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Proliferating Cell Nuclear Antigen Is a Novel Inhibitory Ligand for the Natural Cytotoxicity Receptor NKp44

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NK cells play an important role in the early immune response to cancer. The NKp44 activating receptor is the only natural cytotoxicity receptor that is expressed exclusively by primate NK cells, yet its cellular ligands remain largely unknown. Proliferating cell nuclear Ag (PCNA) is overexpressed in cancer cells. In this study, we show that the NKp44 receptor recognizes PCNA. Their interaction inhibits NK cell function through NKp44/ITIM. The physical interaction of NKp44 and PCNA is enabled by recruitment of target cell PCNA to the NK immunological synapse. We demonstrate that PCNA promotes cancer survival by immune evasion through inhibition of NKp44-mediated NK cell attack. The Journal of Immunology, 2011, 187: 5693–5702.

N atural killer cells contribute substantially to the innate immune response by providing crucial early defense against cancers and pathogens (1). NK cell activity is a balance between signals delivered by inhibitory and activating receptors. Engagement of inhibitory receptors by MHC class I molecules on normal cells suppresses NK cell effector functions (2–4). Major activating receptors include NKGD2D and the natural cytotoxicity receptors (NCRs) NKp30, NKp46, and NKp44 (5, 6). NKp44 (CD336, NCR2) is expressed by activated NK cells (7) and reportedly triggers cytolytic activity against both tumor and virus-infected cells through DAP12-binding domain in its transmembrane region (8, 9). Importantly, the NCR2 gene encoding NKp44 could not be located in the mouse genome; therefore, there is no suitable mouse model for exploration of NKp44 (10). We have shown that NKp44 is involved in the functional recognition of H1, H3-, and H5-subtypes of influenza virus, the hemagglutinin-neuraminidase of Sendai virus, and the envelope glycoprotein of dengue virus and H5-subtypes of influenza virus, the hemagglutinin-neuraminidase region (8, 9). Importantly, the NCR2 gene encoding NKp44 could not be located in the mouse genome; therefore, there is no suitable mouse model for exploration of NKp44 (10). We have shown that NKp44 is involved in the functional recognition of H1, H3-, and H5-subtypes of influenza virus, the hemagglutinin-neuraminidase of Sendai virus, and the envelope glycoprotein of dengue virus and West Nile virus (9, 11–13). Diverse experiments had shown that NKp44 and NCRs in general have a major role in the NK-mediated lysis of various tumors, including carcinomas, melanomas, neuroblastomas, myeloid and lymphoblastic leukemia, multiple myeloma, and EBV-transformed B cells. Remarkably, the involvement of a given NCR in triggering cytolyis of a distinct transformed target cell varies among cells of the same or different histotype. This would imply that the three NCRs might recognize different cellular ligands and that transformed cells might express variable surface densities of different NCR ligands (8). We and others reported that tumor membrane-associated heparan sulfate (HS) serves as a coligand for all three NCRs, and the HS epitopes recognized by the NCRs could be different (14–17). A recent study showed that the B7 family member B7-H6 expressed on the membrane of K562 cells is a ligand for NKp30 (18). Interestingly, two ligands reported for NKp30, namely human CMV structural protein pp65 and HLA-B–associated transcript 3 (BAT3), are not membrane-associated proteins, but are considered to be nuclear/cyttoplasmic. Arnon et al. (19) showed an antagonistic effect of the main CMV tegument protein, pp65, on NKp30, since extracellular-released pp65 dissociated TCR-β from NKp30 and consequently reduced killing. Others have reported that BAT3 overexpression enhanced release of BAT3-containing exosomes from dendritic cells and activated NK cells (20). In this study, we report the interaction between NKp44 and proliferating cell nuclear Ag (PCNA). PCNA is a prominent cancer-associated protein, and as a homotrimer forms a clamp around DNA, facilitating its replication by the DNA polymerases (21). It is highly expressed in proliferating cells, including cancer cells (22). PCNA overexpression is associated with enhanced malignancy (23). We show that the interaction of NKp44 with target cell-expressed PCNA paradoxically inhibits lysis and IFN-γ secretion by NK cells. The inhibition is mediated by an ITIM on the NKp44 cytoplasmic domain, and the nuclear/cyttoplasmic PCNA in the target cell is recruited to the NK immunological synapse (NKIS) when NKp44 is expressed on the NK cells.

Materials and Methods

Cells

Cell lines used in this work were as follows: Panc1, human pancreatic ductal carcinoma (American Type Culture Collection CRL-1469); DU 145, human prostate carcinoma (HTB-81); MCF-7, human mammary gland adenocarcinoma (HTB-22); U-251, human glioblastoma (HTB-17); A375,
human melanoma (CRL-1872); HeLa, human cervical adenocarcinoma (CCL-2); BW5147, murine thymus cells (TIB-48); NK-92, a human NK lymphoma (CRL-2407); and HEK293T, SV40 large T Ag-transfected HEK293 cells (CRL-11268). The NK-92 cell lines, transfected by retrovirus to express high levels of wild-type and mutated NKp44 (designated as NK92-44, NK92-44.D204E, and NK92-44.Y238F), were characterized in detail elsewhere (24). The NK1L human NK cell line was described elsewhere (25).

