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Differential Regulation of Human NK Cell-Mediated Cytotoxicity by the Tyrosine Kinase Itk

Dianne Khurana, Laura N. Arneson, Renee A. Schoon, Christopher J. Dick, and Paul J. Leibson

NK cells are effector lymphocytes that can recognize and eliminate virally infected and transformed cells. NK cells express distinct activating receptors, including an ITAM-containing FcR complex that recognizes Ab-coated targets, and the DNAX-activating protein of 10 kDa-containing NKG2D receptor complex that recognizes stress-induced ligands. The regulatory role of specific tyrosine kinases in these pathways is incompletely understood. In this study, we show that, in activated human NK cells, the tyrosine kinase IL-2-inducible T cell kinase (Itk), differentially regulates distinct NK-activating receptors. Enhanced expression of Itk leads to increases in calcium mobilization, granule release, and cytotoxicity upon stimulation of the ITAM-containing FcR, suggesting that Itk positively regulates FcR-initiated cytotoxicity. In contrast, enhanced Itk expression decreases cytotoxicity and granule release downstream of the DNAX-activating protein of 10 kDa-containing NKG2D receptor, suggesting that Itk is involved in a pathway of negative regulation of NKG2D-initiated granule-mediated killing. Using a kinase mutant, we show that the catalytic activity of Itk is required for both the positive and negative regulation of these pathways. Complementary experiments where Itk expression was suppressed also showed differential regulation of the two pathways. These findings suggest that Itk plays a complex role in regulating the functions initiated by distinct NK cell-activating receptors. Moreover, understanding how these pathways may be differentially regulated has relevance in the setting of autoimmune diseases and antitumor immune responses where NK cells play key regulatory roles. The Journal of Immunology, 2007, 178: 3575–3582.

Natural killer cells are innate effector lymphocytes that eliminate cells infected by viruses. NK cells also have the ability to destroy cells that have undergone malignant transformation. The principal mechanism used by NK cells to kill target cells is the secretion of preformed granules containing perforin and granzymes that together induce apoptosis of the target cell. Upon direct contact with a target, NK cells can also activate apoptosis by engaging death receptor pathways. In addition, inflammatory cytokines, including IFN-γ and TNF-α, are secreted by activated NK cells promoting NK cell cytotoxicity and shaping the innate and adaptive immune responses. NK cell function is regulated by a variety of activating and inhibitory cell surface receptors. Thus, NK cell activation results from the triggering of activating receptors together with reduced signaling through inhibitory receptors. Distinct activating receptors on NK cells include the low-affinity receptor for IgG, FcγRIIIA (or CD16), hereafter referred to as FcR, which recognizes Ab-coated target cells during Ab-dependent cell-mediated cytotoxicity, and the NKG2D receptor that recognizes stress-inducible ligands on cells.

The FcR and NKG2D receptor both transmit an activation signal, resulting in granule release and cytotoxicity by NK cells, although these receptor-initiated events are differentially regulated (1, 2). The signal transducing subunit for the FcR contains an ITAM that recruits Syk family kinases upon activation, whereas the DNAX-activating protein (DAP10)3 signal transducing subunit for the NKG2D receptor contains a motif that binds to both PI3K and Grb2 and triggers cell-mediated killing independently of Syk kinase activation. Grb2 couples to Vav1, phospholipase C (PLC)γ2, and SLP-76, whereas PI3K activation leads to Erk phosphorylation (2, 3). The downstream pathways leading to granule release remain incompletely characterized.

To understand the regulatory role of specific tyrosine kinases in these pathways, we studied the Tec family tyrosine kinase IL-2-inducible T cell kinase (Itk). Tec kinases are nonreceptor tyrosine kinases that modulate signals downstream of many hemopoietic surface receptors, including AgRs, cytokine receptors, integrins, and G protein-coupled receptors (4). Tec kinases are important components of signaling pathways leading to activation of PLCγ and MAPKs, regulation of the actin cytoskeleton, adhesion, migration, and transcriptional activation (5), pathways potentially relevant to NK cell conjugate formation, granule polarization, and exocytosis. Therefore, we hypothesized that Itk, the major Tec family kinase expressed in NK cells, would have an important regulatory role in NK cell-mediated killing. Itk is expressed in T cells, mast cells, and NK cells. In addition to its kinase activity, Itk possesses multiple noncatalytic domains, including a pleckstrin homology (PH), Tec homology, Src homology 2 (SH2), and SH3 domain, which have the capacity to bind to specific protein motifs. A number of potential ligands and mediators of Itk function have

