

Generation and Preclinical Characterization of an NKp80-Fc Fusion Protein for Redirected Cytolysis of Natural Killer (NK) Cells against Leukemia*

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Background: AICL, as a ligand for NKp80, is highly expressed on leukemia cells and may be a target for leukemia immunotherapy.

Results: NKp80-Fc can increase NK target cell conjugation, induce the ADCC effect, and trigger NK cell killing.

Conclusion: NKp80-Fc amplifies NK cell anti-leukemia effects through induction of the ADCC effect.

Significance: NKp80-Fc may be a promising drug for immunotherapy of leukemia.

The capacity of natural killer (NK) cells to mediate Fc receptor-dependent effector functions, such as antibody-dependent cellular cytotoxicity (ADCC), largely contributes to their clinical application. Given that activation-induced C-type lectin (AICL), an identified ligand for the NK-activating receptor NKp80, is frequently highly expressed on leukemia cells, the lack of therapeutic AICL-specific antibodies limits clinical application. Here we explore a strategy to reinforce NK anti-leukemia reactivity by combining targeting AICL-expressing leukemia cells with the induction of NK cell ADCC using NKp80-Fc fusion proteins. The NKp80-Fc fusion protein we generated bound specifically to leukemia cells in an AICL-specific manner. Cell binding assays between NK and leukemia cells showed that NKp80-Fc significantly increased NK target cell conjugation. In functional analyses, treatment with NKp80-Fc clearly induced the ADCC effect of NK cells. NKp80-Fc not only promoted NK-mediated leukemia cell apoptosis in the early stage of cell conjugation but also enhanced NK cell degranulation and cytotoxicity activity in the late stage. The bifunctional NKp80-Fc could redirect NK cells toward leukemia cells and triggered NK cell killing *in vitro*. Moreover, NKp80-Fc enhanced the lysis of NK cells against tumors in leukemia xenograft non-obese diabetic/severe combined immunodeficiency

mice. Taken together, our results demonstrate that NKp80-Fc potentially amplifies NK cell anti-leukemia effects *in vitro* and *in vivo* through induction of the NK cell ADCC effect. This method could potentially be useful for molecular targeted therapy, and the fusion proteins may be a promising drug for immunotherapy of leukemia.

Natural killer (NK)³ cells are innate immune effector cells capable of recognizing and killing tumor cells through the release of cytotoxic enzymes and cytokines. The importance of NK cell-mediated tumor immunosurveillance has been underlined in NK cell-deficient mouse models, but limited information is available regarding humans. The function of recognizing tumor cells as targets suggests possibilities for NK cell therapy for cancer (1). The capacity of NK cells to kill tumor cells depends on the combined effect of suppressive and stimulatory signals delivered through surface receptors. Inhibitory signals result from the interaction of NK inhibitory receptors and human leukocyte antigen molecules on potential target cells, whereas activating receptors engaged by ligands that are expressed predominantly by virally infected and tumor cells provoke activating signals that ultimately cause target cell killing (2).

Notably, various immunoregulatory molecules, including receptors involved in missing and induced self-recognition, influence NK reactivity (3, 4). The main activating receptors expressed on human NK cells include FcγRIIIa (CD16), NKG2D, DNAM-1, and the natural cytotoxicity receptors containing the receptors NKp30, NKp44, and NKp46. Along with the deepening research on NK cells, the number of identified activating receptors and their ligands is increasing gradually, and their properties and functions are slowly becoming clearer.

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³ The abbreviations used are: NK, natural killer; AICL, activation-induced C-type lectin; ADCC, antibody-dependent cellular cytotoxicity; NOD, non-obese diabetic; SCID, severe combined immunodeficiency; SF, serum-free; SC, serum-containing; PE, phycoerythrin; hlgG, human IgG; E:T, effector:target; PMA, phorbol-12-myristate-13-acetate.

In this paper, we will investigate another important receptor for NK cell activation, NKp80, a type II transmembrane-anchored C-type lectin-like protein expressed in almost all human NK cells and in some T cell subsets. Similar to NKG2D, NKp80 stimulates NK cell cytotoxicity and induces calcium influx in human NK cells after being triggered by the appropriate antibodies (5). In contrast to NKG2D, NKp80 lacks a homolog in rodents, which impedes the investigation of NKp80 function *in vivo* (6).

Recently, activation-induced C-type lectin (AICL) has been identified as a myeloid-specific activating receptor capable of binding NKp80 (7). The only known ligand for NKp80 to date is expressed by hematopoietic cells, especially by malignant myeloid cells in acute myeloid leukemia and chronic myeloid leukemia, and by non-hematopoietic cells, including carcinoma and melanoma cells (8). Researchers have already demonstrated that expression of AICL, which engages NKp80, increases the susceptibility of myeloid cells to NK cell-mediated cytotoxicity. However, NK cell-mediated cytotoxicity of autologous LPS-activated monocytes was decreased or absent (7). Importantly, there are currently no available therapeutic antibodies specifically targeting AICL to enhance NK reactivity against autologous leukemia cells.

For some time, chimeric or humanized monoclonal antibodies have been used successfully in cancer therapy. For example, treatment with rituximab and herceptin leads to considerably improved outcomes. However, these therapeutic antibodies have their own limitations (9, 10). Therefore, numerous strategies are being evaluated to increase the efficacy of antitumor antibodies and humanized Fc fusion proteins (11). One of the most important antitumor effects is improving the ability to recruit Fc receptor-bearing immune cells (12). Currently, various antibodies and humanized Fc fusion proteins are in early clinical development. These agents mediate markedly enhanced antibody-dependent cellular cytotoxicity (ADCC) against tumor cells. However, in many diseases, including myeloid leukemia, efforts to explore effective antibody therapy have not yet been successful (13).

On the basis of the fact that AICL is selectively overexpressed by malignant myeloid cells in acute myeloid leukemia and chronic myeloid leukemia, and because there are no available therapeutic antibodies specifically targeting AICL, AICL can be a promising target for immunotherapeutic approaches. Therefore, we generated NKp80-Fc fusion proteins that enable targeting of leukemic cells and demonstrated the feasibility of using tumor-associated expression of AICL for tumor immunotherapy by amplifying the ADCC effect of NK cells.

Materials and Methods

Mice, Cell Lines, and Reagents—Female 6- to 8-week-old NOD/SCID mice were purchased from Vital River Laboratories (Beijing, China) and housed under specific pathogen-free conditions according to the experimental animal guidelines of the University of Science and Technology of China. All experiments involving mice were approved by the Animal Care and Use Committee at the University of Science and Technology of China.

The CHO-K1, U937, THP-1, and HeLa cell lines were purchased from the ATCC. All fluorescein-conjugated antibodies

and the respective isotype controls were purchased from BD Biosciences. Functional anti-NKp80 (clone 5D12) and anti-human IgG-Fc mAb and human IgG were obtained from BioLegend. The chromium (^{51}Cr) solution was purchased from Perkin Elmer Life Sciences.

Production and Purification of NKp80-Fc Fusion Proteins—The recombinant plasmid hIL-2ss-hIgG1-Fc-NKp80ED on the basis of pcDNA3.1 was stably transfected into CHO-K1 cells, and positive clones were selected using 700 $\mu\text{g}/\text{ml}$ hygromycin B (Roche). The NKp80-Fc fusion proteins were purified from the large-scale serum-free CHO culture supernatant (SF) or serum-containing culture supernatant (SC) from positive clone CHO-Fc-NKp80 D1 by protein A affinity chromatography (GE Healthcare). Purity was determined by non-reducing and reducing SDS-PAGE, Western blotting, and size exclusion chromatography.

Preparation of Human NK Cells—Human NK cells were obtained from peripheral blood mononuclear cells of healthy donor buffy coats using Ficoll-Paque density gradient centrifugation (Solarbio). Non-NK cells were depleted using an NK cell isolation kit according to the instructions of the manufacturer (Miltenyi Biotec). Freshly isolated human NK cells were used for functional assays or cultured in complete RPMI 1640 medium (HyClone) in the presence of IL-2 (100–200 units/ml). Cell culture was performed at 37 °C in a 5% CO_2 humidified atmosphere. Functional experiments were performed when the purity of NK cells ($\text{CD}56^+ \text{CD}3^-$) was higher than 90% as determined by flow cytometry.

