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# Proliferating Cell Nuclear Antigen is a novel inhibitory ligand for the natural cytotoxicity receptor NKp44

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## Abstract

Natural Killer (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 Antigen (PCNA) is overexpressed in cancer cells. We show that the NKp44 receptor recognizes PCNA. Their interaction inhibits NK cell function through the NKp44-Immunoreceptor Tyrosine-based Inhibitory Motif (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.

## Introduction

Natural killer (NK) 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 cells effector functions (2–4). Major activating receptors include NKG2D 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 trans-membrane 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 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

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various tumors, including carcinomas, melanomas, neuroblastomas, myeloid and lymphoblastic leukemia, multiple myeloma and Epstein–Barr virus-transformed B cells. Remarkably, the involvement of a given NCR in triggering cytolysis 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 co-ligand 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 cytomegalovirus (CMV) structural protein pp65 and HLA-B associated transcript 3 (BAT3), are not membrane-associated proteins, but are considered to be nuclear/cytoplasmic. Arnon *et al* showed an antagonistic effect of the main CMV tegument protein, pp65, on NKp30, since extracellular-released pp65 dissociated TCR-ζ from NKp30 and consequently reduced killing (19). Others reported that BAT3 over-expression enhanced release of BAT3-containing exosomes from dendritic cells and activated NK cells (20). Here, we report the interaction between NKp44 and proliferating cell nuclear antigen (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 over-expression is associated with enhanced malignancy (23). We show that the interaction of NKp44 with target cell-expressed PCNA paradoxically inhibits lysis and IFNγ secretion by NK cells. The inhibition is mediated by an immunoreceptor tyrosine-based inhibitory motif (ITIM) on the NKp44 cytoplasmic domain, and the nuclear/cytoplasmic 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: PANC-1 – human pancreatic ductal carcinoma (ATCC no. 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 thymoma cells (TIB-48), NK-92 - a human natural killer lymphoma (CRL-2407), and HEK293T- SV40 large T antigen-transfected HEK293 cells (CRL-11268). The NK-92 cell lines, transduced by retrovirus to express high levels of wild-type and mutated NKp44 (designated as NK92-44, NK92-44.Δ204E and NK92-44.Y238F), were characterized in detail elsewhere (24). NKL– human NK cell line was described elsewhere (25).

### Antibodies and fusion-Ig proteins

The following antibodies were used: anti-NKp44 mAb, anti-NKp30 mAb (R&D SYSTEMS), anti-His tag, anti-PCNA (Santa Cruz Biotechnology), APC-conjugated anti-CD56 (Biolegend), FITC-conjugated anti-CD3 (DAKO) and biotin-conjugated mouse anti-human CD107a/LAMP-1 mAb (SouthernBiotech). APC-conjugated $F(ab')_2$ goat anti-human-IgG, APC-conjugated F(ab')2 goat anti-mouse IgG, and APC-conjugated Streptavidin (Jackson ImmunoResearch). 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 NKG2D-Ig was previously described (26, 27). Human IgG1 (hIgG1 kappa, PHP010, Serotec), and

Human IgG was purchased from Sigma. DiO, DiI, and DiD Vybrant$^{tm}$ Cell-Labeling Solution (v22889, Molecular Probes).

### Production of recombinant soluble PCNA and HNF-4

To produce soluble PCNA and HNF-4 we employed a β-gal induced bacterial system. The pET-28 vector, which carries PCNA coding sequence and C-terminal Histidine tag (His-Tag) sequence, was used to transduce *E. coli* BL21 competent cells (primers are described in Table S1). Following transformation, selection and isopropyl β-D-1-thiogalactopyranoside-based induction, bacteria cells were lysed, sonicated and loaded on a Ni column. 0.5M Imidazole buffer was used to elute the protein. HNF-4 coding sequence in the same His-tag-containing pET-28 vector was kindly provided by Prof. Boaz Shaanan, Ben Gurion University, and the protein was produced with the same protocol.

### Constructs and transfections

Human PCNA (Acc. No. CAG46598) was cloned with specific primers (Sigma-Aldrich) from a HeLa-derived cDNA library (Table S1). For production of BW-PCNA, PCNA was cloned in-frame into a pCDNA3.1+ ζ-chain containing cassette (19); BW5147 cells were then transfected by electroporation (field strength 0.95kV/cm, voltage 250V, capacity 970μF) to produce BW5147-PCNA-ζ 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 (SGLRSRAQALQGQGQRS) 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.

### siRNA-based post transcriptional silencing

Human PCNA, BAT3 and control siRNA (Santa Cruz) were used according to the manufacturer's protocol to down-regulate protein levels in several human cancer cell lines. Approximately 48 hrs post-transfection, 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 0–4μg/ml of recombinant His-tag PCNA or HNF4. After blocking, 4μg/ml of various Ig-fusion NK proteins were added. HRP-conjugated goat anti-human IgG, Fcγ fragment-specific (Jackson) Ab (0.2μg/ml) was used for detection. Alternatively, plates were coated with 0–4μg/ml of the various Ig-fusion NK receptors. After blocking, 4μg/ml of recombinant His-tag PCNA or HNF4 were added. Anti-His mAb and HRP-conjugated goat anti-mouse IgG, Fcγ fragment-specific (Jackson) Ab (0.2μg/ml) were used for detection, as measured via optical density at 650 nm with a Dynex Technologies MRX Microplate reader. For blocking assay, NKp44-Ig was pre-incubated 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), 100mM NaCl, 0.1% CHAPS, 1mM DTT, 0.1mM EDTA protease inhibitor cocktail (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) pre-coated with NKp44-Ig or LIR-Ig, and incubated with either cell lysates or recombinant His-tag PCNA or His-tag HNF4. 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 the 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 APC-conjugated anti-human-IgG Ab. Cells were labeled with anti-NKp44 mAb, or anti-PCNA mAb, washed, and stained with APC-conjugated anti-mouse IgG. Dead cells were detected with propidium iodide (PI). Flow cytometry was performed using a FACSCanto II (BD Bioscience) 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 more then 90% ($CD3^-$ $CD56^+$). Purified NK cells were cultured in CellGro SCGM serum-free medium (CellGenix) supplemented with 10% heat inactivated human plasma from healthy donors, 1 mM sodium pyruvate, 2 mM L-glutamine, 1X MEM non-essential amino acids, 1% penicillin/streptomycin, 10 mM HEPES (Gibco®) and 300 IU/ml of human IL-2 (Biological Industries).