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been identified from studying the interactions of its various domains in T cells in vitro. The PH domain of Itk has been demonstrated to bind to G protein βγ subunits, a number of different protein kinase C (PKC) isoforms and inositol phospholipids (6, 7). Furthermore, the PH domain targets Itk to the cell membrane (8). Membrane localization may be needed to regulate Itk function in response to Src family tyrosine kinases (8, 9). The proline-rich region in the Tec homology domain can bind to Grb2 and the SH3 domain of Src family members, including Fyn, Lyn, and Hck (10). Several proteins, including CD28, CD11c, and Wiskott-Aldrich syndrome protein among others, have been shown to interact with the Itk SH3 domain in vitro (11, 12). The SH2 domain of Itk has been shown to bind to the SLP-76 adaptor (13).

Thus, it is apparent that Itk is involved in multiple signaling pathways, although its potential involvement in granule-dependent cell-mediated cytotoxicity is unknown. We report here the use of genetic, biochemical, and functional approaches to study the regulatory role of Itk downstream of two NK cell-activating receptors that trigger cytotoxicity by recruiting different proximal kinases. Our analyses suggest that this Tec kinase has different regulatory roles downstream of the FcR and the NKG2D receptor in human NK cells.

Materials and Methods
Reagents, cells, and Abs

Unless otherwise indicated, reagents were obtained from Sigma-Aldrich. The murine mastocytoma P815 was obtained from American Type Culture Collection. Human NK cells were cloned and passaged as described previously (14). The 3G8 mAb to human FcγRIIA and the anti-EE mAb were purified from ascites by affinity chromatography over protein A-agarose. Monoclonal anti-NKG2D (R&D Systems), monoclonal Itk (BD Pharmingen), monoclonal anti-CD4 (BD Biosciences), purified mouse myeloma protein IgG1 (Bioentics), and anti-phosphorylated tyrosine conjugated (4G10; Upstate Signaling Solutions) were also used. Rabbit polyclonal antisera to PLC-γ1 (Cappel) and anti-PLC-γ2 (Upstate Signaling Solutions) were also used. Rabbit polyclonal Abs to PLC-γ2, Vav1, and Zap70 have been described previously (15–17).

Recombinant vaccinia

Full-length Itk containing an EE-epitope tag was subcloned into the pSP11 vector as described previously (18). A point mutation in the Itk kinase domain (K391R), which prevents ATP binding and thereby Itk activation, was generated with the QuiChange Site-Directed Mutagenesis kit (Stratagene) in accordance with the manufacturer’s instructions. Recombinant vaccinia viruses encoding the wild-type Itk or the kinase inactive form of Itk (K391R) were generated by insertion of the pSP11 constructs into the WR strain of vaccinia virus via homologous recombination (18–20). The Flag-tagged CD4 chimeric receptors were generated by PCR and standard molecular biology techniques as described previously (1).

Ca2+ mobilization assays

Changes in the levels of intracellular Ca2+ were monitored by flow cytometry using cells loaded with the Ca2+-sensitive fluorescent dye, indo-1-AM (Calbiochem-Novabiochem). To express specific proteins, human NK cells were infected with the indicated nonrecombinant (WR) or recombinant vaccinia viruses at a multiplicity of infection of 20:1 for 5 h in a humidified 37°C incubator. For the last hour of the infection, the cells were loaded with 5 μM indo-1. In suppression experiments, NK cells were nucleofected with either negative control dsRNA (Qiagen) or Itk small interfering RNA (siRNA) (SMARTpool; Upstate Signaling Solutions) 48 h earlier or were incubated for 60 min at 37°C with 5 μM indo-1. The cells were washed and kept on ice until analyzed. For analysis, the indo-1-loaded NK cells were warmed for 10 min in a 37°C water bath and then stimulated with Abs to either the endogenous FcR or NKG2D receptor, together with goat anti-mouse IgG (Fab’2) (Cappel). The samples were analyzed immediately by flow cytometry using a UV laser for excitation with violet (405 nm) and blue (530 nm) fluorescence emissions recorded. The data plots were generated using the FlowJo software program (Tree Star). Ca2+ data are presented as mean values.

