Regulation of AKT dependent cell survival by Syk and Rac

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The abbreviations used are: NK, natural killer; PI 3-kinase, phosphatidylinositol 3-kinase; ERK, extracellular regulatory kinase; MAPK, mitogen-activated protein kinase; H2B, histone 2B; MBP, myelin basic protein; SH2, Src homology 2.
Abstract

IL2 prevents cell apoptosis and promotes survival but the involved mechanisms have not been completely defined. Although PI 3-kinase has been implicated in IL2-mediated survival mechanisms, none of the three chains of the IL2 receptor (IL2R) expresses a binding site for PI 3-kinase. However, IL2Rβ does express a Syk-binding motif. By utilizing an IL2-dependent natural killer (NK) cell line, followed by validation of the results in fresh human NK cells, we identified Syk as a critical effector essential for IL2-mediated prosurvival signaling in NK cells. Down-regulation of Syk by piceatannol treatment impaired NK cellular viability and induced prominent apoptosis as effectively as suppression of PI 3-kinase function by LY294002. Expression of kinase-deficient Syk or pretreatment with piceatannol markedly suppressed IL2-stimulated activation of PI 3-kinase and AKT, demonstrating that Syk is upstream of PI 3-kinase and AKT. However, constitutively active PI 3-kinase reversed this loss of AKT function caused by kinase-deficient Syk or piceatannol. Thus, Syk appears to regulate PI 3-kinase which controls AKT activity during IL2-stimulation. More importantly, we observed Rac1 activation by IL2 and found that it mediated PI 3-kinase activation of AKT. This conclusion came from experiments where dominant-negative Rac1 significantly decreased IL2-induced AKT activation while constitutively-active Rac1 re-elevated AKT activity in not only Syk- but also PI 3-kinase-impaired NK cells. These results constitute the first report of a Syk → PI3K → Rac1 → AKT signal cascade controlled by IL2 that mediates NK cell survival.
IL2 plays a critical role in the regulation of immune responses. It not only functions as a growth factor for NK and T cells, but also serves to promote cell survival, thus affecting the amplification of an immune response against an antigen. The IL2 receptor (IL2R) comprises a heterotrimeric complex consisting of a high affinity cytokine-binding subunit, IL2Rα, which associates with IL2Rβ, and the common IL2Rγ that is shared by other cytokine receptors, including IL-4, IL-7, IL-9 and IL-15. The IL2-IL2R interaction triggers a multitude of signaling molecules, beginning with protein tyrosine kinases. The Src family kinases, Lck, Fyn and Lyn, and the Janus kinases (Jaks) closely associate with the IL2R. The Jaks have been well-documented for initiating signaling of IL2. The current concept is that IL2-activated JAKs recruit critical SH2-containing signaling mediators leading to several cascades including the STATs, Ras/Raf/MEK/MAPK, and PI 3-kinase. These events then regulate the function of key transcription factors and regulators implicated in cell proliferation and differentiation.

Despite the insight into the IL2 signaling pathways that trigger activation, those that promote cell survival in lymphocytes have not been completely elucidated. The PI 3-kinase/AKT pathway promoting survival has been implicated in IL2-activated T cells and BAF/3 cells. However, there is no direct binding site for PI 3-kinase in the IL2R, yet PI 3-kinase is readily activated by IL2. Thus, the activation of PI 3-kinase must rely on other upstream effector(s) that associate with the IL2R. In searching for molecules that would fit this function, we narrowed in on Syk that has long been known to directly bind IL2Rβ. Although much effort on Syk has been in B cells, monocytes, and mast cells, primarily delineating its key role in activating the PLCγ and Ras/MAPK pathways,
some reports did suggest that Syk was involved in PI 3-kinase activation, such as the case in oxidative stress- and insulin-induced cell survival \(^{21,22}\), and \(\alpha IIb\beta 3\) integrin signaling in platelet/megakaryocyte cells \(^{23}\). To critically address the issue if a specific Syk-directed pathway essentially dictates IL2 survival signaling, we utilized an IL2-dependent NK cell line and freshly isolated human large granule lymphocytes (LGLs), and examined whether Syk links IL2 to cell survival. We also attempted to identify what the downstream signal components might be. Here we demonstrated that Syk, together with PI 3-kinase, are both required for IL2-mediated NK survival and made the important discovery that Syk is one major upstream effector for PI 3-kinase/AKT in IL2 signaling. Another important finding is the critical role of Rac1 in mediating PI 3-kinase activation of AKT.
Material and Methods

**Cell Culture, Antibodies and reagents.** NK92 cells, provided by Dr. H.G. Klingeman, Terry Fox Laboratory, Vancouver, Canada, were maintained in α-MEM supplemented with 20% fetal calf serum, 100 units/ml IL2, and 5 x 10^-5 M of 2-mercaptoethanol. Large granular lymphocytes (LGL) possessing high NK activity were freshly-isolated from normal donor blood by percoll gradient centrifugation and cultured in IL2 as described \(^{24}\). Highly-enriched LGLs of 85-90% purity were routinely obtained.