Flow Cytometry and Cellular Conjugation Assay—Cells were labeled with NKp80-Fc purified from serum-free and serum-containing culture supernatant followed by PE-conjugated anti-human IgG-Fc antibody and then analyzed by BD Biosciences FACSCalibur. To test cellular conjugation, freshly purified NK cells from healthy donors were labeled with CD56 and mixed with CD33-labeled U937 cells for the indicated times (0–60 min) at 37 °C at an E:T ratio of 2:1. Next, the mixed cells were fixed, and double-positive binding cells were analyzed by flow cytometry. Cellular conjugation was denoted as binding rate, which was calculated as the ratio of $\text{CD}56^+ \text{CD}33^+$ double-positive cells among $\text{CD}56^+$ single-positive cells.

FACS-based Leukemia Cell Apoptosis Assay—CD56-labeled, freshly purified NK cells from healthy donors pretreated with NKp80-Fc or control IgG were mixed with CD33-labeled U937 cells at an E:T ratio of 2:1 and incubated at 37 °C for 1.5 h. After the incubation, FITC-Annexin V was added at 10 min to assess apoptosis of the target U937 cells. Then the frequency of $\text{CD}56^- \text{CD}33^+ \text{AnnexinV}^+$ apoptotic U937 cells among $\text{CD}56^- \text{CD}33^+$ U937 cells under the NKp80-Fc or IgG treatment was analyzed by flow cytometry.

NK Cell Degranulation and Cytotoxicity Assay—Freshly purified NK cells from healthy human donors pretreated with NKp80-Fc or control human IgG were mixed with U937 cells at an E:T ratio of 2:1 or incubated alone in the presence of FITC-CD107a antibody and stimulated with PMA/ion at 37 °C for 4 h. The frequency of $\text{CD}107a^+ \text{CD}56^+$ cells among $\text{CD}56^+$ NK cells in the IgG-treated and NKp80-Fc-treated groups was analyzed by flow cytometry. For a classic 4-h ^{51}Cr release cytotoxicity assay, NK cells were preincubated with NKp80-Fc at 37 °C

for 45 min and mixed with the ^{51}Cr -labeled U937 targets at ratios of 4:1 and 1:1. After a 4-h incubation period, the supernatant of each group was collected and analyzed. The specific ^{51}Cr release was calculated as a percentage using the following formula: (^{51}Cr release in the presence of effector cells – spontaneous release in the absence of effector cells) / (total ^{51}Cr release from target cells incubated with 1% Triton X-100 – spontaneous release in the absence of effector cells) $\times$ 100%. The spontaneous release did not exceed 10% of the maximum release.

Leukemia Xenograft Model—After irradiation with 2 Gy, female 6- to 8-week-old NOD/SCID mice were inoculated subcutaneously with 2.0×10^6 U937 cells/mouse. After 3 days, NKp80-Fc purified from serum-free culture supernatant or human IgG was mixed with 1.0×10^7 /ml freshly isolated human NK cells in a final concentration of 0.1 mg/ml. Then 200 μl of the mixture was injected subcutaneously into the same tumor area. The mice were euthanized, and final tumors were isolated from mice 26 days after tumor inoculation. Images of the tumors were taken, and tumor length, width, and weight were measured. The volume of tumors was calculated by using the formula volume = (length $\times$ width $\times$ width) / 2.

Statistical Analysis—Data were expressed as mean $\pm$ S.D., and significance was denoted as follows: *, $p < 0.05$; **, $p < 0.01$; and ***, $p < 0.001$. Calculations were performed using GraphPad Prism software with Student's t test.

Results

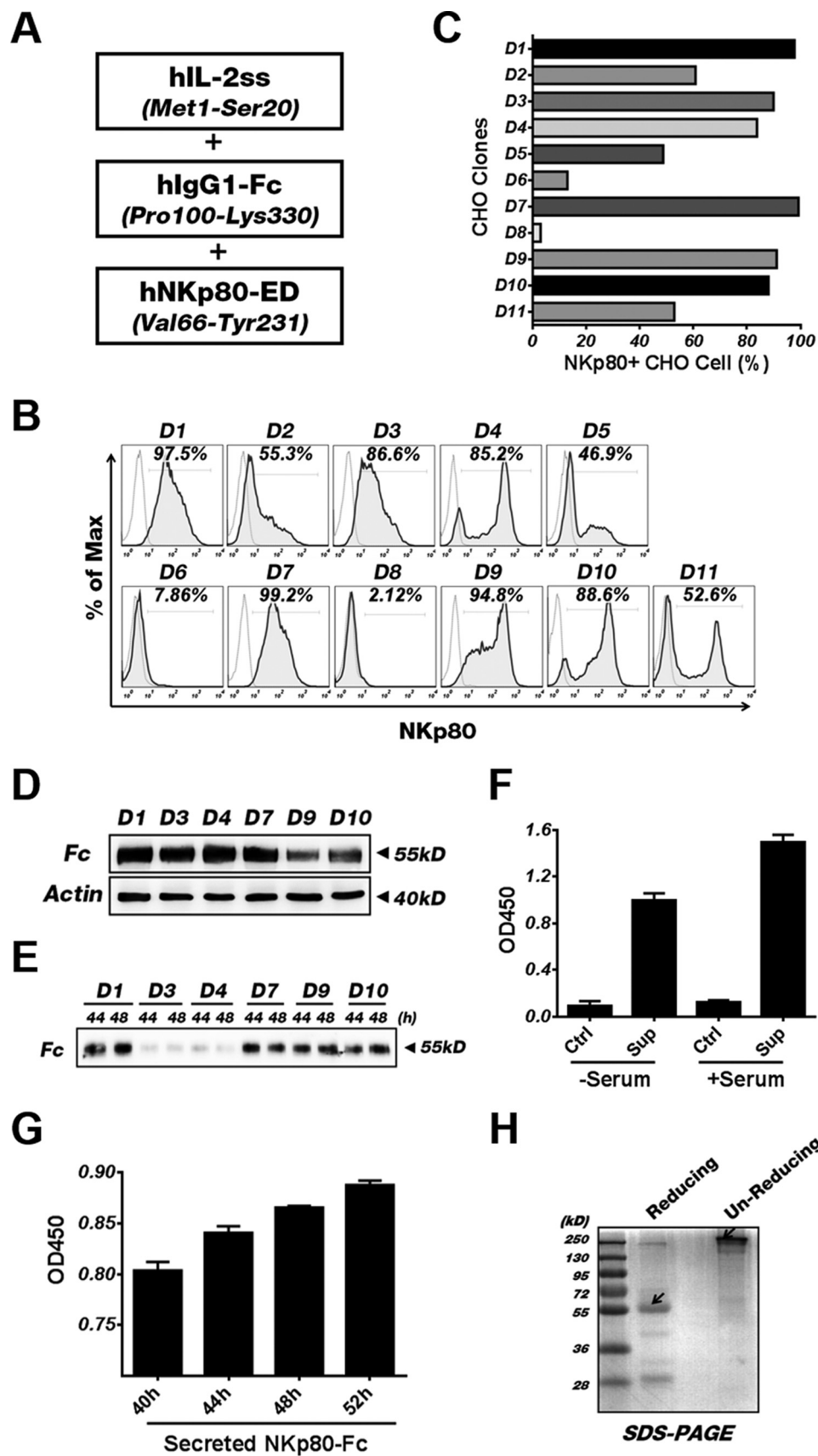
Expression and Purification of Recombinant NKp80-Fc Fusion Proteins—The NKp80 ligand, a potential therapeutic target, has been reported to be highly expressed by leukemia cells and solid tumors. Given the current lack of an available effective therapeutic antibody, we attempted to produce an NKp80-Fc fusion protein and explore its therapeutic efficacy against leukemia cells that highly express NKp80 ligand. On the basis of the secondary structure and functional domain analysis, the constant domain (Fc) from human IgG1 (Pro 100 -Lys 330) and the ectodomain from the NKp80 receptor (Val 66 -Tyr 231) was identified, amplified by PCR along with an additional N-terminal human IL-2 signal sequence (hIL-2ss) for the secretion of NKp80-Fc, and inserted into the pcDNA3.1 Hygro (+) vector (Fig. 1A). The recombinant plasmid hIL-2ss-hIgG1Fc-NKp80ED was stably transfected into CHO-K1 cells, and positive cells were selected with 700 $\mu\text{g}/\text{ml}$ hygromycin B. NKp80-Fc mRNA expression in the CHO-Fc-NKp80 transfectants was identified by RT-PCR. After a limiting dilution, positive clones were isolated and analyzed for NKp80 expression. Six clones (D1, D3, D4, D7, D9, and D10) highly expressing the intracellular NKp80 ectodomain were identified among the 11 positive clones by flow cytometry (NKp80 $^+$ cells > 80%; Fig. 1, B and C) and Western blotting (Fig. 1D). Furthermore, of these six clones, four (D1, D7, D9, and D10) were confirmed to efficiently secrete the Fc fusion protein by an anti-human IgG-Fc mAb. The recombinant fusion protein had a molecular mass of ~ 55 kDa under reducing conditions, which is consistent with its estimated monomer size (Fig. 1, D and E). To prevent contamination with massive bovine IgG in the following purification procedure, we chose a serum-free CD-CHO medium (Life Technologies) and the D1 clone for the following large-scale