### IFNγ secretion and CD107a degranulation assays for NK cell activity

IFNγ assays: 96 well plates were pre-coated with 0.25 μg/mL anti-NKp44 mAb. NK effector cells ($5 \times 10^4$ cells/well) were mixed with target cells ($1.5 \times 10^5$ cells/well) and incubated for 18hr. IFNγ concentrations in the supernatants were then assayed by standard ELISA assay. NK degranulation assay was performed as previously described (15, 16).

### Cytotoxicity and *in-vivo* killing assays

Cytotoxicity assays: Target cells were labeled with $S^{35}$ for 12 hrs. Then, the cytotoxic activity of NK92 and NK92-44 cell lines and primary human NK cells was assessed in a 5-h $S^{35}$-release assay, as previously described (29). In all experiments shown, the spontaneous release was less than 25% of maximal release. The flow cytometry cytotoxicity assay was previously described (30). Target cells were labeled with either DiO, DiI, or DiD. Analysis of 7-AAD was performed using FACSCantoII and data was analyzed by FlowJo. *In-vivo* killing assay: Target cells were labeled with either DiI or DiD, and mixed in a 1:1 ratio. Effector NK cells were added immediately before intra-peritoneum (IP) injection into C57BL/6 mice. All injections were performed in 300μl PBS, $1\times10^6$ of each target cell population and $12\times10^6$ of NK cells per mouse. Six hrs after the injections, mice were killed and the peritonea 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 pre-incubated 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 chip 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 100mM of EDC and 25mM of S-NHS over the chip for 300sec. Estimated activation was 150RU for all channels. 0.145μg of His-tag PCNA was immobilized on the chip. Binding levels were 200 RU and 100 RU, respectively. Different concentrations (from 1μM to 0.03μM) of analyte: NKp44-Ig, NKp46D2-Ig, Np30-Ig and Human-Fc were injected. Kinetic measurements: Data processing was done using the 1:1 Langmuir binding model with mass transfer and baseline

drift. The $\chi^2$ 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 20units/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 60X Oil NA:1.35), random fields were selected and time lapse images were acquired every 2 minutes for 6 hours. Zero Drift Correction (ZDC) was used to avoid loss of the focal plane in the Z direction during time lapse acquisition. In post acquisition analysis; immunological synapse-positive conjugates were defined as stable effector-target interactions that lasted for more then 6 min.

## Results

### Direct binding of recombinant NKp44 to recombinant PCNA

We explored possible ligands to NCRs by expressing various ligand candidates on yeast cell surfaces and testing binding of recombinant NCRs. As a negative control, we employed yeast cells displaying the nuclear protein PCNA on their cell surface. Interestingly, the negative control manifested considerable binding to recombinant NKp44 (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, B). Other recombinant NK receptors, LIR1, NKG2D, KIR2DL4, NKp30 and NKp46 bound only marginally and their weak background binding was not affected by the amount of PCNA (Fig. 1A, B). Alternatively, we coated wells with titrated quantities of NKp44-Ig or other recombinant NK receptors and assayed the binding of soluble PCNA or hepatocyte nuclear factor 4 (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 antibodies significantly suppressed the interaction between NKp44 and PCNA, but 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 Figure 1F. NKp44-Ig displayed a characteristic binding curve to PCNA ($K_D$ = $3.4 \times 10^{-9}$ M), however, no significant interaction could be detected with the other NCRs-Ig. To summarize, we have shown direct *in vitro* interaction between recombinant NKp44 and PCNA.

### Binding of recombinant NKp44 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, while 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 nor NKp46-Ig, manifested enhanced binding to BW cells transfected with membrane-expressed PCNA, as compared to parental BW cells (Fig. 2C–E). To summarize, we have shown direct interaction between recombinant NKp44 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 accordance with previous studies with GFP-fused PCNA (32, 33). On the other hand, 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 sub-cloning; Figure 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 times greater compared to 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 antibody, NKG2D-Ig, NKp30-Ig, and NKp46-Ig revealed no difference between the two transfected pools (HeLa-PCNA-GFP and HeLa-GFP, Fig. S1). NKp44-Ig staining also showed no difference (Fig. S1) 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 (i) NK-92 cells that express low levels of NKp44, (ii) NKp44-transduced NK-92 cells that express high levels of wild-type NKp44 (designated as NK92-44), (iii) IL-2 cultured primary human NK cells that express NKp44, and (iv) NKL cells that do not express NKp44. Figure 3D shows the different NKp44 expression levels by the NK effector cells. We studied IFNγ secretion from anti-NKp44-activated NK effectors co-incubated with either HeLa-GFP or HeLa-PCNA-GFP target cells (Fig. 3E). PCNA overexpression correlated with suppression of IFNγ secretion by NKp44-expressing NK cells; incubation of $NKp44^{high}$ NK92-44 and primary NK cells with HeLa-PCNA-GFP target cells resulted in significantly reduced IFNγ secretion as compared to incubation with HeLa-GFP target cells (Fig. 3D, E). In contrast, no significant difference in IFNγ secretion was observed for $NKp44^{null}$ effector NKL cells or $NKp44^{dull}$ NK-92 cells (Fig. 3D, E). Therefore, PCNA-overexpression in the HeLa cells resulted in reduced IFNγ 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, G), but lysis of HeLa-PCNA-GFP target cells by NK92-44 effector cells was significantly suppressed as compared to lysis of HeLa-GFP target cells. In contrast, lysis of the two target cell lines by NK-92 was similar (Fig. 3F, G). 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 antibodies, but not with mock antibodies, 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 antibodies, but not with mock antibodies, 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 upon NKp44 expression by NK cells, and is abolished by antibodies to NKp44.