**Abs and fusion-Ing proteins**

The following Abs were used: anti-NKp44 mAb, anti-NKp30 mAb (R&D Systems), anti-His tag, anti-PCNA (Santa Cruz Biotechnology), allophycocyanin-conjugated anti-CD56 (BioLegend), FITC-conjugated anti-CD3 (Dako), and biotin-conjugated mouse anti-human CD107a/ALAM-1 mAb (SouthernBiotech). Allophycocyanin-conjugated F(ab')2 goat anti-human IgG, allophycocyanin-conjugated F(ab')2 goat anti-mouse IgG, and allophycocyanin-conjugated streptavidin (Jackson ImmunoResearch Laboratories) were also used. Generation of mouse polyclonal anti-NKp44 or mock serum was previously described (9, 14). The production of NKp44-Ig, NKp30-Ig, and NKp46D2-Ig was described elsewhere (14, 17), and the generation of LIR1-Ig and NKGD2-Ig was previously described (26, 27). Human IgG1 (hIgG1k, PBP010; Serotec) and human IgG were purchased from Sigma-Aldrich. DIO, DIL, and DiD Vybrant cell-labeling solution (v228890) was obtained from Molecular Probes.

**Production of recombinant soluble PCNA and hepatocyte NF-4**

To produce soluble PCNA and hepatocyte NF-4 (HNF-4) we employed a β-gal-induced bacterial system. The pET-28 vector, which carries PCNA coding sequence and C-terminal His-tag sequence, was used to transduce Escherichia coli BL21 competent cells (primers are described in Supplemental Table I). Following transformation, selection, and isolation β-1-thio-galactopyranoside–based induction, bacteria cells were lysed, sonicated, and loaded on a Ni column. Imidazole buffer (0.5 M) was used to elute the protein. HNF-4 coding sequence in the same His-tag–containing pET-28 vector was provided by Prof. Boaz Shaanan (Ben Gurion University), and the protein was produced with the same protocol.

**Constructs and transfections**

Human PCNA (accession no. CAG46598) was cloned with specific primers (Sigma-Aldrich) from a HeLa-derived cdNA library (Supplemental Table I). For production of BW-PCNA, PCNA was cloned in-frame into a pCDNA3.1+ 3′-chain–containing cassette (19); BW5147 cells were then transfected by electroporation (field strength, 0.95 kV/cm; voltage, 250 V; capacity, 970 μF) to produce BW5147-PCNA-3′ cells expressing PCNA monomer on the cell surface (BW-PCNA). For HeLa-GFP-PCNA cells, PCNA was cloned into the pEGFP-C1 vector at the C terminus of GFP using a flexible linker (SGRLRRAQLQQGQQQRS) as previously described (28). HeLa cells were transfected using TurboFect transfection reagent (Fermentas) with PCNA-linker-GFP or empty vector to obtain HeLa-GFP-PCNA and HeLa-GFP, respectively.

**Small interfering RNA-based posttranscriptional silencing**

Human PCNA, BAT3, and control small interfering RNA (siRNA) (Santa Cruz Biotechnology) were used according to the manufacturer’s protocol to downregulate protein levels in several human cancer cell lines. Approxi-mately 48 h posttransfection, cells were used as experimental target cells and a fraction of the sample was analyzed for protein expression.

**ELISA assays**

Two ELISA assays were performed. First, plates were coated with 0–4 μg/ml recombinant His-tag PCNA or HNF-4. After blocking, 4 μg/ml various Ig-fusion NK proteins were added. HRP-conjugated goat anti-human IgG, Fcy fragment–specific Ab (0.2 μg/ml; Jackson ImmunoResearch Laboratories) was used for detection. Alternatively, plates were coated with 0–4 μg/ml various Ig-fusion NK receptors. After blocking, 4 μg/ml recombinant His-tag PCNA or HNF-4 was added. Anti-His mAb and HRP-conjugated goat anti-mouse IgG, Fcy fragment–specific Ab (0.2 μg/ml; Jackson ImmunoResearch Laboratories) were used for detection, as measured by OD at 650 nm with a Dynex Technologies MRX microplate reader. For blocking assay, NKp44-Ig was preincubated with mouse polyclonal anti-NKp44 Ab or mock serum (both diluted 1:200).

**Immunoprecipitation and Western blotting**

Cells were lysed with 50 mM HEPES (pH 7.4), 100 mM NaCl, 0.1% CHAPS, 1 mM DTT, and 0.1 mM EDTA protease inhibitor mixture (Calbiochem) containing buffer. Protein concentration was determined by Bradford assay (Bio-Rad). Proteins were electrophoresed, transferred to membranes, stained with anti-PCNA or anti–β-actin mAbs and goat anti-mouse HRP, and imaged on an XRS+ imaging system (Bio-Rad). Immunoprecipitation was performed with protein A-Sepharose beads (GE Healthcare) precoated with NKp44-Ig or LIR1-Ig and incubated with either cell lysates or recombinant His-tag PCNA or His-tag HNF-4. Eluates were used for immunoblotting with anti-PCNA mAb for cell lysates and anti-His mAb for recombinant proteins. Western blot quantification was performed by measuring band intensity with ImageJ freeware (National Institutes of Health, Bethesda, MD).