culture. As shown in Fig. 1F, compared with the medium control, the fusion protein from the serum-free or serum-containing culture supernatant from the D1 clone reacted with the plate-bound antibody to NKp80 (anti-NKp80) and the anti-hIgG-Fc mAb in a sandwich ELISA assay, indicating successful expression of the recombinant human NKp80-Fc chimera in CHO-K1 cells. Moreover, the kinetic analysis showed that NKp80-Fc expression increased in 40–52 h (Fig. 1G), indicating that a 52-h, serum-free culture could be an optimal expression strategy for the fusion protein. The large-scale, serum-free culture supernatant from the D1 clone was purified using a protein A column and SDS-PAGE. The Western blot showed a purified fusion protein band of ~ 55 kDa under reducing conditions, which was consistent with the estimated monomer size (Fig. 1H).

NKp80-Fc Specifically Binds to AICL on Leukemia Cells—To assess the binding activity of the soluble NKp80-Fc fusion protein with its identified ligand, AICL, on leukemia cells, the AICL-positive leukemia cell lines U937 (a human histiocytic leukemia cell line) and THP-1 (a human monocytic leukemia cell line) and AICL-negative HeLa cells were pre-treated with excess human IgG to block Fc γ R (CD32 and CD64). The cell lines were then incubated with the soluble NKp80-Fc protein, which was followed by labeling with an anti-hIgG-Fc mAb. After ruling out the possibility of the hIgG1-Fc portion from NKp80-Fc binding with Fc γ R (CD32 and CD64) in the leukemia cell lines, the soluble NKp80-Fc fusion protein showed increased binding with U937 cells in a concentration-dependent manner compared with the hIgG control (Fig. 2A). Apparently, the protein purified from the serum-free supernatant showed more efficient binding activity than that from the serum-containing supernatant (Fig. 2B). However, HeLa cells could not bind the NKp80-Fc protein from the serum-free or serum-containing supernatants (Fig. 2C).

To further determine whether the NKp80-Fc protein binds to leukemia cells through the AICL ligand, a blocking assay on the basis of flow cytometry was performed. Using an AICL-specific rabbit polyclonal antibody and fluorescein-conjugated secondary anti-rabbit IgG, U937 and THP-1 both showed high AICL expression, but preincubation with the purified NKp80-Fc fusion protein markedly blocked the anti-AICL staining (Fig. 2, D and E). These results suggest that the NKp80-Fc fusion protein binds specifically to AICL on leukemia cells.

NKp80-Fc Potently Increases Cellular Conjugation between NK and Leukemia Cells—Binding of the humanized Fc portion to the Fc receptor (Fc γ R) can lead to phagocytosis or lysis of targeted cells via ADCC. To ascertain whether the humanized NKp80-Fc fusion protein could mediate a cytolytic function similar to ADCC, we analyzed the effect of the NKp80-Fc protein on NK cell-U937 target cell binding. Freshly purified NK cells from healthy donors were labeled with CD56 and mixed with CD33-labeled U937 cells for the indicated times (0–60 min) at 37 $^{\circ}\text{C}$ at an E:T ratio of 2:1. Next, the mixed cells were fixed, and the double-positive binding cells were analyzed by flow cytometry. Cellular conjugation was denoted as binding rate, which was calculated as the ratio of CD56 $^+$ CD33 $^+$ double-positive cells among CD56 $^+$ single-positive cells.



NKp80-Fc Redirects NK Cells against Leukemia Cells

As shown in Fig. 3A, the binding rate increased markedly during the 10- to 60-min incubation, peaking at 10 min (Fig. 3B). To further explore the role of NKp80-Fc in the formation of NK-U937 conjugates, U937 cells labeled with CD33 were pretreated with NKp80-Fc proteins from five purification batches in parallel groups (groups 1–5) at 37 °C. Then the CD33-labeled U937 cells were mixed with the CD56-labeled purified NK cells and incubated for 10 min, and the cellular conjugation assay was performed as described above. As described in Fig. 3, C and D, compared with the hIgG control group, the five parallel NKp80-Fc preincubation groups presented varying increases in binding rates (Fig. 3C). Statistical analysis of three independent experiments confirmed these results (Fig. 3D). Many ligand-receptor systems are known to participate in the cellular conjugation between NK and leukemia cells. Under normal conditions, the hIgG groups showed a relatively low conjugate ratio, partly because of the direct NKp80-AICL interaction. Notably, treatment with our construct significantly enhanced the cellular conjugation, not only by specifically targeting AICL on leukemia cells but also because of the highly increased affinity to CD16. Therefore, these results suggest that NKp80-Fc fusion proteins can potentially increase the cellular conjugation between NK and leukemia cells.

NKp80-Fc Promotes NK Cell-mediated Apoptosis of Leukemia Cells—Because we found that the highly increased affinity to Fc γ R resulted in increased conjugation, we explored whether the increased conjugation led to an increased killing effect. To further determine whether the purified NKp80-Fc fusion protein could mediate ADCC, we performed a FACS-based cytotoxicity assay to assess the impact of NKp80-Fc on NK cell cytotoxicity toward leukemia cells, as described under “Materials and Methods.” The frequency of Annexin V⁺ apoptotic U937 cells among the CD56⁺CD33⁺ U937 cells in the NKp80-Fc-treated and IgG-treated groups was analyzed statistically.

To rule out self-effects in the apoptosis assay, we assessed apoptotic U937 cells under normal conditions without NK cells. As shown in Fig. 4A, only a small portion (9.31%) of U937 cells presented spontaneous apoptosis. At the beginning stage of incubation with NK cells, the apoptotic cell proportion remained below 10% (9.61%). However, after 1.5 h of incubation, a significant increase was demonstrated in either IgG- or NKp80-Fc-treated cells (Fig. 4B). Of note is that, compared with IgG treatment (76.9%), U937 cell apoptosis was increased remarkably with NKp80-Fc treatment (85.3–93.0%). Statistical analysis confirmed these results (Fig. 4C).

To explore the apoptosis of CD56⁺CD33⁺ cells and conjugated CD56⁺CD33⁺ cells, we assessed the frequency of Annexin V⁺ apoptotic cells among the two populations. Similar to CD56⁺CD33⁺ cells, NKp80-Fc treatment also promoted apoptosis of the conjugated CD56⁺CD33⁺ cells (data not shown),

although the ratio of the conjugated CD56⁺CD33⁺ cells was very low (<5%) in NK/U937 incubation (Fig. 4D). However, the apoptotic proportion of CD56⁺CD33⁺ NK cells was <5% and had no change after NKp80-Fc treatment (data not shown), although the ratio of CD56⁺CD33⁺ NK cells was up to 50% (Fig. 4D). These results indicated that the apoptosis of leukemia cells was initiated at the stage of NK-leukemia cell conjugation. After being detached from one apoptotic cell, the NK cell could recognize and bind with another non-apoptotic leukemia cell and then induce its apoptosis. NKp80-Fc could enhance the NK-leukemia cell conjugation and increased NK cell-mediated apoptosis of leukemia cells.