## Down-regulation 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-down-regulation through an siRNA approach. siRNA-mediated down-regulation 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 (Pogge von Strandmann et al., 2007), siRNA down-regulation resulted in reduced lysis by NK cells (Fig. 4A). siRNA down-regulation of PCNA was confirmed by western blot (Fig. 4A). We then exmained NK IFNγ secretion to demonstrate the effects of siRNA-mediated downregulation of endogenous target cell PCNA in an assay independent of target cell lysis. Down-regulation of endogenous PCNA in target cancer cells enhanced IFNγ secretion upon incubation with NKp44 expressing primary human NK cells or with NK92-44 cells; in contrast, incubation of PCNA-downregulated target cells with $NKp44^{null}$ NKL cells resulted in no enhancement of IFNγ 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 target PCNA effect on NK function is not limited to HeLa cells, we tested 5 more human target cancer cells, derived from different tissues, employing siRNA-mediated down-regulation of PCNA (Fig. 4D–H). Down-regulation 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 (i) transient siRNA-based down-regulation of PCNA did not affect proliferation or viability of target cancer cells (data not shown); (ii) all target cell lines tested expressed considerable levels of endogenous PCNA (Fig. S2); and (iii) Staining with anti HLA class I antibody and NKG2D-Ig revealed no difference between control-siRNA and PCNA-siRNA treatment (checked for PANC-1 cells). To summarize, successful down-regulation of endogenous PCNA resulted in enhanced lysis and IFNγ 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 (data not shown). Therefore we next tested whether the 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 NK92-44, NK92-44Δ204E and NK92-44 Y238F when assayed with an anti-NKp44 mAb (Fig. 5B). Figure 5C–E shows the summary of two experiments assessing IFNγ secretion, lytic activity, and CD107a de-granulation by these 3 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 to that of NK92-44Δ204E or NK92-44Y238F (Fig. 5C–E). To summarize, the removal or mutation of the ITIM decreased the effect of PCNA overexpression on NK function.

## PCNA accumulates in the natural killer 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 while the nuclear factor BAT3 was shown to interact with NKp30 through the exosomal fraction released from iDCs (20). Therefore, we studied whether soluble PCNA, target cell-conditioned medium, or target

cells separated from NK cells by trans-wells could mediate PCNA inhibition of NK cell function. None of these conditions could mimic inhibition of NK cell function by PCNA-expressing target cells (Fig. S3A–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 (Fig. S3D). To verify that direct interaction between NK cell and target cell 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. Figure 6A shows that HeLa-PCNA-GFP cells were lysed to a lower extent as compared to 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 to 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 injected IP together with either NK92 or NK92-44 effector cells into a C57BL/6 mice for 6 hrs (Fig. 6B, C). The HeLa-PCNA-GFP/HeLa-GFP ratio was enhanced significantly when NK92-44 effectors were injected as compared to 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; yet, we could not detect any accumulation of PCNA at the tip (target side) of the NK cell nanotubes (Fig. S3E). Therefore, we investigated whether PCNA is accumulated in the NKIS using live imaging confocal 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). Note that PCNA recruitment was observed not earlier than 2 hours post incubation start. In sharp contrast, PCNA-GFP was not recruited to the NK cell-target cell interaction surface in the synapses with NKp44$^{dull}$-NK92 cells. In addition, GFP was not recruited to the synapse in any HeLa-GFP cells conjugated with any of the NK effector cells (Fig. 6E). Note that cell surface expression of PCNA was detected only upon direct interaction of HeLa-PCNA-GFP with NK cells. Therefore, detectable recruitment of target cell PCNA to the NK cell-target cell interaction surface correlates with a high surface density of NKp44 on the NK cell. Taken together, our results indicate that PCNA is recruited to the NKIS and the presence of NKp44 enables its accumulation.

## Discussion

Natural killer 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 over-expressed 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; 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-over-expressing K562 subpopulation was resistant to lysis by NK cells as compared to parental NK-sensitive K562 cells (38). However, the molecular mechanism explaining this correlation was not previously defined. In the current study, we identify PCNA as an inhibitory ligand for NKp44. We have shown

(i) direct interaction between recombinant NKp44 and soluble, transfected, or endogenous PCNA, (ii) the inhibition of NK cell function by PCNA-NKp44 interactions, (iii) the involvement of the NKp44-ITIM in the inhibitory effect of PCNA, and (iv) 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 interferon producing cells (IPCs) from tonsils. NKp44 activation in those IPCs did not trigger IPC-mediated cytotoxicity but paradoxically, inhibited their IFNα 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 trans-membrane domain. Our previous work showed that NK cell activation through antibody cross-linking of NKp44 was not influenced by the cytoplasmic ITIM (24). In accordance, we observed here that NK92-44Δ204E and NK92-44Y238F cells retained full NKp44-induced triggering capacity but lacked PCNA-mediated inhibition. In addition, 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 non-physiological antibody engagement of NKp44, but 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). Yet, 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 is recruited to 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 (out of the scope of this paper) 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 micro-evolution in the specific host. NKp44 receptor expression is restricted to primates (10); yet, we hypothesize that the PCNA-NKp44 interaction did not evolve in primates due to the cancerous state. Interestingly, the non-cancerous 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 decidual tissue in pregnant women (42–44). Decidual NK cells comprise 50% to 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 DC 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.

