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Anja Fuchs; ... et. al

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CUTTING EDGE

# Cutting Edge: CD96 (Tactile) Promotes NK Cell-Target Cell Adhesion by Interacting with the Poliovirus Receptor (CD155)

*Anja Fuchs, Marina Cella, Emanuele Giurisato, Andrey S. Shaw, and Marco Colonna*[1]

***The poliovirus receptor (PVR) belongs to a large family of Ig molecules called nectins and nectin-like proteins, which mediate cell-cell adhesion, cell migration, and serve as entry receptors for viruses. It has been recently shown that human NK cells recognize PVR through the receptor DNAM-1, which triggers NK cell stimulation in association with $\beta_2$ integrin. In this study, we show that NK cells recognize PVR through an additional receptor, CD96, or T cell-activated increased late expression (Tactile). CD96 promotes NK cell adhesion to target cells expressing PVR, stimulates cytotoxicity of activated NK cells, and mediates acquisition of PVR from target cells. Thus, NK cells have evolved a dual receptor system that recognizes nectins and nectin-like molecules on target cells and mediates NK cell adhesion and triggering of effector functions. As PVR is highly expressed in certain tumors, this receptor system may be critical for NK cell recognition of tumors. The Journal of Immunology,* 2004, 172: 3994–3998.***

Natural killer cells recognize virally infected and tumor cells using multiple receptors with diverse structures, specificities, and signaling properties (1, 2). These receptors activate cytoplasmic protein tyrosine kinases, phosphoinositol kinases, and mitogen-activated protein kinases, which trigger NK cell secretion of cytotoxic granules and IFN-$\gamma$ (3). NK cell functions are also critically dependent on cell surface molecules that mediate adhesion of NK cells to other cells (4). Typically, these adhesion molecules include $\beta_2$ and $\beta_1$ integrins, which interact with ICAM-1 and -2 and VCAM, respectively. By promoting NK cell-target cell adhesion, integrins allow triggering of activating NK cell receptors by their cognate ligands. In turn, some activating receptors further strengthen the NK cell adhesion mediated by integrins (5).

The close cooperation between activating receptors and adhesion molecules in stimulating NK cells is exemplified by the activating receptor DNAM-1, also called CD226 (6, 7). DNAM-1 is a member of the Ig superfamily that stimulates NK cells by recruiting the protein tyrosine kinase Fyn (6, 7). In leukocyte adhesion-deficient patients that lack $\beta_2$ integrin, DNAM-1 does not deliver a stimulatory signal, indicating that DNAM-1 is physically and functionally coupled with $\beta_2$ integrin (8). It has been recently shown that DNAM-1 recognizes the poliovirus receptor (PVR,[2] or CD155) and the poliovirus-related receptor 2 (PRR2 or CD112) on target cells (9). PVR and PRR2 belong to a large family of Ig-like molecules called nectins and nectin-like proteins, which mediate cell-cell adhesion, cell migration, and cell polarization by homotypic contact or heterotypic interaction with other nectins (10). In addition, nectins and nectin-like proteins serve as entry receptors for poliovirus and HSVs (11). To date, it is unknown whether the multiplicity of nectins and nectin-like proteins is matched by a comparably diverse assortment of activating receptors and adhesion molecules on NK cells.

In this study, we show that CD96 or T cell-activated increased late expression (Tactile) (12) is another NK cell receptor for PVR. CD96 promotes NK cell adhesion to target cells expressing PVR, stimulates cytotoxicity of activated NK cells, and mediates acquisition of PVR from target cells. These results indicate that NK cells have evolved a dual receptor system to recognize nectins and nectin-like molecules on target cells, which mediates NK cell adhesion and triggering of NK cell effector functions.

