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Suppressor of Cytokine Signaling 2 Regulates IL-15–Primed Human NK Cell Function via Control of Phosphorylated Pyk2

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NK cells are capable of killing virus-infected or tumor cells and producing IFN-γ. Resting NK cells, however, have only minimal cytolytic activity and secrete a low level of IFN-γ. The cytokine IL-15 can promote the expression of effector functions by resting NK cells. In this study, we demonstrate that suppressor of cytokine signaling 2 (SOCS2) has a novel role in IL-15–primed human NK cell function. SOCS2 expression was upregulated in NK cells following stimulation with IL-15. During IL-15–mediated NK cell priming, SOCS2 interacted with phosphorylated proline-rich tyrosine kinase 2 (Pyk2) at tyrosine 402 (p-Pyk2^{Tyr402}) and induced the proteasome-mediated degradation of p-Pyk2^{Tyr402} via ubiquitination. Knockdown of SOCS2 resulted in the accumulation of p-Pyk2^{Tyr402} and blocked NK cell effector functions. In addition, NK cell cytolytic activity and IFN-γ production were inhibited by overexpression of the wild-type of Pyk2 but not by the overexpression of tyrosine 402 mutant of Pyk2. These results suggest that SOCS2 regulates human NK cell effector functions via control of phosphorylated Pyk2 depending on IL-15 existence. ***The Journal of Immunology*****, 2010, 185: 917–928.**

Natural killer cells are large granular lymphocytes that play an important role in innate immunity (1), contributing to the elimination of tumor or virus-infected cells (2). The major mechanism used by NK cells to destroy target cells is the focused secretion of lytic granules containing granzymes and perforin at the immune synapse (3, 4), with perforin creating pores in the cell membrane that allow the granzymes to move into the target cells and induce death through caspase-dependent or -independent apoptosis (5). Activated NK cells are also a major source of IFN-γ, with IL-12 and IL-18 the major stimulators of IFN-γ secretion by NK cells (6). In addition, activating receptors that transmit signals through ITAMs have the ability to induce IFN-γ production (7). IFN-γ in turn promotes the activation of macrophages (8), shapes the innate and adaptive immune responses (9), and suppresses the proliferation of tumor and virus-infected cells (10). Freshly isolated NK cells from humans and mice have only minimal cytotoxicity and secrete a low level of IFN-γ (11). Prior to receiving stimulatory signals that induce NK cell effector functions, resting NK cells need additional priming signals. IL-15 is essential for the priming of NK cells and to maximize their effector functions (12, 13).

The suppressor of cytokine signaling (SOCS) family, which is known to regulate receptor-mediated signal transduction, comprises eight members (SOCS1–SOCS7 and cytokine-inducible Src homology 2 [SH2] domain-containing protein). The SOCS proteins interact with major molecules of signaling complexes to block further signal transduction, in part by inducing proteasome-mediated degradation of target proteins via ubiquitination (14). SOCS2 has been shown to regulate the growth hormone (GH), insulin growth factor I and prolactin signaling pathways (15). Depending on its concentration, SOCS2 can function as both a positive and a negative regulator of GH signaling (16, 17). Recently, SOCS2 has been shown to target TNFR-associated factors 2 and 6 for proteasome-mediated degradation in dendritic cells (DCs) (18). However, little is known about the role of SOCS2 in other immune cells, and there have been no reports about the function of SOCS2 in NK cells.

In this study, we demonstrate a novel role of SOCS2 in regulating the functions of human NK cells. SOCS2, which is induced by IL-15, interacts with phosphorylated proline-rich tyrosine kinase 2 (Pyk2) at tyrosine 402 (p-Pyk2^{Tyr402}) and promotes proteasome-mediated degradation of p-Pyk2^{Tyr402} via ubiquitination. When the SOCS2-mediated regulation of Pyk2 was blocked by the knockdown of SOCS2 using RNA interference, NK cell effector functions were significantly compromised. Taken together, our data indicate that SOCS2 is a novel regulator for IL-15–primed NK cell cytotoxicity and IFN-γ production.

Materials and Methods

Cell culture and reagents

Umbilical cord blood (UCB) was collected from umbilical veins after neonatal delivery, with informed consent from the pregnant mothers and following the guidance of the local Institutional Review Board. Human primary cord blood NK cells were isolated from UCB using the human NK

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The online version of this article contains supplemental material.

Abbreviations used in this paper: DC, dendritic cell; FAK, focal adhesion kinase; GH, growth hormone; HA, hemagglutinin; HSC, hematopoietic stem cell; mNK, mature NK cell; NCR, natural cytotoxicity receptor; p-Pyk2^{Tyr402}, phosphorylated proline-rich tyrosine kinase 2 at tyrosine 402; Pyk2, proline-rich tyrosine kinase 2; Pyk2-K457A, proline-rich tyrosine kinase 2 in which lysine 457 had been mutated to alanine; Pyk2-Y402F, proline-rich tyrosine kinase 2 in which tyrosine 402 had been mutated to phenylalanine; SH2, Src homology 2; SHP, Src homology region 2 domain-containing phosphatase; shRNA, small hairpin RNA; siRNA, small interfering RNA; SOCS, suppressor of cytokine signaling; UCB, umbilical cord blood; WT, wild-type.

Cell Isolation Kit (Miltenyi Biotec, Auburn, CA). The isolated primary cord blood NK cell populations were >97% $CD56^+CD3^-$ and cultured in Myelocult H5100 (StemCell Technologies, Vancouver, British Columbia, Canada) supplemented with 10 ng/ml IL-15. To prepare the hematopoietic stem cell (HSC)-derived mature NK (mNK) cells, $CD34^+$ HSCs were isolated from UCB using a CD34 MicroBead Kit (Miltenyi Biotec). $CD34^+$ HSCs were differentiated into NK cell precursors by incubating the cells in Myelocult H5100 supplemented with 30 ng/ml stem cell factor and 50 ng/ml Flt3 ligand for 14 d. NK cell precursors were differentiated into mNK cells by stimulation with 30 ng/ml IL-15 for an additional 14 d. mNK cells (>97% $CD56^+CD3^-$ cells) were maintained in Myelocult H5100 with 10 ng/ml IL-15 and were used for functional assays. The human NK cell line NK-92 (American Type Culture Collection, Manassas, VA) was cultured in α-MEM (Life Technologies, Rockville, MD), supplemented with 20% heat-inactivated FBS (Hyclone, South Logan, UT), 2 mM L-glutamine, and 10 ng/ml IL-15. The HEK293T, MCF7, and A549 cell lines (American Type Culture Collection) were cultured in DMEM (Life Technologies) supplemented with 10% heat-inactivated FBS (Hyclone). The K562 and Jurkat T cell lines (American Type Culture Collection) were cultured in RPMI 1640 (Life Technologies) supplemented with 10% heat-inactivated FBS (Hyclone). Recombinant human stem cell factor, Flt3 ligand, IL-7, IL-12, IL-15, IL-18, and IL-21 were purchased from PeproTech (Rocky Hill, NJ). The proteasome inhibitor, MG-132 (Calbiochem, San Diego, CA), was dissolved in DMSO. The ERK-, JNK-, and p38-specific inhibitors PD98059, SP600125, and SB203580 were purchased from Calbiochem.

Lentiviral infection and small interfering RNA nucleofection

For lentiviral infection of NK-92 cells, the pLKO.1-nontarget small hairpin RNA (shRNA) control vector (SHC002) and pLKO.1-SOCS2 shRNA vector (TRCN0000057058) were purchased from Sigma-Aldrich (St. Louis, MO), and the pLL3.7 vector was obtained from Addgene (Cambridge, MA). To generate the pLVX-AcGFP-Pyk2 vector, the human Pyk2 cDNA was amplified from an NK-92 cDNA library by PCR with specific primers. After digestion with XhoI and EcoRI, the Pyk2 cDNA was cloned in the XhoI-EcoRI–digested pLVX-AcGFP-C1 vector. Lentiviruses were produced using a third-generation packaging system (pMDLg/pRRE, pRSV-Rev, and pMD2.G) in HEK293T cells. The lentivirus-containing supernatants were cleared by centrifugation at 3000 rpm for 5 min at 4°C, passed through a 0.45-μm filter, and concentrated by ultracentrifugation at 50,000 × *g* for 90 min at 4°C. Upon infection, GFP-expressing NK-92 cells were sorted (FACSAria; BD Biosciences, San Jose, CA), and shRNA-expressing NK-92 cells were selected with 2 μg/ml puromycin for 4 wk before functional analysis. The nucleofections of NK cell precursors and mNK cells were performed using an Amaxa Human CD34 Cell Nucleofector Kit (program U-08), and the nontarget control small interfering RNA (siRNA), the SOCS2-specific siRNA, the SOCS3-specific siRNA, the SOCS4-specific siRNA, and the Pyk2-specific siRNA SMARTpool were purchased from Dharmacon (Chicago, IL).

NK cell functional assays

Cytotoxicity was examined using a standard 4-h [^{51}Cr] release assay. ^{51}Cr-labeled target cells (3×10^5 cells/well) and serial dilution of IL-15–primed NK cells were plated in triplicate. The [^{51}Cr] released into the supernatant was measured using a gamma counter. The percentage of specific lysis was calculated using the formula: ([experimental release − spontaneous release]/[maximum release − spontaneous release]) × 100. For evaluation of IFN-γ secretion, IL-15–primed NK cells were stimulated in duplicate for 16 h with plate-bound Abs, 10 ng/ml IL-12, or 30 ng/ml IL-18. The secretion of IFN-γ in the supernatant was measured by ELISA (Assay Designs, Ann Arbor, MI).