Mouse mAbs to Syk, PI 3-kinase were from Upstate Biotechnology, Lake Placid, NY. Rabbit antibodies to Syk and PI 3-kinase were from Santa Cruz Inc., Santa Cruz, CA. Rabbit anti-phospho-AKT (Ser\(^{473}\)) and panAKT were from Cell Signaling Technology, Inc., Beverly, MA. Piceatannol, wortmannin, and LY294002 were purchased from Calbiochem, La Jolla, CA.

**Apoptosis assay.** NK92 and LGL cells were cultured in complete medium containing IL2 at a density of 3 x 10^5 cells/ml in the absence or presence of 25 µM of piceatannol or LY294002. The cells were collected at 0, 12, 24, 48 h, washed in wash buffer and stained with Annexin-V-FITC in combination with PI according to the manufacturer’s recommendation (PharMingen, San Diego, CA). Cells stained positively by flow cytometry for Annexin-V-FITC were considered apoptotic. These experiments were performed in triplicate.

**Assessment of apoptotic cell morphology and DNA fragmentation.** NK cells, cultured in complete medium containing IL2, were treated with 25 µM of piceatannol or LY294002 for 24 or 48 h. Cells were resuspended at a concentration of 2 x 10^5 cells/0.1 ml, and cytospins were made in triplicate. The slides were then stained with modified
Wright Giemsa. Cells demonstrating nuclear condensation or nuclear bodies were considered apoptotic. Each slide was examined by two individuals with one person blinded. For DNA fragmentation, cellular DNA was prepared, analyzed on 1.4% agarose gel and visualized by ethidium bromide staining for the presence of DNA ladders.

**Vaccinia virus construction and gene delivery.** The plasmid containing p85(DN), which is a dominant-negative form of the regulatory subunit of PI 3-kinase, was kindly provided by Dr. Masato Kasuga, Kobe University School of Medicine, Kobe, Japan; Myc-tagged p110* mutant, which acts as a constitutively active component of PI 3-kinase, was kindly provided by Dr. Anke Klippel (Chiron Corporation, Emeryville, CA) 25. Recombinant vaccinia virus encoding p85(DN) or Myc-p110* was constructed in the pSC11 vector. Vaccinia virus expressing kinase-deficient SykT, encoding a truncated kinase domain, with a molecular weight of approximately 50 kD, was kindly provided by Dr. Andrew M. Scharenberg, Harvard Medical School, Boston 26.

The procedure of vaccinia virus infection has been described 27,28. Briefly, cells were incubated with recombinant vaccinia for 2 h at 37°C at a multiplicity of infection of 5, then washed three times and cultured in serum-free medium containing 0.5% BSA for another 2 h prior to the activation assays. For those experiments combining pharmaceutical inhibitors and viral infection, cells were pretreated for 30 min at 37°C with 25 µM of the inhibitor prior to infection as described above, except that the inhibitor was added back for the last 2 h of the infection before the evaluation of survival function. The virally-transferred protein expression of SykT, Myc-p110, and p85(DN) was examined in the infected cells by western blotting with specific antibodies and detected by enhanced chemiluminescence (Amersham Pharmacia Biotech, Piscataway, NJ).
**Immunoprecipitation and immunoblotting.** Cells were lysed in a lysis buffer containing 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% NP-40, 1 mM phenylmethylsulfonyl fluoride, 1.5 µg each of aprotinin and leupeptin per ml, 10 mM NaF, 3 mM sodium vanadate, and 15 mM glycerolphosphate. Lysates were centrifuged at 13,000 × g for 10 min at 4°C prior to pre-clearance and immunoprecipitation or Western blotting. Equal amounts of the lysates were analyzed for protein phosphorylation and enzyme activity. For immunoprecipitation, lysates were incubated with the antibody in the presence of 100 µl of protein A-protein G (1:1) agarose beads (Sigma, St. Louis, MO) overnight at 4°C. The immunoprecipitates were then washed 4 times with lysis buffer prior to activity analysis. Protein phosphorylation was examined by western blot analysis with phospho-specific antibodies.

**In vitro protein kinase assay.** The kinase assays were performed as previously described. Briefly, following immunoprecipitation the reaction was carried out in the presence of 10 µCi of [γ-32P]-ATP and 5 µM unlabeled ATP in 40 µl of reaction buffer containing 20 mM Tris-HCl (pH 7.4), 10 mM MgCl₂, 10 mM MnCl₂, and 1 mM dithiothreitol. After incubation at room temperature for 20 min, the reaction was stopped and the mixture was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The relative amounts of incorporated radioactivity were determined by autoradiography and quantitated with a PhosphorImager (Molecular Dynamics, Amersham Pharmacia Biotech, Piscataway, NJ). The amount of protein immunoprecipitates in each reaction was also examined by western blot analysis to ensure equal loading.
Detection of PI 3-kinase activities. Cells were lysed and immunoprecipitated with anti-p85 antibody overnight, then incubated with protein A-protein G (1:1) agarose beads for 2 h at 4°C. The immunoprecipitates were washed 4 times with lysis buffer, twice with kinase reaction buffer, and then divided into two equal aliquots. One aliquot was used to check for equal loading and the other aliquot was analyzed for PI 3-kinase activity by incubating the p85 immunoprecipitates with reaction buffer containing 100 µM ATP, 10 μCi of [γ-32p]-ATP, 20 µg L-phosphatidylinositol-4,5-bisphosphate (PI(4,5)P2), 25 mM HEPES (pH 7.4) and 15 mM MgCl2, for 25 min at 25°C. The reaction was stopped by adding 200 µl of 1 M HCl. The reaction mixture was extracted with CHCl3-MeOH. The phosphorylated inositol was differentiated by thin-layer chromatography as described 29. The conversion of PI(4,5)P2 to PI(3,4,5)P3 was determined by autoradiography and quantitated with a PhosphorImager (Molecular Dynamics, Amersham Pharmacia Biotech, Piscataway, NJ).