## Materials and Methods

*Cells and transfectants*

NK92 cells were kindly provided by M. L. Botet (University Pompeu-Fabre, Barcelona, Spain). Human NK cells were obtained from $CD56^+CD3^-$ PBMC as described (13). CD96, DNAM-1, and PVR full-length cDNAs were amplified by RT-PCR, cloned into pEF6/V5-His A (Invitrogen, Carlsbad, CA), and transfected into P815 murine mastocytoma cells (P815-CD96 and P815-DNAM-1), human Jurkat T cells (Jurkat-PVR), or human Daudi B cells (Daudi-PVR).

Department of Pathology and Immunology, Washington University School of Medicine, St. Louis, MO 63110

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[1] Address correspondence and reprint requests to Dr. Marco Colonna, Department of Pathology and Immunology, Washington University School of Medicine, 660 South Euclid, St. Louis, MO 63110. E-mail address: mcolonna@pathology.wustl.edu

[2] Abbreviations used in this paper: PVR, poliovirus receptor; PRR, poliovirus-related receptor; Tactile, T cell-activated increased late expression; ED, ectodomain; GFP, green fluorescent protein; CEACAM, carcinoembryonic Ag-related cell adhesion molecule; ITIM, immunoreceptor tyrosine-based motif.



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*PVR-ED-IgG and PVR-D1-IgG*

The PVR ectodomain (PVR-ED) and membrane distal Ig domain (PVR-D1) were amplified from PVR cDNA by PCR using a common forward primer (5′-ATGGCCCGAGCCATGGCCGCCGCGTGG-3′) and specific reverse primers (PVR-ED: 5′-GTTACGGGACATGCCTGAGTGC-3′, PVR-D1: 5′-CTTGGCAAGCACTCGGAGCCA-3′). Amplified products were cloned into pHuIgG1 and expressed as human IgG fusion proteins as previously described (14). Binding of PVR-ED-IgG and PVR-D1-IgG (200 $\mu$g/ml) to cells was detected by flow cytometry using a biotinylated goat anti-human IgG-Fc followed by streptavidin-allophycocyanin (Molecular Probes, Eugene, OR).

*Antibodies*

We obtained mAbs against PVR (clone SKII.4, mouse IgG1), DNAM-1 (clone 11A8, mouse IgG1), and CD96 (clone NK92.39, mouse IgG1) by immunizing mice with SK-N-S1 human neuroblastoma cells (American Type Culture Collection, Manassas, VA), human NK cell lines, and NK92 cells, respectively. We selected hybridomas that blocked NK cell-mediated lysis of various targets (SKII.4 and 11A8) or binding of PVR to NK92 (NK92.39). Abs against CD56, CD2, CD4, CD8, CD20, CD14, CD16, and BDCA-2 were mouse IgG2a (Beckman Coulter/Immunotech, Brea, CA and Miltenyi Biotec, Auburn, CA). Primary Abs were detected with FITC or PE-labeled goat anti-mouse IgG1 or IgG2a (Southern Biotechnology Associates, Birmingham, AL).

*Cell conjugations*

P815, P815-CD96, and P815-DNAM-1 were labeled with CFSE (Molecular Probes). Jurkat and Jurkat-PVR were stained with anti-CD45-allophycocyanin (Beckman Coulter/Immunotech). Labeled P815 cells ($2 \times 10^5$) were mixed with $2 \times 10^5$ Jurkat cells, spun down, and incubated at 37°C for 1 h. Conjugates were gently resuspended in a small volume of medium for flow cytometric analysis on FACSCalibur (BD Biosciences, San Jose, CA).

*Down-regulation of CD96 and acquisition of PVR by NK92*

NK92 cells ($10^5$) were mixed with Daudi-PVR or Daudi ($5 \times 10^4$), spun down in a 96-well round-bottom plate and incubated for 2 h either at 37°C or at 0°C. Cell conjugates were dissociated by repeated pipetting, cells were stained on ice with anti-CD96 or anti-PVR mAbs followed by goat anti-mouse IgG1-PE (Southern Biotechnology Associates) and analyzed by flow cytometry. Additional staining with anti-HLA-DR-FITC (mouse IgG2a; BD Biosciences) was performed to distinguish Daudi cells from NK92 in the conjugates.