To further definitively determine the impact of NKp80-Fc-induced ADCC on leukemia cells, we counted the remaining survived U937 cells after 4-h incubation of NK and U937 cells. As shown in Fig. 4E, compared with IgG treatment, NKp80-Fc treatment induced a significant reduction in the number of surviving U937 cells. Therefore, NKp80-Fc is capable of exerting ADCC through NK cells, thereby promoting the apoptosis of leukemia cells in NK-leukemia cell cross-talk.

NKp80-Fc Enhances NK Cell Degranulation after Stimulation with Leukemia Cells—To assess the role of NKp80-Fc in NK cell degranulation, CD107a expression on NK cells was analyzed by flow cytometry. The frequency of CD107a⁺ CD56⁺ cells among NK cells in the IgG-treated and NKp80-Fc-treated groups was analyzed. As shown in Fig. 5, A and B, compared with the IgG-treated group, the NKp80-Fc-treated groups showed a remarkable increase of CD107a⁺ degranulated NK cells (Fig. 5A). The histogram in Fig. 5B also confirmed these results. Similar to previous data, our constructs induced efficient degranulation activity toward leukemia cells. In theory, the soluble NKp80 protein lacking the Fc domain should reduce NK cell degranulation activity because of blocking of the NKp80-AICL interaction. However, our chimeric Fc fusion induced stronger NK activity because of its highly increased affinity to CD16. Therefore, the stronger induction of ADCC by Fc fusion overcomes the loss of activating signals via NKp80, which agrees with data on the hierarchically organized potential of NK receptor activation.

NKp80-Fc Enhances NK Cell Cytotoxicity against Leukemia Cells—To determine whether NKp80-Fc fusion proteins affect the NK cell effector function against leukemia cells, a classic 4-h ⁵¹Cr release cytotoxicity assay was conducted. NK cells were preincubated with NKp80-Fc fusion protein (SF/SC) or IgG at 37 °C for 45 min and mixed with the ⁵¹Cr-labeled U937 targets at ratios of 4:1 and 1:1. After 4 h of incubation, the supernatant of each group was collected and analyzed. As shown in Fig. 6, A and B, compared with the IgG-treated group, NK cells pretreated with NKp80-Fc purified from serum-free culture supernatant (NKp80-Fc (SF), Fig. 6A)

FIGURE 1. Construction, expression, and purification of recombinant NKp80-Fc fusion protein. A, schematic of the NKp80-Fc fusion protein. B, intracellular staining of NKp80-Fc protein expression in CHO-K1 colonies D1–D11 stably transfected with hIL-2ss-hlgG1Fc-NKp80ED. C, frequency of NKp80⁺ cells in stably transfected colonies D1–D11 as described in B. D, NKp80-Fc fusion protein in the whole cell lysate from the six clones highly expressing NKp80-Fc clones (D1, D3, D4, D7, D9, and D10) determined using the anti-human IgG-Fc mAb by Western blotting with β -actin as a control. E, the NKp80-Fc fusion protein in the supernatant from the six clones after 44 and 48 h of serum-free culture as determined by Western blotting. F, relative production of the NKp80-Fc chimera in serum-free or serum-containing culture supernatant from the D1 clone (Sup) and medium only (Ctrl) as determined by sandwich ELISA assay. The supernatant and control samples incubated with plate-bound antibody to NKp80 (anti-NKp80) were then reacted with the anti-human IgG-Fc mAb, and A₄₅₀ was analyzed. G, relative production of the secreted NKp80-Fc chimera in the supernatant from the D1 clone after 40–52 h of serum-free culture as determined by sandwich ELISA assay and as described in F. H, SDS-PAGE analysis of the purified NKp80-Fc protein from serum-free supernatant of the D1 clone under reducing and non-reducing conditions. The arrow indicates the purified fusion protein band of ~55 kDa under reducing conditions. Data were collected from three independent experiments and analyzed by Student's t test.

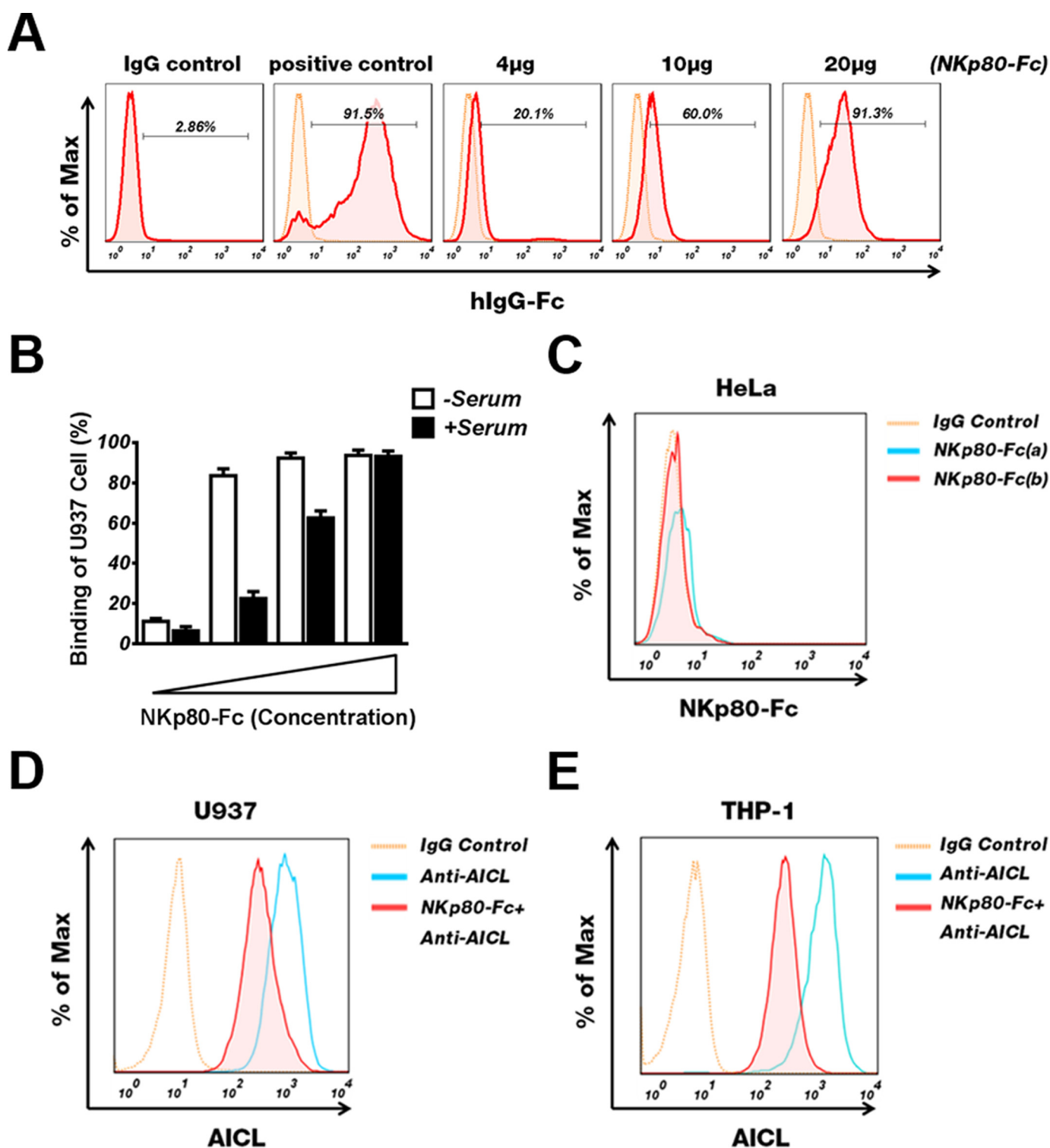


FIGURE 2. NKp80-Fc specifically binds to AICL on leukemia cells. *A*, flow cytometry analysis for the binding of purified NKp80-Fc protein to U937 cells with hlgG as a negative control and anti-CD33 as a positive control. *B*, frequency of NKp80-Fc⁺ cells among U937 cells labeled with NKp80-Fc purified from serum-free and serum-containing culture supernatant. *C*, flow cytometry analysis of HeLa cells stained with NKp80-Fc (a: serum-free; b: serum-containing) followed by a PE-conjugated anti-hlgG-Fc mAb. *D* and *E*, flow cytometry analysis for AICL staining of U937 (*D*) and THP-1 (*E*) cells after preincubation with purified NKp80-Fc. Data are representative of three independent experiments.

and NKp80-Fc purified from serum-containing culture supernatant (NKp80-Fc (SC), Fig. 6*B*) both showed potently enhanced target cell lysis activity. Additionally, similar to previous data, treatment with our chimeric Fc fusion induced pronounced NK-mediated lysis activity toward leukemia cells. In agreement with

the hierarchically organized potential of the various activating receptors governing NK reactivity, and on the basis of its highly increased affinity to CD16, the NKp80-Fc fusion protein potently enhances NK cell reactivity against leukemia cells through induction of the ADCC effect of NK cells.