Detection of Rac1 activity by PAK1 PBD affinity assay. Cells were lysed with Mg2+ lysis/wash buffer provided with the Rac activation kit (Upstate Biotechnology, Lake Placid, NY). The active form of Rac1, Rac1-GTP, was affinity-precipitated from the lysates by incubation with 15 µg of PAK1 PBD-linked agarose, washed 3 times with the wash buffer provided, and then subjected to 12.5% SDS-PAGE. The activated Rac1 that bound with PAK1 PBD was examined by western blotting with anti-Rac provided in the kit and detected by enhanced chemiluminescence.
Results

Requirement for Syk in IL2-dependent survival. Syk has been reported to associate with IL2R in peripheral blood lymphocytes, implicating its role in c-myc gene activation and cellular proliferation\(^\text{16}\). PI 3-kinase/AKT is also known to be involved in the IL2 anti-apoptotic signaling in T and BAF/3 cells\(^\text{13,14}\). We therefore examined whether IL2 maintains survival either via Syk or PI 3-kinase in an IL2-dependent NK cell line. NK92 cells, cultured in complete medium containing IL2, were treated with 25 µM of piceatannol (Pic), 25 µM of LY294002 (LY) or DMSO (used as the solvent). Piceatannol is a naturally-occurring plant polyphenol (trans-3,4,3”",5”"-tetrahydroxystilbene, also known as 3-hydroxyresveratrol) with tyrosine kinase inhibitory activity that selectively targets Syk\(^\text{30}\). LY294002, 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one, is a derivative of quercetin which specifically abolishes PI 3-kinase activity but not PI 4-kinase or other protein and lipid kinases\(^\text{31}\). Cell viability and survival was evaluated by trypan blue exclusion every 24 h. Medium and DMSO control cells possessed high viability that ranged from 94% to 99% at all time points. In contrast, interference with either Syk or PI 3-kinase significantly impaired cellular viability, which was reduced to 66%, 33%, 19% for piceatannol, and 73%, 43%, 27% for LY294002 at 24, 48 and 72 h, respectively (Fig. 1A).

To determine whether these cells underwent apoptosis, we examined their morphological changes. Two typical features of apoptosis, the condensation of nuclei and the formation of nuclear bodies, were assessed. In medium and DMSO controls only 2-4% of cells appeared to be apoptotic (Fig. 1 B). In contrast, a robust increase of nuclei condensation and nuclear bodies was observed after piceatannol or LY294002 treatment,
amounting to 43% and 65%, or 38% and 57%, at 24 h and 48 h intervals (Fig. 1B). In addition, analysis of nuclear DNA also revealed that piceatannol- and LY294002-treatment induced significant internucleosomal DNA fragmentation, another indicator of apoptosis (data not shown). These data suggest that both Syk and PI 3-kinase are required for sustaining NK viability.

**Inactivation of Syk induces apoptosis.** One of the earliest markers of apoptosis is the appearance of phosphoserine on the cell surface prior to DNA degradation. The role of Syk and PI 3-kinase in IL2-mediated prosurvival and antiapoptotic signaling was thus further evaluated by flow cytometric analysis using FITC-labeled annexin-V that binds phosphoserine and propidium iodide that detects fragmented DNA. A rapid increase in the percentage of apoptotic cells was seen with piceatannol- or LY294002- treatment in comparison to the controls (Fig. 1C). At 12 h, 24 h, and 48 h respectively, 29%, 47%, and 79% of IL2-cultured NK cells showed apoptosis when treated with piceatannol, while 26%, 43%, and 71% of IL2-cultured and LY294002-treated cells were apoptotic (p < 0.05). The slight difference between these Annexin-V results and that from morphologic examination (Fig. 1B) might stem from the fact that detection of phosphoserine has a higher sensitivity than microscopic observation. These data strongly support the involvement of Syk and PI 3-kinase in IL2-mediated cell survival and delay of apoptosis.

**Inhibition of Syk activation by piceatannol and SykT.** In light of the observation that piceatannol reduced NK survival and induced apoptosis, we analyzed whether Syk was activated by IL2 in NK cells. Following IL2 stimulation (100 u/ml), Syk was immunoprecipitated from NK cells and evaluated for kinase activity by analyzing Syk auto-phosphorylation and Syk phosphorylation of myelin basic protein (MBP) as the **in**
vitro kinase assay substrate. Activation of Syk appeared within 5 min of IL2 exposure, demonstrated by Syk phosphorylation of MBP, as well as itself, which was markedly suppressed by piceatannol treatment (Fig. 2A). To have further proof that Syk is activated by IL2, we introduced kinase-deficient Syk, SykT<sup>26</sup>, or an irrelevant control, CD56, into NK92 cells via vaccinia virus-mediated gene transfer. SykT, but not CD56, significantly suppressed Syk activation by IL2 (Fig. 2A). Western blotting of the same filter showed an equal amount of endogenous Syk immunoprecipitated from each sample, and a significant level of virally expressed truncated SykT<sup>26</sup>, with a molecular weight around 50 KD (Fig. 2A, bottom panel).

