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Preparation and functional identification of a monoclonal antibody against the recombinant soluble human NKp30 receptor

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ABSTRACT

NKp30 is an important activating receptor of human natural killer (NK) cells that participates in NK cell activation and cytotoxicity against tumor and infected cells. To study the function of NKp30, anti-human NKp30 monoclonal antibody was prepared. The human NKp30 ectodomain (rhNKp30) was expressed in *Escherichia coli* as inclusion bodies and refolded using the dilution method. The refolded rhNKp30 was purified by immobilized metal affinity chromatography. The activity of soluble rhNKp30 was confirmed by flow cytometry and NK cytotoxicity assays. Four hybridoma cell lines producing monoclonal antibodies against rhNKp30 were obtained. One of the monoclonal antibodies, designated as "3G5", was highly specific and could be used in western blotting, immunoprecipitation, ELISA, and flow cytometry assays. The preparation of soluble rhNKp30 and a monoclonal antibody against NKp30 may provide useful tools for further functional studies of human NKp30.

1. Introduction

Natural killer (NK) cells are important effectors of the human innate immune system [1] that play a major role in host defense, including the control of infectious pathogens and the killing of neoplastic cells [2]. NK cell function is dependent upon different cell-surface receptors, which, through interactions with their ligands on target cells, control cytotoxicity [3]. NK cells have both inhibitory and activating receptors, which discriminate between normal and HLA class-deficient cells. As a result, autologous normal cells can protect themselves against NK cell cytotoxicity [3]. Conversely, NK-mediated cytotoxicity against virally infected cells or tumor cells is dependent on non-HLA-restricted activating receptors. The major activating receptors are NKG2D, a member of the lectin subfamily, and the natural cytotoxicity receptors (NCRs), which belong to the immunoglobulin superfamily [4,5]. Of the NCRs, NKp46, NKp44 and NKp30 were recently discovered [6–8]. These NK surface receptors cooperatively contribute to NK cell activation and cytotoxicity against target cells [4].

NKp30 is a 30-kDa glycoprotein characterized by a signal peptide (18 amino acids) and an extracelluar, IgV-like domain containing region of 120 amino acids that is selectively expressed by resting and activated NK cells. NKp30 signal transduction is mediated by its association with the immunoreceptor tyrosine-based activation motif (ITAM)-bearing protein CD3ξ, which, upon receptor-engagement, becomes tyrosine-phosphorylated [8]. Similar to NKp46 and NKp44, NKp30 plays an important role in NK cell cytotoxicity [8]. Furthermore, recently accumulating evidence indicates that NKp30 is involved in immune regulation through crosstalk between NK cells and dendritic cells (DC) [9–11].

Activated NKp30 has been shown to interact with heparan sulfate as a co-receptor [12–14]. The HCMV tegument protein pp65 was the first identified ligand of NKp30. However, pp65 inhibits NKp30 activation by causing the dissociation of the adaptor molecule CD3ξ from NKp30 [15]. Subsequently, human leukocyte antigen-B-associated transcript 3 (BAT3) was identified as an activating ligand of NKp30. However, BAT3 is a nuclear protein that is released from tumor cells or DC cells upon heat shock treatment [16]. Recently, the B7 family member B7-H6 was reported to be an activating ligand of NKp30 on tumor cells, and B7-H6-expressing tumor cells were shown to strongly induce NKp30-dependent cell activation and cytotoxicity of NK cells [17].

In this study, we successfully cloned and expressed the human NKp30 ectodomain in *Escherichia coli (E. coli)*. Then, we refolded the rhNKp30 protein using the dilution procedure and purified the refolded rhNKp30 protein using Ni^{2+}-affinity chromatography. Furthermore, we tested the activity of the refolded rhNKp30 by flow cytometry and neutralization of NK cell cytotoxicity. Using soluble rhNKp30 as an antigen, we vaccinated mice and produced four hybridoma cell lines to generate antibodies against rhNKp30. One of the four mAbs that was generated against NKp30, named 3G5, was selected for further study by virtue of its good characteristics and was shown to be functional in western blotting, immunoprecipitation, ELISA and flow cytometry assays.

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2. Materials and methods

2.1. Antibodies

Mouse anti-His-tag mAb (clone 2A8) was from Abmart. Alexa Fluor® 488 mouse anti-human CD56 (clone B159), PE-Cy5 mouse anti-human CD3 (clone UCHT1) and PE-mouse-anti-human NKp30 (clone p30-15) were purchased from BD PharMingen. PE-goat anti-mouse IgG (PE-GaM) and purified anti-NKp30 mAb (clone y4E6) were from Santa Cruz Biotechnology, Inc. Purified mIgG1κ and anti-NKp30 mAb (clone P30-15) were from Biolegend. HRP-conjugated goat anti-mouse IgG secondary antibody was from Wuhan Boster Biological Technology, LTD.

2.2. Bacteria strains and cell lines

The *E. coli* strains DH5α and Rosetta (DE3) and the cell lines NK-92, Hela, HEK293, HL-60, Raji, BA/F3 and SP2/0 were stored in our laboratory. DH5α cells were grown in LB medium. Rosetta (DE3) cells were grown in LB medium supplemented with 34 μg/ml chloramphenicol. *E. coli* culture was performed on a rotary shaker at 37 °C. NK-92 cells were cultured in α-MEM (Gibco) containing 12.5% heat-inactivated fetal bovine serum (FBS, Gibco), 12.5% equine serum (HyClone), 2 mM L-glutamate, 0.1 mM 2-mercaptoethanol, and supplemented with 100 U/ml rhIL-2 (Changchun Institute of Biological Products, China). Hela, HEK293 and BA/F3 cells were cultured in Dulbecco's modified Eagle's medium (Gibco) supplemented with 10% FBS and 2 mM L-glutamate. HL-60, Raji and SP2/0 cells were cultured in RPMI-1640 medium supplemented with 10% FBS and 2 mM L-glutamate. Cell culture was performed at 37 °C in a 5% CO2 humidified atmosphere.