Inositol 1,4,5-trisphosphate (IP3) measurements

NK cells were infected with control vaccinia (WR) or recombinant vaccinia expressing Itk and stimulated with cross-linking anti-FcR or anti-NKG2D mAb as described previously (21). The reactions were stopped with ice-cold 20% perchloric acid, followed by 20-min incubation on ice. The samples were centrifuged at 14,000 rpm for 15 min at 4°C, and supernatants were decanted and neutralized to pH 7.5 with ice-cold 1.5 M KOH. KClO4 was sedimented by further centrifugation at 14,000 rpm for 15 min, and the decanted supernatants were used for measuring the amount of IP3 extracted. IP3 was measured using a competitive tritiated thymidine ([3H]IP3 binding assay (Amersham Biosciences) in accordance with the manufacturer’s instructions. The amount of IP3 in each sample was determined from measurement of the radioactivity and interpolation from a standard curve.

Cytotoxicity assays

The 51Cr release assays were done as described previously (14). In all experiments, spontaneous release did not exceed 10% of maximum release. In redirected cytotoxicity assays (2), NK clones killed the FeR+ P815 target cells only in the presence of the designated triggering mAbs. Lytic units (LUs) per 106 effector cells were calculated on the basis of 20% lysis of target cells, as expressed by the formula: number of LUs/106 effectors = 10^6/TXp, where T is the number of target cells, p is the reference lysis level (20%), and Xp is the E:T ratio required to lyse 20% of the targets (22).

Cell stimulation and immunoblot analysis

Vaccinia infection of NK cells, cell stimulation, protein immunoprecipitation, and detection of tyrosine phosphorylation were conducted as described previously (21).

Measurements of granule release

High-affinity binding 96-well plates (Costar) were incubated overnight at 4°C with either 1 and 5 μg/ml mAb to FcR or NKG2D or with IgG1 at the same concentrations in 0.05 M carbonate buffer (pH 9.6) (100 μl per well in triplicate). After washing the plates, NK cell suspensions (4 × 105 cells/ml) were added to each well (150 μl/well) in RPMI 1640 medium (In-vitrogen Life Technologies) containing 10% calf serum. After incubation at 37°C for 4 h, supernatants were evaluated for secretion using a standard N-benzoyloxycarbonyl lysine thiobenzyl ester (BLET) esterase assay (23). For maximum secretion, 150 μl of cells was freeze-thawed three times in liquid nitrogen. Spontaneous secretion was from cells in wells with medium alone. Percent secretion was calculated as (sample – spontaneous)/(maximum – spontaneous) × 100%.

Itk suppression

Itk-specific SMARTpool siRNA was obtained from Upstate Signaling Solutions and transduced into NK cells using the Amaxa nucleofector system as previously described (2). Nucleofected cells were returned to a fresh flask of the original culture medium that had been reserved from the day the cells were passed. Nonsilencing dsRNA (AF488; Qiagen) was used as a negative control. Functional assays with whole-cell lysates were performed 48 h after nucleofection. Cell viability at this time was measured with a Vi-cell viability analyzer (Beckman Coulter), and in all experiments, viability after nucleofection exceeded 75%. Protein suppression was quantified by densitometry using the LabWorks analysis software (UVP).

Statistics

In assays in which the percent 51Cr release is plotted, error bars represent SD of triplicate samples. Where LUs are plotted, error bars represent SD obtained from nonlinear regression analysis to the exponential fit y = A × (1 – e–kx) as described previously (24). Where normalized LUs or secretion are plotted, error bars represent the SD of the averages of multiple experiments. Comparison between groups was performed with the paired Student t test. A p value of <0.05 was considered statistically significant.

Results

Tyrosine phosphorylation of Itk after stimulation of the FcR and NKG2D receptor in NK cells

Upon FcR ligation, NK cells initiate Ab-dependent cell-mediated cytotoxicity, whereas a pathway of natural cytotoxicity is triggered after NKG2D ligation (25). Although each pathway is initiated by...
Contribution of Itk to PLCγ2 activation, IP3 release, and calcium signaling after stimulation of either the FcR or NKG2D