**Flow cytometry**

Cells were incubated on ice with various fusion-Igs (40 μg/ml), washed, and stained with allophycocyanin-conjugated anti-human-IgG Ab. Cells were labeled with anti-NKp44 mAb or anti-PCNA mAb, washed, and stained with allophycocyanin-conjugated anti-mouse IgG. Dead cells were detected with propidium iodide. Flow cytometry was performed using a FACS Canto II (BD Biosciences) and results were analyzed using FlowJo software (Tree Star).

**Isolation and culture of primary NK cells**

NK cells were isolated from the peripheral blood of healthy donors using the human NK cell isolation kit (Miltenyi Biotec). NK purity was >90% (CD3+CD56+). Purified NK cells were cultured in CellGro stem cell serum-free growth medium (CellGenix) supplemented with 10% heat-inactivated human plasma from healthy donors, 1 mM sodium pyruvate, 2 mM l-glutamine, 1 × MEM nonessential amino acids, 1% penicillin/streptomycin, 10 mM HEPES (Life Technologies), and 300 U/ml human IL-2 (Biological Industries).

**IFN-γ secretion and CD107a degranulation assays for NK cell activity**

For IFN-γ assays, 96-well plates were precoated with 0.25 μg/ml anti-NKp44 mAb. NK effector cells (5 × 105 cells/well) were mixed with target cells (5 × 104 cells/well) in a 1:5 virus–concentrating supernatants were then assayed by standard ELISA assay. An NK degranulation assay was performed as previously described (15, 16).

**Cytotoxicity and in vivo killing assays**

For cytotoxicity assays, target cells were labeled with [35S] for 12 h. Then, the cytotoxic activity of NK92 and NK92-44 cell lines and primary human NK cells was assessed in a 5-h [35S]-release assay, as previously described (29). In all experiments shown, the spontaneous release was <25% of maximal release. The flow cytometry cytotoxicity assay was previously described (30). Target cells were labeled with either DIO, DIL, or DiD. Analysis of 7-aminoactinomycin D was performed using a FACSCanto II and data were analyzed by FlowJo. For in vivo killing assays, target cells were labeled with either DIO or DIL and mixed in a 1:1 ratio. Effector NK cells were added immediately before i.p. injection into C57BL/6 mice. All injections were performed in 300 μl PBS, 1 × 105 of each target cell population, and 12 × 106 of NK cells per mouse. Six hours after the injections, mice were killed and the peritoneum washed out with PBS. The peritoneal lavage and the equivalent in vitro mixed target culture were then both analyzed by flow cytometry. All experiments were done in the animal facilities of Ben-Gurion University, according to guidelines of the Ethical Committee. For blocking experiments, NK cells were preincubated with mouse polyclonal anti-NKp44 Ab or with mock serum before being combined with target cells.

**Kinetic analysis by surface plasmon resonance**

We employed the ProteOn XPR36 protein interaction array system, GLC, and ProteOn Manager 2.1.2 version 2.1.2.05 (Bio-Rad) to measure the affinity of NKp44-Ig fusion protein to PCNA. Activation was performed by running 100 mM 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride and 25 mM N-hydroxysulfosuccinimide over the chip for 300 s. Estimated activation was 150 resonance units (RU) for all channels. His-tag PCNA (0.145 μg/ml) was immobilized on the chip. Binding levels were 200 and 100 RU, respectively. Different concentrations (from 1 to 0.03 μM) of antibody (NKp44-Ig, NKp46D2-Ig, IgG3-Jg, and human-Fc) were injected for kinetic measurements. Data processing was done using the 1:1 Langmuir binding model with mass transfer and baseline drift. The R2 value is a standard statistical measure of the closeness to fit, representing the mean square of the signal noise.
Live imaging confocal microscopy

HeLa-PCNA-GFP or HeLa-GFP target cells were cultured in chambered μ-slide (80862; ibidi) and incubated overnight at 37°C. Effector cells were counted, labeled with DiD, suspended in culture medium containing 20 U/ml IL-2, and added to wells containing the target cells in a 3:1 E:T ratio. Using the Olympus FluoView FV1000 (Olympus) multi-area time lapse feature (UPLSAPO ×60 oil; numerical aperture, 1.35), random fields were selected and time lapse images were acquired every 2 min for 6 h. Zero drift correction was used to avoid loss of the focal plane in the Z direction during time lapse acquisition. In postacquisition analysis, immunological synapse-positive conjugates were defined as stable effector/target interactions that lasted for >6 min.

Results

Direct binding of rNKp44 to rPCNA

We explored possible ligands to NCRs by expressing various ligand candidates on yeast cell surfaces and testing binding of rNCRs. As