2.3. Construction of recombinant vectors

The cDNA (570 bp) encoding full-length human NKp30 (GenBank accession no. AJ223153) was amplified by RT-PCR from total RNA from NK-92 cells and cloned into the pMD18-T simple vector (Takara). Using the pMD18-T-NKp30 construct as the template, the fragment encoding the NKp30 ectodomain (residues 19–130) was amplified using the following primers: 5′-GGAATTCCATATGCATCATCATCATCATCATCATCATCTCTGGGTGTCCCAGCCCCC-3′ (Nde I, 8×His codons, forward) and 5′-CCGCTCGAGTTACTATCATTCTTTCTCCACCACCAGCCGAGTCCCATTCCCTGTCCC-3′ (Xho I, three stop codons, reverse). The PCR fragment was subcloned into the Nde I and Xho I (Ferments) restriction sites of the pET-22b(+) vector (Novagen) to generate the recombinant vector pET-22b(+)-8His-NKp30. The inserted nucleotide sequence was confirmed by DNA sequencing (ABI PRISM 377 DNA sequencer) by the Shanghai Sangon Company.

2.4. Expression, refolding, purification and identification of the recombinant protein

The recombinant plasmid pET-22b(+)-8His-NKp30 was transformed into Rosetta (DE3) competent cells. Single bacterial colonies were grown in medium until the OD_{600nm} reached 0.6 and then induced with 1 mM isopropy-β-D-thiogalactoside (IPTG) for 6 h at 37 °C. Bacteria were harvested and disrupted with high-pressure homogenization, and then inclusion bodies were separated by centrifugation. The inclusion bodies were washed three times in wash buffer (50 mM Tris–HCl, 500 mM NaCl, 2 M urea, 1% TritonX-100, 5 mM EDTA, pH 8.5) and resolved in 8 M urea. The inclusion bodies were then refolded by a dilution procedure as previously described [18–21]. The supernatant was slowly diluted in refolding buffer (50 mM Tris–HCl, 100 mM NaCl, pH 9.0, 0.5 M L-arginine, 3 mM reduced glutathione and 0.3 mM oxidized glutathione) and left for 48 h. The refolded protein was concentrated using the Labscale TEF system (Millipore), dialyzed overnight against 50 mM Tris–HCl, 100 mM NaCl, pH 9.0 and purified by immobilized metal affinity chromatography using nickel-nitrilotriacetic acid agarose (Qiagen). The samples were then concentrated by ultrafiltration. The protein concentration was measured using the Bio-Rad protein assay (BIO-RAD).

2.5. Preparation of murine monoclonal antibodies against NKp30

The procedures for the preparation of mAbs were performed as in a previous report [22]. Briefly, 8–10 week-old BALB/c female mice were immunized 5 times over the course of two weeks with refolded rhNKp30. For the first immunization, 40–60 μg of rhNKp30 and an equal volume of complete Freund's adjuvant were injected intraperitoneally. For subsequent immunizations, 40–60 μg rhNKp30 was injected intraperitoneally with an equal volume of incomplete Freund's adjuvant. Serum antibody titers were measured by indirect enzyme-linked immunosorbent assay (ELISA). After a final booster with 40–60 μg rhNKp30 (without any adjuvant), the mice were killed, and splenocytes were collected. Then, the splenocytes were fused with SP2/0 myeloma cells using the PEG method, and hybridoma cells were selected in HAT medium (RPMI1640 supplemented with 20% FBS, 10 mM sodium hypoxanthanine, 40 mM aminopterin and 1.6 mM thymidine). Positive clones were confirmed by indirect ELISA. Positive hybridoma cell lines were obtained after three cycles of subcloning.

To generate anti-NKp30 monoclonal antibodies, hybridoma cells were injected intraperitoneally into liquid paraffin-primed female BALB/c mice (8–10 weeks old) with approximately $1–2\times10^6$ cells per mouse. Approximately 6–8 days later, ascites were collected, and the antibodies were purified by Protein G affinity chromatography (GE Healthcare). Monoclonal antibody isotypes were determined using the IsoQuick (TM) kit for mouse monoclonal isotyping (Sigma-Aldrich).

2.6. ELISA

Antibody titers were determined by indirect enzyme-linked immunosorbent assay (ELISA) as previously described [23]. Briefly, 100 μl of rhNKp30 (2 μg/ml) was added to microtiter plates, and the plates were incubated overnight at 4 °C. After washing 3 times with PBST (PBS plus 0.05% Tween 20), the plates were blocked with 3% BSA in PBST for 2 h at 37 °C. Different dilutions of serum from immunized mice, cell culture supernatant or ascites were added to the plates for 2 h at 37 °C. The plates were then incubated with HRP-conjugated anti-mouse antibody for 1 h and with TMB as substrate for 15–20 min. The colorimetric signal was measured at OD_{450nm} and OD_{630nm}. The specification and affinity constant of the monoclonal antibodies were determined by competitive ELISA as previously described [24,25].

2.7. Cell isolation and flow cytometry

Fresh human peripheral blood mononuclear cells (PBMCs) were isolated from blood using Ficoll-Paque according to the manufacturer's instructions (Solarbio, China). Human NK cells were purified from PBMCs using an NK cell isolation kit (Miltenyi Biotech), and the purity of NK cells reached 95%. IL-2 activated NK cells were cultured as previously described [26].

Cells were washed twice with cold PBS, blocked and incubated with the appropriate concentrations of recombinant proteins or Abs for 30 min at 4 °C. Cells were washed 4 times after incubation, and then analyzed using a FACSCalibur cytometer (Becton Dickinson).