Flow cytometry

Single-cell suspensions were stained with FITC- or PE-conjugated Abs. The conjugated Abs specific to the following Ags were obtained from BD Biosciences and BioLegend (San Diego, CA): CD56, NKp30, NKp44, NKp46, IL-12Rβ, IL-18Rα, NKG2D, CD45, perforin, and granzyme B. An Annexin V^{FITC} Apoptosis Detection Kit (BD Pharmingen, San Diego, CA) was used for the measurement of apoptosis. Data were collected on a FACSCalibur (BD Biosciences) and analyzed using CellQuest Pro software (BD Biosciences).

Real-time PCR

Total RNA was extracted using TRIzol (Invitrogen, Carlsbad, CA) and reverse transcribed into cDNA using Moloney murine leukemia virus reverse transcriptase (Promega, Madison, WI) with random primers (Takara Bio, Otsu, Japan). Real-time PCR was performed using a Dice TP 800 Thermal Cycler and the SYBR Premix Ex Tag (Takara Bio). The data were normalized to the amount of the GAPDH transcript. The primer sequences were as follows: 5′-agagcttcgactgcctcttc-3′ and 5′-ctcaggtagtcgcggaggac-3′ for SOCS1, 5′-taaaagaggcaccagaaggaac-3′ and 5′-tcgatcagatgaaccacactg-3′ for SOCS2, 5′-gccacctactgaaccctcct-3′ and 5′-acggtcttccgacagagatg-3′ for SOCS3, 5′-acatgacccctgtgtcttcc-3′ and 5′-gcagggaaaaagggaaagtc-3′ for SOCS4, 5′-acccagagttcattggatgc-3′ and 5′-cccacagtatcctgcaacct-3′ for SOCS5, 5′-accattgctacctccaatgc-3′ and 5′-tcacaaccatgggaccacta-3′ for SOCS6, 5′-tgcagtctttccccctacc-3′ and 5′-cccccaatacccaaccacac-3′ for SOCS7, 5′-gtccaacgcaaagcaataca-3′ and 5′-ctcttcgacctcgaaacagc-3′ for IFN-γ, and 5′-cagcctcaagatcatcagca-3′ and 5′-gtcttctgggtggcagtgat-3′ for GAPDH.

Plasmids and transient transfection

To generate GST-SOCS2, the cDNA encoding human SOCS2 was amplified from an NK-92 cDNA library by PCR with specific primers. The PCR product was cloned into the BamHI and ClaI sites of the pEBG vector. GST-SOCS2-ΔSOCS was generated by ligation of a 451-bp BamHI-ClaI fragment of SOCS2, after the SOCS box was deleted, into the BamHI-ClaI–digested pEBG vector. GST-SOCS2-ΔSH2 was generated using a 135-bp BamHI-ClaI fragment of SOCS2 after the SH2 domain and SOCS box were deleted. To generate Flag-Pyk2, the human Pyk2 cDNA was amplified by PCR with specific primers. The PCR product was cloned into the EcoRI and SalI sites of the pBICEP-CMV-Flag vector. Flag-Pyk2-Y402F, AcGFP-Pyk2-Y402F, and AcGFP-Pyk2-K457A were generated by creating an Y402F mutation or a K457A mutation in the pBICEP-CMV-Flag-Pyk2 construct or pLVX-AcGFP-Pyk2 construct using the QuickChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA). For transient expression, HEK293T cells (~80–90% confluence) were transfected with the vectors indicated in Fig. 5 using Lipofectamine 2000 (Invitrogen), according to the manufacturer's instructions.

Western blot analysis

Cells were washed twice with ice-cold PBS and lysed in radioimmunoprecipitation assay buffer (50 mM Tris-HCl [pH 7.4], 150 mM NaCl, 0.25% SDS, 1% Nonidet P-40, and 1 mM EDTA, supplemented with a protease inhibitor mixture tablet and a phosphatase inhibitor mixture tablet from Roche [Basel, Switzerland]). The cell lysates were resolved using 10 or 12% SDS-PAGE gels and transferred to a polyvinylidene difluoride membrane (Millipore, Bedford, MA). The membrane was probed with Abs specific to the following molecules: SOCS2, p-$STAT5^{Tyr694}$, STAT5, p-Src^{Tyr416}, Src, p-$Syk^{Tyr525,526}$, Syk, p-$ERK^{Thr202,Tyr204}$, ERK, p-$JNK^{Thr183,Tyr185}$, JNK, p-$p38^{Thr180,Tyr182}$, p38, p-$Pyk2^{Tyr402}$, Pyk2, Src homology region 2 domain-containing phosphatase (SHP)-1, SHP-2, ubiquitin, or GST (Cell Signaling Technology, Beverly, MA); Flag (Sigma-Aldrich); β-actin or hemagglutinin (HA) (Santa Cruz Biotechnology, Santa Cruz, CA); or GAPDH (Assay Designs). After incubation with peroxidase-conjugated anti-rabbit or anti-mouse IgG (Jackson ImmunoResearch Laboratories, West Grove, PA), the signals were detected using SuperSignal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL) or Immobilon Western Chemiluminescent HRP Substrate (Millipore).

Immunoprecipitation and GST pull down

For the immunoprecipitation, cell lysates were incubated with different Abs for 4 h at 4°C. The Ag–Ab complexes were precipitated by incubation at 4°C overnight with protein G-conjugated agarose (Roche). The immunoprecipitated complexes were washed and analyzed by Western blot. For the GST–pull-down assay, cell lysates were incubated with glutathione Sepharose 4B (GE Healthcare, Piscataway, NJ) for 4 h at 4°C. The glutathione Sepharose 4B pellets were washed and analyzed by Western blot.

Statistical analysis

Comparisons were analyzed for statistical significance by the Student *t* test using Microsoft Excel software. A value of $p < 0.05$ was considered significant.

Results

IL-15 induces SOCS2 expression

Using a serial analysis of gene expression screen to assess potential regulatory genes involved in NK cell differentiation or activation (19), we found that the expression of SOCS2 was upregulated during IL-15–mediated in vitro differentiation of mouse NK cells (Supplemental Table I). This upregulation was also observed during IL-15–mediated human NK cell differentiation (Fig. 1*A*, *left panel*) at both the mRNA (Fig. 1*A*, *right panel*) and protein (Fig. 1*B*) level. To determine whether SOCS2 upregulation was

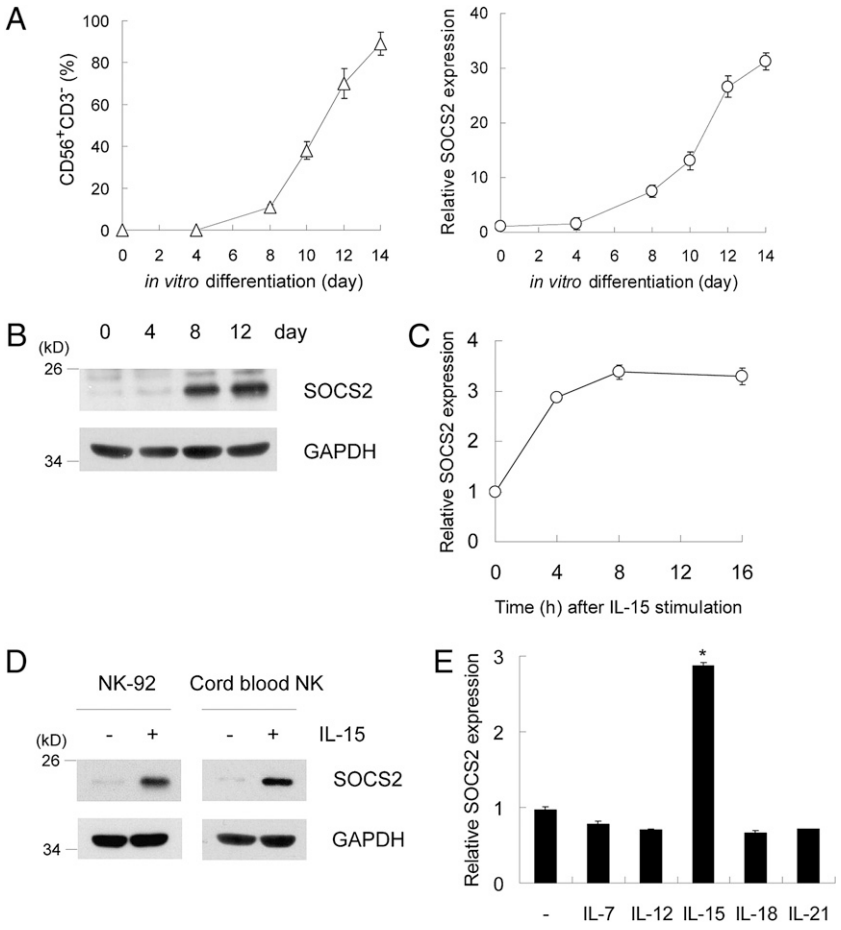

FIGURE 1. Expression of SOCS2 in human NK cells. *A*, In vitro differentiation of human NK cell. NK cell precursors were stimulated with IL-15 (30 ng/ml) for the indicated time, after which the cells were collected and analyzed for CD56 expression by flow cytometry (*left panel*), the relative mRNA expression of SOCS2 to GAPDH by real-time PCR (*right panel*), and SOCS2 protein expression by Western blot analysis (*B*). *C*, NK-92 cells were deprived of IL-15 for 24 h and then restimulated with IL-15 (10 ng/ml) for the indicated time. The relative mRNA expression of SOCS2 to GAPDH was analyzed by real-time PCR. *D*, IL-15–deprived NK-92 cells (*left panel*) and human primary cord blood NK cells (*right panel*) were stimulated with IL-15 (10 ng/ml) for 18 h. SOCS2 protein expression was detected by Western blot analysis. *E*, IL-15–deprived NK-92 cells were stimulated with IL-7 (10 ng/ml), IL-12 (10 ng/ml), IL-15 (10 ng/ml), IL-18 (20 ng/ml), or IL-21 (20 ng/ml) for 6 h. The relative mRNA expression of SOCS2 to GAPDH was analyzed by real-time PCR. $*p < 0.05$. The data are representative of three independent experiments, and the error bars represent the SD of triplicates.