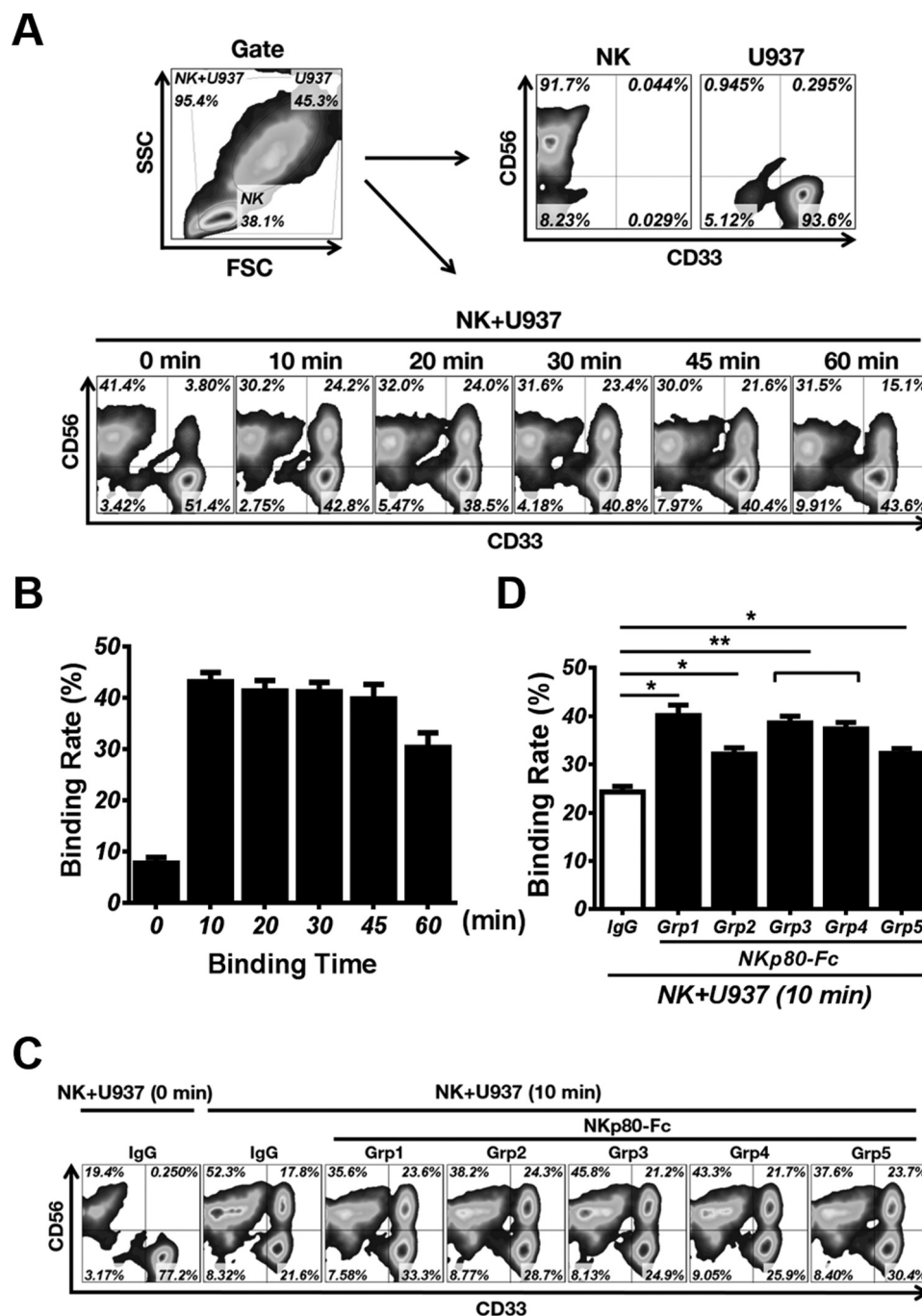


FIGURE 3. NKp80-Fc potentially increases cellular conjugation between NK and leukemia cells. *A*, representative flow cytometry analysis of the cellular conjugation between freshly purified NK cells and U937 cells. CD56-labeled NK cells and CD33-labeled U937 cells were mixed for the indicated times (0–60 min) at 37 °C at an E:T ratio of 2:1. Then the mixed cells were fixed and analyzed by flow cytometry. *FSC*, forward scatter; *SSC*, side scatter. *B*, NK-U937 cell conjugates at the indicated times (0–60 min) were determined by flow cytometry. Cellular conjugation was denoted as binding rate, which was calculated as the ratio of CD56⁺CD33⁺ double-positive cells among CD56⁺ single-positive cells. The data are representative of at least three independent experiments. *C*, NKp80-Fc potentially increases cellular conjugation between NK and U937 cells. U937 cells labeled with CD33 were pretreated with NKp80-Fc proteins from five purification batches in parallel groups (groups (Grp) 1–5) at 37 °C. Then CD33-labeled U937 cells were mixed with CD56-labeled purified NK cells and incubated for 10 min, and the cellular conjugation assay was performed as described above. *D*, NK-U937 cell conjugates denoted as binding rate at 10 min in five NKp80-Fc-pretreated groups. Data are representative of at least three independent experiments and were analyzed by Student's *t* test. *, *p* < 0.05; **, *p* < 0.01.

NKp80-Fc Amplifies NK Cell Anti-leukemia Effects in Vivo— The leukemia xenograft mouse model was used to assess whether NKp80-Fc could induce ADCC of NK cells and inhibit tumor progression. Irradiated NOD/SCID mice were injected subcutaneously with 2.0×10^6 U937 cells/mouse. After 3 days, about 2.0×10^6 freshly isolated human NK cells with NKp80-Fc protein or

human IgG were injected subcutaneously into the same tumor area. Mice were euthanized, and the tumors were isolated on day 26 after tumor inoculation. The volumes and weights of tumors were calculated. As shown in Fig. 7, the tumors in U937 xenograft mice without NK cell treatment grew rapidly within a couple of weeks. The volumes and weights of tumors were reduced after NK

