immunoprecipitation, and phosphorylation assays

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 PMSF, 1% Triton X-100, 0.5 M NaCl, 0.1% SDS, and 0.1% sodium deoxycholate (mixed buffers)). The preparation was centrifuged at 14,000 × g at 4°C for 15 min and the lysate was precleared by incubation with protein A/G-agarose at 4°C for 30 min. The protein concentration of the lysates was measured using Bradford method and equal amounts were used in Western blot analysis or immunoprecipitation. For immunoprecipitation, the lysates were incubated overnight at 4°C with a 1/1000 dilution of the relevant Ab. The immune complexes were collected by addition of protein A/G-agarose, the pellet was washed three times in immunoprecipitation buffer before suspension in SDS sample buffer. Proteins were separated on SDS-PAGE, transferred onto PVDF membranes, and blocked with 5% skim milk in Tris-buffered saline containing 0.05% Tween 20, except for tyrosine phosphorylation experiments in which 1% BSA was added to the 0.05% Tween 20. The membranes were incubated with primary Ab in blocking buffer, at 4°C overnight, then with secondary Ab, conjugated to alkaline phosphatase or HRP, 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 m.w. standards were used (Bio-Rad). Phosphorylation assays were performed by immunoprecipitating the NK lysate with the indicated Abs and immunoblotting with anti-phosphotyrosine.

Rac activation assay

Rac activation was measured by preparing lysates from normal and XLP NK cells, unstimulated or stimulated with anti-2B4, and active Rac (Rac-GTP) was immunoprecipitated with p21-activated protein kinase (PAK)-1 binding domain (PBD) agarose (Upstate Biotechnology). PBD bound proteins were separated by SDS-PAGE gel, transferred onto PVDF membrane, and immunoblotted with mouse anti-Rac mAb followed by goat anti-mouse HRP-conjugated Ab. Cell lysates were also incubated with 1 mM GDP as a negative control and 10 µM final concentration of GTPγS as a positive control. For total protein, lysates prepared as previously described were immunoprecipitated with anti-Rac and immunoblotted with anti-Rac.

Kinase activity assays

Raf-1, MEK-1, MEK-2, and phosphoinositide-dependent kinase (PDK)-1 activity were measured using specific kinase activity kits (Upstate Biotechnology). Briefly, Raf-1 activity was measured by the phosphotransferase activity in a kinase reaction that requires active Raf-1. Raf-1 was immunoprecipitated from normal or XLP NK cell lysates with anti-Raf-1 (or control Ig, negative control). These lysates were either used with or without active MEK-1 (recombiant full-length human MEK-1) added as a positive control to specifically assess Raf-1 kinase activity. Briefly, recombinant inactive MEK-1 was used as a substrate for Raf-1 phosphorylation. The resulting reaction was incubated with inactive Erk-2. Under conditions in which MEK-1 is phosphorylated and activated, inactive Erk-2 is activated and when combined with a specific substrate, myelin basic protein (MBP) and [γ-³²P]ATP, produces phosphorylated MBP. The end product, [³²P]MBP, was transferred to phosphocellulose paper, washed in 0.75% phosphoric acid and acetone, then subjected to a scintillation counting. For MEK-1/2 activity assays, MEK-1/MEK-2 immunoprecipitates or active MEK-1/MEK-2 (positive controls) were incubated with or without inactive MAPK-GST (negative control) in the presence of MG132/³²P ATP buffer that leads to MAPK-2 activation. Activated MAPK-2 phosphorylates MBP in the presence of [γ-³²P]ATP, which was measured as discussed. PDK-1 activity was measured by quantifying the ability of Akt/PDK-1 to activate serum- and glucocorticoid-induced protein kinase (SGK). Immunoprecipitates of PDK-1, control Ig (negative control) or active PDK-1 (positive control) were then combined with inactive SGK. An Akt/SGK substrate peptide (RPRRAATF) and [γ-³²P]ATP are added, and the reaction products [³²P]RPRRAATF are measured by scintillation assay.