dependent on IL-15, we investigated SOCS2 mRNA expression after IL-15 stimulation in IL-15–deprived NK-92 cells and found expression of SOCS2 was upregulated following IL-15 stimulation in a time-dependent manner (Fig. 1*C*). Moreover, the increased expression of SOCS2 was confirmed at the protein level after IL-15 stimulation of either NK-92 cells (Fig. 1*D*, *left panel*) or primary human cord blood NK cells (Fig. 1*D*, *right panel*). The effects of several other cytokines, which can also regulate the activation or differentiation of NK cells, on SOCS2 expression were evaluated. However, SOCS2 expression was increased by IL-15 and not by other cytokines (Fig. 1*E*). NK-92 cells expressed SOCS4 and SOCS5 in addition to SOCS2 (Supplemental Fig. 1*A*, *upper*). Similarly, mNK cells expressed SOCS2, SOCS3, SOCS4, and SOCS5 (Supplemental Fig. 1*A*, *lower*). Thus, we investigated whether IL-15 enhances the expression of other SOCS family members in human NK cells. Treatment with IL-15 did not induce expression of SOCS3, SOCS4, and SOCS5 in mNK cells, but IL-21 increased SOCS3 expression (Supplemental Fig. 1*B*). Moreover, upregulation of SOCS1 or SOCS3 mRNA was not detected after IL-15 stimulation of primary cord blood NK cells (Supplemental Fig. 1*C*). Overall, these results show that SOCS2 expression is upregulated by IL-15 in human NK cells.

SOCS2 does not affect IL-15R signaling or IL-15–mediated NK cell differentiation and survival

SOCS2 is a member of the SOCS family, which is known to act as negative feedback regulators in cytokine receptor-mediated signaling pathways (14, 20). Because IL-15 is an essential cytokine for NK cell development and survival (21–23), we examined whether knockdown of SOCS2 influences IL-15–mediated NK cell differentiation by measuring the percentage of CD56-positive and CD3-negative ($CD56^+CD3^-$) NK cells differentiated in vitro from control siRNA- or SOCS2 siRNA-nucleofected NK cell precursors (Fig. 2*A*, 2*B*). The siRNA-treated NK cell precursors differentiated into similar proportions of $CD56^+CD3^-$ NK cells (Fig. 2*B*), suggesting that SOCS2 does not affect IL-15–mediated NK cell differentiation. In addition, the control shRNA- and SOCS2 shRNA-expressing NK-92 cells exhibited similar survival (Fig. 2*C*) and proliferation rates (Fig. 2*D*). Also, studies have reported that IL-15 stimulation activates Jak3 and subsequently induces phosphorylation of STAT5, an essential transcription factor that mediates IL-15R signaling (24). To assess whether SOCS2 affects IL-15R signaling in NK cells, we examined the level of STAT5 phosphorylation in NK-92 cells expressing control shRNA or SOCS2 shRNA with or without added IL-15. We observed that control shRNA or SOCS2 shRNA-expressing NK-92 cells had similar levels of phosphorylated STAT5 following IL-15 stimulation (Fig. 2*E*). Furthermore, both shRNA-expressing cells expressed perforin, granzyme B, and various activating receptors at equivalent levels (Fig. 2*F*). In addition, SOCS2 knockdown did not affect the expression level of other SOCS family members (Supplemental Fig. 1*D*). Therefore, SOCS2 is not a direct negative regulator of IL-15R signaling in human NK cells.

SOCS2 is required for IL-15–primed NK cell cytotoxicity and IFN-γ production

IL-15 is an essential cytokine to prime NK cells and to maximize their effector functions (12, 13). Accordingly, we investigated whether IL-15–induced upregulation of SOCS2 is involved in NK cell effector functions. To examine the cytolytic activity of IL-15–primed NK

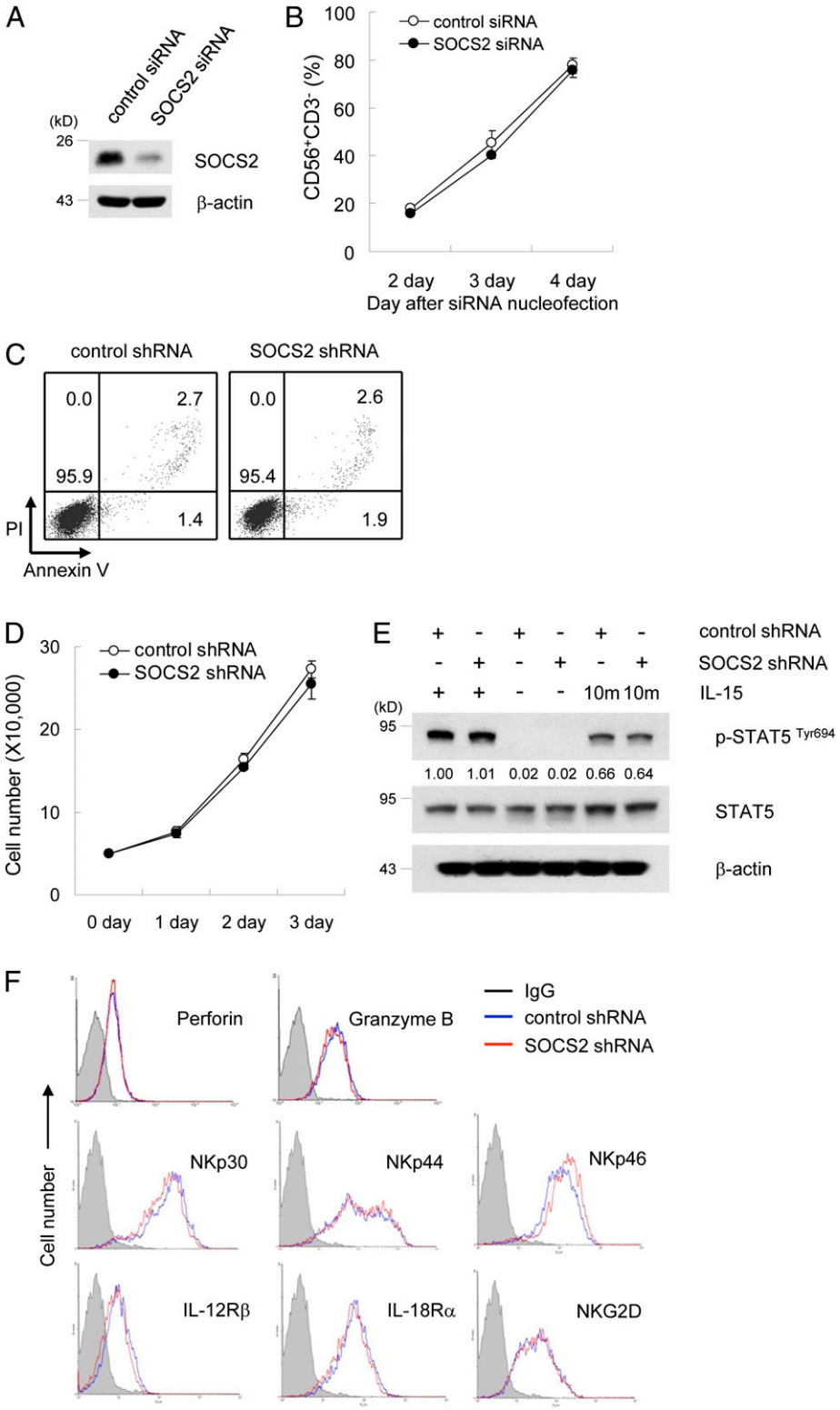

FIGURE 2. Effect of SOCS2-knockdown on IL-15R signaling, IL-15–mediated NK cell differentiation, and survival. *A*, NK cell precursors that had been cultured with IL-15 (30 ng/ml) for 6 d were nucleofected with either the control or SOCS2 siRNA. After 3 d, the nucleofected NK cell precursors were collected to analyze the protein expression of SOCS2. *B*, The nucleofected NK cell precursors were cultured with IL-15 (30 ng/ml) for the indicated time, after which CD56 expression was evaluated by flow cytometry. *C*, NK-92 cells were transduced with lentiviral expression construct encoding a control shRNA or a SOCS2 shRNA. The infected cells were selected in 2 μg/ml puromycin. The shRNA-transduced NK-92 cells were stained with annexin V and propidium iodide and were analyzed by flow cytometry. *D*, Approximately 50,000 transduced NK-92 cells were seeded and cultured with IL-15 (10 ng/ml) for the indicated time. At each indicated day, the cells were counted via the trypan blue dye exclusion method using a hemacytometer. *E*, Control shRNA- or SOCS2 shRNA-expressing NK-92 cells were cultured with IL-15 (10 ng/ml; *left*), then cultured without IL-15 (for 24 h; *middle*), and then restimulated with IL-15 (10 ng/ml for 10 min; *right*). p-STAT5^{Tyr694} and STAT5 were detected by Western blot analysis. The normalized intensities of the phosphorylated STAT5 relative to total STAT5 were quantified with ImageJ software and are presented. *F*, The transduced NK-92 cells were stained for perforin, granzyme B, NKp30, NKp44, NKp46, IL-12Rβ, IL-18Rα, and NKG2D and analyzed by flow cytometry. The data are representative of three independent experiments, and the error bars represent the SD of triplicates.