2.8. Cytotoxicity assay

The cytotoxicity of NK-92 cells and IL-2 activated NK cells against target cells was measured using a standard 4-hour ^{51}Cr release assay, as previously described [27]. Samples of 1×10^6 target cells were labeled with 200 μCi sodium [^{51}Cr] chromate (PerkinElmer) for 1 h at

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37 °C and then washed 3–4 times with PBS. Labeled target cells (1×10^4cells/100 μl/well) blocked with BSA or rhNKp30 were incubated with different concentration of protein for 1 h at 37 °C in a 96-well U-bottom plate, and then varying amounts of NK-92 cells were added. The varying numbers of NK-92 cells or IL-2 activated NK cells blocked with the mAb were preincubated with the indicated concentration of anti-NKp30 (clone P30-15) or 3G5 mAb for 1 h at 37 °C before addition of target cells. Maximum ^{51}Cr release was determined by incubation of target cells with 2% Triton X-100. To measure spontaneous ^{51}Cr release, target cells were incubated without effector cells in assay medium alone. All experiments were performed in triplicate. Plates were briefly spun and incubated at 37 °C for 4 h. After 4 h, cells were pelleted, and the amount of ^{51}Cr release in 100 μl of supernatant was counted using a gamma counter. The percentage of specific ^{51}Cr release was calculated from the formula ([experimental release-spontaneous release]/[maximum release-spontaneous release])×100%. Spontaneous release did not exceed 10% of the maximum release in all experiments.

2.9. SDS-PAGE and western blotting

Bacterial extracts or lysates of cells were separated by SDS-PAGE electrophoresis under reducing or non-reducing conditions on a 12% polyacrylamide gel and then transferred onto PVDF membranes (Millipore) by electroblotting in vertical buffer tanks. The membranes were blocked with 5% non-fat milk in TBST buffer (50 mM Tris ± HCl, pH 7.4, 0.9% NaCl, and 0.1% Tween 20) and then incubated with primary antibodies for 2–3 h at room temperature or overnight at 4 °C. After HRP-conjugated secondary antibodies were added for 1 h, bands on the membranes were detected with the enhanced chemiluminescent system (Pierce).

2.10. Immunoprecipitation

Cells were collected, washed in ice-cold PBS, and resolved in lysis buffer containing 1% Triton X-100, 25 mM Tris–HCl, pH 7.5, 10 mM $MgCl_2$, 100 mM NaCl, 10 mM NaF, 1 mM PMSF (Sigma), 2 mM EDTA, and protease inhibitor cocktail (Roche) for 30 min on ice with a 10-min interval of vortexing for 30 sec. The cell lysates were precleared with protein A/G plus agarose (Santa Cruz) at 4 °C for 4 h. The supernatant was decanted into a fresh tube with 20 μg of 3G5 mAb or mouse IgG1κ and incubated at 4 °C for 2 h. Then, protein A/G plus agarose and 5% BSA were added, and samples were incubated on ice for 2 h. The protein-bound beads were washed three times with cell lysis buffer and resuspended in PBS. Then, the suspension was mixed with 2× SDS-loading buffer, boiled and centrifuged to remove the pellet.