Statistical analysis

Applicable data were analyzed using Student’s t test.
Results
Stimulation of 2B4 phosphorylates Vav-1 and induces Rac-GTP binding in normal but not XLP NK cells

Our previous work showed that PI3K associates with 2B4 following 2B4 stimulation in human NK cells, that the association is dependent on SAP, and that NK cells carrying the SAP mutation were incapable of synthesizing 3’ phosphoinositides, which are products of PI3K (10). To determine the downstream consequences of defective PI3K activity in 2B4-stimulated cells, we studied proteins that might be related to this signaling pathway. A time course experiment was performed to determine the kinetics of Vav-1 phosphorylation. These experiments indicated that tyrosine phosphorylation of Vav-1 was at maximum 5–10 min after 2B4 stimulation (Fig. 1A). Vav-1 phosphorylation was present 10 min after 2B4 stimulation in normal but not XLP NK cells (Fig. 1B). We then investigated potential targets of Vav including the small G proteins, Ras and Rac-1, and the serine-threonine kinase Raf. We found that 2B4 stimulation did not affect Ras activity in either normal or XLP NK cells (data not shown), but that Rac-1 activity was augmented in normal but not XLP NK cells (Fig. 1C). Because Rac-1 is known to act on Raf-1 through the activation of PAK (28) we also determined the activity of Raf-1 in normal and XLP NK cells. After 2B4 stimulation, the activity of Raf-1 was significantly increased in normal NK cells but not XLP NK cells, indicating that Raf-1 mediates signals downstream of the 2B4-SAP interaction (Fig. 1D).

Stimulation of 2B4 potentiates the activity of MAPK in normal but not XLP NK cells

Rac-1 has previously been shown to synergize with Raf to promote activation of the MAPK via mechanisms that involve PAK1 phosphorylation of MEK-1 (29). Therefore, to investigate the downstream signaling of Rac-1, we examined the roles of Rac-1, MEK-1, and MEK-2 and the MAPKs Erk-1 and Erk-2 for any alteration of activity. To determine whether activation of Vav-1 and Raf-1 affect the MAPK cascade, we measured the activity of MEK-1 and MEK-2 as well as the phosphorylation of MAPKs Erk-1 and Erk-2 for any alteration of activity. To determine whether activation of Vav-1 and Raf-1 affect the MAPK cascade, we measured the activity of MEK-1 and MEK-2 as well as the phosphorylation of MAPKs Erk-1 and Erk-2. Primary NK cells were incubated with either control Ig or with anti-2B4 and the activity of MEK-1 and MEK-2 present in NK cell lysates was assayed. Although MEK-1 activity was unchanged following 2B4 stimulation, its constitutive activity in unstimulated normal NK cells significantly exceeded that of unstimulated XLP NK cells suggesting that MEK-1 is not involved in 2B4 signaling (Fig. 2A). In contrast, the activity of MEK-2 was significantly enhanced following 2B4 ligation of normal cells but not XLP cells indicating that the 2B4–SAP PI3K pathway lies upstream of MEK-2 (Fig. 2B). Activation of MEK2 has been shown previously to lead to phosphorylation of multiple threonine and tyrosine sites of Erk1/2 (30). Therefore, we examined NK cell lysates for evidence of Erk1/2 tyrosine phosphorylation following 2B4 stimulation. In normal but not XLP cell lysates, ligation of 2B4 led to a significant increase in the phosphorylation of Erk-1 and to a lesser extent Erk-2 demonstrating that Erk1/2 activation is disrupted in XLP (Fig. 2C).

NK cells with a mutation of SAP fail to phosphorylate and inactivate GSK-3

To determine protein phosphorylation defects that might be present in NK cells from individuals with XLP, phosphoprotein and protein kinase immunoblot arrays were performed comparing 2B4-stimulated NK cells obtained from an XLP patient carrying a mutation of SAP (31) with those from two healthy controls (data not shown). From the immunoblot arrays, two phosphoprotein bands that were present in normal NK cells following 2B4 stimulation but were absent in XLP cell lysates were chosen for this study. These bands corresponded to two isoforms of GSK-3, GSK-3α, and GSK-3β. To confirm the array findings, control and XLP NK cells were stimulated with anti-2B4, immunoprecipitated with either anti-GSK-3α or anti-GSK-3β, and immunoblotted with anti-phosphotyrosine Ab (Fig. 3). Expression of phosphorylated GSK-3
isoforms was low in XLP NK cells and did not increase following 2B4 stimulation. In contrast, phosphorylated GSK-3 was constitutively present in healthy NK cells and increased dramatically following 2B4 ligation. Therefore, stimulation of NK cells through 2B4 produces GSK-3 phosphorylation in normal but not XLP NK cells and XLP NK cells lack constitutive phosphorylation of GSK-3.