**Suppressive effects of inactivation of Syk on IL2-induced AKT function.** The PI 3-kinase/AKT pathway is activated by IL2R triggering in lymphocytes, despite the fact that the IL2R has no binding capacity for them<sup>7,8,14,32,33</sup>. We thus investigated if Syk, which does directly bind IL-2Rβ, could be involved in PI3-kinase/AKT activation by IL2. In analyzing AKT activation by its phosphorylation at Ser437, we found that piceatannol pretreatment for 30 min sharply reduced AKT phosphorylation in IL2-triggered NK92 cells, even with the lowest concentration of piceatannol at 10 µM (Fig. 2B). These data suggest that Syk is involved in IL2 regulation of AKT.

**Similar effects of Syk and PI 3-kinase inhibitors on AKT.** We next examined whether LY294002 and wortmannin had the same effects as piceatannol on AKT activation in IL2-triggered NK cells. Wortmannin is a fungal metabolite which specifically inactivates PI 3-kinase by modifying Lys 802, a residue required in the phosphate transfer reaction in the ATP binding site<sup>34</sup>. Inhibitors of both Syk and PI 3-kinase were equally effective in suppressing IL2-induced AKT phosphorylation (Fig.
2C). To check that AKT function, and not simply AKT phosphorylation, was affected, the same lysates were immunoprecipitated with anti-panAKT and analyzed for kinase activity using histone 2B (H2B) as a substrate. The results verified that AKT kinase function was also lost by piceatannol, LY294002 or wortmannin treatment in IL2-stimulated NK92 cells (Fig. 2C). These results strongly support our proposal that Syk regulates AKT in IL2 signaling.

**Inhibition of IL2-induced AKT activation by function-deficient Syk.** To further explore the contribution of Syk to IL2-induced AKT activation, we introduced function-deficient SykT or dominant-negative p85 (p85(DN)) into NK92 cells prior to IL2 stimulation. Both SykT and p85(DN) remarkably suppressed IL2-induced AKT phosphorylation (Fig. 2D). Correspondingly, *in vitro* kinase assays showed similar impairment of IL2-stimulated AKT kinase by SykT and p85(DN) (Fig. 2D).

**Syk control of IL2-stimulated PI 3-kinase activation.** Thus far, our data clearly revealed that both Syk and PI 3-kinase are the critical modulators located upstream of AKT in IL2 survival signaling, but gave no clue to whether they operated within the same pathway to control AKT. Based on information from other systems where Syk regulates PI 3-kinase, we explored the impact of Syk on PI 3-kinase in IL2 signaling. NK92 cells were evaluated for PI 3-kinase-mediated inositol kinase activity by *in vitro* kinase assay with phosphatidylinositol (3,4) diphosphate (PI(3,4)P₂) as the substrate and thin-layer chromatography. Within 5 min of IL2 stimulation, PI 3-kinase immunoprecipitated by anti-p85 from NK92 cells displayed high capacity to produce PI(3,4,5)P₃ (Fig. 3). Piceatannol pretreatment substantially reduced this capacity; so did the expression of SykT. In contrast, expression of CD56, as a control, did not interfere with IL2-stimulated
PI 3-kinase activation (Fig. 3). Western blot analysis of the p85 immunoprecipitates indicates equal loading of all samples. Thus, Syk appeared to control PI 3-kinase activation in IL2 signaling.

**Reversal of Syk inhibition of AKT by constitutively active PI 3-kinase.** As an additional proof that Syk regulates PI 3-kinase/AKT, we resorted to Myc-p110*, a constitutively-active mutant of the p110 subunit of PI 3-kinase. We reasoned that if PI 3-kinase lies downstream of Syk for AKT activation, p110* expression in Syk-inactivated cells should restore AKT activation triggered by IL2. We therefore constructed the vaccinia virus encoding Myc-p110* and examined if piceatannol-mediated AKT suppression can be overcome by vaccinia virus-transferred p110*. NK92 cells were pretreated with 25 µM of piceatannol, then infected with vaccinia virus encoding Myc-p110* or CD56 prior to IL2 engagement. Virally transduced Myc-p110* was readily expressed in the cells (Fig. 4A, top panel). IL2 elicted AKT phosphorylation in medium and DMSO+CD56 control groups. As noted before, piceatannol impaired IL2-induced AKT activation. However, this impairment was markedly reversed by the expression of Myc-p110*, whereas Myc-p110* also elevated the basal level of AKT phosphorylation (Fig. 4A, middle panel). Equal loading of AKT was confirmed by western blotting (Fig. 4A, bottom panel).