*Confocal microscopy*

To visualize NK cells, we transfected NK92 with a cDNA encoding the adapter DAP12 (3) cloned into the retrovirus pMX-IRES-green fluorescent protein (GFP) (15). NK92 cells expressing the DAP12-GFP bicistronic transcript were conjugated to Daudi or Daudi-PVR cells at 1:1 to 1:10 ratios, briefly spun down, and incubated for 5–30 min at 37°C. After conjugation, cells were gently resuspended, placed onto poly-L-lysine-coated glass slides for 1 h at room temperature, stained with the anti-PVR mAb SKII.4, followed by Cy3-conjugated goat anti-mouse IgG1 (Jackson ImmunoResearch Laboratories, West Grove, PA). Cell conjugates were visualized using a Zeiss LSM 510 laser-scanning confocal microscope (Oberkochen, Germany). We examined ≥30 conjugated NK92 cells per slide.

# Results

*CD96 is a receptor for PVR*

It was recently shown that human NK cells recognize PVR on target cells through DNAM-1 (9). To further investigate NK cell recognition of PVR, we tested a variety of human NK cell lines for expression of DNAM-1 and their capacity to specifically bind the ectodomain of PVR expressed as an IgG fusion protein (PVR-ED-IgG). Remarkably, the NK cell line NK92 did not express DNAM-1 (Fig. 1*A*), yet strongly bound PVR-ED-IgG (Fig. 1*B*). This result suggested that NK92 expresses an as yet unknown receptor for PVR. Among potential PVRs, CD96 (also called Tactile) (12) and carcinoembryonic Ag-related cell adhesion molecule (CEACAM) (16) were particularly attractive candidates in view of their similarity to DNAM-1. Nectin and nectin-like family members, which include PVR, were also plausible receptors for PVR as they mediate adhesion by heterotypic interactions with other nectins (10).

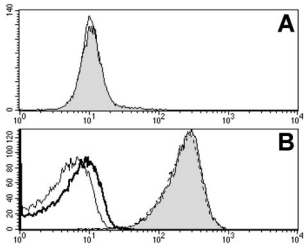


**FIGURE 1.** NK92 does not express DNAM-1 but binds PVR-ED-IgG. *A*, Staining of NK92 with the anti-DNAM-1 mAb 11A8 (gray profile) coincides with background staining (solid line). *B*, The soluble PVR ED fused with human IgG Fc (PVR-ED-IgG) binds NK92 (gray profile). Binding is inhibited by the anti-PVR mAb SKII.4 (thick solid line) but not by the anti-DNAM-1 mAb 11A8 (dashed line that overlaps with the gray profile). Background staining of NK92 is indicated (thin solid line).

To identify the receptor for PVR expressed on NK92 we immunized mice with NK92, produced anti-NK92 mAbs and, among these, selected the mAb NK92.39, which specifically blocked the interaction between PVR-ED-IgG and NK92 (Fig. 2*A*). Flow cytometric analysis revealed that the NK92.39 Ag is