**PDK-1 or RSK-1 are not engaged in the inactivation of GSK-3**

Because both PDK-1 and the serine-threonine kinase, Rsk, have been shown to lie upstream of GSK-3 (32–35), we investigated phosphorylation (activation) of Rsk and measured PDK-1 activity in NK cells from normal as well as XLP subjects suggesting that PDK-1 or RSK-1 are not engaged in the inactivation of GSK-3.

**Constitutively active GSK-3 has been shown to bind the transcriptional coactivator β-catenin resulting in its phosphorylation and degradation. Conversely, phosphorylation of GSK-3 results in its inactivation, in the dephosphorylation of β-catenin, dissociation from GSK-3, and the accumulation of β-catenin in the cytoplasm.** Subsequently, β-catenin is translocated to the nucleus to interact with transcription factors such as TCF-1, Lef-1, and NFATc (36–38). To investigate the effect of GSK-3 dysfunction on β-catenin, 2B4-stimulated NK cell lysates from normal and XLP individuals were separated into cytoplasmic and nuclear fractions and immunoblotted with a specific Ab to the active form of β-catenin (37, 34). In control NK cells, stimulation of 2B4 resulted in the cytoplasmic accumulation and nuclear translocation of β-catenin. However, NK cells derived from XLP patients failed to accumulate β-catenin in either the cytoplasm or nucleus following 2B4 stimulation (Fig. 5).

**Phosphorylation and inactivation of GSK up-regulate NK cell cytotoxicity**

To determine the effect that GSK-3 and β-catenin have on cellular function, NK cells were treated with a specific inhibitor of GSK-3β, TDZD-8 (a thiaazolidinone), and cytotoxicity was determined against the NK target cell lines K562 and Raji by chromium release assay (Fig. 6). TDZD-8 is a highly selective, non-ATP inhibitor of GSK-3β that phosphorylates and inactivates kinase activity (39, 40). Treatment of NK cells with GSK-3β inhibitor should lead to dissociation of β-catenin from GSK-3, its translocation to the nucleus, and increased cytotoxicity by NK cells. In agreement with previous findings, XLP NK cells exhibited deficient cytotoxicity against both target cell lines and cytotoxicity was not augmented following stimulation through 2B4 (Fig. 6, B and D). As anticipated, pretreatment of NK cells with the GSK-3 inhibitor produced significant increases in the cytotoxicity of NK cells derived from normal as well as XLP subjects suggesting that no effect on normal NK cells before or after stimulation (Fig. 4B). Because PDK-1 is known to phosphorylate Rsk leading to GSK-3 phosphorylation (32, 33), it does not appear that Rsk itself is involved in this process following 2B4 stimulation.

**FIGURE 3.** Differential phosphorylation of GSK-3 in normal and XLP NK cells following 2B4 stimulation. NK cells from a healthy individual (Normal) and an XLP patient (XLP) were left unstimulated (−) or were stimulated (+) with 0.2 μg/ml anti-2B4 plus 10 U/ml IL-2. Cell lysates were prepared as mentioned and immunoprecipitated with anti-GSK-3α or anti-GSK-3β then immunoblotted with anti-phosphotyrosine (top) or anti-GSK3 (bottom). Arrow indicates phosphorylated GSK-3α and GSK-3β (∼52 and 46 kDa, respectively).
In addition to cytotoxicity, a major function of NK cells is to release IFN-γ. In healthy individuals (Normal) were stimulated with 0.2 μg/ml anti-2B4 plus 10 U/ml IL-2 and the cell lysates were assayed for PDK-1 activity as described in Materials and Methods. Negative control Ig (clg) and positive control (active PDK-1) for PDK-1 activity are indicated. B, Phosphoserine sites of Rsk remain unphosphorylated following 2B4 stimulation in normal NK cells. Cells from two healthy individuals (C1 and C2) were unstimulated (-) or incubated with anti-2B4 (+), and lysates were prepared as mentioned. The lysates were immunoprecipitated with anti-Rsk-1 and immunoblotted with either specific anti-phosphoserine 363/anti-phosphoserine 380 (top) or with anti-Rsk-1 (bottom).