Analyses of AKT kinase activity, with anti-AKT immunoprecipitates using H2B as a substrate, demonstrated a similar pattern. IL2 sharply induced AKT function in all control groups, i.e., medium, DMSO and CD56 (Fig. 4B). SykT effectively blocked AKT kinase function, as expected. Piceatannol + CD56-treated cells showed similarly reduced AKT activation; however, piceatannol + Myc-p110*-treated cells showed high AKT kinase
activity, comparable to those of the control groups (Fig. 4B), indicating that Myc-p110* overcomes the inhibition by piceatannol. These results re-confirm that both PI 3-kinase and AKT are downstream of Syk in IL2 signaling.

**Regulation of IL2-induced AKT activation by Rac1.** Rac1 has been coupled to mediating PI 3-kinase signaling in various functions, including cell mobility, actin rearrangement, membrane ruffling. However, Rac1 has also been implicated upstream of PI 3-kinase to regulate AKT activation following TCR engagement. We therefore determined if Rac1 was involved in AKT activation in IL2 signaling. Rac1 was significantly activated by IL2, and this activation could be blocked by either SykT or piceatannol, or by p85(DN) or LY294002 (Fig. 5A). Thus, both Syk and PI 3-kinase appear to control IL2-stimulated Rac1 activation. We then expressed mutant Rac1 in the cells and examined their effects on IL2-induced AKT activation. When dominant-negative Rac1, N17Rac1, was introduced into NK92 cells, IL2 could no longer activate AKT phosphorylation (Fig. 5B). In contrast, constitutively-active Rac1, V12Rac1, markedly elevated AKT phosphorylation, even in the absence of IL2. Thus, Rac1, like Syk and PI 3-kinase, is required for IL2-induced AKT function.

**Counteracting the inactivation of Syk by constitutively active Rac1** Several possibilities could occur, with one possibility being that Rac1 and Syk act independently on PI 3-kinase in parallel non-intersecting pathways to result in AKT activation. A second possibility is that Rac1 and Syk are within the same pathway to modulate PI 3-kinase. If this is the case, then it is essential to define if Syk is upstream of Rac1 or vice versa, and to identify the specific sequence of this signaling cascade. With the combination of V12Rac1, piceatannol and LY294002, we were able to address this issue.
NK92 cells, pretreated with piceatannol, LY294002 or DMSO, were infected with vaccinia virus encoding V12Rac1. These cells were then stimulated with IL2 for 5 min. While DMSO, DMSO plus CD56, and DMSO plus V12Rac1 controls all exhibited a high level of AKT activity, piceatannol-, and LY294002 abrogated this activation (Fig. 5C). However, V12Rac1 remarkably counteracted the inhibitory effect of piceatannol to restore AKT function (Fig. 5C). Moreover, V12Rac1 also restored AKT activation lost in LY294002-treated NK cells, demonstrating that Rac1 was downstream of both Syk and PI 3-kinase (Fig. 5C). Taken together, these data clearly suggest that a specific signaling cascade is triggered by IL2 that involves sequential activation of Syk, PI 3-kinase, Rac1 that leads to AKT activation.

Validation of this Syk-dependent IL2 signaling in human NK cells. To confirm that the same pathway is physiologically relevant and not isolated to the NK92 cell line, we extended our observations to fresh LGL cells from normal donors cultured in IL2-containing medium. Again, we found a marked induction of apoptosis in piceatannol- or LY294002-treated LGLs, demonstrated by Annexin V labeling (Fig. 6A). Internuclear DNA fragmentation showed the same results (data not shown).

Inspection of AKT phosphorylation and kinase function correspondingly showed similar results as those obtained from NK92 cells. Piceatannol notably suppressed IL2-induced AKT phosphorylation and kinase function in LGLs, which were significantly re-elevated by expression of V12Rac1 (Fig. 6B, C). Taken together, these results demonstrated that this Syk-directed IL2 signaling pathway also operates in normal human NK cells, where PI 3-kinase, Rac1 and AKT interact and function downstream of Syk.
Discussion

The key role of Syk in IL2-maintained NK survival has, to date, not been addressed, although it has been documented that Syk physically associates with IL2R in peripheral lymphocytes. IL2 modulates multiple cellular processes, including lymphocyte maturation, proliferation, survival, homeostasis as well as lytic function. While multiple signaling and effector pathways have been implicated in IL2 signaling in independent observations, the contribution of each of them to guide a specific biological function is still incompletely understood. Prior to our work, little was known about the important role of Syk in sustaining NK survival; neither the correlation between Syk and IL2-directed PI 3-kinase/AKT cascade nor that between Syk and NK survival has been reported.