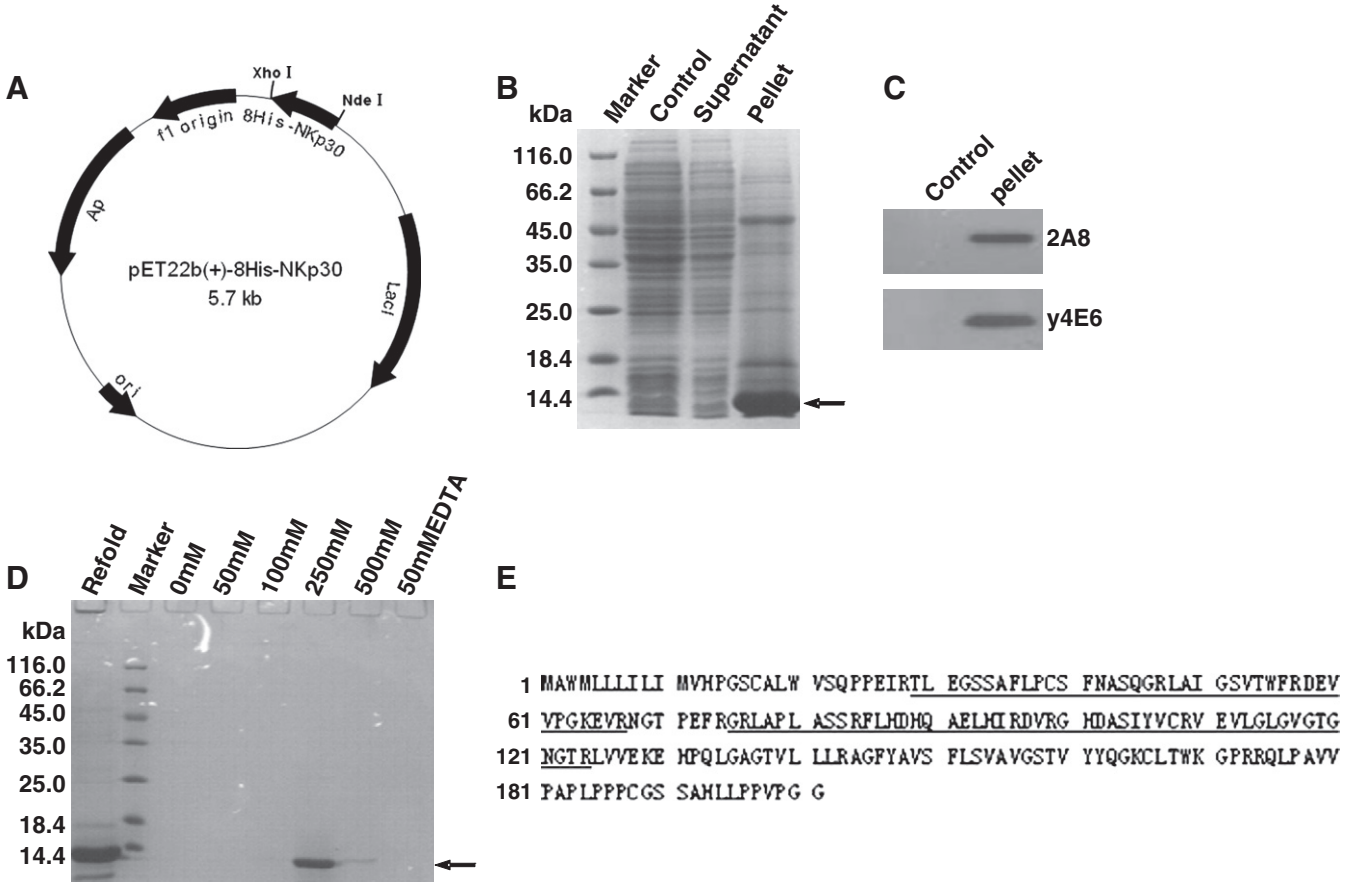

Fig. 1. Recombinant expression and purification of the human NKp30 protein. (A) The strategy for the construction of the recombinant vector pET-22b(+)-8His-NKp30. With pMD18-T-NKp30 as a template, PCR was performed to amplify the target fragment. The gene coding the NKp30 ectodomain with an N-terminal 8× His-tag was then inserted into the pET22b(+) vector. (B) Expression of the recombinant protein was detected by SDS-PAGE analysis. Cell lysates from control Rosetta(DE3)/pET22b(+) (lane 2), supernatant (lane 3) and pellet (lane 4) from lysates from Rosetta(DE3)/pET22b(+)-8His-NKp30 were induced with 1 mM IPTG at 37 °C for 6 h. Proteins were separated by 12% SDS-PAGE gel electrophoresis. Arrowheads indicate the position of the recombinant protein. (C) The recombinant NKp30 protein was detected by western blotting. The separated proteins were transferred onto a PVDF membrane and probed with mouse anti-His-Tag mAb (2A8) and mouse anti-NKp30 mAb (y4E6), respectively. (D) Purified rhNKp30 was detected by SDS-PAGE analysis. The refolded rhNKp30 protein was purified by Ni+ affinity chromatography. The refolded recombinant NKp30 (lane 1), protein molecular weight marker (lane 2); elution buffer containing 0 mM–500 mM of imidazole (lanes 3–7), and elution buffer containing 50 mM EDTA (lane 8) were analyzed on a 12% SDS-PAGE gel under non-reducing conditions. Arrowheads indicate the position of the rhNKp30 protein. (E) Sequencing of rhNKp30 by LC-MS. The underlined amino acid residue sequences were identified using LTQ Mass Spectrometer.

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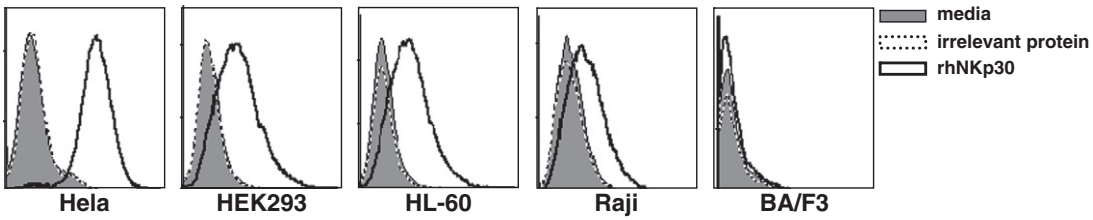

Fig. 2. Flow cytometric analysis of binding of soluble rhNKp30 with its ligands on tumor cells. The NKp30 ligand-positive tumor cell lines Hela, HEK293, HL60, and Raji and the NKp30 ligand-negative cell line BA/F3, which was used as a non-binding control, were incubated with media, an irrelevant protein TEV-6His or rhNKp30 protein for 45 min, followed by staining with anti-His-Tag mouse mAb (2A8) and PE-labeled goat anti-mouse IgG. The samples were analyzed by flow cytometry. Data are representative of three independent experiments.

The supernatant was stored at −20 °C for SDS-PAGE and western blotting analysis.

2.11. Statistical analysis

Statistical analysis was performed using the Student's *t* test. All p-values were two-tailed, and $p<0.05$ was considered statistically significant.

3. Results

3.1. Recombinant expression and purification of the ectodomain of the human NKp30 receptor

Based on secondary structure and functional domain analysis, the NKp30 receptor ectodomain was identified, amplified by PCR along with an additional N-terminal 8×His sequence and inserted into the pET-22b(+) vector (Fig. 1A). The recombinant plasmid pET-22b(+)–8His-NKp30 DNA was sequenced, and the sequence was consistent with NKp30. The recombinant plasmid pET-22b(+)–8His-NKp30 was then transformed into *E. coli* Rosetta (DE3)–competent cells, and the recombinant-expressed protein band was observed in the pellet lane on an SDS-PAGE gel in comparison with control. The recombinant protein had a molecular weight of around 14 kDa, which is consistent with its estimated size (Fig. 1B), and it existed in the form of inclusion bodies. To further characterize the recombinant protein, western blotting was performed using a mouse anti-His-Tag mAb (2A8) and a mouse anti-NKp30 mAb (y4E6). The recombinant protein reacted with both the anti-His-Tag mAb (2A8) and the anti-NKp30 mAb (y4E6), indicating successful expression of recombinant human NKp30 protein (rhNKp40) in Rosetta (DE3) cells (Fig. 1C). The recombinant protein was refolded and purified under different conditions, as described in Fig. 1D. The protein band was only observed upon purification with 250 mM imidazole, which suggests that this is the optimal strategy for purification of the rhNKp30 protein (Fig. 1D). Furthermore, the amino acid sequence of the refolded rhNKp30 protein was analyzed by LC-MS using an LTQ mass spectrometer (Research Centre for Life Sciences, University of Science and Technology of China, Hefei Anhui, China). Twenty one different peptides were found, covering 44.28% of the total NKp30 residue sequences and nearly all the extracellular region of rhNKp30 (Fig. 1E).