A key downstream effect of Tec kinases in hemopoietic cells is to fully activate PLCγ contributing to a sustained release of calcium in the cell (26, 27). PLCγ-dependent calcium mobilization in NK cells is required for granule exocytosis and killing. The specific contribution of Itk to FcR or NKG2D-mediated phosphorylation of PLCγ2, the predominantly expressed isoform in NK cells, has not been described previously. When we expressed recombinant Itk and stimulated either the FcR or NKG2D, tyrosine phosphorylation of PLCγ2 was enhanced downstream of both pathways compared with either cells infected with the control WR vaccinia virus or with cells expressing the kinase inactive (K391R) mutant (Fig. 2A). The enhanced activation of PLCγ2 was principally seen at 5 min after receptor stimulation for each pathway, a finding consistent with the ability of Tec kinases to sustain calcium signaling. The level of tyrosine phosphorylation of PLCγ2 in cells infected with control WR vaccinia vs cells expressing the recombinant kinase mutant (K391R) was comparable in five independent experiments (data not shown).

Itk is tyrosine phosphorylated after both FcR and NKG2D stimulation. A, Cloned human NK cells were stimulated for the indicated times at 37°C with either isotype control, anti-FcR mAb, or anti-NKG2D mAb, followed by a secondary goat anti-mouse IgG (Fab’),. Itk immunoprecipitates were resolved by SDS-PAGE, transferred to a membrane, and probed with either anti-phosphotyrosine mAb (upper panel, pTyr) or anti-Itk mAb (lower panel, Itk). B, NK cells were infected with vaccinia virus encoding the EE-Itk construct. Using anti-EE mAb, expressed EE-Itk was immunoprecipitated from NK cells after anti-FcR or anti-NKG2D cross-linking for the indicated times at 37°C. The immunoprecipitates were resolved, transferred, and blotted with either anti-phosphotyrosine mAb (upper panel, pTyr) or anti-EE mAb (lower panel, EE). The data shown are representative of three independent experiments.

Plots show tyrosine phosphorylation of PLCγ2, IP3 release, and intracellular calcium downstream of both the FcR and NKG2D receptor. A, NK cells were infected with control vaccinia (WR) or with recombinant vaccinia encoding either wild-type Itk or the catalytically inactive Itk mutant (EE.K391R). Cells were then stimulated for the indicated times with either anti-FcR or anti-NKG2D, and then PLCγ2 immunoprecipitates were resolved and blotted. Densitometry (bar graph) was expressed as the ratio of the density of the tyrosine-phosphorylated band to the density of the corresponding band in the PLCγ2 blot. Numbers refer to individual lanes. The data shown are representative of five independent experiments. B, NK cells infected with control vaccinia or recombinant vaccinia encoding Itk were stimulated with cross-linking anti-FcR or anti-NKG2D mAb for the indicated times. IP3 release in stimulated samples was determined. The data shown are representative of two independent experiments. C, NK cells infected with the indicated nonrecombinant (WR) or recombinant vaccinia viruses were labeled with indo-1 and then stimulated with either anti-FcR mAb or anti-NKG2D mAb, followed by goat anti-mouse IgG (Fab’). Calcium release was measured by flow cytometry. Expression of Itk was determined by immunoblotting (inset): lane 1, control WR vaccinia; lane 2, recombinant vaccinia encoding Itk; and lane 3, recombinant vaccinia encoding catalytically inactive Itk (K391R). The data are representative of two independent experiments.
Regulation of NK cell-mediated cytotoxicity and granule release by Itk

To determine whether the enhancement of FcR- and NKG2D-induced calcium signals with Itk overexpression had a functional consequence, we assessed the influence of Itk on NK cell-mediated killing. We infected NK cells with either control vaccinia or recombinant vaccinia encoding Itk. To specifically initiate NK cell killing through either the FcR or NKG2D, infected NK cells were used as effectors in a redirected cytotoxicity assay against P815 tumor targets that bind either anti-FcR mAb or anti-NKG2D mAb. We found that, consistent with increased calcium, cells expressing recombinant Itk enhanced FcR-initiated killing (Fig. 3A). Surprisingly, the same cells expressing recombinant Itk decreased killing triggered through NKG2D (Fig. 3A). We tested over 20 different NK clones in independent experiments and consistently observed this differential regulation of cytotoxicity with Itk overexpression (data not shown). To determine whether the catalytic activity of Itk was required for the modulation of killing, the catalytically inactive form of Itk was expressed. As seen before, wild-type Itk enhanced killing of P815 cells triggered through the FcR and suppressed cytotoxicity initiated through NKG2D (Fig. 3B). Mutation of the catalytic site fully abrogated that modulation, suggesting that activation of Itk is required for both the positive and negative regulation of cytotoxicity (Fig. 3B). Although Itk has the potential to positively regulate both pathways through calcium, only FcR-initiated killing is enhanced, whereas NKG2D-initiated cytotoxicity is actively suppressed. Interestingly, NK cell killing of the 721 tumor, which does not express the NKG2D ligands MICA/B (data