We chose to focus on deciphering the IL2 signaling code that controls survival and apoptosis, and uncovered a novel, specific Syk-regulated pathway that controls PI 3-kinase/AKT and cell survival. First, we demonstrated that intact function of Syk and PI 3-kinase is essential for IL2-regulated NK survival; down-regulation of either of them affected cell viability and resulted in apoptosis. Second, we discovered that Syk regulates IL2-dependent AKT activation via its critical control over PI 3-kinase. Syk inhibition by dominant-negative SykT expression or piceatannol pretreatment abrogated PI 3-Kinase as well as AKT activities in IL2-stimulated NK cells. Impaired AKT function in Syk-inhibited NK cells can be restored by constitutively active PI 3-kinase, indicating that PI 3-kinase is the intermediate signal molecule between Syk and AKT. These findings suggest that Syk, which associates with IL2R, is critical for PI 3-kinase-dependent AKT activation. Previous studies with PI 3-kinase in IL2 function have implicated numerous
pathways regulated downstream of PI 3-kinase. These include MEK/MAPK activation\textsuperscript{39}, p70\textsuperscript{56K}, E2F induction and T lymphocyte proliferation\textsuperscript{9,10,40}, cytoskeleton alterations\textsuperscript{36}, and survival\textsuperscript{9,10,13,14}. No information has been available on what signal component could act upstream of PI 3-kinase. Our results represent the first report on Syk linkage to PI 3-kinase in IL2 signaling for NK cell survival. Of note, Syk-deficient, and in fact, Syk-/- Zap70-/- mice have been reported to express normal NK cell development and function\textsuperscript{41,42}. It is known that NK cell development and function is complex and depends on a combination of cytokines, including c-kit ligand, Flt-3 ligand, IL7, IL12 and IL15 besides IL2\textsuperscript{43-46}. The likely explanation for normal NK cell development and function in Syk-/- and Syk-/- Zap70-/- is that some of these other cytokines can utilize signals independent of Syk or Zap70 and thus provide redundant mechanisms for growth and survival.

The mechanism by which Syk activates PI 3-kinase is not yet studied, but several possibilities come to mind. As a direct substrate of Syk, Shc appears to be a good candidate, which can either recruit the Ras/MAPK cascade via Grb2/SOS or the PI 3-kinase pathway\textsuperscript{47}. In BAF3 cells, engagement of the GMCSF or IL-3 receptor leads to Shc phosphorylation, which leads to Gab2 tyrosine phosphorylation and its binding with the p85 subunit of PI 3-kinase\textsuperscript{48}. Additionally, gene delivery of Syk into 293T cells results in Gab2 phosphorylation\textsuperscript{49}. Thus, Syk can activate Shc, which phosphorylates Gab2, creating a binding site for PI 3-kinase, resulting in its activation. IL2 also has been reported to activate Gab2 via Shc\textsuperscript{48,49}. In addition, cross-linking of CD2 and CD16 in NK3.3 cells induced both Syk and PI 3-kinase activation, with the latter correlating with the tyrosine phosphorylation of Shc\textsuperscript{50}. On the other hand, other systems may also exist that link Syk to PI 3-kinase. The p85 has been reported to bind Jak1 and this association
is suggested to be another means by which PI 3-kinase becomes activated. A novel B cell adaptor for PI3K, termed BCAP, which triggers BCR-associated protein tyrosine kinase-induced PI3K activation, has been implicated in BCR-induced PI 3-kinase signaling, whose phosphorylation mediated by Syk and Btk, provides binding site(s) for p85. In T cells p36/38 linker protein also provides a bridge to the recruitment/activation of PI 3-kinase via its phosphorylation by Syk or a Syk-regulated kinase following TCR-triggering. It remains to be seen which pathway mediates Syk activation of PI 3-kinase in IL2 signaling, but it seems more likely that Gab2 is responsible via Shc, particularly based on the report that IL2 induces Gab2 activation.

AKT is conventionally considered to be activated in a PI 3-kinase-dependent manner. Our findings indicate that AKT activation by IL2 is also Syk-dependent. This observation is supported in other systems. BCR-induced AKT activation requires Syk, while Lyn acts as an endogenous antagonist for this activation, implying a mechanism for the delicate adjustment on BCR-triggered AKT. Syk is also required for the activation of the PI 3-kinase/AKT survival pathway induced by oxidative stress in B cells. Additionally, Syk regulates AKT activation in platelet/megakaryocyte-specific αIIbβ3 integrin signaling in fibrinogen-adherent cells.

Although AKT dependency on Syk is becoming known, its reliance on Rac1 is a novel finding. This conclusion came from experiments here in this study whereby dominant-negative Rac1 suppressed AKT activation in IL2-treated NK cells. In addition, constitutively-active Rac1 could induce AKT activation in either piceatannol- or LY294002-treated NK cells, indicating that Rac1 can work downstream of both Syk and PI 3-kinase. Rac1 is particularly noted for its role in cytoskeletal organization, membrane
Our data suggest that Rac1 is also critical for cell survival via activation of AKT. Despite the well-accepted concept that AKT activation requires PDK1 and/or ILK, we now have supportive data to indicate that AKT activation, at least in IL2 signaling, requires Rac1. It is possible that the Rac1 pathway for AKT activation may be restricted to cells of the immune system, because two recent reports have also demonstrated AKT as a downstream target of Rac1 in TCR signaling and FceRI stimulation. It should be noted that Rac1 can also apparently act upstream of PI 3-kinase to activate AKT in certain cell systems. Some systems, such as membrane ruffling, appear to utilize a similar pathway as ours in that Rac1 worked downstream of PI 3-kinase. Interestingly, PTEN (Phosphatase and tensin homolog deleted on chromosome 10), a tumor suppressor possessing the phosphatase activity on phosphatidylinositol (PI) 3,4,5-trisphosphate (PI(3,4,5)P3), a product of PI 3-kinase, which decreases PDK1 and AKT activation, remarkably downregulates Rac1 and Cdc42 function to negatively control cell motility, providing another important insight that Rac1 might mediate PI 3-kinase effects on AKT. We do not know yet how Rac1 can function on either side of PI 3-kinase. It is conceivable that the divergence between our observation and that of other groups might stem from the different signaling components inherent in the systems of choice. It is also possible that the variations in the respective functions between different systems influenced the upstream signaling that regulates AKT.