3.2. The binding of rhNKp30 protein with its ligands on the cell surface

To verify the binding activity of the soluble rhNKp30 with its ligands on tumor cells, the NKp30-ligand (B7-H6) positive tumor cell lines Hela, HEK293, HL-60 and Raji and the NKp30-ligand negative cell line BA/F3 [17] were incubated with soluble rhNKp30 protein, followed by labeling with anti-His mAbs. His+ cells were analyzed by flow cytometry. As shown in Fig. 2, the NKp30-ligand positive tumor cell lines were distinctly stained by anti-His mAbs compared with control. However, BA/F3 cells could not be stained by anti-His mAbs. These results suggest that rhNKp30 specifically binds to NKp30 ligands on tumor cells.

3.3. Inhibition of NK-92 cytolysis by soluble rhNKp30

To test the ability of soluble rhNKp30 to block the interaction between NKp30 and its ligands, an NK cell cytotoxicity assay was performed. It has been reported that NK-92 cells and IL-2 activated NK cells can kill Hela cells in an NKp30-dependent manner [8,28]. As expected, the cytotoxicity of NK-92 cells against Hela cells was significantly inhibited with an NKp30 blocking antibody (P30-15).

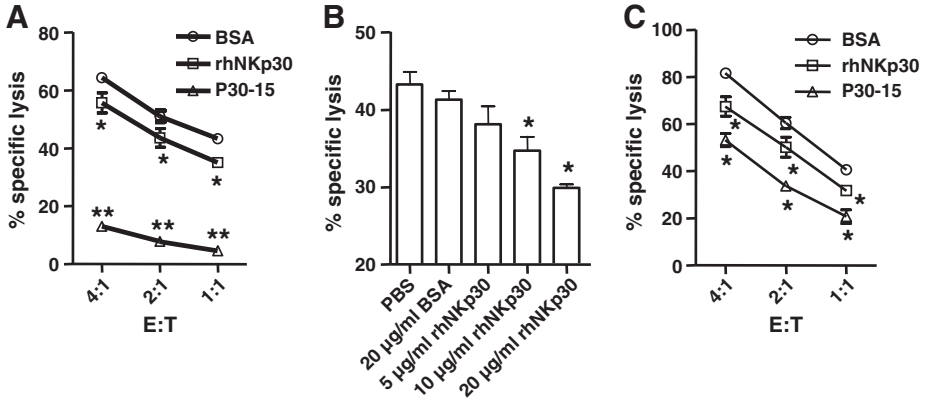

Fig. 3. Inhibition of NK cell-induced cytolysis of Hela cells by soluble rhNKp30. (A) Hela cells (target cells) were labeled with ^{51}Cr and incubated with 10 μg/ml of the irrelevant protein BSA, rhNKp30 or NKp30 blocking mAb (P30-15) for 1 h. Then, NK-92 cells (effectors) were added at the indicated effector/target ratios (E:T). (B) Hela cells were labeled with ^{51}Cr and incubated with 20 μg/ml BSA or 5–20 μg/ml rhNKp30 protein for 1 h. Then, NK-92 cells were added at an effector/target ratios of 1:1. (C) Hela cells were labeled with ^{51}Cr and incubated with 10 μg/ml BSA, rhNKp30 and P30-15 for 1 h, and then IL-2 activated human NK cells (effectors) were added at the indicated effector/target ratios (E:T). Error bars represent the mean ± SD of triplicates. Data are representative of three independent experiments. Data were analyzed by Student's *t* test, *, $p<0.05$; **, $p<0.01$ compared with BSA.

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Table 1
Features of the 3G5 mAb.

	3G5 mAb
Isotype	IgG1κ
Titer (culture supernatant)	7.81×10^{-5}
Titer (ascitic fluid)	9.77×10^{-8}
Affinity constant (L/M)	8.64×10^{7}
Applications	western blotting, ELISA, immunoprecipitation, flow cytometry

Compared to the irrelevant protein BSA, the cytotoxicity of NK-92 cells against Hela cells was partially inhibited by soluble rhNKp30 at different effector/target ratios (Fig. 3A). Soluble rhNKp30 inhibited the cytotoxicity of NK-92 cells against Hela cells in a dose-dependent manner at an effector/ target ratio of 1:1 (Fig. 3B). The cytotoxicity of IL-2 activated NK cells against Hela cells was also partially inhibited by soluble rhNKp30 at different effector/target ratios (Fig. 3C). These results indicate that the soluble rhNKp30 is functional.

3.4. Preparation and features of an anti-NKp30 monoclonal antibody

To further study the function of NKp30, murine monoclonal antibodies against rhNKp30 were prepared. After immunization of mice with soluble rhNKp30 as an antigen, followed by cell fusion, hybridoma cell cloning and subcloning, four hybridoma cell lines, named 2H9, 2H12, 2 G12 and 3G5, were obtained, which all stably produced anti-rhNKp30 mAbs. One of the four hybridoma cell lines, 3G5, was further selected for subsequent tests by virtue of its high growth characteristics in vitro and high reactivity with rhNKp30 in an ELISA assay. The features of the 3G5 mAb are shown in Table 1. Using the IsoQuick™ kit for mouse monoclonal isotyping (Sigma, ISOQ5), the 3G5 mAb was determined to be of the IgG1κ isotype. The titer of the 3G5 mAb in the culture supernatant and the ascetic fluid and the affinity constant of the antibody are summarized in Table 1. Highly concentrated 3G5 mAb was prepared from ascites of BALB/c mice and purified by Protein G affinity chromatography. The purity and molecular weight of the 3G5 mAb were determined by SDS-PAGE analysis. The concentration of the purified mAb was about 2.05 mg/ml, and the purity was estimated to be greater than 95%. The amino acid sequence of the 3G5 mAb was identified by LC-MS using a LTQ mass spectrometer.