FIGURE 1. NKp44-Ig binds human PCNA specifically. A and B, ELISA plates were coated with titrated concentrations of rPCNA, followed by incubation with 4 μg/ml different human Fc-chimeric NK receptors and detection with HRP-conjugated anti-human Fc Ab. C, ELISA plates were coated with titrated concentrations of NK receptors, followed by incubation with 4 μg/ml His-tag PCNA (left panel) or His-tag HNF-4 (right panel) and detection with mouse anti-His mAb and HRP-conjugated anti-mouse Ig Ab. D, ELISA plates were coated with PCNA or HNF-4, followed by NKp44-Ig preincubated with anti-NKp44 or mock mouse serum and detection with HRP-conjugated anti-human Fc Ab. Bars 6 SD. *p < 0.05, ANOVA. E, Protein-A Sepharose beads were coated with NKp44-Ig or LIR1-Ig, followed by incubation with 2.8 μg His-tagged PCNA or HNF-4 in 100 μl wash buffer and thorough wash. Eluates were immunoblotted with anti-His mAb. F, Kinetics of interactions with immobilized PCNA. Results from the ProteOn analysis are expressed in RU against time. Human Ig (hlg) (upper left), NKp44-Ig (upper right), NKp30-Ig (bottom right), and NKp46-Ig (bottom left) were injected over a PCNA-immobilized surface at increasing concentrations, ranging from 0 to 1 μM. K_D was calculated using the 1:1 Langmuir binding model with mass transfer and baseline drift. χ^2 < 0.05 of R_max. The results are from one representative experiment of at least two performed.
a negative control, we employed yeast cells displaying the nuclear protein PCNA on their cell surface. Interestingly, the negative control manifested considerable binding to rNKp44 (data not shown). In parallel, NKp30 was reported to bind to the nuclear protein BAT3 (20, 31). We thus further studied the direct in vitro binding of NCRs to PCNA. NKp44-Ig bound to wells coated with soluble PCNA protein, and the amount of binding was correlated with the amount of PCNA coating (Fig. 1A, 1B). Other rNK receptors, LIR1, NK2G2D, KIR2DL4, NKp30, and NKp46 bound only marginally and their weak background binding was not affected by the amount of PCNA (Fig. 1A, 1B). Alternatively, we coated wells with titrated quantities of NKp44-Ig or other rNK receptors and assayed the binding of soluble PCNA or HNF-4. Soluble PCNA, but not HNF-4, manifested binding to NKp44-Ig and did not bind to the other NK receptors (Fig. 1C). Blocking of NKp44 with anti-NKp44 Abs significantly suppressed the interaction between NKp44 and PCNA, but it did not affect its background binding to HNF-4 (Fig. 1D). NKp44-Ig–coated beads precipitated soluble PCNA, but not soluble HNF-4, and neither protein was precipitated by LIR1-Ig–coated beads (Fig. 1E). We further studied the binding of NKp44-Ig and PCNA using surface plasmon resonance analysis. A comparison of NKp44-Ig and other NCRs-Ig or human Fc binding to PCNA immobilized to a sensor chip is shown in Fig. 1F. NKp44-Ig displayed a characteristic binding curve to PCNA (K_D = 3.4 × 10^{-9} M); however, no significant interaction could be detected with the other NCRs-Ig. To summarize, we have shown direct in vitro interaction between rNKp44 and PCNA.

**Binding of rNKp44 to endogenous and membrane-associated PCNA**

We further tested the binding of NKp44 toward endogenous native PCNA. NKp44-Ig–coated beads precipitated native PCNA from HeLa and HEK293 cell lysates, whereas LIR1-Ig–coated beads did not (Fig. 2A). Binding specificity was confirmed by staining for β-actin, which was not precipitated by NKp44-Ig from the same lysates (Fig. 2A). Next, we studied whether NKp44-Ig can bind PCNA presented on the cell surface, by expressing PCNA fused to the TCR-ζ-chain. Membrane-associated PCNA expression was detected on the surface of transfected BW cells (Fig. 2B). NKp44-Ig, but not NKp30-Ig or NKp46-Ig, manifested enhanced binding to BW cells transfected with membrane-expressed PCNA, as compared with parental BW cells (Fig. 2C–E). In summary, we have shown direct interaction between rNKp44 and endogenous or transfected PCNA in multiple cellular contexts.

**Target cell PCNA interaction with NKp44 is associated with reduced NK function**

To study the functional interaction of intracellular PCNA and NK-expressed NKp44, we expressed PCNA fused to GFP in HeLa cells (HeLa-PCNA-GFP, Fig. 3A). PCNA-GFP manifested the characteristic distribution of endogenous PCNA during the cell cycle. Thus, PCNA-GFP expression did not alter cell phenotype (Fig. 3B and data not shown), in agreement with previous studies with GFP-fused PCNA (32, 33). Additionally, GFP distribution in control GFP-transfected HeLa cells (HeLa-GFP) was uniform and did not change during the cell cycle (Fig. 3B). Note that PCNA-GFP– and GFP-transfected HeLa cells were selected as pools of GFP-positive cells and were not subjected to subcloning; Fig. 3A shows the diverse expression level of PCNA-GFP and GFP in the transfected pools. Western blot analysis with anti-PCNA mAb showed that the expression level of transfected PCNA-GFP was 1.5-fold greater compared with endogenous PCNA (Fig. 3C). We first tested whether cell surface density of ligands to NCRs and other NK receptors was different between HeLa-PCNA-GFP and HeLa-GFP cells. Staining with anti HLA class I Ab, NKp30-Ig, and NKp46-Ig revealed no difference between the two transfected pools (HeLa-PCNA-GFP and HeLa-GFP, Supplemental Fig. 1). NKp44-Ig staining also showed no difference (Supplemental Fig. 1) in contrast to the BW-PCNA cells (Fig. 2C).