cells in which expression of SOCS2 was silenced, we measured the ability of NK cells to lyse K562 (a human erythromyeloblastoid leukemia cell line), Jurkat (a human acute lymphoblastic leukemia cell line), MCF-7 (a human breast adenocarcinoma cell line), and A549 (a human lung adenocarcinoma cell line) target cells. Both IL-15–primed, SOCS2 shRNA-expressing NK-92 cells (Fig. 3*A*) and SOCS2 siRNA-nucleofected mNK cells (Fig. 3*B*) had a reduced capacity to lyse target cells. Next, we tested the effect of SOCS2 knockdown on IFN-γ production. IL-15–primed NK cells were stimulated with IL-12 or IL-18, the major stimulatory cytokines of IFN-γ production, or Abs specific for the following natural cytotoxicity receptors (NCRs): NKp30, NKp44, and NKp46. SOCS2 shRNA-expressing NK-92 cells showed a marked reduction in IFN-γ secretion (Fig. 3*C*) and mRNA generation (Fig. 3*D*) in response to stimulation with Abs against the NCRs. However, IL-12– or IL-18–induced IFN-γ production was normal in SOCS2 shRNA-expressing NK-92 cells (Fig. 3*C*, 3*D*). Similarly, NCR-mediated IFN-γ production, but not IL-12– or IL-18–induced

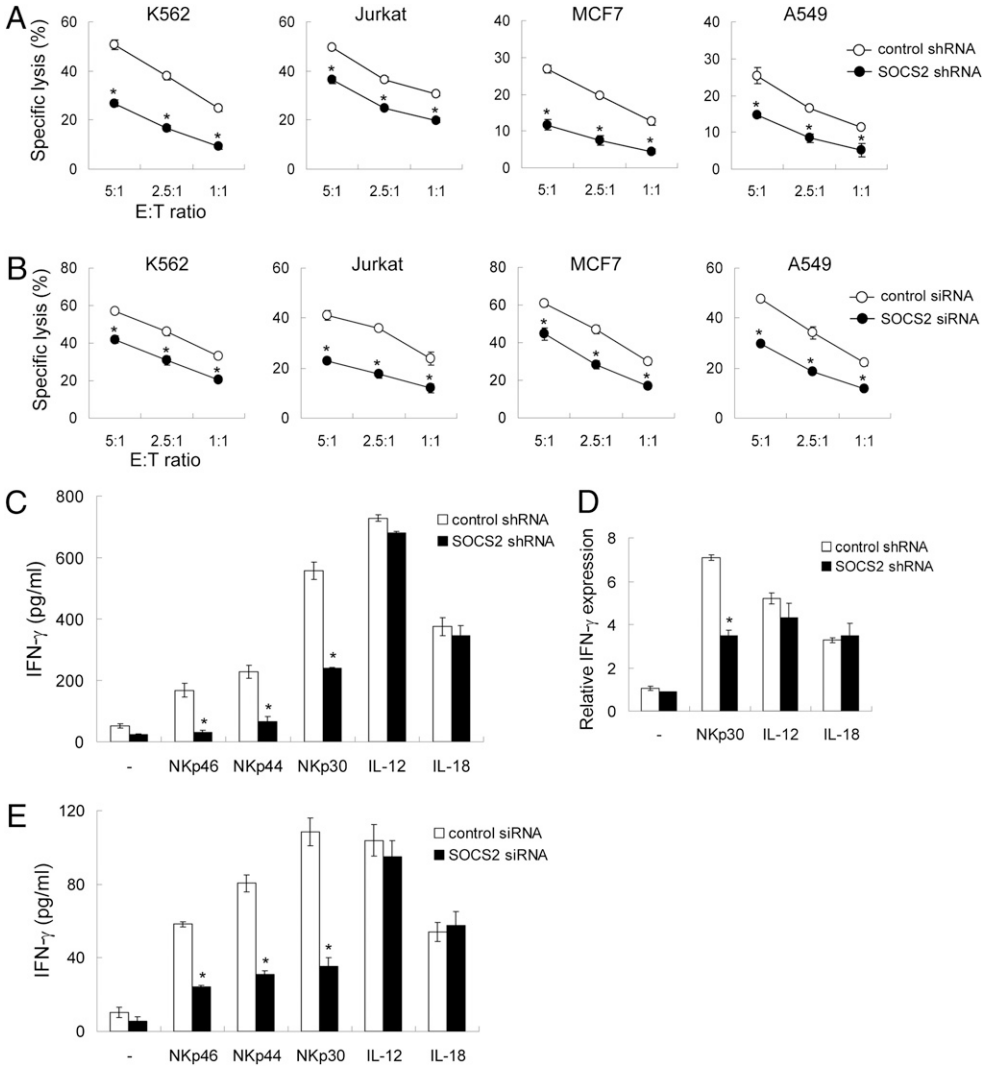

FIGURE 3. Impaired cytolytic activity and IFN-γ production in SOCS2-knockdown NK cells. *A*, IL-15–primed, control shRNA- or SOCS2 shRNA-expressing NK-92 cells were incubated with ^{51}Cr-labeled target cells at the indicated E:T ratios. $*p < 0.05$. *B*, IL-15–primed, control siRNA- or SOCS2 siRNA-nucleofected mNK cells were used in a 4-h [^{51}Cr] release assay. $*p < 0.05$. *C*, IL-15–primed shRNA-transduced NK-92 cells were stimulated with plate-bound Abs to NCRs (NKp30, NKp44, and NKp46; 10 μg/ml) or were stimulated with IL-12 (10 ng/ml) or IL-18 (30 ng/ml) in the culture medium. After 16 h, the IFN-γ released into the supernatant was measured by ELISA. $*p < 0.05$. *D*, shRNA-transduced NK-92 cells were stimulated with anti-NKp30 mAb (10 μg/ml), IL-12 (10 ng/ml), or IL-18 (30 ng/ml) for 6 h, and the relative mRNA expression of IFN-γ to GAPDH was analyzed by real-time PCR. $*p < 0.05$. *E*, As described in *C*, the IFN-γ released into the supernatant from IL-15–primed control siRNA- or SOCS2 siRNA-nucleofected mNK cells was quantified by ELISA. $*p < 0.05$. The data are representative of three independent experiments, and the error bars represent the SD of triplicates (*A*, *B*) or duplicates (*C*–*E*).

IFN-γ production, was decreased in SOCS2 siRNA-nucleofected mNK cells compared with control siRNA-nucleofected mNK cells (Fig. 3*E*). However, knockdown of SOCS3 or SOCS4 (Supplemental Fig. 2*A*) affected neither NK cytolytic activity (Supplemental Fig. 2*B*) nor NKp30-mediated IFN-γ production (Supplemental Fig. 2*C*). Therefore, the results indicate that SOCS2 is important for IL-15–primed NK cell effector functions but is not involved in regulating all NK stimulatory signals.

SOCS2 knockdown results in a reduction of NK receptor signaling events

NK cell cytotoxicity and IFN-γ production result from the activation of NK cell receptors. Engagement of the ITAM-bearing NK receptors activates the Src and Syk family of protein tyrosine kinases. Subsequently, signal transduction is transmitted via various adaptor proteins leading to activation of the MAPKs: ERK, JNK, and p38 (25). These MAPKs are involved in granule polarization and secretion during NK cell cytotoxicity (26–29). In addition, they regulate IFN-γ gene expression by modulating the activation of transcription factors, such as c-Jun and Elk-1 (30–32). Therefore, we examined whether SOCS2 knockdown influences NK receptor-mediated signaling pathways. After incubation with K562 target cells, the phosphorylation of Src, Syk, and JNK was reduced in IL-15–primed, SOCS2 shRNA-expressing NK-92 cells (Fig. 4*A*, Supplemental Fig. 3*A*). However, the control shRNA- and SOCS2 shRNA-expressing NK-92 cells had similar levels of phosphorylated ERK and p38. In addition, the levels of Src and JNK phosphorylation were substantially decreased, and p38 phosphorylation was slightly reduced in SOCS2 shRNA-expressing NK-92 cells after NKp30 receptor engagement (Fig. 4*B*, Supplemental Fig. 3*B*). Among the three MAPKs, we observed that JNK phosphorylation was significantly reduced in SOCS2 shRNA-expressing NK-92 cells. To confirm the role of JNK as an important MAPK for NK cell effector functions, we used specific pharmacological inhibitors