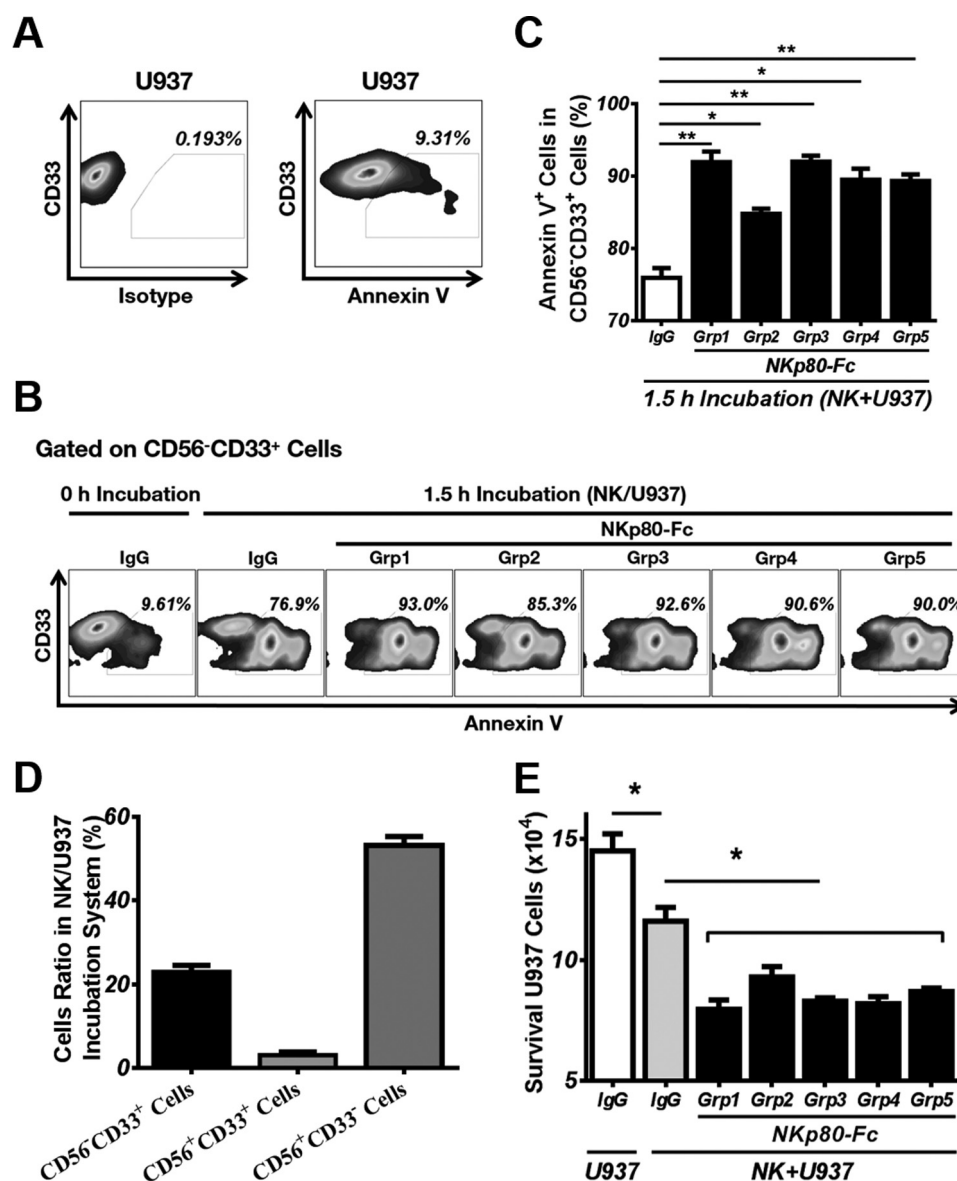


FIGURE 4. NKp80-Fc promotes NK cell-mediated apoptosis of leukemia cells. *A*, spontaneous apoptotic phenotype of U937 cells under normal conditions without NK cells. *B* and *C*, NKp80-Fc promotes NK cell-mediated apoptosis of U937 cells after 1.5 h of incubation. CD56-labeled, freshly purified NK cells from healthy donors were pretreated with control IgG or NKp80-Fc proteins from five purification batches in parallel groups (groups 1–5). Then the NK cells were mixed with CD33-labeled U937 cells at an E:T ratio of 2:1 and incubated at 37 °C for 0 or 1.5 h. After the incubation, FITC-Annexin V was added at 10 min to assess apoptosis of the target U937 cells. Then the frequency of AnnexinV⁺ apoptotic U937 cells among CD56⁺CD33⁺ U937 cells in the NKp80-Fc-treated and IgG-treated groups was analyzed by flow cytometry. *D*, the ratio of the three populations (CD56⁺CD33⁺, CD56⁺CD33⁺, and CD56⁺CD33⁺ cells) in NK/U937 cell incubation after the apoptosis assay. *E*, the remaining surviving U937 cells were counted and analyzed statistically after 4-h incubation of NK and U937 cells. Data are representative of at least three independent experiments and were analyzed by Student's *t* test. *, *p* < 0.05; **, *p* < 0.01.

cell treatment, indicating that NK cells could inhibit tumor progression (Fig. 7, *B* and *C*). Importantly, compared with NK- or NK-hIgG-treated mice, there was a significant reduction of tumor volume (Fig. 7*B*) and weight (Fig. 7*C*) in NK-NKp80-Fc-treated mice. These results indicated that NKp80-Fc enhanced the sensitivity of U937 tumors to NK cells *in vivo*.

Discussion

The activity of both tumor and immune effector cells, including NK cells, is influenced substantially by various members of the C-type lectin-like receptors, including NKG2D and NKp80 and its ligand AICL. AICL is expressed

preferentially on the surface of myeloid cells, especially on malignant leukemia cells, but not on nonmyeloid hematopoietic cells or nonhematopoietic cells (8). AICL expression increases the susceptibility of myeloid cells to NK cell-mediated cytotoxicity (7). However, so far there are no available therapeutic antibodies specifically targeting AICL for NK cell therapy against malignant leukemia cells.

Several studies conducted on NK cells from human leukemia samples and solid tumors indicate that their phenotype and function are altered greatly (14, 15). For example, NK cells present in the microenvironment of non-small cell lung carcinoma display a strongly altered phenotype with decreased expression of NKp30,

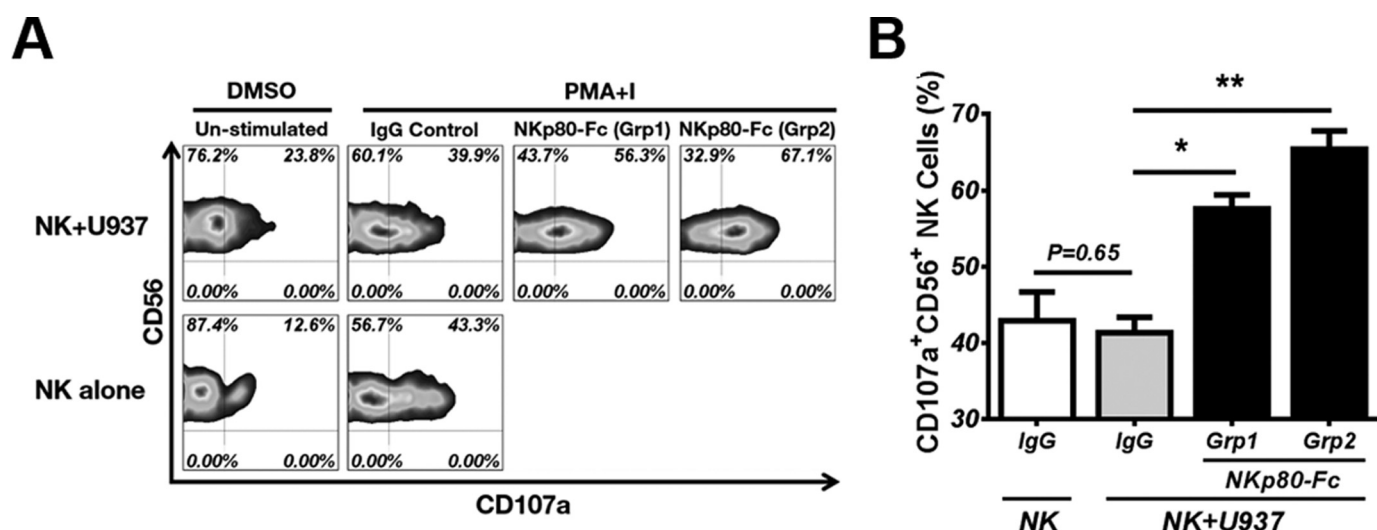


FIGURE 5. NKp80-Fc enhances NK cell degranulation after stimulation with leukemia cells. A, representative flow cytometry analysis of NK degranulation toward U937 cells in the presence of NKp80-Fc. Freshly purified NK cells from healthy human donors were pretreated with NKp80-Fc proteins from two purification batches in parallel groups (groups (Grp) 1 and 2) or control human IgG and were then mixed with U937 cells at an E:T ratio of 2:1 or incubated alone in the presence of FITC-CD107a antibody and stimulated with PMA/ion at 37 °C for 4 h. The frequency of CD107a⁺CD56⁺ cells among CD56⁺ NK cells in the IgG-treated and NKp80-Fc-treated groups was analyzed by flow cytometry. DMSO, dimethyl sulfoxide. B, the frequency of CD107a⁺CD56⁺ cells among NK cells in the IgG-treated and NKp80-Fc-treated groups was analyzed for statistical significance. Data are representative of at least three independent experiments and were analyzed by Student's *t* test. *, *p* < 0.05; **, *p* < 0.01.

