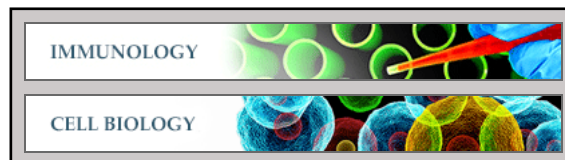


**Immunology:**  
**CD226 Involves in Immune Synapse  
Formation and Triggers NK Cell Activation  
via Its First Extracellular Domain**

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## **CD226 involves in immune synapse formation and triggers NK cell activation via its first extracellular domain**

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Running title: *First extracellular domain of CD226 in Natural Killer cells activation*

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**KeyWords:** Natural killer (NK) cell; Cell junctions; phosphorylation; Cell biology; ERK

**Background:** CD226 is an activating receptor on NK cells that mediates NK cell cytotoxicity.

**Results:** The first extracellular domain of CD226 (CD226-ECD1) mediates NK cell recognition, adhesion, immune synapse formation and cytotoxicity against target cells.

**Conclusion:** CD226-ECD1 retains almost all functions of the full-length CD226 protein.

**Significance:** The conclusion is helpful to understand the mechanism by which CD226 recognizes its ligands.

### **ABSTRACT**

CD226, an activating receptor that interacts with the ligands CD155 and CD112, activates natural killer (NK) cells via its immunoreceptor tyrosine-based activatory motif (ITAM). There are two extracellular domains of CD226, however, the comparative functional relevance of these domains remains unknown. In this study, two different deletion mutants, rCD226-ECD1 (the first extracellular domain) and rCD226-ECD (full extracellular domains), were recombinantly expressed. We observed that rCD226-ECD1, similar to rCD226-ECD, specifically bound to

ligand-positive cell lines and that this interaction could be competitively blocked by an anti-CD226 mAb. In addition, rCD226-ECD1 was able to block the binding of CD112 mAb to tumor cells in a competitive binding assay. Importantly, based on surface plasmon resonance (SPR), we determined that rCD226-ECD1, similar to rCD226-ECD, directly bound to its ligand CD155 on a protein chip. Functionally, NK cell cytotoxicity against K562 or HeLa cells was blocked by rCD226-ECD1 by reducing the expression of CD69 and granzyme B, indicating the critical role of ECD1 in NK cell activation. We also examined the role of rCD226-ECD1 in effector/target interactions by using rCD226-ECD to block these interactions. Using flow cytometry, we found that the number of conjugates between IL-2-dependent NKL cells and HeLa cells was reduced and observed that the formation of immune synapses was also decreased under confocal microscopy. In addition, we prepared two anti-rCD226-ECD1 agonistic antibodies, 2E6 and 3B9. Both 2E6 and 3B9 antibodies could induce the phosphorylation

**of ERK in NK-92 cells. Taken together, our results show that CD226 functions via its first extracellular domain.**

Natural killer cells recognize and eliminate virus-infected or abnormally transformed cells via cytotoxic effects (1-3). Multiple receptors have been identified that play important roles in the biological functions of natural killer cells (4, 5). NK cell cytotoxicity is triggered by the cooperation of activating receptors and adhesion molecules (6), and activating signals activate NK cells to secrete cytotoxic granules (7, 8). CD226 is an activation receptor on NK cells and T cells. It was initially discovered by Burns and named T lineage-specific activation antigen (TLiSA) (also called DNAM-1). Previous studies have revealed that CD226 plays an important role in the NK cell-mediated cytotoxicity of tumor cells (9-14).

Previously, CD226 was mainly considered to be an adhesion molecule that is involved in immune synapse formation in T cells and NK cells during cell cytotoxicity. During the formation of the immune synapse, CD226 transmits a signal and then induces the aggregation of LFA-1 (15-17). CD226 has two ligands, CD155 and CD112 (18), both of which belong to the immunoglobulin super family (IgSF) and are type I transmembrane proteins. Many members of the immunoglobulin superfamily have been reported to be involved in cell adhesion and activation in the immune system (19, 20). CD155 and CD112 have three IgV-like domains in their N-terminal extracellular regions. CD155 is also called the poliovirus receptor (PVR) and binds poliovirus via the first IgV-like domain at its N-terminus (21). CD112 is also known as nectin-2, a member of the nectin family, which is composed of four members, nectin-1 to -4. There are two extracellular domains of CD226 (22), and a recent structural study implies that CD226 binds its ligands via its first N-terminal Ig V-like domain (23); however, which extracellular

domain is functionally important remains unknown.

In this study, two different deletion mutants of the CD226 protein, the first extracellular domain (rCD226-ECD1) and both extracellular domains (rCD226-ECD), were recombinantly expressed and purified. We compared the function of rCD226-ECD1 and rCD226-ECD and found that these two regions function similarly with respect to ligand binding, cell adhesion, immune synapse formation and NK cell activation. Therefore, CD226 is involved in NK cell activation, likely via its first extracellular domain.

## EXPERIMENTAL PROCEDURES

**Antibodies**--The monoclonal antibodies (mAbs) used in this study are as follows: FITC-conjugated anti-CD226 and anti-CD112; PE-conjugated anti-CD11a, anti-ICAM-1, anti-CD112, anti-CD155, anti-CD69 and anti-granzyme B; PE-Cy5-conjugated anti-CD3, APC-conjugated anti-CD56, purified anti-CD226 (DX11) and purified anti-CD112 (R2.525). These antibodies were purchased from BD Biosciences (San Diego, CA, USA). An Alexa Fluor 488-conjugated penta-His mAb was purchased from Qiagen (GmbH, Germany). An Alexa Fluor 488-conjugated goat anti-mouse antibody and goat serum were purchased from Invitrogen (Carlsbad, CA, USA). Anti-ERK1/2 and anti-phospho-ERK1/2 T202/Y204 antibodies were purchased from Cell Signaling (Beverly, MA, USA). An anti- $\beta$  actin antibody, HRP-conjugated goat anti-mouse IgG secondary antibody and HRP-conjugated goat anti-rabbit IgG secondary antibody were purchased from Wuhan Boster Biological Technology, Ltd. (Wuhan, China).