3.5. Application of the 3G5 mAb in detection of the human NKp30 receptor

First, we examined the ability of the 3G5 mAb to detect NKp30 in western blotting assays. As shown in Fig. 4A, the 3G5 mAb can specifically detect rhNKp30, but not the unrelated recombinant protein 8His-NKG2F. Importantly, 2 ng of rhNKp30 could be detected by the 3G5 mAb, but not by the commercial anti-NKp30 mAb, y4E6 (Santa Cruz), demonstrating that the 3G5 mAb has a higher affinity for rhNKp30 than the y4E6 mAb. Furthermore, the 3G5 mAb could detect a specific band corresponding to NKp30 in NK-92 cells lysates (an NKp30 positive cell line) but not in the lysates from YT cells (an NKp30 negative cell line) (Fig. 4B). These results suggest that the 3G5 mAb can be specifically used to detect NKp30 by western blotting.

Second, to test whether the 3G5 mAb could be used in immunoprecipitation assays, we used the 3G5 mAb to immunoprecipitate proteins from NK-92 cell lysates. Mouse IgG1κ was used as a negative control. Western blotting was performed to analyze the products from the immunoprecipitation assays. As expected, a specific band of about 30 kDa was detected in the 3G5 mAb immunoprecipitation reaction; however, no 30-kDa band was seen in the mIgG1κ immunoprecipitation reaction (Fig. 4C). The amino acid sequence of the 30-kDa protein was confirmed as NKp30 by LC-MS using a LTQ mass spectrometer.

Third, to test the 3G5 mAb for use in ELISAs, plates were coated with different concentrations of rhNKp30, and the 3G5 mAb was used as the primary antibody. The $\triangle OD_{450nm}$ values for different protein concentrations were calculated. The standard curve developed by the indirect ELISA was able to detect rhNKp30 over a broad concentration range (Fig. 5A).

Fourth, to test whether the 3G5 mAb could be used for flow cytometry, the NK cell lines NK-92 and YT were used as target cells for staining. As expected, compared to the isotype control group (mIgG1κ), the 3G5 mAb could stain NK-92 specifically, while YT cells could not be stained (Fig. 5B). To further characterize the 3G5 mAb, fresh human PBMCs were isolated and stained with the 3G5 mAb or mouse IgG1κ as a control. The expression of NKp30 in different PBMC subgroup was analyzed by flow cytometry. The NK cell (CD3−CD56+) subgroup, but not the T cell (CD3+CD56−) subgroup, could be stained by the 3G5 mAb specifically (Fig. 5C). These results suggest that the 3G5 mAb can be used in flow cytometry for specifically staining the NKp30 receptor. However, the 3G5 mAb was not as efficient as the anti-NKp30 mAb (P30-15) in flow cytometry.

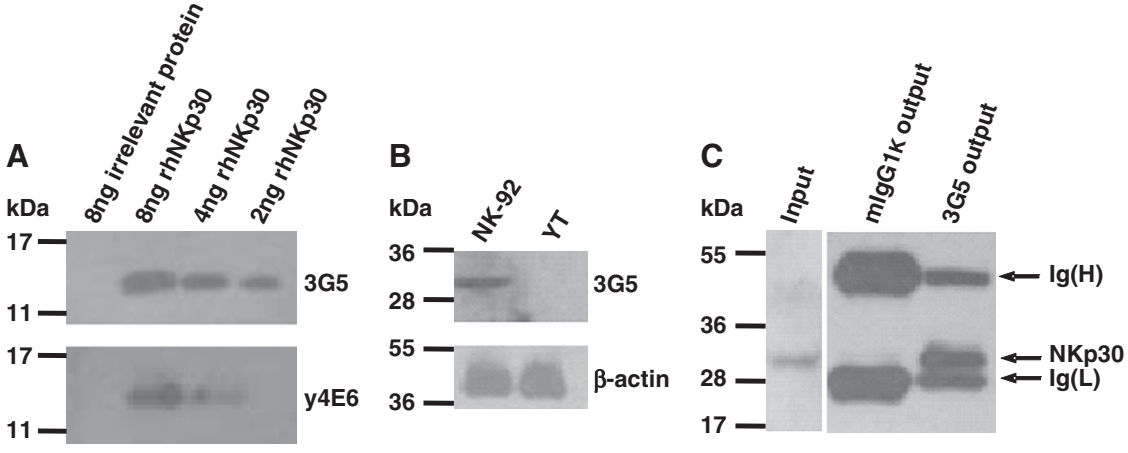

Fig. 4. Ability of the 3G5 mAb to detect NKp30 in western blotting and immunoprecipitation assays. (A) The specificity of the 3G5 mAb against soluble rhNKp30 protein was verified by western blotting. Samples containing 2–8 ng/ml of the rhNKp30 protein or 8 ng/ml of the irrelevant protein 8His-NKG2F were probed with the 3G5 or y4E6 mAbs. (B) Cell lysates of the NKp30 receptor-positive cell line NK-92 and the NKp30 receptor-negative cell line YT were separated by SDS-PAGE gel electrophoresis and analyzed by western blotting using the 3G5 mAb. (C) NK-92 cell lysates were immunoprecipitated with the 3G5 mAb or control mIgG1κ mAb, and NKp30 was detected in immune complexes by western blotting with the 3G5 mAb. Input, 50 μg of protein from NK92 lysates; mIgG1κ output, 10 μg of products immunoprecipitated with control mIgG1κ; 3G5 output, 10 μg of products immunoprecipitated with the 3G5 mAb.