We next examined the effect of target cell PCNA overexpression on NK function and its correlation to NKp44 expression levels by various NK cells. As NK effector cells, we employed 1) NK-92 cells that express low levels of NKp44, 2) NKp44-transduced NK-92 cells that express high levels of wild-type NKp44 (designated as NK92-44), 3) IL-2 cultured primary human NK cells that express NKp44, and 4) NKL cells that do not express NKp44. Fig. 3D shows the different NKp44 expression levels by the NK effecter cells. We studied IFN-γ secretion from anti-NKp44–activated NK effectors cocubated with either HeLa-PCNA-GFP or HeLa-GFP target cells (Fig. 3E). PCNA overexpression correlated with suppression of IFN-γ secretion by NKp44-expressing NK cells; incubation of NKp44null NK-92-44 and primary NK cells with HeLa-PCNA-GFP target cells resulted in significantly reduced IFN-γ secretion as compared with incubation with HeLa-GFP target cells (Fig. 3D, 3E). In contrast, no significant difference in IFN-γ secretion was observed for NKp44null effector NKL cells or NKp44null NK-92 cells (Fig. 3D, 3E). Therefore, PCNA overexpression in the HeLa cells resulted in reduced IFN-γ secretion by effector NK cells expressing high levels of NKp44. We further tested the lysis of HeLa-PCNA-GFP and HeLa-GFP target cells by NK effector cells. Elevated NKp44 expression enhanced lysis of target HeLa cells (Fig. 3F, 3G), but lysis of HeLa-PCNA-GFP target cells by NK92-44 effector cells was significantly suppressed as compared with lysis of HeLa-GFP target cells. In contrast, lysis of the two target cell lines by NK-92 was similar (Fig. 3F, 3G). We next assayed whether anti-NKp44 polyclonal Ab blockade could affect the differential killing of target cells.
Incubation of NK92-44 with soluble anti-NKp44 Abs, but not with mock Abs, abolished the differences in lysis (Fig. 3H). The NKp44 PCNA-mediated suppression of lysis was also observed in human primary NK cells. These NKp44-expressing primary NK cells (Fig. 3D) lysed HeLa-PCNA-GFP target cells significantly less efficiently than HeLa-GFP target cells (Fig. 3I). Again, blockade of the primary NK cells with anti-NKp44 Abs, but not with mock Abs, abolished the difference in lysis (Fig. 3I). To summarize, we have demonstrated that overexpression of PCNA by target cells is associated with reduction of NK function that is dependent on NKp44 expression by NK cells and is abolished by Abs to NKp44.

**Downregulation of endogenous PCNA enhances lysis by NK cells**

As a result of our observations regarding the effect of PCNA overexpression on NK cell function, we investigated the effect of endogenous PCNA downregulation through an siRNA approach. siRNA-mediated downregulation of endogenous PCNA in HeLa cells resulted in enhanced lysis of the target cells by NKp44-expressing primary human NK cells (Fig. 4A). This enhancement of lysis was specific to siRNA targeting PCNA. For example, in the case of BAT3, a reported activator of NK cells through NKp30 (31), siRNA downregulation resulted in reduced lysis by NKp44-expressing primary human NK cells (Fig. 4A).

**FIGURE 3.** PCNA overexpression is associated with NKp44-mediated suppression in NK function. HeLa PCNA-GFP or HeLa-GFP target cells images. A, Images of HeLa-PCNA-GFP (left panel) and HeLa-GFP cells (right panel). Scale bars, 40 μm. B, Cropped images taken throughout the division cycle of a single cell: HeLa-PCNA-GFP (upper panel) and HeLa-GFP (lower panel). Scale bars, 10 μm. C, Lysates of HeLa-PCNA-GFP and HeLa-GFP were immunoblotted with anti-PCNA mAb. D, Cell surface staining of the various NK effector cells with anti-NKp44 mAb. E, HeLa-PCNA-GFP and HeLa-GFP target cells were incubated with various anti-NKp44–activated NK effectors for 18 h. IFN-γ levels in the culture supernatants are shown. F–I, Target cells were radioactively labeled with [35S] for 12 h, washed, and incubated with various NK effector cells. Then, the lysis of the various targets by NK cells was assayed in a standard 5-h [35S]-release assay. Shown is lysis by NK92 (F) and NK92-44 (G), NK92-44 in the presence of anti-NKp44 or mock serum (H), and primary human NK (I) in the presence of anti-NKp44 or mock serum. The results are from one representative experiment of two to four performed. Bars ± SD. *p < 0.05, **p < 0.01, ANOVA.
NK cells (Fig. 4A). siRNA downregulation of PCNA was confirmed by Western blot (Fig. 4A).

We then examined NK IFN-γ secretion to demonstrate the effects of siRNA-mediated downregulation of endogenous target cell PCNA in an assay independent of target cell lysis. Downregulation of endogenous PCNA in target cancer cells enhanced IFN-γ secretion upon incubation with NKp44 expressing primary human NK cells or with NK92-44 cells; in contrast, incubation of PCNA-downregulated target cells with NKp44null NKL cells resulted in no enhancement of IFN-γ secretion (Fig. 4B). Similar lysis enhancement results were obtained when NK92-44 cells were employed as effectors on target HeLa cells (Fig. 4C). To verify that the target PCNA effect on NK function is not limited to HeLa cells, we tested five more human target cancer cells, derived from different tissues, employing siRNA-mediated downregulation of PCNA (Fig. 4D–H). Downregulation of PCNA in PANC-1 (pancreas), MCF-7 (breast), DU145 (prostate), and U251 (brain) resulted in enhanced lysis by NK92-44 cells (Fig. 4D–G). PCNA levels in A375 melanoma cells were not reduced following siRNA treatment and, in accordance, their lysis by NK92-44 cells was not enhanced (Fig. 4H). Note that 1) transient siRNA-based downregulation of PCNA did not affect proliferation or viability of target cancer cells (data not shown); 2) all target cell lines tested expressed considerable levels of endogenous PCNA (Supplemental Fig. 2); and 3) staining with anti-HLA class I Ab (W6/32) and NKG2D-Ig revealed no difference between control-siRNA and PCNA-siRNA treatment (checked for PANC-1 cells, data not shown). In summary, successful downregulation of endogenous PCNA resulted in enhanced lysis and IFN-γ secretion by NKp44-expressing NK cells.