We recently reported that Rac1, acting downstream of Syk and PI 3-kinase, controls PAK1, MEK and ERK function in human NK cells, which resulted in the mobilization of lytic granules towards the engaged tumor cell. Thus, Rac1 may have multiple...
downstream effectors, depending on the kinases that are ultimately regulated. At least, in NK cells, we were able to dissect out 2 distinct pathways utilizing Rac1, depending on the receptor. NK receptor engagement by tumor cells triggered Rac1-dependent ERK that drives lytic function, while IL2R engagement elicited Rac1-dependent AKT activation that controls survival. Of note, is that both NK receptor engagement and IL2R engagement triggered Syk activation leading initially to activation of PI 3-kinase, followed by Rac1. However, substrates downstream of Rac1 differ depending on either lytic function or cell survival.

This is the first report documenting a novel linkage between Syk and Rac1 in control of IL2-mediated NK survival. These data have provided functional and biochemical evidence pertaining to the sequential and specific interaction of Syk, PI 3-kinase, Rac1 and AKT in NK cells to prevent apoptosis, while the cytotoxic function of human NK cells also requires the sequential interaction of these molecules.

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References


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Figure legend

FIG. 1. Inhibition of IL2-mediated survival by inactivating Syk and PI 3-kinase. (A) NK92 cells, cultured in complete medium containing 100 units/ml of IL2, were incubated with 25 µM piceatannol or 25 µM LY294002 or DMSO vehicle control, and examined for cell viability by trypan blue exclusion at 0 h, 24 h, 48 h and 72 h. Experiments were performed in triplicate. The percentage of viable cells calculated per experiment is shown (p<0.05). (B) NK92 cells obtained from (A) were examined for cell morphological changes at 24 h. Experiments were performed in triplicate. The % apoptotic cells, as well as the total number of cells counted per experiment is shown on the bottom of each panel. Statistical analysis using a χ² test revealed that piceatannol and LY294002 significantly induced apoptotic morphology in NK cells (p < 0.05). Nuclear condensation and nuclear body (arrow) are indicated. (C) NK92 cells from A were examined for apoptosis by Annexin-V-FITC binding and PI uptake. The cells were collected at 24 and 48 h in each experiment, washed in sample wash buffer and stained with annexin-V-FITC in combination with PI. The annexin-V-FITC binding in piceatannol-, LY294002-, or DMSO-treated, and untreated NK92 cells is shown.

FIG. 2. Inhibition of IL2-induced AKT function by inactivating Syk. (A) NK92 cells, rested in IL2-free medium for 4 h at 37°C, were treated with 25 µM piceatannol, or DMSO for 30 min at 37°C. In parallel, IL2-starved cells were also infected with recombinant vaccinia virus encoding SykT or CD56 irrelevant control gene. The cells were then stimulated with IL2 (100 units/ml) for 5 min at 37°C and analyzed for Syk protein kinase activation by Syk auto-phosphorylation and by its ability to phosphorylate
myelin basic protein (MBP) as the substrate in an *in vitro* kinase assay. After autoradiography, the same membrane was probed with anti-Syk to check for equal loading which were shown at the bottom of the panel. (B) NK92 cells, rested in IL2-free medium for 4 h at 37°C, were treated with 10, 25, or 50 µM piceatannol, or DMSO for 30 min at 37°C, then stimulated with IL2 (100 units/ml) for 5 min at 37°C and analyzed for AKT phosphorylation with Ser473-specific antibody. The same membranes were stripped and re-probed with anti-panAKT to check for equal loading. (C) IL2-starved NK92 cells were treated with 25 µM piceatannol, or 25 µM LY294002, or 25 nM wortmannin, or DMSO for 30 min at 37°C. The cells were then stimulated with IL2 (100 units/ml) for 5 min at 37°C prior to western blot analysis of AKT phosphorylation at Ser473. The cells were also analyzed for AKT kinase activation by *in vitro* kinase assay with histone H2B as the substrate. After autoradiography the same membrane was probed with anti-panAKT to check for equal loading. (D) IL2-starved NK92 cells, infected with recombinant vaccinia virus encoding SykT, or P85(DN), or CD56 irrelevant control gene, were stimulated with IL2 (100 units/ml) for 5 min at 37°C and then analyzed for AKT phosphorylation at Ser473 as well as AKT kinase activation. The same membranes were stripped and re-probed with anti-panAKT. These results represent one of four independent experiments.