To summarize, this work demonstrate a new ligand for NKp44, reveal a novel immune evasion mechanism involving NKp44-PCNA interaction and re-emphasize the role of nuclear proteins as ligands to natural cytotoxicity receptors. Yet, it also point to a plausible mechanism involved in the tolerization of decidual NK during pregnancy.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

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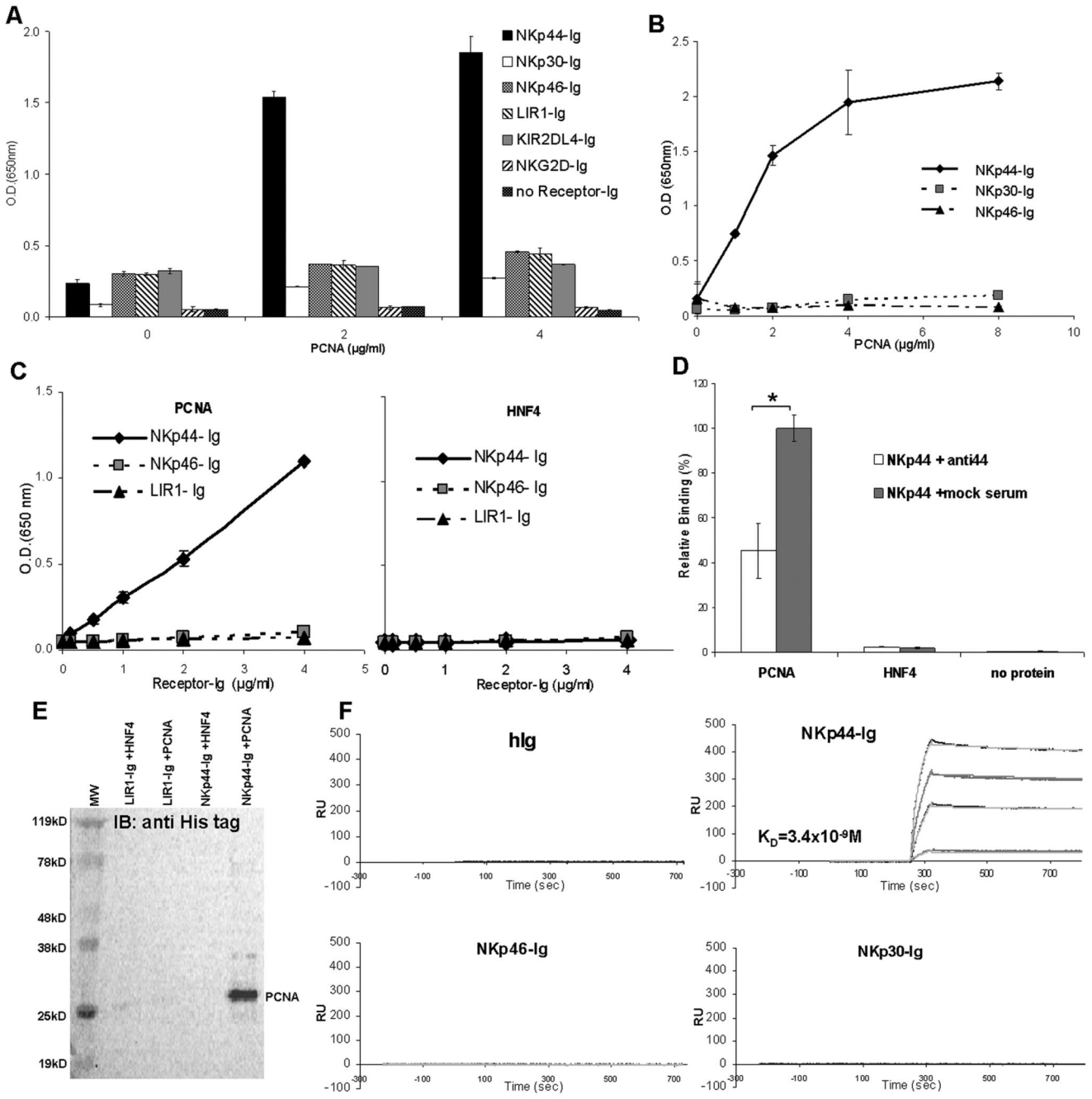


**Figure 1. NKp44-Ig binds human PCNA specifically**

(A, B) ELISA plates were coated with titrated concentrations of recombinant PCNA, followed by incubation with 4µg/ml of different human Fc-chimeric NK receptors and detection with HRP-conjugated anti-human Fc Ab. (C) ELISA plates were coated with titrated concentrations of recombinant NK receptors, followed by incubation with 4µg/ml His-tag PCNA (C left panel) or His-tag HNF-4 (C 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 pre-incubated with anti-NKp44 or mock mouse serum and detection with HRP-conjugated anti-human Fc Ab. Bars ± SD. * P-value < 0.05, ANOVA. (E) Protein-A sepharose beads were coated with NKp44-Ig or LIR1-Ig, followed

by incubation with 2.8μg of His-tagged PCNA or HNF-4 in 100μl 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 Resonance Units (RU) against time. Human Ig (hIg) (upper left panel), 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 1μM. $K_D$ was calculated using the 1:1 Langmuir binding model with mass transfer and baseline drift. $\chi^2$<0.05 of Rmax. The results are from one representative experiment of at least two performed.