FIGURE 3. Itk differentially regulates cell-mediated cytotoxicity. A, NK cells were infected with either control WR vaccinia or recombinant vaccinia encoding Itk. Infected NK cells were then incubated with 51Cr-labeled P815 cells and either 0.14 μg/ml anti-FcR mAb or anti-NKG2D mAb. The E:T ratios used were 20:1, 10:1, 5:1, and 2.5:1. Supernatants were assayed for 51Cr release after 4 h at 37°C. The data are expressed as LU per 1 SD. Data shown are representative of multiple independent cytotoxicity experiments. B, NK cells infected with the indicated vaccinia viruses were incubated with P815 cells coated with anti-FcR or anti-NKG2D as in A. Itk expression was determined by immunoblotting (inset); lane 1, control WR vaccinia; lane 2, recombinant vaccinia encoding Itk; and lane 3, recombinant vaccinia encoding kinase inactive Itk (K391R). C, Infected cells were incubated with 51Cr-labeled 721 target cells (natural cytotoxicity assay). Data shown are representative of three independent experiments.

Activated PLCγ hydrolyzes its substrate, phosphatidylinositol 4,5-bisphosphate, generating the second messengers IP3 and diacetylglycerol (28). When we measured IP3 release in NK cells expressing recombinant Itk and stimulated through either the FcR or NKG2D, an ∼1.5-fold increase in IP3 was observed downstream of either pathway compared with the amount of IP3 generated in the stimulated control infected cells (Fig. 2B). Because IP3 acts to open intracellular calcium stores promoting an initial, transient rise in intracellular calcium, we next compared calcium fluxes in NK cells. Consistent with the increased tyrosine phosphorylation of PLCγ2 and IP3 generation observed, we found that intracellular calcium was enhanced for both the FcR and NKG2D pathways when recombinant Itk was expressed (Fig. 2C). Notably, the calcium waveform was different downstream of the NKG2D receptor as has been reported previously (21). The waveform had a relatively delayed onset and lower amplitude; however, its amplitude was increased by recombinant Itk expression. Calcium fluxes were the same in NK cells infected with control vaccinia or in cells expressing the recombinant kinase mutant, consistent with their similar tyrosine phosphorylation of PLCγ2. Taken together, these results suggest that Itk enhances the proximal signaling pathways leading to calcium release downstream of both the FcR and NKG2D receptor.
not shown), was also enhanced by recombinant Itk (Fig. 3C). However, the receptors and ligands that trigger natural cytotoxicity in this assay are not known.

To determine whether the difference in killing seen with enhanced Itk expression was a result of the signaling motifs in the cytoplasmic tails of each receptor, recombinant vaccinia encoding Flag-tagged CD4 chimeric receptors were generated. The cytoplasmic tails of chimeric receptors either had ITAMs (γ, ζ, and DAP12) or the PI3K/Grb2 motif YINM (DAP10) (Fig. 4A). We have previously shown that these chimeric receptors, when expressed in human NK cells and cross-linked with a CD4 Ab, initiate the same signaling pathways as the endogenous ITAM- or non-ITAM-coupled receptors (1). The different signaling pathways downstream of the chimeric receptors are each activated with the same Ab, thereby excluding possible differences in Ab stimulation of the endogenous receptors. In NK cells expressing the chimeric receptors, coexpression of recombinant Itk enhanced cytotoxicity triggered through each of the ITAM-containing receptors. In contrast, cytotoxicity initiated through either the DAP10 chimeric receptor or the endogenous NKG2D receptor was suppressed in cells expressing recombinant Itk (Fig. 4B). These results suggest that Itk differentially regulates ITAM- vs DAP10-mediated cytotoxicity.