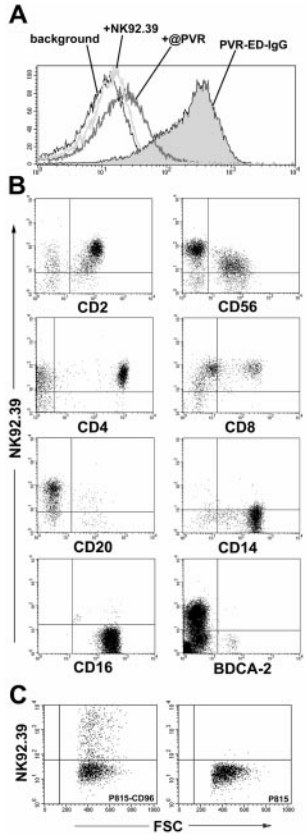


**FIGURE 2.** mAb NK92.39 blocks binding of PVR-ED-IgG to NK92 and recognizes CD96. *A*, mAb NK92.39 blocks binding of PVR-ED-IgG to NK92 cells as the anti-PVR mAb does. *B*, Cell surface expression of NK92.39 Ag on PBMC. We analyzed lymphocytes, monocytes, and granulocytes using separate forward scatter (FSC)/side scatter gates with the exception of natural IFN-producing cells. $CD2^+$ lymphocytes, $CD56^+$ NK cells, $CD4^+$ and $CD8^+$ T cells and a few $CD20^+$ B cells express the NK92.39 Ag. $CD14^+$ monocytes, $CD16^+$ granulocytes, and $BDCA2^+$ IFN-producing cells lack the NK92.39 Ag. *C*, mAb NK92.39 stains P815-cell transiently transfected with CD96 cDNA but not untransfected control cells. Transfected and untransfected cells stained with a control Ab fell into the *lower right quadrant*.

expressed on all peripheral blood NK cells as well as all $CD4^+$ and $CD8^+$ T cells and a few B cells. In contrast, mAb NK92.39 did not stain monocytes, granulocytes, IFN-producing cells (Fig. 2*B*) or epithelial cell lines (data not shown). This cellular distribution was more consistent with that reported for CD96 (12) than CEACAM or other nectins. Staining of P815 cells transiently expressing CD96 cDNA with mAb NK92.39 confirmed that the NK92.39 Ag is CD96 (Fig. 2*C*).

To corroborate that CD96 is a receptor for PVR, we tested binding of P815 cells stably transfected with CD96 cDNA (P815-CD96) with PVR-ED-IgG and a PVR-IgG fusion protein that included only the membrane-distal Ig domain of PVR (PVR-D1-IgG). PVR-ED-IgG and PVR-D1-IgG bound P815-CD96 equally well and the binding was inhibited by NK92.39 and anti-PVR Abs (Fig. 3). In addition, both PVR-ED-IgG and PVR-D1-IgG specifically bound P815 cells expressing DNAM-1 (Fig. 3). We conclude that CD96 specifically recognizes PVR and that the first Ig domain of PVR is sufficient to mediate interaction with CD96 and DNAM-1.

*CD96-PVR interaction mediates cell-cell adhesion*

To determine whether CD96-PVR interactions mediate cell-cell adhesion, we mixed P815-CD96 cells with Jurkat T cells stably transfected with PVR cDNA (Jurkat-PVR) or control Jurkat. After a 30-min incubation at 0°C or 37°C, we measured formation of conjugates by two-color flow cytometry. Under both conditions, P815-CD96 made abundant conjugates with PVR-Jurkat but not with Jurkat (Fig. 4). Conjugate formation was either entirely or partially blocked by anti-PVR and anti-CD96 Abs, confirming the specificity of the interaction (data not shown). The conjugation frequency of CD96-PVR was similar to that obtained with P815-DNAM-1 transfectants and Jurkat-PVR (Fig. 4). No significant conjugation of untransfected cells was observed (Fig. 4).

We also analyzed adhesion of NK92 with PVR transfectants, particularly with those made in the human B cell Daudi, as Daudi can be easily distinguished from NK92 within conjugates by its characteristic expression of MHC class II. As expected, NK92 cells formed abundant conjugates with Daudi-PVR (data not shown). Interestingly, expression of CD96 on