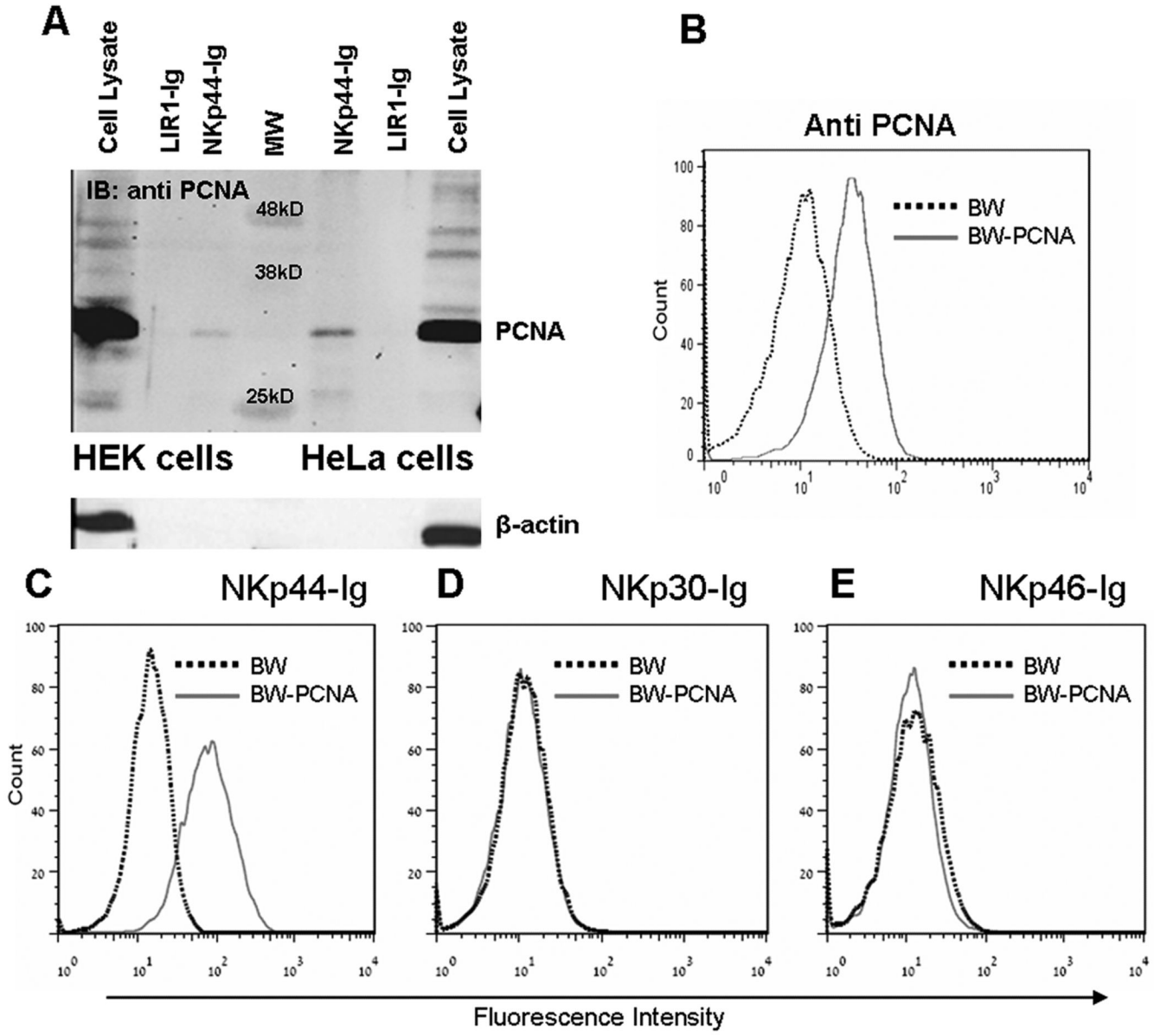


**Figure 2. Direct binding of NKp44-Ig to endogenous and transfected PCNA**

(A) Lysates from HeLa and HEK293T cells were immunoprecipitated with protein-A sepharose beads coated with NKp44-Ig or LIR1-Ig. Eluates were immunoblotted with anti-PCNA mAb or anti β-actin mAb as a control. (B) BW and BW-PCNA cells were stained with anti-PCNA mAb to assay PCNA monomer expression on the cell surface. (C–E) Cell surface staining of BW and BW-PCNA cells with NKp44-Ig (C), NKp30-Ig (D) and NKp46-Ig (E). Staining results were analyzed by flow cytometry. The results are from one representative experiment of 3 performed.