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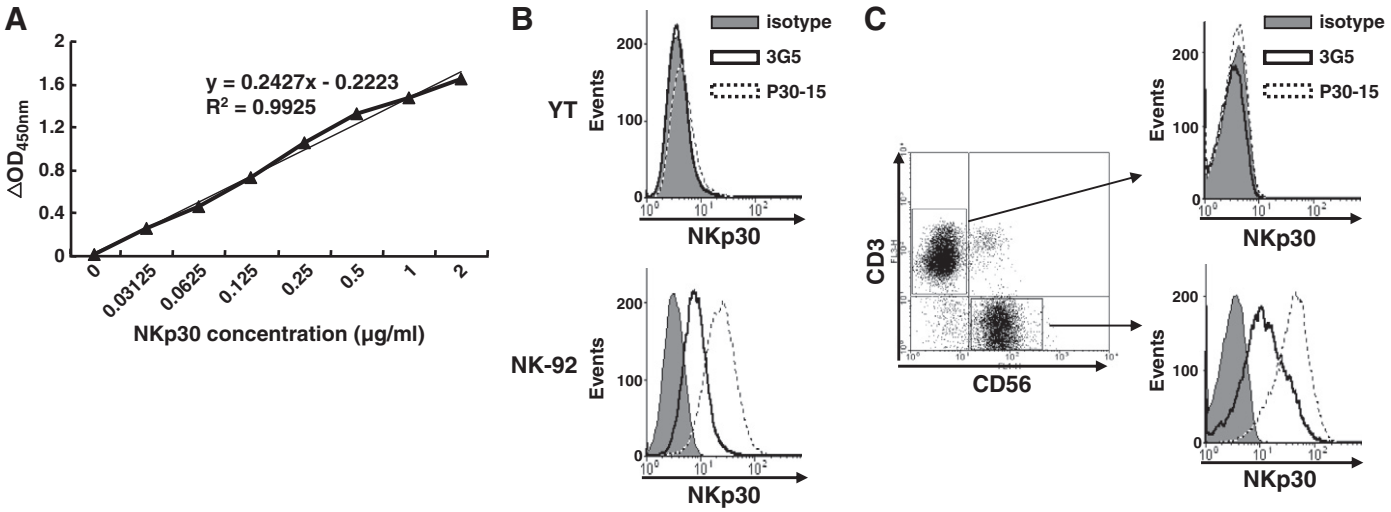

Fig. 5. Ability of the 3G5 mAb to detect NKp30 by ELISA and flow cytometry. (A) ELISA plates were coated with different concentrations of rhNKp30 and binding was assayed using the 3G5 mAb. The optical density (OD) at 450 nm (OD_{450nm}) and 630 nm (OD_{630nm}) were measured. $\triangle OD_{450nm} = OD_{450nm} - OD_{630nm}$ were calculated. (B) The NKp30 receptor-positive cell line NK-92 and the NKp30 receptor-negative cell line YT were incubated with the 3G5 mAb, the P30-15 mAb or the isotype control (mIgG1κ) mAb followed by staining with PE-labeled goat anti-mouse IgG and analysis by flow cytometry. (C) Freshly isolated PBMCs were incubated with the 3G5 mAb, the P30-15 mAb or the isotype control (mIgG1κ) mAb followed by staining with PE-labeled goat anti-mouse IgG, mouse anti-human CD56 and CD3. NKp30+ cells were analyzed for CD3+CD56− or CD3−CD56+ cell populations by flow cytometry. Data are representative of three independent experiments.

Finally, to test the ability of the 3G5 mAb to block the interaction between NKp30 and its ligands, an NK cell cytotoxicity assay was performed. As shown in Fig. 6A, the cytotoxicity of NK-92 cells against Hela cells was significantly inhibited with an NKp30 blocking antibody (P30-15) but not with the 3G5 mAb at different effector/target ratios. The cytotoxicity of NK-92 cells was not inhibited with increased concentration of 3G5 (Fig. 6B). The cytotoxicity of IL-2 activated NK cells against Hela cells was also not inhibited by the 3G5 mAb (Fig. 6C). These results indicate that the 3G5 mAb does not affect the binding of NKp30 to its ligands. As shown in Fig. 6D, the binding of NKp30 by the