**ITIM in the cytoplasmic tail of NKp44 is involved in PCNA-mediated suppression of NK function**

We first postulated that PCNA could mediate suppression of NK function through binding competition with activating cellular ligands to NKp44. The only cellular ligand described for NKp44 is HS (15, 17), but soluble PCNA did not interfere with HS binding to NKp44. The only cellular ligand described for NKp44 is HS (15, 17), but soluble PCNA did not interfere with HS binding to NKp44 (data not shown). Therefore, we next tested whether the

![FIGURE 4. PCNA downregulation in various tumor cells enhances lysis by NK cells. A, HeLa cells transfected either with control-siRNA, BAT3-siRNA, or with PCNA-siRNA were labeled radioactively and incubated with NKp44-expressing primary NK cells. NC, untransfected HeLa cells were used as an additional negative control. The lysis of the various targets by NK cells was assayed in a standard 5-h [35S]-release assay (left panel). Effect of siRNA transfection on protein expression, including control β-actin, was measured by Western blotting (right panel). B, Target PANC-1 cells, transfected with either control-siRNA or with PCNA-siRNA, were incubated with various anti-NKp44–activated NK effectors for 18 h. Relative IFN-γ levels in the culture supernatants are shown. C–H, Various cancer cell lines were transfected either with control siRNA or with PCNA-siRNA and assayed for lysis by NK92-44 cells. Effect of siRNA transfection on protein expression, including control β-actin, was measured by Western blotting (inset). As measured by ImageJ freeware, the silencing of PCNA in the target cell lines relative to β-actin in the lysis assays were: HeLa, 61%; PANC-1, 60%; MCF7, 37%; DU145, 67%; U251, 63%; and A375, 8%. The results are from one representative experiment of two performed. Bars ± SD. *p < 0.05, ANOVA.](http://www.jimmunol.org/Download)
suppression might involve the ITIM located in the cytoplasmic tail of NKp44 (7, 24). We employed NK-92 cells transduced with NKp44 in which the cytoplasmic tail is either truncated prior to the ITIM (NK92-44Δ204E) or mutated at the tyrosine within the ITIM (NK92-44Y238F) (Fig. 5A). Previous work showed that removal of the cytoplasmic tail or mutation of the ITIM did not affect NKp44-mediated activation when the receptor was cross-linked with mAbs or through “redirected” cytotoxicity assays (24). Cell surface expression levels of NKp44 were similar between NK92-44Δ204E and NK92-44 Y238F when assayed with an anti-NKp44 mAb (Fig. 5B). Fig. 5C–E shows the summary of two experiments assessing IFN-γ secretion, lytic activity, and CD107a degranulation by these three effector cells in response to HeLa-PCNA-GFP or HeLa-GFP target cells. The overexpression of PCNA resulted in significantly reduced NK92-44 function as compared with that of NK92-44Δ204E or NK92-44Y238F (Fig. 5C–E). In summary, the removal or mutation of the ITIM decreased the effect of PCNA overexpression on NK function.

**PCNA accumulates in the NK immunological synapse**

Several mechanisms have been suggested to be involved in the interaction of membrane-associated NK cell receptors with cytoplasmic or nuclear proteins. In particular for NKp30, CMV-pp65 was shown to be secreted to the supernatant whereas the NF plasmic or nuclear proteins. In particular for NKp30, CMV-pp65 was shown to interact with NKp30 through the exosomal fraction released from immature dendritic cells (20). Therefore, we studied whether soluble PCNA, target cell-conditioned medium, or target cells separated from NK cells by Transwells could mediate PCNA inhibition of NK cell function. None of these conditions could mimic inhibition of NK cell function by PCNA-expressing target cells (Supplemental Fig. 3 A–C). We then tested for PCNA-containing exosomal secretion from HeLa-PCNA-GFP cells. Both endogenous PCNA and transfected PCNA-GFP could be observed in target-secreted exosomes by Western blot analysis (data not shown), but exosomal fractions of HeLa-PCNA-GFP cells did not inhibit NK cell function (Supplemental Fig. 3D).

To verify that direct interaction between NK cells and target cells is imperative for PCNA-mediated effects, DiD labeled HeLa-PCNA-GFP and DiI labeled HeLa-GFP cells were mixed and applied as targets to NK92-44 effectors. As controls, labeled target cells were separately combined with NK cells. Fig. 6A shows that HeLa-PCNA-GFP cells were lysed to a lower extent as compared with HeLa-GFP cells either when used as mixed or separate targets. Therefore, the presence of HeLa-PCNA-GFP cells mixed with the HeLa-GFP target cells did not inhibit the lysis of HeLa-GFP cells, as compared with the lysis of HeLa-GFP cells as the only target. To further corroborate this result, using in vivo conditions, target cells from both lines were i.p. injected together with either NK92 or NK92-44 effector cells into C57BL/6 mice for 6 h (Fig. 6B, 6C). The HeLa-PCNA-GFP/HeLa-GFP ratio was enhanced significantly when NK92-44 effectors were injected as compared with NK92. These in vitro and in vivo results strongly suggest that the PCNA effect is mediated through direct interaction of the NK cell and the PCNA-expressing target cell.