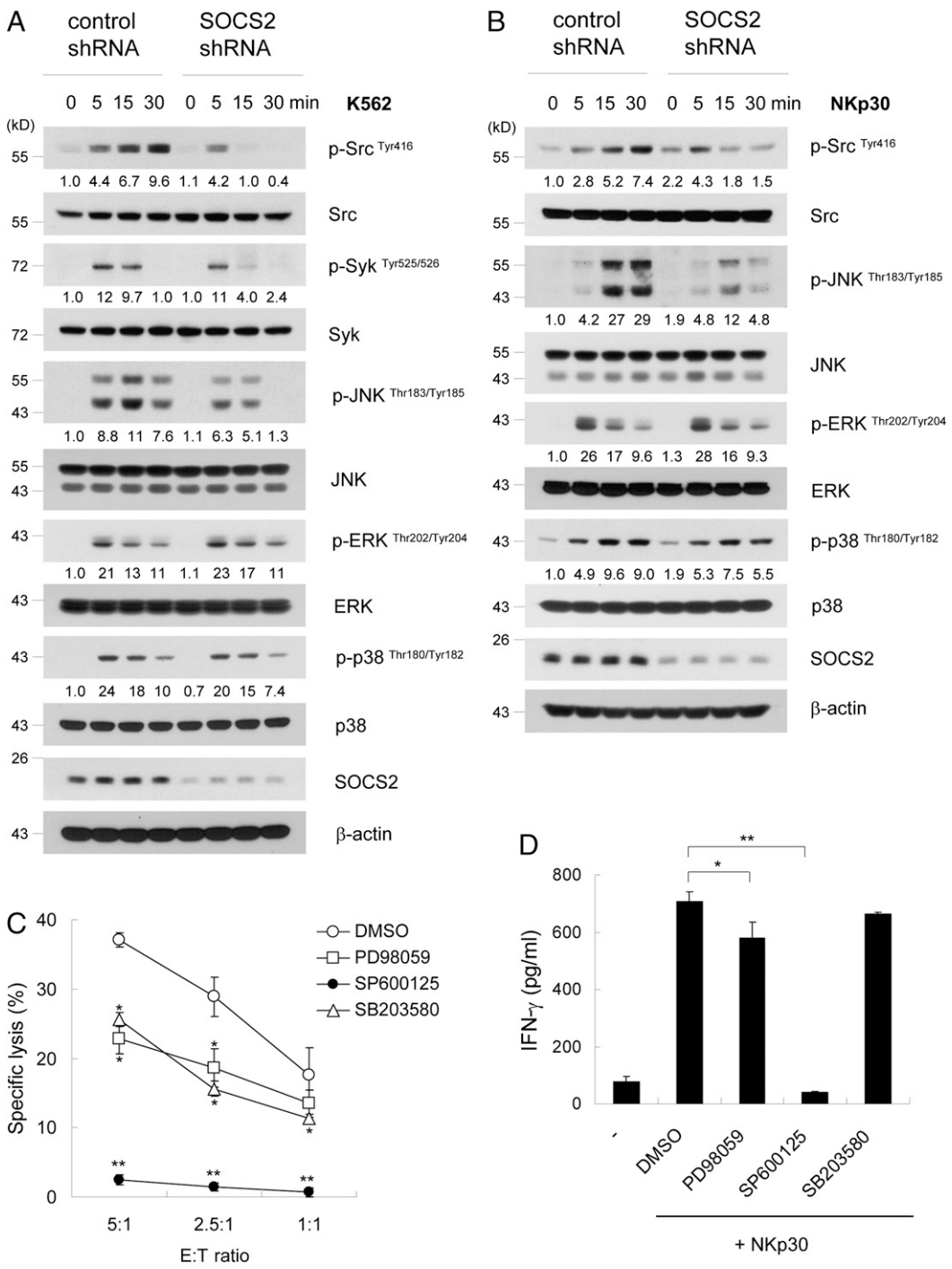

FIGURE 4. Reduced signal transduction in SOCS2-knockdown NK cells. *A*, IL-15–primed NK-92 cells (1×10^6 cells/sample) that were transduced with the shRNA-expressing lentiviruses and target K562 cells (1×10^5 cells/sample) were incubated together for the indicated time at 37°C. The phosphorylation of Src, Syk, JNK, ERK, and p38 was detected by Western blot analysis. The normalized intensities of the phosphorylated Src, Syk, JNK, ERK, and p38 relative to their total forms are presented for each condition. *B*, IL-15–primed shRNA-transduced NK-92 cells were stimulated with plate-bound anti-NKp30 mAb (10 μg/ml) for the indicated time at 37°C. The phosphorylation of Src, JNK, ERK, and p38 was detected by Western blot analysis. The normalized intensities of the phosphorylated Src, JNK, ERK, and p38 relative to their total forms are presented for each condition. *C*, The inhibition of the NK cell-mediated lysis of K562 by the ERK inhibitor (10 μM PD98059), the JNK inhibitor (SP600125; 10 μM), or the p38 inhibitor (10 μM SB203580). $*p < 0.05$; $**p < 0.01$. *D*, The inhibition of IFN-γ secretion upon stimulation with plate-bound anti-NKp30 mAb by the ERK inhibitor (10 μM PD98059), the JNK inhibitor (10 μM SP600125), or the p38 inhibitor (10 μM SB203580). $*p < 0.05$; $**p < 0.01$. The data are representative of three independent experiments, and the error bars represent the SD of triplicates (*C*) or duplicates (*D*).

of MAPKs in NK cell functional assays. Treatment with SP600125, a JNK-specific inhibitor, significantly reduced K562 target cell lysis (Fig. 4*C*) and NKp30-mediated IFN-γ production (Fig. 4*D*). Also, treatment with PD98059, an ERK inhibitor, or SB203580, a p38 inhibitor, reduced NK cytolytic activity moderately (Fig. 4*C*), and treatment with PD98059, not SB203580, decreased NKp30-mediated IFN-γ production slightly (Fig. 4*D*). In addition, treatment with PD98059, SP600125, or SB203580 did not affect cell viability in this condition (Supplemental Fig. 4). Thus, these results demonstrate that knockdown of SOCS2 results in an

impairment of NK activating receptor-mediated signaling events to evoke NK cell effector functions.

SOCS2 interacts with phosphorylated Pyk2

SOCS2 is known to interact with tyrosine phosphorylated target proteins via an SH2 domain and to induce the ubiquitination of target proteins using the SOCS box (15, 18). Hence, we hypothesized that SOCS2 might be regulating a target protein involved in IL-15–primed NK cell function and that this control might be lost in SOCS2 knockdown NK cells. To identify target proteins of SOCS2, we performed a yeast two-hybrid screen and identified Pyk2, a member of the focal adhesion kinase (FAK) family, as a binding partner of SOCS2 (Supplemental Fig. 5). To verify the interaction between SOCS2 and Pyk2, GST-tagged SOCS2 and Flag-tagged Pyk2 were coexpressed in HEK293T cells, and the interaction was examined using a GST pull down (Fig. 5*A*). Interestingly, treatment with the proteasome inhibitor MG-132 increased the amount of Pyk2 that coprecipitated with SOCS2. These data suggest that SOCS2 interacts with Pyk2 and induces its proteasome-mediated degradation. As might be expected for a protein interacting with SOCS2, ubiquitination of Pyk2 was observed in Flag-immunoprecipitated lysates of cells coexpressing GST-SOCS2 (Fig. 5*B*, Supplemental Fig. 6*A*). To determine which region of SOCS2 interacted with Pyk2, SOCS2 deletion mutants were generated. Flag-tagged Pyk2 precipitated GST-tagged full-length SOCS2 and a GST-tagged SOCS box deletion mutant (GST-SOCS2-ΔSOCS) but not a GST-tagged SH2 domain-deletion mutant (GST-SOCS2-ΔSH2) (Fig. 5*C*). In addition, Flag-tagged Pyk2 coexpressed with a GST-tagged SOCS box-deletion mutant was not ubiquitinated. The endogenous interaction between SOCS2 and Pyk2 was confirmed in NK-92 cells (Fig. 5*D*). Moreover, phosphorylation of Pyk2 at tyrosine 402 was observed in the lysates immunoprecipitated with the SOCS2-specific Ab. To investigate whether SOCS2 interacts with Pyk2 in a tyrosine 402 phosphorylation-dependent manner, Flag-tagged mutant Pyk2 in which tyrosine 402 had been mutated to phenylalanine (Pyk2-Y402F) was transfected into HEK293T cells, and its interaction with GST-tagged SOCS2 was analyzed. Phosphorylation of Pyk2 at tyrosine 402 was not detected in the Pyk2-Y402F protein (Fig. 5*E*), and in contrast to wild-type (WT) of Pyk2, Pyk2-Y402F failed to interact with GST-tagged SOCS2. Also, ubiquitination of Pyk2 was not observed in cells coexpressing Pyk2-Y402F and GST-tagged SOCS2 (Fig. 5*E*). Moreover, we demonstrated that phosphorylated Pyk2 at tyrosine 402 (p-Pyk2^{Tyr402}) is the major ubiquitinated fraction of Pyk2 protein (Supplemental Fig. 6*B*). These data suggest that SOCS2 interacts with p-Pyk2^{Tyr402} using the SH2 domain and induces the SOCS box-dependent ubiquitination of Pyk2.