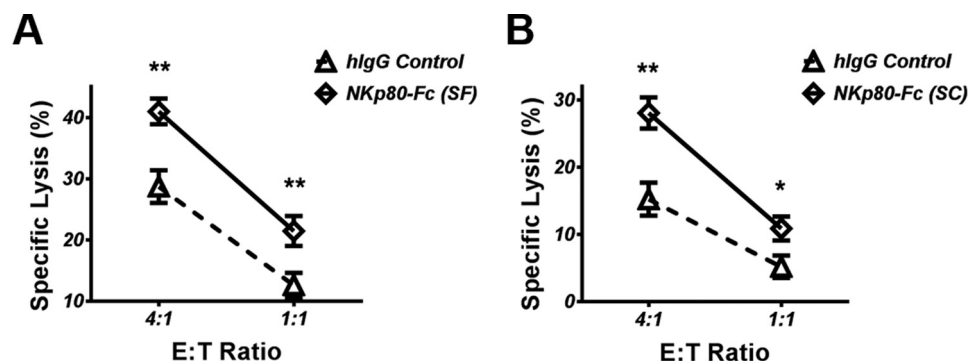


FIGURE 6. NKp80-Fc enhances NK cell cytotoxicity against leukemia cells. A and B, cytotoxicity assay of NK cells against leukemia U937 cells in the presence of NKp80-Fc purified from serum-free culture supernatant (NKp80-Fc (SF), A) or NKp80-Fc purified from serum-containing culture supernatant (NKp80-Fc (SC), B) during a classic 4-h ⁵¹Cr-release assay. NK cells were preincubated with NKp80-Fc fusion protein (SF/SC) or IgG at 37 °C for 45 min and mixed with the ⁵¹Cr-labeled U937 targets at ratios of 4:1 and 1:1. After 4 h of incubation, the supernatants were collected, and the released ⁵¹Cr was counted using a γ counter. Data were collected from three independent experiments and analyzed by Student's *t* test. *, *p* < 0.05; **, *p* < 0.01.

NKp80, DNAM-1, CD16, and ILT2 and impaired cytotoxic functions (1, 16). The possible mechanisms underlying these defects remain unclear. As a triggering receptor on NK cells, NKp80 may be down-regulated in leukemia patients, resulting in impaired anti-leukemia reactivity through reduced NKp80-AICL interaction (17). However, with a potent effect on NK cell reactivity, CD16 provides an effective means for leukemia therapy through NK cell ADCC, which constitutes an effector mechanism by which NKp80-Fc contributes to anti-tumor immunity.

To improve this situation, we developed a therapeutic strategy that, to our knowledge for the first time, takes advantage of the tumor-restricted expression of AICL by using it as a target for the induction of NK cell ADCC. To this end, we generated fusion proteins containing the extracellular domain of NKp80 and a humanized IgG Fc portion for induction of ADCC by NK cells. These proteins are capable of targeting leukemia cells through the NKp80-AICL interaction and, at the same time, potentially inducing NK cell ADCC against AICL-expressing leukemia cells.

We utilized this approach for several reasons. AICL is selectively overexpressed by malignant leukemia cells, for which no immunotherapeutic antibodies are available. In contrast to a specific mAb that would recognize only AICL, our NKp80-Fc binds all of the NKp80 ligands containing AICL and other potential, so far undiscovered ligands. Another advantage is that ADCC has been recognized as a major mechanism by which antitumor antibodies and humanized Fc fusion proteins mediate their effects, and strategies to fuse the NK cell receptor with the Fc domain to recruit Fc receptor-bearing immune cells are clinically efficacious (18). Most importantly, the inevitable reduction of activating signals mediated by NKp80 caused by the binding of our construct to AICL should be compensated for by the potential of CD16 to stimulate NK cells. Last, but not least, this approach avoids the impairment of anti-leukemia reactivity through the direct NKp80-AICL interaction resulting from decreased expression of NKp80 in leukemia patients. Therefore, CD16 provides an effective means for leukemia therapy by mediating NK cell ADCC.

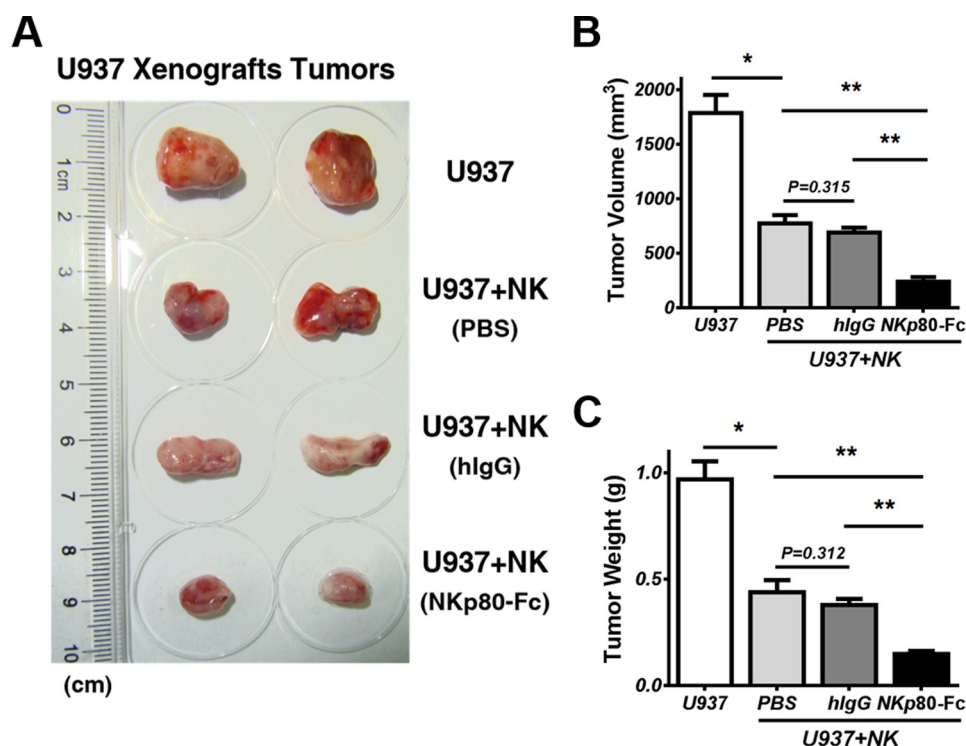


FIGURE 7. NKp80-Fc amplifies NK cell anti-leukemia effects *in vivo*. A, NKp80-Fc increases NK cell lysis against U937 xenograft tumors in NOD/SCID mice. Female 6- to 8-week-old irradiated NOD/SCID mice were inoculated subcutaneously with 2.0×10^6 U937 cells/mouse. After 3 days, NKp80-Fc or human IgG was mixed with 2.0×10^6 freshly isolated human NK cells in a final concentration of 0.1 mg/ml. Then the mixture was injected subcutaneously into the same tumor area. The mice were euthanized, and the tumors were isolated from the mice on day 26 after tumor inoculation. Images of the tumors are shown. B and C, the volumes (B) and weights (C) of the tumors were calculated. Data are representative of at least three independent experiments and were analyzed by Student's *t* test. *, $p < 0.05$; **, $p < 0.01$.

In our study, our constructed fusion proteins specifically bound to leukemia cells and significantly increased NK target cell conjugation. In the functional analyses of NK and leukemia cells, the treatment with NKp80-Fc clearly induced the NK cell ADCC effect. The fusion proteins not only promoted NK-mediated target cell apoptosis in the early stage of cell conjugation but also enhanced NK cell degranulation and cytotoxicity activity in a target antigen-dependent manner in the relatively late stage. More importantly, NKp80-Fc potently amplifies NK cell anti-leukemia effects *in vitro* and *in vivo* through induction of the NK cell ADCC effect. In theory, the soluble NKp80 protein lacking the Fc domain should reduce NK cell degranulation activity by blocking the activating signals via the NKp80 receptor through direct NKp80-AICL interaction. However, our chimeric Fc fusion induced more pronounced NK-mediated degranulation and lysis activity toward leukemia cells through potent ADCC because of its highly increased affinity to CD16. Therefore, the strong induction of ADCC by the NKp80-Fc fusion protein overcomes the loss of activating signals via NKp80, which agrees with previous data on the hierarchically organized potential of activating NK receptors (19–21).