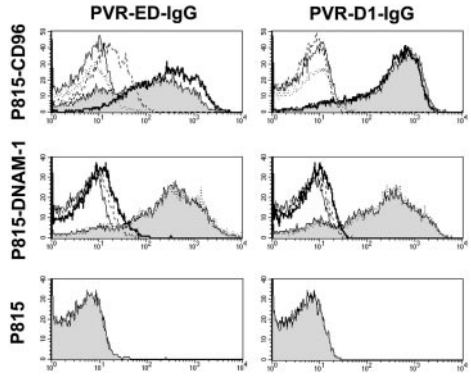


**FIGURE 3.** CD96 binds PVR and the membrane distal PVR Ig domain is sufficient for binding. Gray profiles represent binding of PVR-ED-IgG (*left panels*) or PVR-D1-IgG (*right panels*) to P815-CD96 (*top panels*), P815-DNAM-1 (*middle panels*) and P815 (*lower panels*). Binding was performed in the presence of mAbs against PVR (dashed lines), CD96 (dotted lines), DNAM-1 (thick solid lines). Thin solid lines indicate background staining of transfected and untransfected P815.

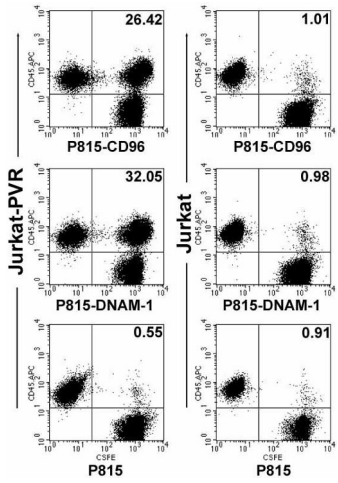


**FIGURE 4.** Conjugate formation between transfectants expressing CD96 and PVR. P815-CD96 transfectants (*top panels*) and P815-DNAM-1 transfectants (*middle panels*) make conjugates with Jurkat-PVR but not with untransfected Jurkat. Percentage of conjugates are indicated in the *upper right quadrants*. P815 did not make conjugates with either Jurkat-PVR or untransfected Jurkat (*bottom panels*).

NK92 was selectively reduced after conjugation at 37°C but not at 0°C (Fig. 5*A*), indicating that CD96 is down-regulated upon interaction with PVR, possibly by an active process of internalization. Moreover, conjugation of NK92 with Daudi-PVR resulted in acquisition of PVR by NK92 (Fig. 5*B*). Interestingly, acquisition of PVR by NK92 was detected after 2 h of conjugation at 0°C and was markedly increased at 37°C (Fig. 5*B*). Thus, NK cells may acquire PVR in part through an active process of transport as well as by detouching it from transfectants when the cells are pulled apart, due to the high affinity of CD96 for PVR. Confocal microscopy of conjugates between NK92 and Daudi-PVR revealed PVR clusters not only at the site of cell-cell contact but also in NK92 cells at sites distal to the contact site, corroborating PVR acquisition by NK92 after CD96-PVR interaction (Fig. 5, *C* and *D*). We conclude that CD96-PVR-mediated adhesion results in exchange of membrane molecules between NK cells and cells expressing PVR, which may also involve internalization of CD96-PVR clusters.

*CD96 triggers cytotoxicity of activated NK cells*

Because DNAM-1 transduces activating signals upon engaging PVR (6, 9), CD96 may also function as an activating receptor for PVR. However, the CD96 cytoplasmic domain contains tyrosine-based motifs that resemble immunoreceptor tyrosine-based motifs (ITIM), which may in fact trigger inhibitory signals (3). To investigate CD96 signaling properties, we tested the cytotoxicity of human polyclonal NK cell lines and NK92 against P815 cells in the presence of Abs that bind the FcR on P815 and engage CD96, DNAM-1, or the activating receptors NKp44 and NKp30 on NK cells. Engagement of CD96 increased the lysis of P815 by human polyclonal NK cell lines, although not as efficiently as did engagement of DNAM-1, NKp30, and NKp44 (Fig. 6*A*). In addition, coengagement of CD96 and NKp30 did not reduce the lysis of P815 triggered by NKp30 alone, demonstrating that CD96 does not transduce inhibitory signals (Fig. 6*B*). However, CD96 did not stimulate cytotoxicity of NK92 cells, suggesting that CD96-mediated