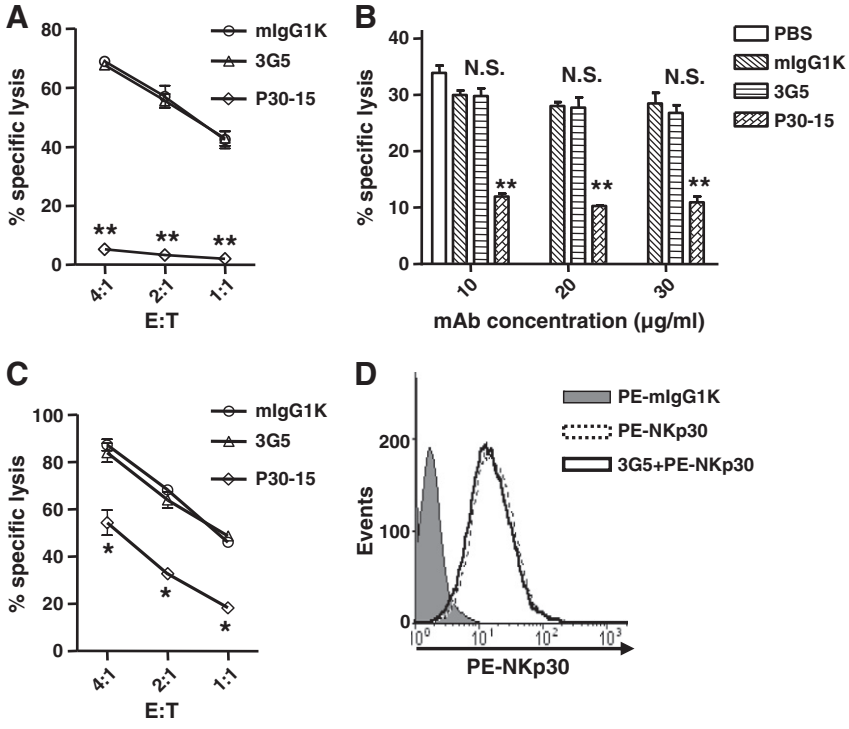

Fig. 6. No inhibitory effect of the 3G5 mAb on NK cell cytolysis against Hela cells. (A) NK-92 cells (effectors) were incubated with 10 μg/ml of the 3G5 mAb, NKp30 blocking mAb (P30-15) or control mIgG1κ mAb for 1 h, and then Hela cells (target cells) were labeled with ^{51}Cr and added at the indicated effector/target ratios (E:T). (B) NK-92 cells (effectors) were incubated with 10–30 μg/ml of the 3G5 mAb, the P30-15 mAb or the mIgG1κ mAb for 1 h. Then, Hela cells (target cells) labeled with ^{51}Cr were added at an effector/ target ratios of 1:1. (C) IL-2 activated human NK cells (effectors) were incubated with 10 μg/ml of the 3G5 mAb, the NKp30 blocking mAb (P30-15) or the control mIgG1κ mAb for 1 h. Then, Hela cells (target cells) labeled with ^{51}Cr were added at the indicated effector/target ratios (E:T). (D) NK-92 cells were incubated with PBS or 10 μg/ml of the 3G5 mAb for 30 min followed by staining with PE-mouse-anti-human NKp30 (PE-NKp30). The samples were analyzed by flow cytometry. Error bars represent the mean ± SD of triplicates. Data are representative of three independent experiments. Data were analyzed by Student's *t* test, *, $p<0.05$; **, $p<0.01$; N.S., not significant compared with mIgG1κ.

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3G5 mAb does not interfere with binding of the NKp30 blocking mAb to NK-92 cells suggesting that the 3G5 and p30-15 mAbs bind to different epitopes.

4. Discussion

NKp30 plays an important role in the cytotoxicity of NK cells against infected cells and tumor tissues. Recently, accumulating evidence indicates that NKp30 also participates in NK-DC cell crosstalk. In our study, we made a soluble recombinant human NKp30 protein and an anti-NKp30 mAb, which could serve as important tools for further studying the function of NKp30.

In this study, the NKp30 ectodomain (residues 19–130), with an N-terminal 8×His-tag, was cloned into the pET22b (+) vector. The recombinant protein was expressed as inclusion bodies in Rosetta (DE3) *E. coli* cells. Several factors were optimized to assist in the refolding of rhNKp30 [29,30]. We found that the presence of high-purity inclusion bodies facilitated the efficient refolding of the recombinant protein, and the presence of L-arginine and GSH–GSSG (reduced glutathione and oxidized glutathione) also aided in protein refolding, similarly to previous reports [20,21]. As described elsewhere, several methods can be used to verify the activity of refolded proteins [31,32]. In our study, flow cytometry and NK cytotoxicity assays were performed. The soluble rhNKp30 protein distinctly and specifically stained NKp30 ligand-positive cells in flow cytometry and NK cytotoxicity assays, which suggests that rhNKp30 could be used as a diagnostic tool to identify NKp30 ligand-positive tumor cells. In a functional assay, we confirmed that the soluble rhNKp30 protein dose-dependently inhibited the cytotoxicity of NK cells against targets, indicating that soluble human NKp30 protein neutralizes the interaction between NKp30 and its ligands. This suggests that this recombinant protein may possibly be used to inhibit NKp30-mediated overactivity of autoimmunity responses, as previously reviewed by Poggi, A. [33]. However, the inhibitory effect of recombinant NKp30 was not as distinct as that seen with the anti-NKp30 antibody (clone P30-15). This may be due to lower affinity of the monomer form of soluble rhNKp30.

To prepare antibodies against rhNKp30, different immunization strategies were performed [22]. In our study, the antigen, refolded rhNKp30 protein, was mixed with Freund's adjuvant, and the complex was used to immunize mice. We obtained one monoclonal antibody designated “3G5” that displayed very high reactivity with rhNKp30 in ELISA assays. The 3G5 mAb could be a powerful tool in functional studies as it can be used in a wide variety of applications including western blotting, immunoprecipitation, ELISA and flow cytometry. However, the 3G5 mAb was not as efficient for flow cytometry as the commercial anti-NKp30 (clone P30-15) mAb from Biolegend. This may due to the different affinities of these mAbs and the specific structure of NKp30 on the cell surface. The anti-NKp30 (clone P30-15) mAb is a conformational antibody and can be used in blocking experiments [28]; however, the 3G5 mAb did not influence the binding of PE-mouse anti-human NKp30 (clone p30-15) to NK-92 cells (Fig. 6D). Results from NK-92 cell and IL-2-activated NK cell cytotoxicity assays suggest that the 3G5 mAb may not influence the binding of NKp30 to its ligands (Fig. 6A–C). Compared to the anti-NKp30 mAb (clone y4E6) from Santa Cruz, the 3G5 mAb we prepared was more sensitive for detecting NKp30 in western blotting assays.

In conclusion, we successfully expressed the human NKp30 ectodomain in *E. coli* and developed an efficient method to produce biologically active rhNKp30. Using the soluble rhNKp30 as an antigen, we prepared monoclonal antibodies against human NKp30. One of the monoclonal antibodies, 3G5, was functional in several applications such as western blotting, immunoprecipitation, ELISA and flow cytometry assays. The soluble rhNKp30 protein and mAbs against NKp30 could be useful tools for further functional research of NKp30 and could potentially be used as diagnostic or therapeutic tools.

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