The induction of NK reactivity by the fusion protein was strictly dependent on the expression of AICL on leukemia cells, thereby confirming that our constructs stimulate NK reactivity in a highly targeted, antigen-restricted manner. With regard to clinical application, it is necessary to consider that expression of AICL may not be restricted to malignant cells. Previous reports have demonstrated that nonmalignant myeloid cells such as monocytes have low expression of AICL and DNAM-1

ligands and are mostly resistant to NK cell-mediated cytotoxicity, in contrast to leukemia cell lines such as U937 and THP-1 (7, 8). The engagement of AICL and NKp80 increases the susceptibility of myeloid cells to NK cell-mediated cytotoxicity. However, although malignant cells are lysed strongly by NK cells, antitumor LPS-activated monocytes showed greatly decreased or even absent NK cell-mediated cytotoxicity (7). Therefore, the potential side effects of the administration of NKp80-Fc fusion proteins to patients would likely be low. Although further work is required before patients with leukemia can be treated with NKp80-Fc, we provide evidence that this method could potentially be useful in further research on molecular targeted therapy, and our data clearly indicate that NKp80-Fc fusion proteins capable of specifically targeting AICL while at the same time potently inducing the ADCC effect *in vitro* and *in vivo* may be promising agents for the immunotherapy of leukemia.

Author Contributions—G. D., X. Z., Z. T., and R. S. conceived and designed the experiments. G. D. and J. Z. performed the experiments. G. D., X. Z., and R. S. analyzed the data. H. W. contributed reagents, materials, and analysis tools. G. D., X. Z., Z. T., and R. S. wrote the manuscript. All authors analyzed the results and approved the final version of the manuscript.

References

- Cremer, I., Fridman, W. H., and Sautès-Fridman, C. (2012) Tumor microenvironment in NSCLC suppresses NK cells function. *Oncoimmunology* 1, 244–246

2. Vivier, E., Raulet, D. H., Moretta, A., Caligiuri, M. A., Zitvogel, L., Lanier, L. L., Yokoyama, W. M., and Ugolini, S. (2011) Innate or adaptive immunity? The example of natural killer cells. *Science* **331**, 44–49
3. Lanier, L. L. (2005) NK cell recognition. *Annu. Rev. Immunol.* **23**, 225–274
4. Moretta, L., and Moretta, A. (2004) Unravelling natural killer cell function: triggering and inhibitory human NK receptors. *EMBO J.* **23**, 255–259
5. Vitale, M., Falco, M., Castriconi, R., Parolini, S., Zambello, R., Semenzato, G., Biassoni, R., Bottino, C., Moretta, L., and Moretta, A. (2001) Identification of NKp80, a novel triggering molecule expressed by human NK cells. *Eur. J. Immunol.* **31**, 233–242
6. Yokoyama, W. M., and Plougastel, B. F. (2003) Immune functions encoded by the natural killer gene complex. *Nat. Rev. Immunol.* **3**, 304–316
7. Welte, S., Kuttruff, S., Waldhauer, I., and Steinle, A. (2006) Mutual activation of natural killer cells and monocytes mediated by NKp80-AICL interaction. *Nat. Immunol.* **7**, 1334–1342
8. Akatsuka, A., Ito, M., Yamauchi, C., Ochiai, A., Yamamoto, K., and Matsumoto, N. (2010) Tumor cells of non-hematopoietic and hematopoietic origins express activation-induced C-type lectin, the ligand for killer cell lectin-like receptor F1. *Int. Immunol.* **22**, 783–790
9. Arteaga, C. L., Sliwkowski, M. X., Osborne, C. K., Perez, E. A., Puglisi, F., and Gianni, L. (2012) Treatment of HER2-positive breast cancer: current status and future perspectives. *Nat. Rev. Clin. Oncol.* **9**, 16–32
10. Keating, G. M. (2010) Rituximab: a review of its use in chronic lymphocytic leukaemia, low-grade or follicular lymphoma and diffuse large B-cell lymphoma. *Drugs* **70**, 1445–1476
11. Beck, A., Wurch, T., Bailly, C., and Corvaia, N. (2010) Strategies and challenges for the next generation of therapeutic antibodies. *Nat. Rev. Immunol.* **10**, 345–352
12. Lazar, G. A., Dang, W., Karki, S., Vafa, O., Peng, J. S., Hyun, L., Chan, C., Chung, H. S., Eivazi, A., Yoder, S. C., Vielmetter, J., Carmichael, D. F., Hayes, R. J., and Dahiyat, B. I. (2006) Engineered antibody Fc variants with enhanced effector function. *Proc. Natl. Acad. Sci. U.S.A.* **103**, 4005–4010
13. Feldman, E. J., Brandwein, J., Stone, R., Kalaycio, M., Moore, J., O'Connor, J., Wedel, N., Roboz, G. J., Miller, C., Chopra, R., Jurcic, J. C., Brown, R., Ehmann, W. C., Schulman, P., Frankel, S. R., De Angelo, D., and Scheinberg, D. (2005) Phase III randomized multicenter study of a humanized anti-CD33 monoclonal antibody, lintuzumab, in combination with chemotherapy, versus chemotherapy alone in patients with refractory or first-relapsed acute myeloid leukemia. *J. Clin. Oncol.* **23**, 4110–4116
14. Carlsten, M., Norell, H., Bryceson, Y. T., Poschke, I., Schedvins, K., Ljunggren, H. G., Kiessling, R., and Malmberg, K. J. (2009) Primary human tumor cells expressing CD155 impair tumor targeting by down-regulating DNAM-1 on NK cells. *J. Immunol.* **183**, 4921–4930
15. Schleypen, J. S., Baur, N., Kammerer, R., Nelson, P. J., Rohrmann, K., Gröne, E. F., Hohenfellner, M., Haferkamp, A., Pohla, H., Schendel, D. J., Falk, C. S., and Noessner, E. (2006) Cytotoxic markers and frequency predict functional capacity of natural killer cells infiltrating renal cell carcinoma. *Clin. Cancer Res.* **12**, 718–725
16. Delahaye, N. F., Rusakiewicz, S., Martins, I., Ménard, C., Roux, S., Lyonnet, L., Paul, P., Sarabi, M., Chaput, N., Semeraro, M., Minard-Colin, V., Poirier-Colame, V., Chaba, K., Flament, C., Baud, V., Authier, H., Kerdine-Römer, S., Pallardy, M., Cremer, I., Peaudecerf, L., Rocha, B., Valteau-Couanet, D., Gutierrez, J. C., Nunès, J. A., Commo, F., Bonvalot, S., Ibrahim, N., Terrier, P., Opolon, P., Bottino, C., Moretta, A., Tavernier, J., Rihet, P., Coindre, J. M., Blay, J. Y., Isambert, N., Emile, J. F., Vivier, E., Lecesne, A., Kroemer, G., and Zitvogel, L. (2011) Alternatively spliced NKp30 isoforms affect the prognosis of gastrointestinal stromal tumors. *Nat. Med.* **17**, 700–707
17. Marras, F., Bozzano, F., and De Maria, A. (2011) Involvement of activating NK cell receptors and their modulation in pathogen immunity. *J. Biomed. Biotechnol.* **2011**, 152430
18. Owen, C., and Stewart, D. A. (2012) Obinutuzumab for the treatment of lymphoproliferative disorders. *Expert Opin. Biol. Ther.* **12**, 343–351
19. Bryceson, Y. T., Ljunggren, H. G., and Long, E. O. (2009) Minimal requirement for induction of natural cytotoxicity and intersection of activation signals by inhibitory receptors. *Blood* **114**, 2657–2666
20. Bryceson, Y. T., March, M. E., Ljunggren, H. G., and Long, E. O. (2006) Synergy among receptors on resting NK cells for the activation of natural cytotoxicity and cytokine secretion. *Blood* **107**, 159–166
21. Steinbacher, J., Baltz-Gharemanpour, K., Schmiedel, B. J., Steinle, A., Jung, G., Kübler, A., André, M. C., Grosse-Hovest, L., and Salih, H. R. (2015) An Fc-optimized NKG2D-immunoglobulin G fusion protein for induction of natural killer cell reactivity against leukemia. *Int. J. Cancer* **136**, 1073–1084

Immunology:

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