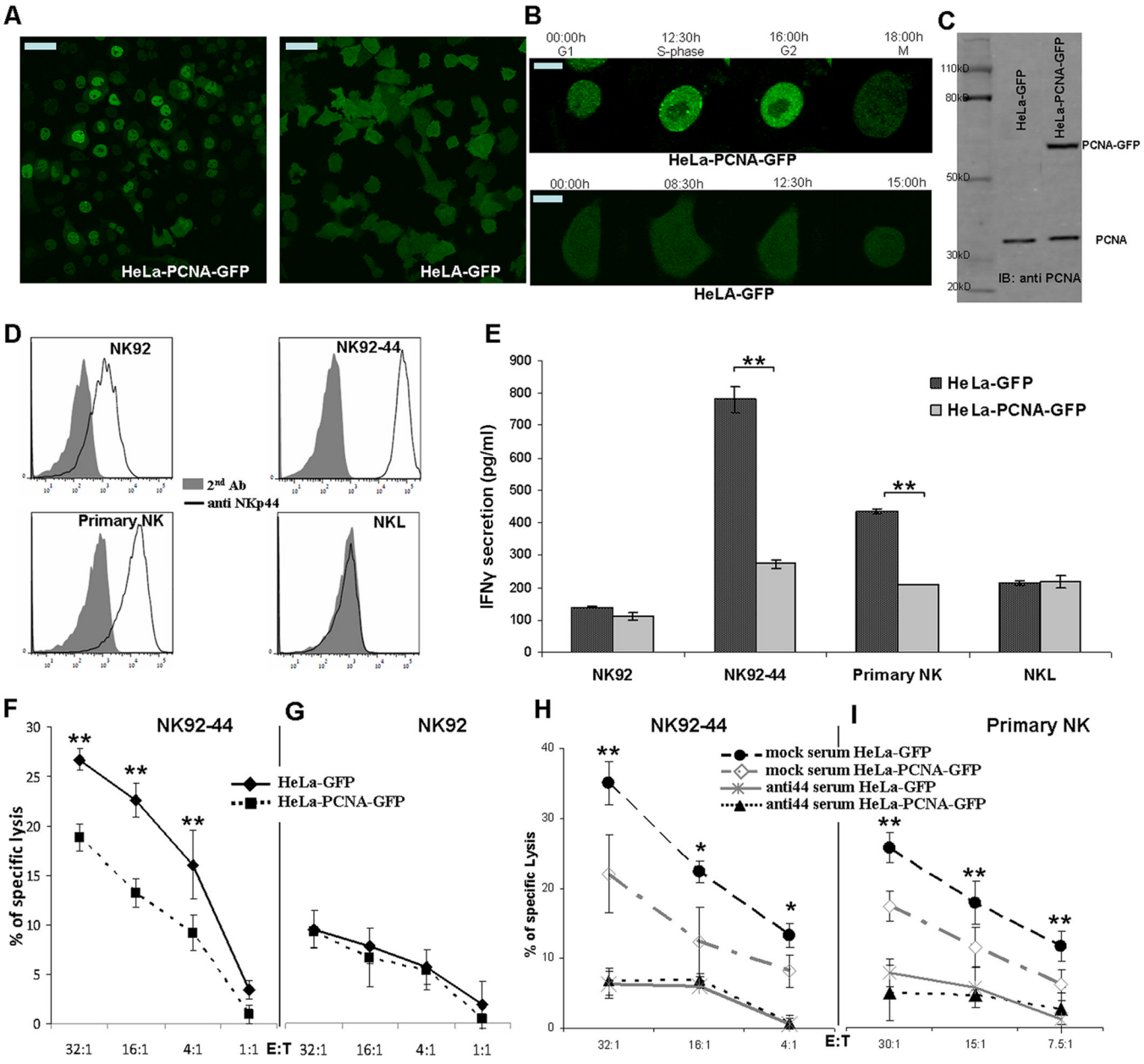


**Figure 3. PCNA overexpression is associated with NKp44-mediated suppression in NK function**

HeLa PCNA-GFP or HeLa-GFP target cells images:(A) Images of HeLa-PCNA-GFP (left panel) and HeLa-GFP cells (right panel), [scale bar = 40μm]. (B) Cropped images taken throughout the division cycle of a single cell: HeLa-PCNA-GFP (upper panel) and HeLa-GFP (lower panel), [scale bar = 10μm]. (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 hrs. IFNγ levels in the culture supernatants are shown. (F–I) Target cells were radioactively labeled with $S^{35}$ for 12 hrs, 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 $S^{35}$ release assay. 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 2–4 performed. Bars ± SD. * P-value < 0.05, ** P-value < 0.01, ANOVA.

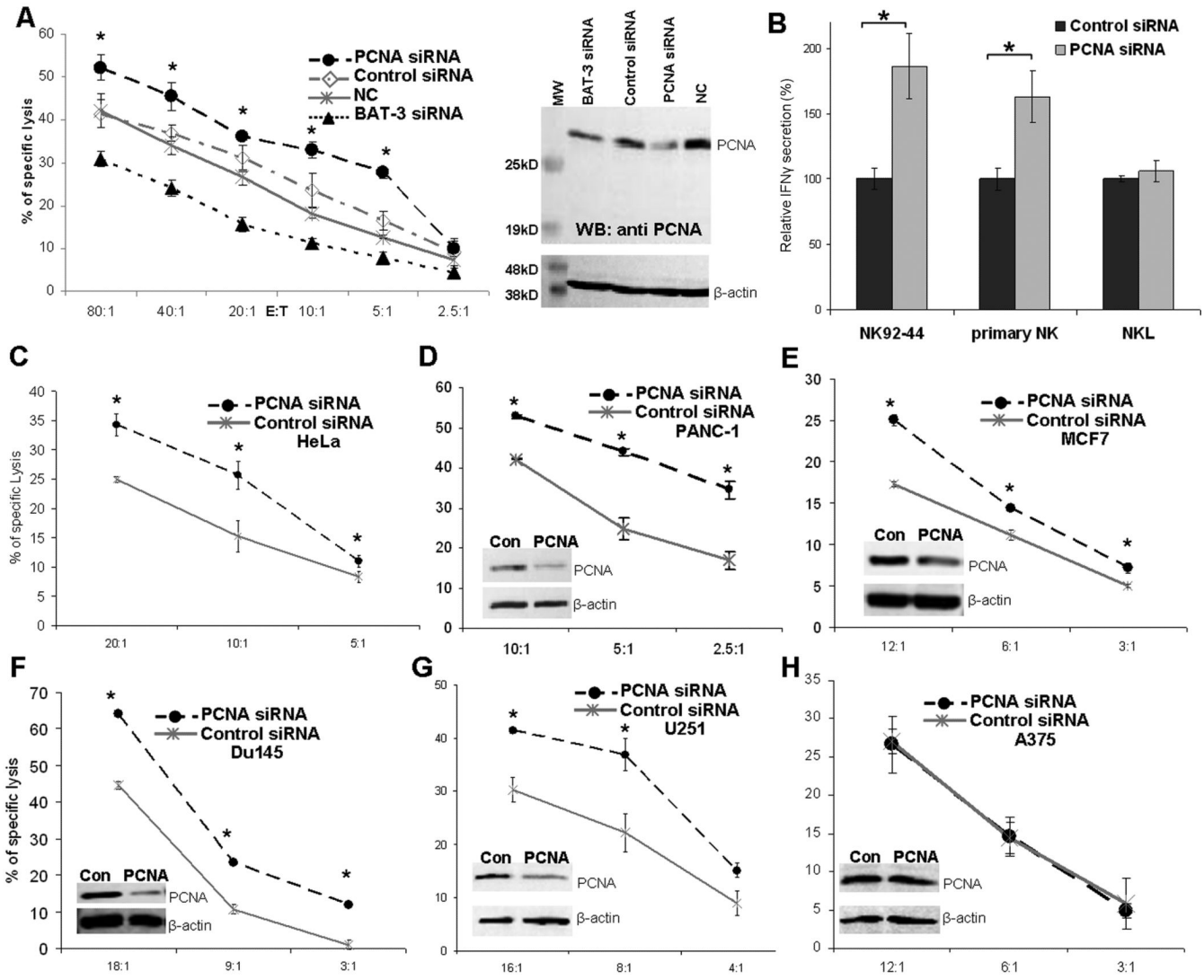


**Figure 4. PCNA down-regulation in various tumor cells enhances lysis by NK cells**

(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 additional negative control. The lysis of the various targets by NK cells was assayed in a standard 5-h $S^{35}$ 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 hrs. Relative IFNγ 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 2 performed. Bars ± SD. * P-value < 0.05, ANOVA.