Inhibition of GSK-3 promotes IFN-γ secretion and granzyme B release by NK cells

In addition to cytotoxicity, a major function of NK cells is to secrete IFN-γ. To determine the role of mutated SAP in secretion of IFN-γ following 2B4 stimulation, healthy and XLP NK cells were assayed by ELISPOT following incubation with K562 target cells (Fig. 7, A and B). K562 target cells were selected because they do not express the 2B4 ligand CD48 (data not shown). As expected, NK cells with mutated SAP produced significantly fewer spots than normal cells and the number of spots did not increase in response to 2B4 stimulation. To determine whether GSK-3 inhibition affects the secretion of IFN-γ in normal or XLP NK cells, cells pretreated with GSK-3 inhibitor were also used in both IFN-γ and granzyme B ELISPOT assays (Fig. 7). As with cytotoxic function, inhibition of GSK-3 resulted in increased frequencies of cells secreting IFN-γ and granzyme B suggesting that GSK-3 mediates IFN-γ secretion and granzyme B release following 2B4 stimulation.

Discussion

We have previously reported that PI3K associates with the 2B4 receptor in NK cells and that XLP patients who have a mutation in SAP lack PI3K lipid kinase activity following 2B4 stimulation (10). To identify relevant protein phosphorylation events that might be disrupted in NK cells with a mutation of SAP, we performed differential phosphoprotein and protein kinase blot arrays comparing NK cells from an XLP patient with those from two healthy controls and we directly investigated molecules downstream of PI3K that might be involved in 2B4 signal transduction. We found that Vav-1 was highly phosphorylated in normal NK cells when stimulated through 2B4 and we speculate that this may be a consequence of the recruitment and/or activation of PI3K in stimulated cells as has been reported following TCR stimulation (41). The phosphorylation of Vav-1 was not detected in XLP NK cells following 2B4 stimulation, supporting a mechanism in which PI3K lipid kinase activity is involved directly or indirectly in activation of Vav-1. Because Vav-1 phosphorylation leads directly to
activation of small G proteins such as Rho family members and Ras, we also investigated whether XLP NK cells were deficient in the activation of Ras and Rac-1 and demonstrated that Rac-1 (but not Ras, data not shown) activation is defective in cells with mutated SAP.

Because we stimulated NK cells with a combination of 2B4 and low dose IL-2, there is a possibility that IL-2 alone may have been responsible for some of the signaling changes. However, we have previously shown that incubation of NK cells with IL-2 alone does not lead to phosphorylation of 2B4 or an association between SAP and 2B4 (10). In addition, NK cells incubated with low dose IL-2 alone did not affect NK cell function (data not shown). Therefore, the SAP-mediated signal transduction changes seen in these experiments are independent of IL-2 signaling.

Interestingly, it has previously been reported that in NK cells, stimulation of 2B4 alone does not result in Vav-1 phosphorylation, but rather serves to enhance LFA-1 mediated Vav-1 phosphorylation (42). Our results using IL-2-stimulated NK cells suggest that Vav-1 is phosphorylated in response to 2B4 stimulation alone implying that in NK cells with an IL-2-activated LAK phenotype, the requirement for LFA-1 may be bypassed. Alternatively, the differences seen may be because we stimulated 2B4 using a mAb and not its natural ligand, CD48. In addition, it was demonstrated that Rac-1 activity is induced in NK cells following LFA-1 mediated Vav-1 phosphorylation (42). Our results with 2B4 parallel those findings and suggest that 2B4 and LFA-1 may use similar transduction pathways in NK cells.

In addition to Vav-1 phosphorylation, activation of Rac-1, Raf-1, MEK-2, and Erk1/2 occurred following 2B4 stimulation. Previous studies using the human NK cell line YT along with specific protein inhibitors support a role for Ras, Raf, and Erk in the 2B4 signaling pathway (9, 43), although in our experiments we failed to find any alteration of Ras activity in either normal or XLP NK cells, suggesting that primary and/or IL-2-activated NK cells may behave differently than YT cells. Nevertheless, in agreement with those findings, we observed that Raf-1 and Erk1/2 were activated following 2B4 stimulation.