To determine whether the modulation of killing was reflected by changes in granule release, the amount of granzyme A being released was quantified in a BLT esterase assay. In this assay, granule secretion is triggered with Abs to each receptor coated on a plate and granzyme A release quantitated by spectrophotometry. In this assay, granule release triggered by either the FcR or NKG2D have comparable sensitivity to calcium as determined by measuring granule release after combining specific receptor stimulations with a range of concentrations of the calcium ionophore ionomycin (data not shown). The same differential regulation of granule secretion occurred with expression of recombinant Itk as with cytotoxicity, i.e., enhancement of granule release downstream of the FcR and suppression of NKG2D-initiated granule secretion (Fig. 5). Similarly, expression of the kinase inactive mutant (K391R) fully abrogated the modulation of granule secretion. Three independent experiments with additional NK clones indicated that Itk overexpression significantly enhanced FcR-initiated secretion (p < 0.05) and significantly inhibited NKG2D-initiated secretion (p < 0.05). Expression of the K391R did not significantly change secretion levels induced by either receptor as compared with the WR control. Expression of either wild-type Itk or the K391R mutant Itk consistently, but modestly (<20%), increased spontaneous granule release (data not shown), suggesting a potential modulatory role for Itk independent of its catalytic activity. Taken together, these results suggest that Itk differentially regulates granule release and killing downstream of the FcR and NKG2D receptor.

**FIGURE 5.** Granule secretion triggered through the FcR and NKG2D receptor is differentially regulated by Itk. Cloned human NK cells infected with the indicated vaccinia viruses were stimulated for 4 h at 37°C with either 1 or 5 μg/ml plate-bound mAb (anti-FcR and anti-NKG2D). Granule release was measured by a BLT esterase assay.

**FIGURE 6.** Suppression of Itk in human NK cells with siRNA. A, We introduced control dsRNA or serial dilutions of Itk-specific siRNA into NK clones with the Amaxa nucleofector system. For titrated Itk siRNA samples, control dsRNA was supplemented to have 300 pmol of total siRNA per sample. Equal protein from whole-cell lysates was resolved by SDS-PAGE 48 h after nucleofection, and Itk expression was determined by immunoblot and densitometry (below blot). The results of three independent experiments are shown. B, The membrane with greatest Itk suppression (experiment 1; 83% suppression) was reblotted with rabbit polyclonal antiserum to PLCγ2, Vav, and Zap70.

**Ik suppression in NK cells using siRNA**

Genetic down-regulation of specific proteins has been useful in discerning the function of many regulatory proteins. However, the in vivo suppression of individual Tec family kinases has often been complicated by redundant functions between the family members (29). Despite this potential limitation, we used siRNA delivered with the Amaxa nucleofector system to suppress Itk in our cells. As a control, nonsilencing dsRNA was introduced into NK cells in parallel. To demonstrate the specificity of the Itk-targeting siRNA, we examined the protein levels 48 h after nucleofection. Expression of Itk was specifically reduced and was titratable (Fig. 6A) whereas the expression of other key signaling molecules was not (Fig. 6B). Semiquantitative RT-PCR also detected suppression of Itk-specific mRNA in the siRNA-treated cells, whereas no changes in Rlk-specific or Tec-specific mRNA were noted (data not shown). When we suppressed Itk in NK cells and compared calcium fluxes, we observed a small but consistent decrease in calcium mobilization downstream of both FcR and NKG2D receptor (Fig. 7). The partial reduction in calcium is consistent with Itk acting as an amplifier of calcium signaling and the potential for redundancy with other nonsuppressed Tec family kinases in the activation of PLCγ2. To determine the activation of other downstream pathways that may be regulated by Itk, we stimulated Itk-suppressed cells through each receptor pathway and used phospho-specific Abs to compare Erk activation. Although Erk was inducibly tyrosine phosphorylated downstream of both receptor pathways, we did not detect any differences in Erk phosphorylation in Itk-suppressed NK cells, despite suppression of Itk by greater than 80% (data not shown). This may indicate potential functional redundancy in the regulation of Erk by other Tec family kinases expressed in NK cells or the potential for receptor-initiated activation of Erk to proceed in a Tec family kinase-independent manner.
We then used Itk-suppressed NK cells as effectors in a redirected cytotoxicity assay, initiating killing through either the FcR or NKG2D receptor. A partial decrease in killing through the FcR was observed in Itk suppressed cells, whereas killing triggered by the same cells through the NKG2D pathway was maintained (Fig. 8A). These trends were confirmed in five independent experiments, which show that the suppression of killing through the FcR in ITK-suppressed cells was significant \((p < 0.005)\), whereas killing through the NKG2D pathway by the same cells was not significantly different (Fig. 8B). In these experiments, increased suppression of Itk correlated with a greater decrease in FcR-initiated killing, whereas killing initiated by the NKG2D receptor remained relatively constant despite suppression of Itk by 84%, which was the highest knockdown achieved. These results are consistent with Itk acting as a positive regulator of FcR-mediated cytotoxicity and suggest that Itk has a complex activity downstream of NKG2D potentially involving both positive and negative regulation. We have found that both receptor pathways display a similar dependence on calcium to trigger granule release and killing (data not shown). Therefore, the involvement of Itk in a pathway of negative regulation downstream of NKG2D is likely, since killing initiated by NKG2D in Itk suppressed cells was maintained despite a decrease in intracellular calcium.