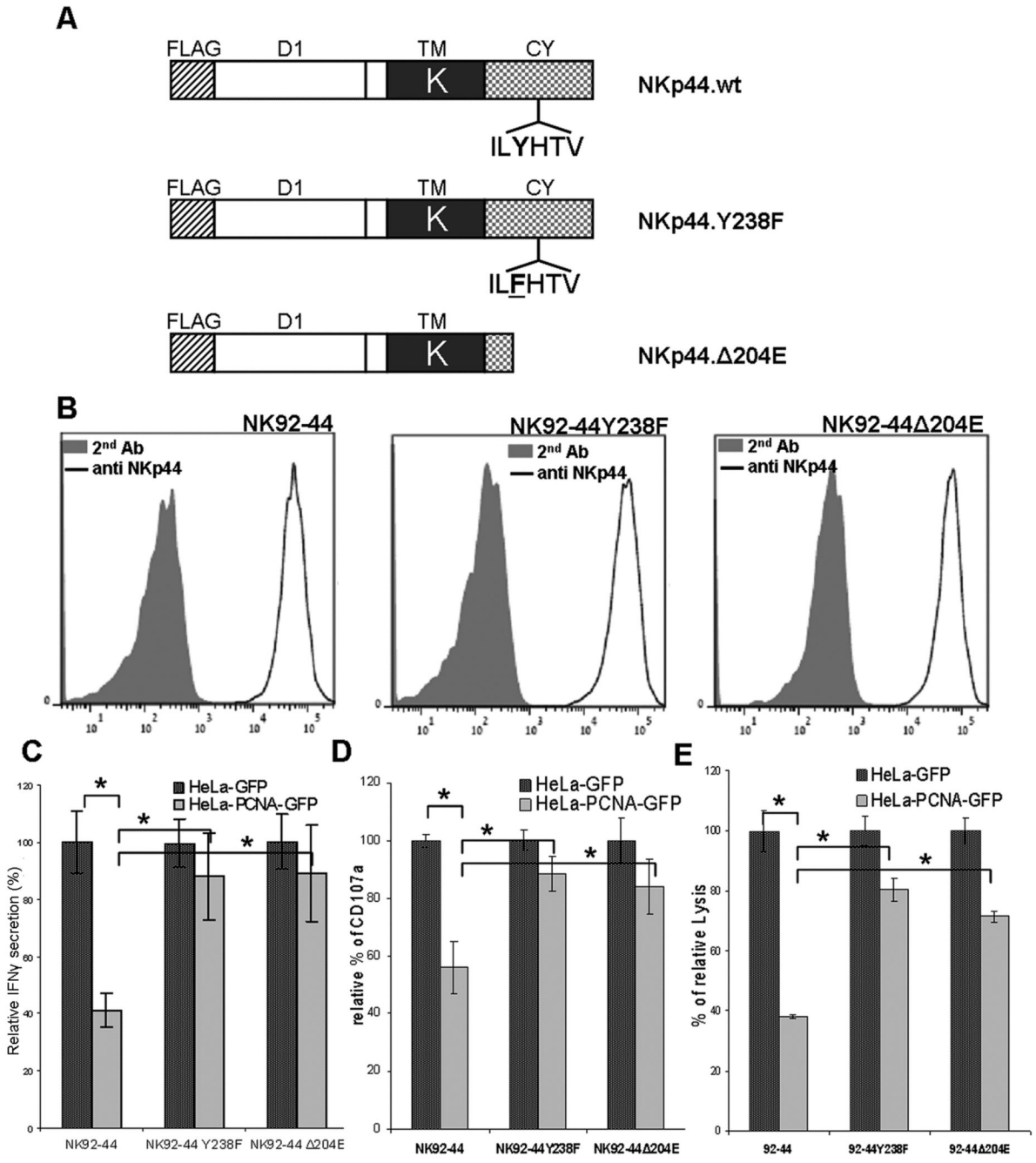


**Figure 5. ITIM in the cytoplasmic tail of NKp44 mediates PCNA-associated inhibition**

(A) Schematic diagram of the wt-NKp44, Δ204E-truncated NKp44 and Y238F-ITIM mutated NKp44. Transmembrane domain (TM), Domain 1 (D1), cytoplasmic domain (CY), DAP12-binding lysine residue is marked (K), and the tyrosine-based ITIM sequences in the cytoplasmic domains. (B) Transduced NK effector cells expressing the various NKp44 forms are NK92-44, NK92-44Δ204E and NK92-44 Y238F 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) IFNγ assay: target cells were incubated with anti-NKp44-activated NK effector cells for 18 hrs; IFNγ levels in the culture supernatants of target and effector cells are shown. (D) Degranulation assay: NK effector cells were co-incubated for 4

hrs 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) for 5 hrs and lysis was assayed by 7AAD incorporation. Functional results are the average of 3 independent experiments, in which results were normalized according to the result of HeLa-GFP target presented as 100%. Bars ± SD. * P-value < 0. 005, ANOVA.