*FIG. 3.* Suppression of IL2-induced PI 3-kinase activation by inhibiting Syk. IL2-starved NK92 cells, treated with 25 µM piceatannol, or infected with recombinant vaccinia virus encoding SykT or CD56 irrelevant control gene, were stimulated with IL2 (100 units/ml) for 5 min at 37°C. The cells were then analyzed for PI 3-kinase activity by *in vitro* kinase assay and thin-layer chromatography analysis with PI(3,4)P2 as the substrate. The fold
increase of IL2-stimulated PI 3-kinase activity compared to basal level is shown at the bottom of the panel. These results represent one of three independent experiments.

FIG. 4. Rescue of IL2-induced AKT activation in Syk-impaired cells by constitutively active PI 3-kinase. (A) IL2-starved NK92 cells, treated with 25 µM piceatannol or DMSO, were infected with recombinant vaccinia virus encoding constitutively active catalytic subunit of PI 3-kinase or CD56 irrelevant control gene, then stimulated with IL2 (100 units/ml) for 5 min at 37°C. The cells were analyzed for AKT activation by western blot analysis with Ser473-specific antibodies. (B) The cells were similarly treated as in (A), the whole cell lysates were analyzed by *in vitro* kinase assays for AKT activation with H2B as the substrate. The same membranes were stripped and re-probed with anti-panAKT. These results represent one of four independent experiments.

FIG. 5. Regulation of IL2-mediated AKT activation by Rac1. (A) IL2-starved NK92 cells, pretreated by LY294002 (25 µM), piceatannol (25 µM), or DMSO control, or infected with recombinant vaccinia virus encoding dominant negative P85 (P85(DN)), or kinase-deficient Syk (SykT), or CD56 irrelevant control gene, were stimulated with IL2 (100 units/ml) for 5 min at 37°C, and analyzed for IL2-triggered Rac1 activation before and after the impairment of Syk function. Rac1 was immunoprecipitated from NK92 cell lysates and examined for activity by affinity precipitation (AP) with PAK1 PBD, which binds only to activated Rac1-GTP, but not inactivated Rac1-GDP. The IL2-activated Rac1, Rac1-GTP, was precipitated by PAK1 PBD agarose, then resolved by 12.5% SDS-PAGE and examined by anti-Rac monoclonal antibody provided in the kit. (B) IL2-starved NK92 cells, infected with recombinant vaccinia virus encoding dominant-negative Rac1 (N17Rac1) or constitutively active Rac1 (V12Rac1), or CD56 irrelevant
control gene, were stimulated with IL2 (100 units/ml) for 5 min at 37\(^{\circ}\)C. The cells were analyzed for AKT activation by western blot analysis. (C) IL2-starved NK92 cells, treated with 25 \(\mu\)M piceatannol or 25 \(\mu\)M of LY294002 or DMSO, were infected with recombinant vaccinia virus encoding constitutively active V12Rac1 or CD56 irrelevant control gene, then stimulated with IL2 (100 units/ml) for 5 min at 37\(^{\circ}\)C. The cells were analyzed for AKT activation by western blot analysis with Ser473-specific antibody. The same membranes were stripped and re-probed with anti-panAKT. In parallel, these NK cells were also analyzed by \textit{in vitro} kinase assays for AKT activation with H\textsubscript{2}B as the substrate. These results represent one of four independent experiments.

FIG. 6. Confirmation of the critical role of Syk in IL2 maintained LGL survival. (A) Human fresh NK (LGL) cells, cultured in complete medium containing 100 units/ml of IL2, were incubated with 25 \(\mu\)M piceatannol or 25 \(\mu\)M LY294002 or DMSO vehicle control, and examined for cellular viability by Annexin-V-FITC binding and PI uptake. The LGL cells were collected at 24 h and 48 h, washed in sample wash buffer and stained with Annexin-V-FITC in combination with PI. The Annexin-V-FITC binding in piceatannol-, LY294002-, or DMSO-treated, and untreated NK92 cells is shown. (B) Inhibition of IL2-induced AKT function by inactivating Syk, as well as the rescue of this inhibition by constitutively active PI 3-kinase. IL2-starved LGL cells, treated with 25 \(\mu\)M piceatannol or DMSO, were infected with recombinant vaccinia virus encoding the constitutively active catalytic subunit of PI 3-kinase, P110*, or CD56 irrelevant control gene, then stimulated with IL2 (100 units/ml) for 5 min at 37\(^{\circ}\)C. The cells were analyzed for AKT activation by western blot analysis with Ser473-specific antibodies. (C) The LGL cells were similarly treated as in (B), the whole cell lysates were analyzed by \textit{in
vitro kinase assays for AKT activation with H$_2$B as the substrate. The same membranes were stripped and re-probed with anti-panAKT. These results represent one of four independent experiments.
Fig. 1
Fig. 2
Fig. 2
**Fig. 3**

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<th>DMSO</th>
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**Fold increase**

|            | 1.0  | 13.3 | 0.7 | 1.0  | 11.8 | 0.9  |

![Western Blot Image]

**WB**

α P85 - IP
Fig. 4
Fig. 5
Fig. 5
**Fig. 6**

A. 

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B. 

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WB

- α phospho-AKT
- α panAKT

C. 

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Fold increase: 1.0 13.9 1.4 1.5 13.8 1.6 1.4 14.5

WB

- α panAKT

H₂B phosphorylation

panAKT IP
Regulation of AKT dependent cell survival by Syk and Rac

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