**Discussion**

We have found that, in human NK cells, Itk plays a differential role in regulating distinct pathways of cell-mediated cytotoxicity. We show in multiple experimental settings that Itk plays a positive regulatory role on ITAM-initiated killing, whereas Itk is involved in a pathway of negative regulation downstream of the NKG2D receptor that initiates signaling through the non-ITAM transmembrane adaptor DAP10. We also show that the regulation of cytotoxicity by Itk involves a differential modulation of granule release and requires the catalytic activity of Itk. Our findings have potentially important clinical implications as elaborated below.

The role of Itk in peripheral cytotoxic lymphocytes has been investigated from studies of CD8\(^+\) T cell function in Itk-deficient mice (30, 31). It has recently been shown that Tec kinases regulate the development of CD8\(^+\) CTLs in this animal model (32, 33). Studies of CD8\(^+\) T cell function in Itk-deficient mice have reported a disparity between the in vitro responses of these cells and the in vivo responses of these mice to viral infections (30, 31). The TCR stimulation of CD8\(^+\) T cells lacking Itk or Itk and Rlk resulted in impaired phosphorylation and activation of PLC\(\gamma1\), Erk, and p38 MAPK and loss of a sustained calcium response. These lead to defects in expansion and effector cytokine production. Despite the substantial defects in CD8\(^+\) T cell responses observed in vitro, antiviral immune responses proceeded efficiently in the absence of Itk and Rlk (31). The authors proposed that the T cell defects could be compensated for by the innate immune response to the infection. However, innate cytotoxic cells are also dependent on the activation of PLC\(\gamma\) and intracellular calcium release for effector function. Our findings that Itk differentially regulates distinct pathways of NK cell-mediated cytotoxicity may provide an explanation for this apparent disparity. It would be predicted from our work that killing of virally infected cells through the NKG2D pathway would be intact and possibly enhanced in Itk-deficient NK cells. It is also possible that Itk has a negative regulatory role in the function of CTLs, which, like NK cells, also express NKG2D. One can also speculate from our data that the antitumor immune responses of NK cells toward tumors bearing ligands for NKG2D may also be enhanced by Itk deficiency. This possibility remains to be tested.

Similar to what we have found in NK cells, a differential role for Itk downstream of distinct T cell pathways has been reported previously. In T cells, Itk has been implicated in signaling pathways from both the ITAM-containing TCR and the CD28 costimulatory receptor that contains a PI3K-binding motif (34, 35). The role of Itk may differ downstream in these T cell pathways. Early characterization of T cell function in Itk-deficient mice showed that CD3-mediated proliferative responses were reduced (36), whereas proliferation of Itk-deficient CD4\(^+\) T cells in response to anti-CD28 stimulation was enhanced, supporting a differential role for
Itk downstream of CD3 and CD28 (37). More recent work suggests that Itk is not essential for CD28 signaling in naive Itk-deficient CD4+ T cells (38), although the ability of Itk to negatively influence CD28 may depend on the state of activation of the cells. It remains to be determined whether Itk has a negative role in pathways downstream of other transmembrane proteins and receptors that contain a PI3K-binding motif in their cytoplasmic tails such as TCR-interacting molecule and ICOS.

In accordance with its involvement in T cell signaling downstream of the TCR and CD28, we show that in NK cells Itk becomes tyrosine phosphorylated downstream of both the FcR and NKG2D receptor that use similar proximal kinases to initiate signaling. Notably, we found that tyrosine phosphorylation of Itk was more prominent in the FcR pathway, which couples to Syk family kinases. Interestingly, earlier studies in Jurkat T cells found that Itk was dependent on the Syk family kinase Zap70 and the transmembrane adaptor linker for activation of T cells for activation (39). This may suggest that the activation of Itk is different downstream of the NKG2D receptor, which, unlike the FcR, does not couple to either Syk family kinases or the adaptor linker for activation of T cells but instead initiates signaling through PI3K and Grb2 (1, 2).