IL-15–induced SOCS2 stimulates the degradation of phosphorylated Pyk2

To assess whether IL-15–induced SOCS2 regulates Pyk2 in NK cells, we examined the level of p-Pyk2^{Tyr402} after IL-15 stimulation of IL-15–deprived NK-92 cells. IL-15 induced the upregulation of SOCS2 in a time-dependent manner (Fig. 6*A*). Conversely, the level of p-Pyk2^{Tyr402} decreased following IL-15 stimulation. In addition, deprivation of IL-15 for 24 h from IL-15–primed primary human cord blood NK cells resulted in an increase in the level of p-Pyk2^{Tyr402} and a decrease in the level of SOCS2 (Fig. 6*B*). In addition, we determined whether SOCS2, which was upregulated by IL-15, induces the ubiquitination and degradation of p-Pyk2^{Tyr402}. Ubiquitinated Pyk2 was detected in IL-15–primed NK-92 cells but not in IL-15–deprived NK-92 cells (Fig. 6*C*). These data suggest that SOCS2 controls the level of p-Pyk2^{Tyr402} during IL-15–dependent NK cell priming, and this regulation might be an important step to prime NK cells. We next investigated whether the regulation of p-Pyk2^{Tyr402} apparently mediated by SOCS2 is blocked in IL-15–primed, SOCS2-knockdown NK cells. Interestingly, p-Pyk2^{Tyr402} accumulated in SOCS2 shRNA-expressing NK-92 cells (Fig. 6*D*, *left panels*) and SOCS2 siRNA-nucleofected mNK cells (Fig. 6*D*, *right panels*). Moreover, the total Pyk2 protein level was increased by the accumulation of p-Pyk2^{Tyr402} in SOCS2 knockdown NK cells (Fig. 6*D*). SOCS2 could potentially be regulating the expression or activity of tyrosine phosphatases, because there was a relatively modest decrease in total levels of Pyk2, compared with a dramatic decrease in the levels of p-Pyk2^{Tyr402} (Fig. 6*A*, 6*B*). Thus, we measured the expression level and activity of tyrosine phosphatases in control-shRNA or SOCS2-shRNA expressing NK-92 cells. The protein expression levels of SHP-1, SHP-2, and CD45, known tyrosine phosphatases that regulate NK cell activity (25, 33, 34), were not altered in SOCS2-shRNA expressing NK-92 cells, compared with control shRNA-expressing NK-92 cells (Supplemental Fig. 7A, 7*B*). Also, SOCS2 knockdown did not affect the activity of tyrosine phosphatases in NK-92 cells (Supplemental Fig. 7*C*). Therefore, IL-15–induced SOCS2 appears to directly regulate the protein level of p-Pyk2^{Tyr402} via inducing ubiquitination of p-Pyk2^{Tyr402}, rather than via the regulation of tyrosine phosphatase activity or expression. In addition, we determined whether SOCS2 overexpression regulates the fate of p-Pyk2^{Tyr402} in NK cells. SOCS2 overexpression further reduced p-Pyk2^{Tyr402} that had been already downregulated by IL-15–induced endogenous SOCS2 in IL-15–primed NK-92 cells (Supplemental Fig. 8*A*). However, SOCS2 overexpression did not affect NK cytolytic activity (Supplemental Fig. 8*B*), NKp30-mediated IFN-γ production (Supplemental Fig. 8*C*), or K562-induced signal transduction (Supplemental Fig. 8*D*). Thus, it seems that IL-15–induced endogenous SOCS2 is enough to regulate p-Pyk2^{Tyr402} for NK cell activity. Therefore, IL-15–induced SOCS2 appears to play a central role in controlling the level of p-Pyk2^{Tyr402} and priming the effector functions of NK cells.

Overexpression of Pyk2-WT or Pyk2-K457A, not Pyk2-Y402F, inhibits NK cell function

To determine whether the accumulation of p-Pyk2^{Tyr402} by SOCS2 knockdown inhibits NK cell effector functions, we overexpressed GFP-tagged Pyk2-WT (GFP-Pyk2-WT), GFP-Pyk2-Y402F (phosphorylation mutant at tyrosine 402), or GFP-Pyk2-K457A (kinase-inactive mutant) in NK-92 cells using a lentiviral expression system (Fig. 7*A*). Phosphorylation of overexpressed Pyk2 at tyrosine 402 was observed in GFP-Pyk2-WT–overexpressing NK-92 cells and GFP-Pyk2-K457A–overexpressing NK-92 cells but not in GFP-Pyk2-Y402F–overexpressing NK-92 cells (Fig. 7*A*). The overexpression of GFP-Pyk2-WT or GFP-Pyk2-K457A reduced the ability of NK-92 cells to lyse K562 target cells (Fig. 7*B*), consistent with a previous report demonstrating that overexpression of WT Pyk2 and kinase-inactive mutant Pyk2 blocked the cytotoxic response of NK cells (35). However, GFP-Pyk2-Y402F overexpression did not inhibit NK cytolytic activity (Fig. 7*B*). Similarly, NKp30-mediated IFN-γ production was reduced by overexpression of GFP-Pyk2-WT or GFP-Pyk2-K457A but not by GFP-Pyk2-Y402F overexpression (Fig. 7*C*). These results demonstrate that the accumulation of p-Pyk2^{Tyr402} inhibits NK cell effector functions regardless of Pyk2 kinase activity, and the regulation of p-Pyk2^{Tyr402} by IL-15–induced SOCS2 is important to perform NK cell effector functions.

Discussion

NK cells are important lymphocytes of the innate immune system not only because of cytotoxic capacity against transformed or virus-infected cells but also because of the ability to produce IFN-γ (1, 2). NK cells had been thought to rapidly respond to target cells