Although these direct experiments implicate Vav-1 and Rac-1 in the transduction of signals from 2B4, the protein blot arrays revealed the unexpected finding that GSK-3 is highly phosphorylated in 2B4-stimulated NK cells of healthy but not XLP individuals. These results also indicate that GSK-3 might be a key signaling molecule in both 2B4-dependent and 2B4-independent cellular cytotoxicity as well as granzyme B and IFN-γ secretion because pretreatment with a specific GSK-3β inhibitor significantly up-regulated these functions in NK cells. Nonetheless, it remains unclear exactly how GSK-3 mediates this activity. Unlike most kinases, serine phosphorylation of GSK-3α and GSK-3β leads to their inactivation, either through signal termination or delivery of a negative signal (39). Both GSK-3α and GSK-3β are expressed ubiquitously in mammalian tissues and have been shown to regulate a wide selection of cell functions including cell growth (44), cell polarity (45), and cell fate (46). In resting B lymphocytes unstimulated through their BCR, GSK-3 phosphorylates the transcriptional regulator β-catenin thus targeting it for degradation (47, 48). However, upon BCR stimulation GSK-3 is phosphorylated and GSK-3 mediated phosphorylation of β-catenin is inhibited. The subsequent accumulation of β-catenin leads to its nuclear translocation and transcriptional activity (47). Interestingly, mutations of β-catenin are frequently associated with NK and T cell lymphoproliferative disorders (49, 50) suggesting that β-catenin defects may also contribute to a loss of lymphocyte homeostasis. Our results showed that in normal NK cells, 2B4 stimulation resulted in cytoplasmic and nuclear accumulation of β-catenin implying that in NK cells, as in B cells, GSK-3 phosphorylation results in β-catenin dephosphorylation, dissociation from GSK-3, and nuclear translocation of β-catenin. The increased level of β-catenin in the nucleus of NK cells may result in the

**FIGURE 7.** Inhibition of GSK-3β augments the frequency of IFN-γ and granzyme B-secreting NK cells. ELISPOT assays were used to determine the frequency of NK cells secreting IFN-γ (■) or granzyme B (□) from normal (A and C) or XLP-derived (B and D) NK cells. NK cells were incubated with K562 target cells in the presence (+) or absence (−) of 0.2 μg/ml anti-2B4 plus 10 U/ml IL-2 with or without GSK-3β inhibition (1 μM TDZD-8). The dosage was chosen based on a GSK-3 inhibition dose-response curve (data not shown). Each data point is the mean value of three assays and error bars represent the mean ± SD. B, GSK-3β inhibitor elevated the basal level of granzyme B in NK cells.

**FIGURE 8.** Proposed signal transduction pathway of 2B4. Activation of 2B4 results in tyrosine phosphorylation within its cytoplasmic domain. Subsequent recruitment of SAP is dependent on the activity of PI3K. Generation of lipid kinase products (P1,3,4,5-P3 → P1,4,5-P3) leads to phosphorylation and activation of the guanine exchange factor Vav-1, then sequential activation of Rac, Raf-1, MEK-2, Erk and finally, phosphorylation of GSK-3. Phosphorylated (inactive) GSK-3 releases active β-catenin, which accumulates in the cytoplasm and translocates to the nucleus. Nuclear translocation allows interaction of β-catenin with transcription factors (TFs) affecting gene transcription. The phosphorylation of GSK-3 following 2B4 ligation appears to be through a pathway involving the MAPK and Erk1/2 rather than a pathway involving PDK-1 and Rsk-1 (54).
activation of transcription factors necessary for cell growth, proliferation, or specific NK functions (51–53). In contrast, in XLP NK cells the β-catenin, although present constitutively in the cytoplasm, was significantly decreased following 2B4 stimulation and undetectable in the nucleus. The lack of nuclear localization of β-catenin in XLP NK cells is compatible with the loss of GSK-3 phosphorylation in these cells following 2B4 stimulation. However, we are unable to explain how active β-catenin was constitutively present in the cytoplasm of unstimulated XLP NK cells.

In conclusion, our findings support a mechanism whereby GSK-3 critically mediates the increased cytotoxic function of 2B4-stimulated NK cells. Phosphorylation of GSK-3 in stimulated NK cells is followed by the translocation of β-catenin to the nucleus. One possible pathway leading to GSK-3 phosphorylation involves Vav-1, Rac-1, Raf-1, MEK-2, and Erk-1/2 (Fig. 8). However, in individuals with XLP, elements of this transduction cascade are dysfunctional, GSK-3 phosphorylation is lost, and the cytotoxic and IFN-γ secretion functions of NK cells are diminished.

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Disclosures

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References