Nanotubes were recently reported as a means for NK cells to sense ligands on target cells (34). We observed nanotubes originating from NK cells and contacting target cells in live imaging confocal microscopy; however, we could not detect any accumulation of PCNA at the tip (target side) of the NK cell nanotubes (Supplemental Fig. 3E). Therefore, we investigated whether PCNA is accumulated in the NKIS using live imaging confocal microscopy. Previous work showed that removal or mutation of the ITIM decreased the effect of PCNA overexpression on NK cell function. The HeLa-PCNA-GFP cells were lysed to a lower extent as compared with HeLa-GFP cells either when used as mixed or separate targets. Therefore, the presence of HeLa-PCNA-GFP cells mixed with the HeLa-GFP target cells did not inhibit the lysis of HeLa-GFP cells, as compared with the lysis of HeLa-GFP cells as the only target. To further corroborate this result, using in vivo conditions, target cells from both lines were i.p. injected together with either NK92 or NK92-44 effector cells into C57BL/6 mice for 6 h (Fig. 6B, 6C). The HeLa-PCNA-GFP/HeLa-GFP ratio was enhanced significantly when NK92-44 effectors were injected as compared with NK92. These in vitro and in vivo results strongly suggest that the PCNA effect is mediated through direct interaction of the NK cell and the PCNA-expressing target cell.

**FIGURE 5.** ITIM in the cytoplasmic tail of NKp44 mediates PCNA-associated inhibition. A, Schematic diagram of the wild-type (wt)-NKp44, Δ204E-truncated NKp44, and Y238F-ITIM-mutated NKp44. Transmembrane domain (TM), domain I (D1), cytoplasmic domain (CY), DAP12-binding lysine residue (K) are marked, and the tyrosine-based ITIM sequences in the cytoplasmic domains are shown. B, Transduced NK effector cells expressing the various NKp44 forms are NK92-44, NK92-44Δ204E, and NK92-44Y238F cells. Cell surface staining of NK effector cells with anti-NKp44 mAb. For functional assays HeLa-PCNA-GFP and HeLa-GFP target cells were used. C, IFN-γ assay. Target cells were incubated with anti-NK44-activated NK effector cells for 18 h. IFN-γ levels in the culture supernatants of target and effector cells are shown. D, Degranulation assay. NK effector cells were coincubated for 4 h with target cells. Cells were then washed and stained for CD107a cell surface expression on CD56+-gated (NK) cells. E, Flow cytometry-based lysis assay. Labeled target cells were incubated with NK effector cells (10:1 E:T ratio) for 5 h and lysis was assayed by 7AAD incorporation. Functional results are the average of three independent experiments, in which results were normalized according to the result of HeLa-GFP target presented as 100%. Bars ± SD. *p < 0.005, ANOVA.
microscopy. We analyzed random fields of time lapse microscopy of DiD-labeled primary human NK cells incubated with HeLa-PCNA-GFP cells or with HeLa-GFP cells and identified the accumulation of PCNA-GFP but not GFP at the interface with conjugated NK cells (Fig. 6D). PCNA-GFP was recruited to the NK cell/target cell interaction surface in 30 and 37% of the synapses counted for NKp44-expressing primary NK and NK92-44 cells, respectively (Fig. 6E). Positive or negative synapses were defined according to the recruitment of GFP or PCNA-GFP to the synapse. Contrast of GFP image in D was enhances to allow a better view (using Olympus FV1000 2.1 software). White and yellow arrows represent positive and negative synapses, respectively (D, left panels). A summary of three independent live-imaging experiments for various NK effector cells with HeLa PCNA-GFP and HeLa-GFP target cells is shown in E. The numbers of monitored synapses were 57 and 56 for primary NK, 53 and 51 for NK92-44, and 12 and 29 for NK92 on HeLa-GFP and HeLa-PCNA-GFP, respectively. For ratio comparison (C), ANOVA was performed on log of the ratio values. *p < 0.001, Fisher exact test.

Discussion

NK cells constitute a key frontline defense against cancer, and NCRs are key activating receptors for tumor recognition. The tumor-associated cellular ligands recognized by NCRs are primarily expressed upon target cell activation, proliferation, or transformation (35). PCNA is commonly overexpressed in cancer cells (36), and its crucial involvement in cellular proliferation and tight association with transformation are well documented (22). PCNA was reported scarcely within the context of NK immunity; an inverse correlation between PCNA index and the ability of NK cells to lyse target cells was observed in cancer patients (37). A...
cisplatin-resistant and PCNA-overexpressing K562 subpopulation was resistant to lysis by NK cells as compared with parental NK-sensitive K562 cells (38). However, the molecular mechanism explaining this correlation was not previously defined. In the present study, we identify PCNA as an inhibitory ligand for NKP44. We have shown 1) direct interaction between rNkp44 and soluble, transfected, or endogenous PCNA; 2) the inhibition of NK cell function by PCNA/NKp44 interactions; 3) the involvement of the NKP44-ITIM in the inhibitory effect of PCNA; and 4) the recruitment of target PCNA to the NKIS that correlates with NKP44 expression on NK cells.