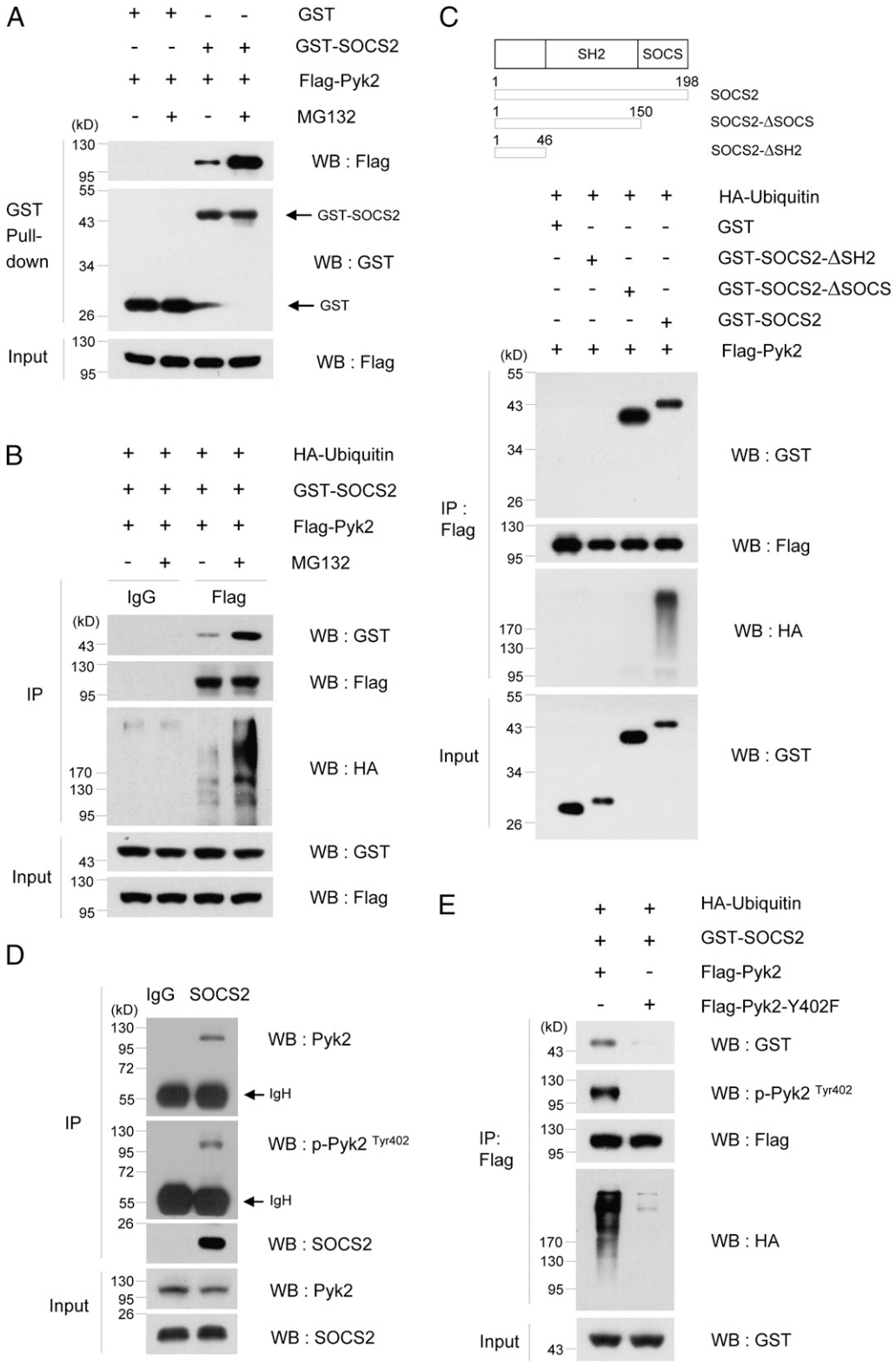

FIGURE 5. Interaction between SOCS2 and Pyk2. *A*, HEK293T cells were transiently transfected with the indicated plasmids (2 μg GST, 2 μg GST-SOCS2, or 2 μg Flag-Pyk2). Twenty-four hours after transfection, the cells were cultured with or without MG-132 (10 μM) for 4 h. The cell lysates were subjected to a pull-down assay with glutathione-Sepharose beads, and the precipitates were analyzed by Western blot using an anti-Flag or anti-GST Ab. Total cell lysates (5% of input) were analyzed by Western blot using an anti-Flag Ab to confirm Pyk2 expression. *B*, HEK293T cells were transiently transfected with the indicated plasmids (2 μg GST-SOCS2, 2 μg Flag-Pyk2, and 0.2 μg HA-ubiquitin). Twenty-four hours after transfection, the cells were cultured with or without MG-132 (10 μM) for 4 h. The cell lysates were immunoprecipitated with the control IgG or an anti-Flag Ab, and the precipitates were analyzed by Western blot with Abs against GST, Flag, or HA. Total cell lysates (5% of input) were analyzed by Western blot using an anti-GST or anti-Flag Ab. *C*, HEK293T cells were transiently transfected with the indicated plasmids (2 μg GST, 2 μg GST-SOCS2-ΔSH2, 2 μg GST-SOCS2-ΔSOCS, 2 μg GST-SOCS2, 2 μg Flag-Pyk2, or 0.2 μg HA-ubiquitin). Twenty-four hours after transfection, cell lysates were immunoprecipitated with an anti-Flag Ab. The precipitated proteins were analyzed by Western blot using Abs against GST, Flag, and HA. Total cell lysates (5% of input) were analyzed by Western blot using an anti-GST Ab to confirm the expression of SOCS2 and the SOCS2 deletion mutants. *D*, IL-15–primed NK-92 cells were pretreated

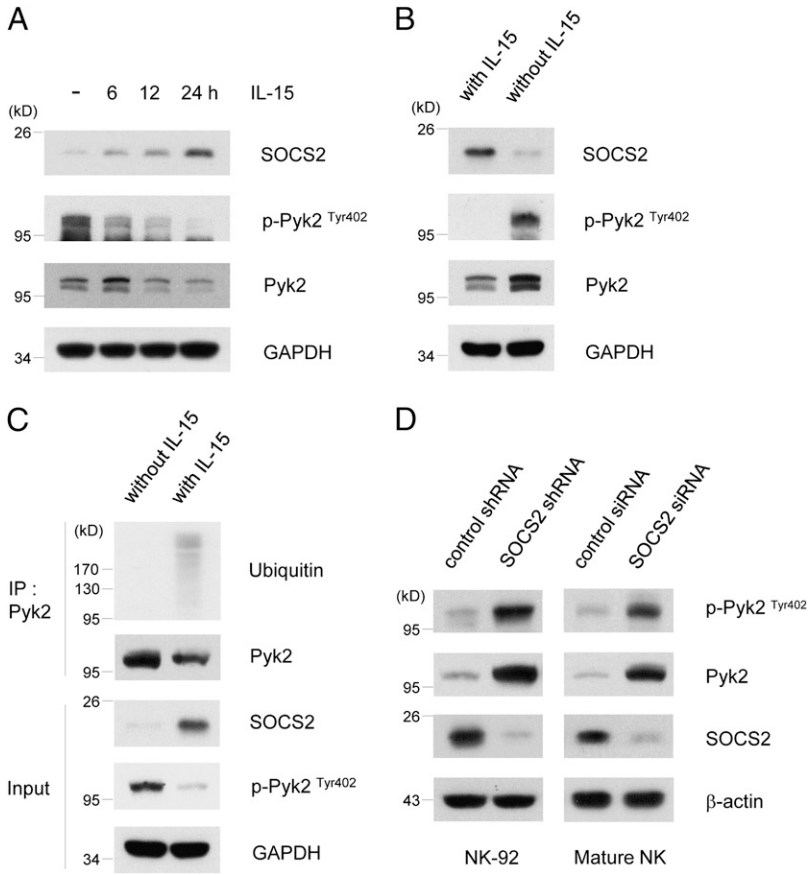

FIGURE 6. SOCS2 controls the protein level of phosphorylated Pyk2 depending on IL-15 in NK cells. *A*, NK-92 cells were deprived of IL-15 for 24 h and restimulated with IL-15 (10 ng/ml) for the indicated time. SOCS2, p-Pyk2^{Try402}, and total Pyk2 was detected by Western blot analysis. *B*, IL-15–primed primary human cord blood NK cells were cultured with IL-15 (10 ng/ml) or without IL-15 for 24 h. SOCS2, p-Pyk2^{Try402}, and total Pyk2 was detected by Western blot analysis. *C*, IL-15–deprived NK-92 cells (deprived for 18 h) and IL-15–primed NK-92 cells (10 ng/ml for 18 h) were treated with MG-132 (10 μM) for 6 h. The cell lysates were immunoprecipitated with an anti-Pyk2 Ab, and the precipitated proteins were analyzed by Western blot using an Ab specific for ubiquitin or Pyk2. Total cell lysates (5% of input) were analyzed by Western blot using an anti-SOCS2 or p-Pyk2^{Try402} Ab. *D*, IL-15–primed control shRNA- or SOCS2 shRNA-expressing NK-92 cells (*left panels*) and IL-15–primed control siRNA- and SOCS2 siRNA-nucleofected mNK cells (*right panels*) were harvested, and the expression of p-Pyk2^{Try402}, Pyk2, and SOCS2 was analyzed by Western blot. The data are representative of three independent experiments.

without priming. However, resting NK cells show only minimal cytotoxicity and secrete only low levels of IFN-γ (11) and need additional priming signals from cytokines, such as IL-15 or IL-2, prior to receiving stimulatory signals for full activation (12, 13). IL-15 is synthesized by DCs, monocytes, and stromal cells, whereas the major source of IL-2 is activated T cells (36). DCs have a key role in priming NK cells in vivo via transpresentation of IL-15. Thus, neutralization of IL-15 using Abs specific for IL-15 reduced DC-mediated NK cell priming, whereas neutralization of IL-2 failed to block priming by DCs (13, 37). Accordingly, we used IL-15 as an NK cell-priming cytokine in this study.

Our studies revealed that SOCS2 expression is upregulated by IL-15 stimulation in resting human NK cells (Fig. 1). SOCS2, a member of the SOCS family, has been implicated in the negative regulation of cytokine activation via binding to key signaling components and/or targeting these components for proteasome-mediated degradation through SOCS box-dependent ubiquitination (14, 15). Knockdown of SOCS2 expression, however, failed to impact direct IL-15R signaling, IL-15–mediated NK cell differentiation, and IL-15–dependent NK cell survival (Fig. 2). Rather, SOCS2 knockdown inhibited IL-15–primed NK cell effector functions and the underlying signaling pathways required for such NK cell functions (Figs. 3, 4).

Previous reports have demonstrated that SOCS2 has a dual role in the positive or negative regulation of GH signaling (16, 17). These observations suggest that SOCS2 might play a role in intracellular homeostasis. Thus, we hypothesized that SOCS2 might be involved in the homeostasis of IL-15–primed NK cell function via regulation of intracellular components. SOCS2 has an SH2 domain that binds to a phosphorylated tyrosine residue in target proteins and a SOCS box that induces ubiquitination of target proteins via recruitment of the Elongin BC complex (14, 15). Therefore, we hypothesized that SOCS2 targets a tyrosine-phosphorylated intracellular component for proteasomal degradation to maintain IL-15–primed NK cell homeostasis. Liu et al. (38). have reported that both SOCS1 and SOCS3 interact with FAK, which is not expressed in NK cells (35), in a FAK-Y397–dependent manner and also demonstrated that SOCS1 and SOCS3 promote the ubiquitination and degradation of FAK in a SOCS box-dependent manner. Similar to the findings in this previous report, we observed that SOCS2 interacts with p-Pyk2^{Tyr402} and promotes degradation of p-Pyk2^{Tyr402} through ubiquitination (Fig. 5). However, despite a dramatic decrease in the p-Pyk2^{Tyr402}, there was a relatively modest decrease in total levels of Pyk2. We hypothesize that this difference reflects the relatively small portion of phosphorylated Pyk2 at tyrosine 402 in total Pyk2 proteins. In NK cells, phosphorylation of Pyk2 is induced by chemokine signaling, integrin activation, and target cell interaction (39, 40). The data in Fig. 6 were produced using NK cells that had been cultured without any other stimulatory signals,

with or without MG-132 (10 μM) for 4 h prior to vanadate treatment (50 μM) for 30 min. Endogenous SOCS2 was immunoprecipitated using an anti-SOCS2 Ab, and the precipitated proteins were analyzed by Western blot using an Ab against Pyk2, p-Pyk2^{Try402}, or SOCS2. Total cell lysates (5% of input) were analyzed by Western blot using an anti-Pyk2 or anti-SOCS2 Ab. *E*, HEK293T cells were transiently transfected with the indicated plasmids (2 μg GST-SOCS2, 2 μg Flag-Pyk2, 2 μg Flag-Pyk2-Y402F, or 0.2 μg HA-ubiquitin). Twenty-four hours after transfection, cell lysates were immunoprecipitated with an anti-Flag Ab. The precipitated proteins were analyzed by Western blot using an Ab against GST, p-Pyk2^{Try402}, Flag, or HA. Total cell lysates (5% of input) were analyzed by Western blot using an anti-GST Ab. The data are representative of three independent experiments.