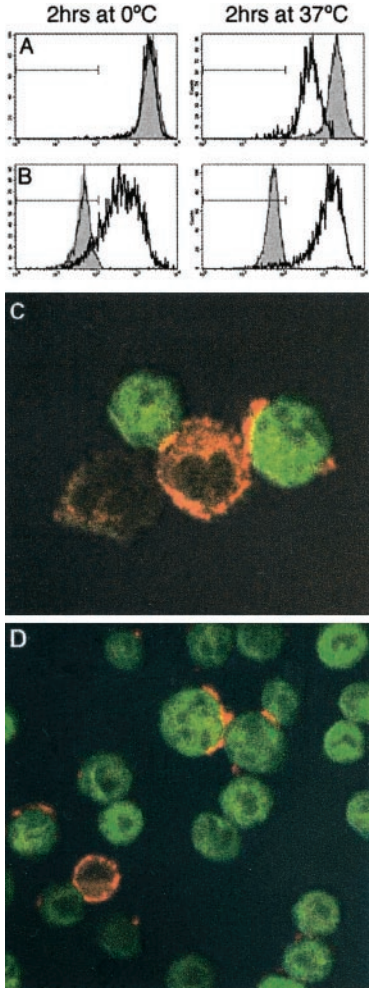


**FIGURE 5.** Down-regulation of CD96 and acquisition of PVR by NK92 during conjugation with PVR transfectants. *A*, Expression of CD96 on NK92 after 2 h of conjugation with Daudi-PVR (thick solid line), Daudi (thin solid line), or no conjugation (gray profile overlapping with thin solid line) at 0°C and 37°C. Cells stained with a control Ab fell within the marker. *B*, PVR expression on NK92 cells after conjugation with Daudi-PVR (thick solid line), Daudi (thin solid line), or no cells (gray profile overlapping with thin solid line). *C*, NK92 cells expressing the DAP12-GFP bicistronic transcript (green) form conjugates with Daudi-PVR (red). PVR (red) appears not only on the cell surface of Daudi-PVR but also on the surface of NK92, especially at the site of cell-cell contact. *D*, PVR (red) acquired by NK92 (green) from PVR-Daudi (red) is clearly detectable at sites distal to the NK92-Daudi-PVR contact site.