NKP44 is considered to be an NK cell activating receptor, which was shown to be expressed in peripheral NK cells that have been stimulated by IL-2 (7). However, inhibitory function was previously reported for NKP44 expressed on a subset of natural IFN producing cells from tonsils. NKP44 activation in those IFN producing cells did not trigger IFN producing cell-mediated cytotoxicity but, paradoxically, inhibited their IFN-α secretion (39). Our results do not contradict previous evidence that NKP44 engages a ligand on certain tumor target cells to stimulate NK cell responses. For example, NK92-44 cells are more potent than NK-92 cells, and NKP44 blockade attenuates lytic function of primary NK cells and results in similarly suppressed lytic function of NK92-44. Nevertheless, we demonstrate that PCNA serves as an inhibitory ligand for NKP44 that is capable of reducing the activating function induced by the receptor. We demonstrated this inhibition in vitro and also when employing in vivo-like conditions.

We showed that the inhibitory function of PCNA is mediated through the ITIM located in the NKP44 cytoplasmic tail. In contrast, NKP44 triggers NK cell activation through DAP12 association with the transmembrane domain. Our previous work showed that NK cell activation through Ab cross-linking of NKP44 was not influenced by the cytoplasmic ITIM (24). In accordance, we observed in this study that NK92-44Δ204E and NK92-44Y238F cells retained full NKP44-induced triggering capacity but lacked PCNA-mediated inhibition. Additionally, these mutants express NKP44 levels similar to NK92-44, while manifesting higher cytotoxic activity toward target cells than NK92-44 (data not shown; this difference could be attributed to the inhibitory effect of endogenous PCNA in the target cells on NK92-44, which is absent for the mutants that lack an ITIM). Our results further indicate that PCNA is not functioning by blocking NKP44 from interacting with an activating ligand. In contrast, our results indicate that PCNA is engaging NKP44 in an alternative manner to trigger an inhibitory signal, which is mediated through the ITIM. This result does not contradict our previous findings (24) that demonstrated only activation signaling upon nonphysiological Ab engagement of NKP44; instead, we have now identified a physiological ligand that triggers a unique inhibitory response through the same receptor.

PCNA is primarily a nuclear/cytoplasmic factor (36). However, the observation that cytoplasmic or nuclear proteins could serve as ligands to membrane-associated NCRs was previously shown for NKP30 and either CMV-pp65 or nuclear BAT3 (19, 31). The mechanisms suggested were either direct secretion (CMV-pp65) or exosomal secretion (BAT3) (20). However, BAT3 was also reported to translocate to the membrane of target cancer cells upon incubation with NK cells (31). We studied different plausible mechanisms for PCNA/NKP44 interaction and demonstrated that PCNA can be recruited into the NKIS upon interaction of target cancer cells with NKP44-expressing NK cells. Membrane localization of PCNA has not been previously reported, yet we have observed it in cancer cells only upon interaction with NK cells, which was not previously examined. Interestingly, a recent study demonstrated that PCNA interacts with annexin A2, which is described as a membrane-associated protein (40) that is also involved in membrane trafficking and recruitment of proteins to lipid rafts (41). Therefore, it could be that the recruitment of PCNA to the NKIS is facilitated through interaction with proteins that are directed to the immune synapse upon the interaction with NK cells. Our observations of PCNA accumulation at the NKIS were obtained by live cell imaging and are therefore limited to PCNA-GFP. Additional studies are needed to further investigate the interaction of endogenous PCNA with NKP44 at the immunological synapse.

PCNA overexpression is correlated with cancer virulence, and its primary contribution to the cancerous state is mediated by its function at the very heart of many essential processes that are necessary for tumor survival, such as DNA replication, repair of DNA damage, chromatin structure maintenance, chromosome segregation, and cell cycle progression (22). Immune evasion through inhibition of NKP44-mediated NK cell attack is an additional mechanism by which PCNA promotes cancer survival through microevolution in the specific host. NKP44 receptor expression is restricted to primates (10); however, we hypothesize that the PCNA/NKP44 interaction did not evolve in primates due to the cancerous state. Interestingly, the noncancerous tissue that is associated with PCNA overexpression is the decidua and the only known NK cell subset that constitutively expresses NKP44 is decidual NK cells that are tolerized toward decidial tissue in pregnant women (42–44). Decidual NK cells comprise 50–90% of the lymphoid cells present in the decidua during the first trimester (44); this correlates with PCNA overexpression in trophoblasts during the first trimester (45) and with PCNA overexpression in a subset of decidual dendritic cells reported to interact with decidual NK cells (46). Therefore, it is possible that PCNA-induced inhibition through NKP44 is one of the mechanisms ensuring the regulatory and immunotolerant phenotype of decidual NK cells during pregnancy.

In summary, this work demonstrates a new ligand for NKP44, reveals a novel immune evasion mechanism involving NKP44/PCNA interaction, and re-emphasizes the role of nuclear proteins as ligands to natural cytotoxicity receptors. Additionally, it also points to a plausible mechanism involved in the tolerization of decidual NK cells during pregnancy.

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Disclosures
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References