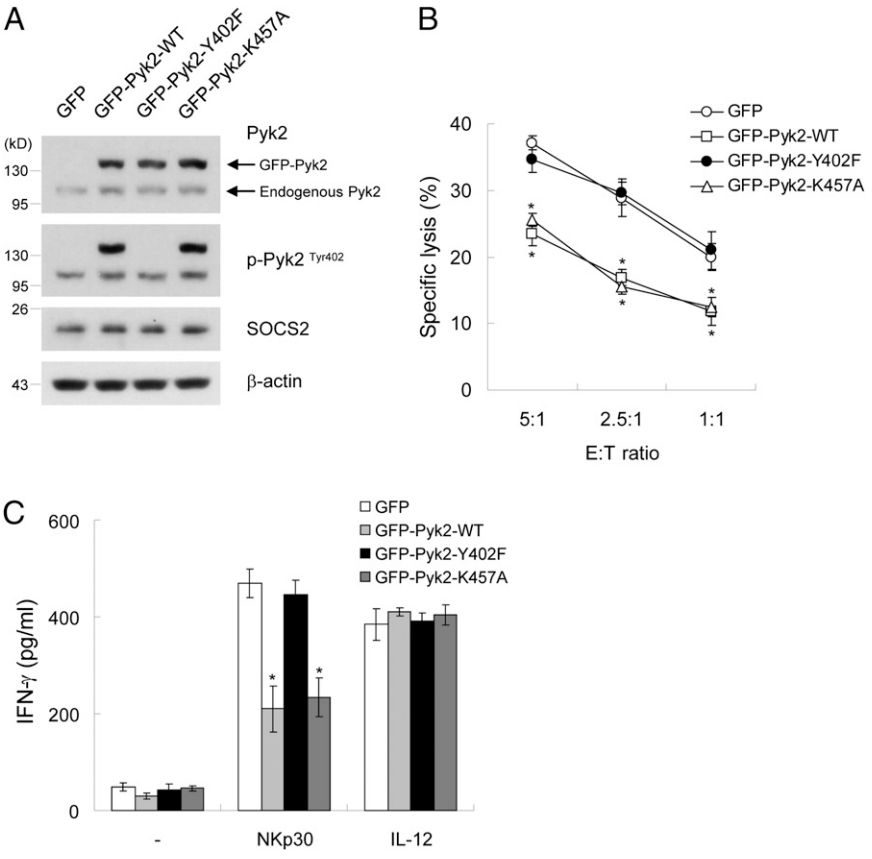

FIGURE 7. Overexpression of Pyk2-WT or Pyk2-K457A, but not Pyk2-Y402F, blocks NK cell effector functions. *A*, IL-15–primed NK-92 cells transduced with a lentivirus encoding GFP, GFP-Pyk2-WT, GFP-Pyk2-Y402F, or GFP-Pyk2-K457A. Pyk2, p-Pyk2^{Tyr402}, and SOCS2 were detected by Western blot analysis. *B*, IL-15–primed transduced NK-92 cells were used in a 4-h [^{51}Cr] release assay with K562 target cells. $*p < 0.05$. *C*, IL-15–primed transduced NK-92 cells were stimulated with plate-bound anti-NKp30 mAb (10 μg/ml) or IL-12 (10 ng/ml). After 16 h, the IFN-γ released into the supernatant was measured by ELISA. $*p < 0.05$. The data are representative of two independent experiments, and the error bars represent the SD of triplicates (*B*) or duplicates (*C*).

except for IL-15. Thus, we thought that p-Pyk2^{Tyr402} detected in Fig. 6 might be spontaneously autophosphorylated Pyk2, because tyrosine 402 of Pyk2 is a main autophosphorylation site. However, phosphorylation at tyrosine 402 was also detected in overexpressed kinase-inactive mutant of Pyk2 (Fig. 7*A*). Therefore, we assumed that the endogenous Pyk2 might phosphorylate the kinase-inactive mutant. In addition, there was a possibility that other kinases activated by IL-15R–mediated signaling (e.g., PI3K or AKT signaling) (41) might be involved in the phosphorylation of Pyk2. Importantly, knockdown of SOCS2 promoted the accumulation of p-Pyk2^{Tyr402} in IL-15–primed NK cells (Fig. 6), and we demonstrated that accumulated p-Pyk2^{Tyr402} inhibited NK cell effector functions using GFP-Pyk2 overexpression system (Fig. 7). There was a possibility that overexpressed GFP-Pyk2 might be ubiquitinated by endogenous SOCS2. However, endogenous SOCS2 was not sufficient to ubiquitinate and degrade all of the overexpressed GFP-p-Pyk2^{Tyr402} (Fig. 7*A*).

In human NK cells, the engagement of the β_1 and β_2 integrins induces the phosphorylation and activation of Pyk2 (42). Hence, Pyk2 may also play a role in the transendothelial migration of NK cells (40). Following the interaction of NK cells with a target cell, Pyk2 localizes to the contact region between the NK cell and the target cell, colocalizing with paxillin and the microtubule organizing center. Interestingly, overexpression of Pyk2 inhibits microtubule organizing center polarization and NK cytotoxicity (35). However, the exact role of Pyk2 in NK cell function, including IFN-γ production, is still unclear. As shown in Figs. 6 and 7, IL-15–induced SOCS2 decreased p-Pyk2^{Tyr402} via the ubiquitin-mediated proteasomal degradation, and accumulated p-Pyk2^{Tyr402} inhibited IL-15–primed NK cell effector functions. Our results (Fig. 7*B*) are concordant with the results of Sancho et al. (35) who reported that overexpression of a WT Pyk2 or a kinase dead mutant Pyk2 could inhibit NK cytotoxicity. However, Gismondi et al. (39, 40) reported that overexpression of kinase dead mutant Pyk2, but not WT Pyk2, inhibits NK cytotoxicity. Sancho et al. (35) suggested Pyk2 may function in part as an adaptor protein in the NK cell receptor signaling pathway, whereas Gismondi et al. (39, 40) suggested Pyk2 functioned as a kinase for NK cell receptor signaling cascades. Our data are most consistent with the hypothesis by Sancho et al. (35) who emphasized an adaptor function of Pyk2. The kinase activity of Pyk2 seems to be not important for NK cell effector functions, because overexpression of kinase-inactive mutant Pyk2 inhibited NK cell effector functions similar to overexpression of WT Pyk2 (Fig. 7). Previous reports demonstrated that the phosphorylation of Pyk2 at tyrosine 402 creates an interaction site for the SH2 domain of Src, resulting in recruitment of Src family kinases to the signaling complex in osteoclasts (43–45). Src family kinases are also important in signal transduction evoked by NK receptor-mediated activation (25), and the phosphorylation of Src was reduced in shRNA-expressing NK-92 cells after activating receptor stimulation (Fig. 4*A*, 4*B*). Therefore, we hypothesize that Pyk2, using phospho-tyrosine at 402 aa as a binding motif, might be a crucial adaptor molecule involved in the recruitment of the Src-related signaling complex following activating receptor stimulation of NK cells. Also, we hypothesize that the accumulation of p-Pyk2^{Tyr402} by SOCS2 knockdown (Fig. 6*D*) or IL-15 deprivation (Fig. 6*A*, 6*B*) might affect the Src-related signaling complex, prior to receiving NK stimulatory signals (e.g., cross-linking of NK-activating receptors or IFN-γ–producing cytokines). However, further investigation is required to demonstrate the precise roles of Pyk2 in NK cell activation as well as the roles of SOCS2 in NK cells.

Ubiquitin-mediated proteasomal degradation of p-Pyk2^{Tyr402} by SOCS2 depends on IL-15, because the expression of SOCS2 was induced by IL-15 stimulation (Fig. 6*A*, 6*B*). The accumulated p-Pyk2^{Tyr402} appears to be a probable cause for the defect in effector functions in IL-15–deprived NK cells and SOCS2 knocked down

NK cells, because overexpression of Pyk2-WT, but not Pyk2-Y402F, inhibited NK cytolytic activity and NKp30-mediated IFN-γ production (Fig. 7). IL-15 is an essential cytokine to prime NK cells, and IL-15–stimulated NK cells undergo a process of priming that rapidly initiates molecular programs (e.g., upregulation of intracellular granzyme B), endowing them with effector functions (13, 37). In fact, deprivation of IL-15 substantially inhibited NK cytolytic activity and IFN-γ production while priming with IL-15 maximized NK cell effector functions (Supplemental Fig. 9). Therefore, we would suggest that the regulation of p-Pyk2^{Tyr402} by SOCS2 during IL-15–mediated priming might be a crucial step in the molecular programs required to prime NK cells. However, knockdown of Pyk2 was not able to enhance NK effector function in nonprimed mNK cells (Supplemental Fig. 10). Thus, we thought that the regulation of p-Pyk2^{Tyr402} by SOCS2, as one of the steps in IL-15–mediated NK cell priming, could not be substituted for whole processes of IL-15–mediated priming, although this regulation by SOCS2 might be an important step in IL-15–mediated NK cell priming. Anyway, that IL-15–dependent regulation of p-Pyk2^{Tyr402} by SOCS2 might provide clues to clarify unidentified molecular programs of NK cell priming.

In conclusion, we have shown that SOCS2 is a novel regulator of IL-15–primed human NK cell function. SOCS2 controls p-Pyk2^{Tyr402} during IL-15–mediated NK cell priming, and this regulation is important for IL-15–primed NK cell cytolytic activity and IFN-γ production.

Disclosures

The authors have no financial conflicts of interest.

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