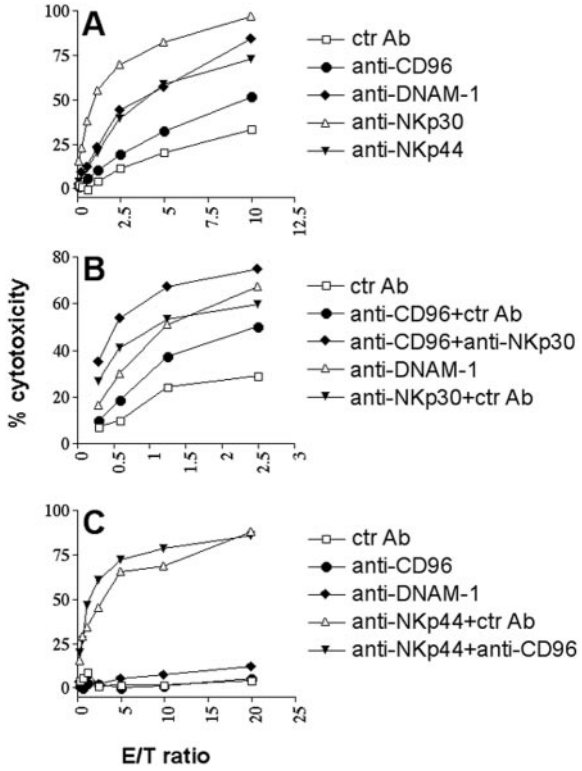


**FIGURE 6.** CD96 stimulates lytic activity of freshly established human NK cell lines. *A*, Lysis of P815 by a human NK cell line is augmented in the presence of Abs against CD96, DNAM-1, NKp30, NKp44 as compared with a control Ab. *B*, When the anti-CD96 and anti-NKp30 mAbs are used together, lysis of P815 is not reduced as compared with that induced by the anti-NKp30 mAb alone. *C*, Anti-CD96 mAb does not activate lysis by NK92, as compared with anti-NKp44 mAb. As expected, anti-DNAM-1 Ab has no effect because NK92 do not express DNAM-1.

stimulation may require expression and/or functional activation of additional molecules that are present in freshly established NK cell lines but not in NK92 (Fig. 6*C*).

## Discussion

Our study presents manifold evidence that CD96 is an NK cell receptor for PVR: 1) our anti-CD96 mAb blocks binding of soluble PVR to NK92; 2) PVR-IgG specifically binds CD96 transfectants; 3) CD96 transfectants effectively conjugate with PVR transfectants; 4) NK cells down-regulate CD96 after conjugation with PVR transfectants. CD96 is a member of the Ig superfamily, with an ectodomain that includes three Ig domains and a long serine/threonine/proline-rich region typical of an *O*-glycosylated domain (12). Our study shows that the membrane distal Ig domain of PVR is sufficient for binding to CD96, as well as to DNAM-1, the other Ig superfamily member that has been shown to recognize PVR (9). Interestingly, CD96, DNAM-1, and PVR share significant sequence identity among themselves and with other members of the nectin and nectin-like families. Thus, this family of receptors is reminiscent of the CD2 family, which includes multiple receptors that mediate NK cell adhesion and activation by homotypic interactions and/or by heterotypic interactions with other CD2 family members (17).

Our results indicate that the predominant function of CD96 is to mediate adhesion of NK cells to other cells expressing PVR. The strong adhesion between CD96 and PVR promoted the exchange of cell surface molecules between NK cells and target cells, in particular, the acquisition of PVR by NK cells as well as possible internalization of CD96 bound to PVR. A similar phenomenon has been observed after interaction of MHC on APCs with the TCR on T cells (18) and the inhibitory receptors on NK cells (19). It has been suggested that transfer of MHC to Ag-specific T cells may stimulate neighboring T cells to kill those expressing captured MHC/Ag peptide, exhausting T cell responses (18). Similarly, the transfer of PVR from target cells to NK cells may make them susceptible to "fratricide". Because some tumors express high levels of PVR (20), transfer of PVR from tumors to NK cells via CD96 may elicit NK cell fratricide, providing tumors with a mechanism of immunoevasion.

We observed that CD96 can also promote NK cell activation, although less efficiently than DNAM-1 and other activating NK cell receptors. As NK cell surface expression of CD96 and

DNAM-1 is similar (data not shown), they may trigger pathways with different activating capability. Notably, CD96 stimulated freshly activated NK cells, but not NK92, suggesting that the stimulatory function of CD96 may require expression and functional cooperation of other molecules that are absent in NK92, just as DNAM-1 requires $\beta_2$ integrin to trigger NK cells (8). Despite the presence of cytoplasmic ITIM-like motifs, CD96 did not initiate inhibitory signals. In fact, these motifs may mediate CD96 down-regulation by promoting receptor internalization. Alternatively, CD96 may be down-regulated by cell surface shedding or other mechanisms.

In conclusion, our study reveals that NK cells express a dual receptor system that recognizes nectins and nectin-like molecules on target cells. During cell-cell contact, CD96, DNAM-1, and their ligands may accumulate at the cell-cell contact site, leading to the formation of a mature immunological synapse between NK cells and target cells. This may not only trigger adhesion and secretion of lytic granules and IFN-$\gamma$, but may also promote NK cell-target cell molecular exchanges, which could subsequently lead to resolution of NK cell responses.

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