Consistent with the differences in proximal kinase activation, we again show that the calcium waveform differs downstream of the endogenous FcR and NKG2D receptor. FcR engagement produces a rapid increase in intracellular calcium, whereas NKG2D activation leads to a relatively delayed low amplitude signal. Intracellular calcium controls a wide variety of cellular functions in addition to granule-mediated killing, including cell adhesion, motility, gene expression, and proliferation. Many of these functions also involve Tec kinases (40–42). Variations in calcium waveforms may have different functional consequences as has been demonstrated in B lymphocytes (43). In these cells, the amplitude and duration of calcium signals has been reported to control differential activation of the proinflammatory transcriptional regulators NF-κB, JNK, and NFAT (44, 45). We show that Itk enhances the proximal signaling pathways leading to calcium release for both the FcR and NKG2D pathways, consistent with the function of the Tec kinase Btk in amplifying proximal AgR signals in B cells (46). The differential regulation of FcR- and NKG2D-initiated pathways by Itk therefore suggests that Itk has additional functions downstream of intracellular calcium or independently of calcium which differ for each receptor. When we stimulate granule release with 12-O-tetradecanoylphorbol-13-acetate and ionomycin to bypass proximal signaling pathways, granule release is inhibited from cells expressing recombinant Itk (data not shown), which is consistent with a potential inhibitory role for Itk in downstream signaling subsequent to calcium. Possibilities include the activation of specific PKC family members, isoforms of which may interact directly or indirectly with Tec family kinases (47, 48). In addition, novel PKC isoforms, which do not rely on calcium for their activation, have been implicated as negative regulators of secretion in other cell types (49, 50). Therefore, it is possible that PKC family members have differential roles in NK cells that may be regulated by Itk. Moreover, we have observed that NKG2D-initiated cytotoxicity is more sensitive to the effects of PKC inhibition than killing initiated by the FcR (D. Khurana and P. J. Leibson, unpublished observations).

It should be noted that a complex modulatory function for Btk in B cells has been reported previously. Btk has been found to be both a positive and negative regulator of apoptosis, where it has been shown to facilitate apoptosis in B cells exposed to reactive oxygen intermediates but to inhibit Fas-activated apoptosis (51). The related Src family kinase Lyn similarly plays both positive and negative regulatory roles in AgR signaling in B cells (52). It is possible that Tec family kinases have the capacity to both amplify and attenuate signaling to fine tune signaling pathways.

There are several implications of our work. First, this study shows that human NK cells, a subpopulation of lymphocytes that play a role in tumor immunosurveillance, autoimmune and inflammatory diseases, and transplantation biology, can be modulated by the Tec family kinase Itk. Specifically, the NKG2D pathway has been shown to have a deleterious effect in the development of certain autoimmune diseases. For example, in rheumatoid arthritis (53) and celiac disease (3), abnormal expression of stress-induced NKG2D ligands in inflamed tissues together with the induction of NKG2D receptors on lymphocytes by local inflammatory cytokines allows these cells to become cytotoxic toward the stressed tissues. Our findings suggest that Itk has a negative regulatory role in NKG2D-initiated killing. In this context, elucidation of the precise mechanism of inhibition of NKG2D-mediated cytotoxicity would be expected to have significant therapeutic implications for these disorders. Second, our finding that expression of a catalytically inactive form of Itk completely abrogated the modulation of cytotoxicity in both the FcR and NKG2D pathways suggests that the kinase domain of Itk may be a therapeutic target for regulating these pathways. Third, our finding that Itk has a complex role downstream of NKG2D involving both positive and negative regulation supports a potential role for this Tec kinase to serve as a modulator of the activity of the NKG2D pathway in both resting and activated cells. Taken together, our analyses suggest that, in human NK cells, Itk plays a differential role in regulating the functions initiated by distinct activating receptors. The modulation of NK cell function by targeting Itk has relevance not only for improving tumor immunosurveillance and viral immunity but also for reducing the severity of autoimmune diseases and graft rejection.

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Disclosures
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References