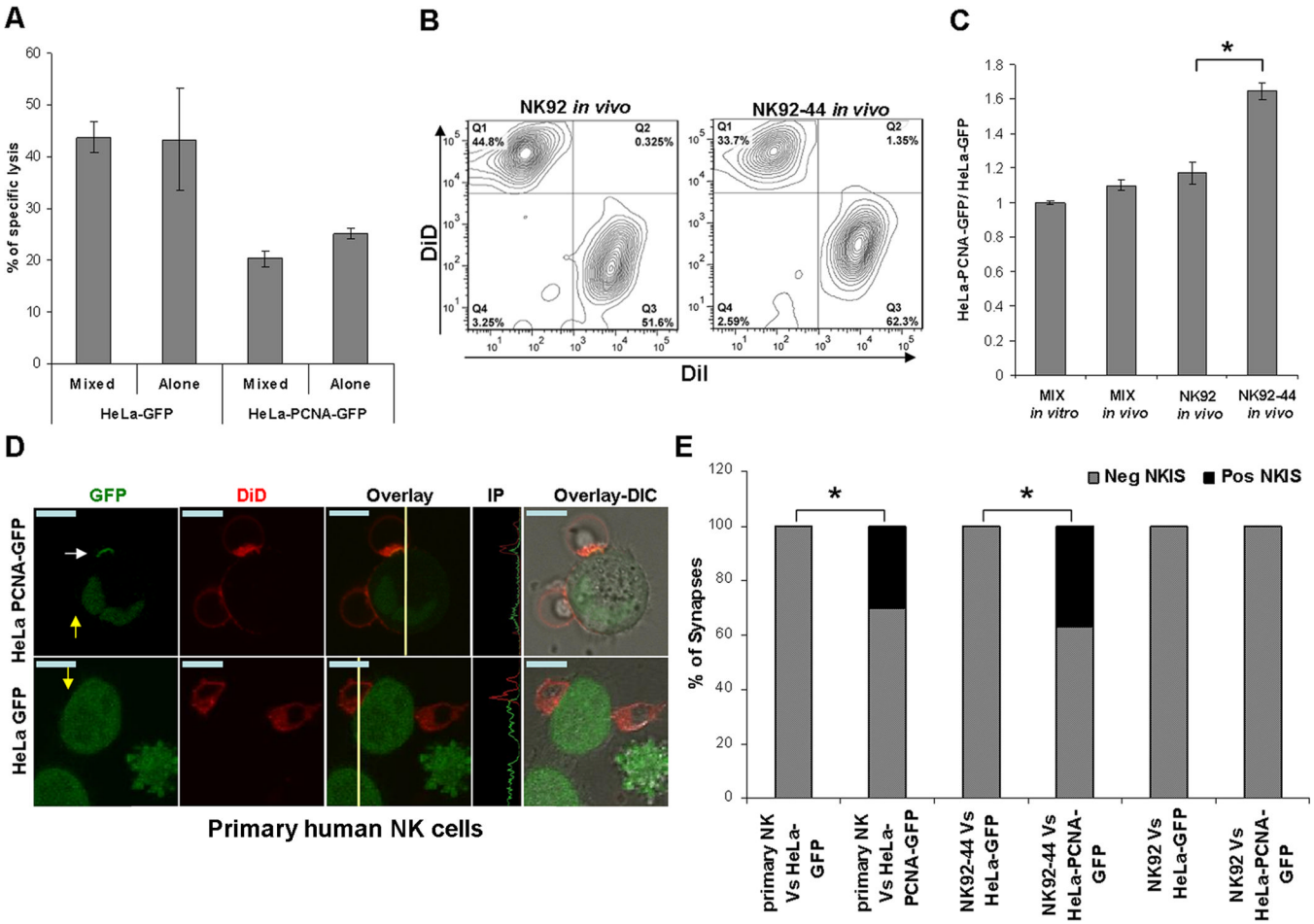


**Figure 6. PCNA is recruited to the NK immunological synapse**

(A) Flow cytometry-based lysis assay: target HeLa-PCNA-GFP and HeLa-GFP were labeled with either DiD or DiI. In repeated experiments, labeling dyes were switched between target cells to avoid dye-mediated bias. Differently-labeled HeLa-PCNA-GFP and HeLa-GFP were either mixed (5×104 cells/well from each) or used separately (105/well). Mixed or separate target cells were incubated with NK92-44 (10:1 E:T) for 5 hrs and lysis was assayed by 7AAD incorporation. (B–C) In vivo lysis: fluorescently-labeled mixed target cells (DiD and DiI) were co-injected IP either with NK92 or NK92-44. Six hrs later, peritoneal lavage was assayed for the presence of target cells. (B) Representative contour plot of peritoneal lavage from mouse inoculated with target mix and NK-92 (left panel) or NK92-44 (right panel). Target mix contained DiI-labeled HeLa-PCNA-GFP and DID-labeled HeLa-GFP and plot was gated on GFP positive cells. (C) Ratio of HeLa-PCNA-GFP/HeLa-GFP target cells in mice (3 mice in each group) peritoneal lavage, the results are from one representative experiment of 2 performed. (D, E) HeLa PCNA-GFP and HeLa-GFP target cells were cultured in a chambered μ-slide and incubated overnight at 37°C. DiD-labeled primary NK cells were added and the slide was mounted to the FV1000 system (objective 60X, NA:1.35). Random fields were selected and time lapse images were acquired every 2 minutes for 6 hours. Representative images are shown in (D) for HeLa-PCNA-GFP (upper panels) and HeLa-GFP (bottom panels) incubated with primary human NK cells. Intensity profile (IP) of green (GFP) and red (DiD) channels is also shown [scale bar = 10μm]. Positive or negative synapses were defined according to the recruitment of GFP or PCNA-GFP to the synapse. Contrast of GFP image in panel D was enhances to

allow better view (using Olympus FV1000 2.1 software). White and yellow arrows represent positive and negative synapses, respectively (D, left panels). A summary of 3 independent live-imaging experiments for various NK effector cells with HeLa PCNA-GFP and HeLa-GFP target cells is shown in (E). Number of monitored synapses was 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. * P-value < 0.001. Fisher's exact test (E). For ratio comparison (C) ANOVA was performed on log of the ratio values.