**Cell lines and bacterial strains**--The NK-92, NKL, K562, HeLa and CHO-K1 cell lines were purchased from the American Type Culture Collection (ATCC). The *Escherichia coli* strain DH5 $\alpha$  was purchased from Invitrogen (Carlsbad, CA, USA). The *E. coli* strain Rosetta (DE3) was purchased from Novagen (Lund, Sweden).

**Cell culture**--The rhIL-2-dependent NK cell line NK-92 was maintained in  $\alpha$ -MEM medium (Gibco) containing 12.5% heat-inactivated fetal bovine serum (FBS; Gibco), 12.5% equine serum (HyClone), 2 mM L-glutamate, 100  $\mu$ g

/mL penicillin and 100 µg/mL streptomycin and supplemented with 100 IU/mL rhIL-2 (Changchun Institute of Biological Products, Ministry of Public Health, People's Republic of China). The IL-2-dependent NK cell line NKL was maintained in RPMI 1640 medium (Gibco) containing 15% heat-inactivated FBS (Gibco), 2 mM L-glutamate, 100 µg/mL penicillin and 100 µg/mL streptomycin and supplemented with 100 IU/mL rhIL-2 (Changchun Institute of Biological Products, Ministry of Public Health, People's Republic of China). The K562 tumor cell line was cultured in RPMI 1640 medium (Gibco) supplemented with 10% heat-inactivated FBS, 100 µg/mL penicillin and 100 µg/mL streptomycin. The HeLa tumor cell line was cultured in RPMI 1640 medium (Gibco) supplemented with 10% heat-inactivated FBS, 100 µg/mL penicillin and 100 µg/mL streptomycin. The non-tumor CHO-K1 cell line was cultured in DMEM medium (Gibco) supplemented with 10% heat-inactivated FBS, 100 µg/mL penicillin and 100 µg/mL streptomycin. Cell culture was performed at 37°C in a 5% CO<sub>2</sub> humidified atmosphere. Cells were harvested at mid-log phase.

**Expression of the recombinant extracellular domain of human CD226**—The amino acid sequence of this protein can be accessed through NCBI Protein Database under NCBI Accession # NP\_006557.2 (18). The sequences of primers are ECD1-F: 5'-ggcgcgcacatggaagaggtgctttggcacaacat-3', ECD1-R: 5'-gacggactcgagactatctgactggaaccacctgtatc-3', ECD-F: 5'-cgggatccgattatcctacttacttttggc-3' and ECD-R: 5'-aactcgagctccagccacaagagggtatat-3'. Two different deletion mutants containing the extracellular domain of human CD226, ECD1 (19-129 aa) and ECD (19-243 aa), were cloned by PCR from NKL cell total mRNA and inserted into the pET22b vector (Novagen, Lund, Sweden) to construct the expression vectors pET22b/CD226-ECD1 and pET22b/CD226-ECD, which encode each ECD protein with an N-terminal His-tag. *E. coli* Rosetta (DE3) cells that were successfully transformed with these expression plasmids were grown in Luria-Bertani medium both containing ampicillin (50 mg/L) and chloramphenicol (34 mg/L). Expression of the recombinant proteins was induced at exponential phase with 1 mM isopropyl β-D-thiogalactoside (IPTG). The transformed *E. coli* cells were cultured for

another 4 h at 37°C and then harvested and lysed. The target proteins were found mainly in the pellets as inclusion bodies. After refolding, the His-tagged proteins were purified with Ni-sepharose (Amersham Biosciences). Next, 12% SDS-PAGE was conducted to separate the purified products. The gels were either stained with Coomassie Brilliant Blue or subjected to western blot analysis.

**Flow cytometry**—The cells to be analyzed were washed twice with PBS and blocked with mouse serum for 30 min at 4°C. Next, the cells were stained with saturating concentrations of the appropriate fluorochrome-conjugated mAbs for 30 min at 4°C. Then, the cells were washed twice with PBS and analyzed using a FACSCalibur flow cytometer (Becton Dickinson). For analysis of CD226 binding, the recombinant protein was first added at a concentration of 1 µg/mL and then detected with an anti-His-tag mAb. For blocking analysis using the recombinant protein, cells were incubated with the recombinant protein for 30 min at 4°C, washed and then stained with the appropriate antibody.

**Cytotoxicity assay**—The cytotoxic activity of NK-92 cells against target cells was measured using a standard 4-h <sup>51</sup>Cr release assay as previously described (24). Briefly, K562 cells were labeled with 200 µCi sodium chromate (<sup>51</sup>Cr) (Perkin Elmer) per 10<sup>6</sup> cells for 1 h at 37°C. NK-92 cells were incubated with these K562 cells in 96-well round bottom plates for 4 h at 37°C and 5% CO<sub>2</sub>. The percentage of specific <sup>51</sup>Cr release was calculated using the following formula: (<sup>51</sup>Cr release in the presence of effector cells - spontaneous release in the absence of effector cells)/(total <sup>51</sup>Cr release from target cells incubated with 1% Triton X-100 - spontaneous release in the absence of effector cells)×100%. Spontaneous release did not exceed 10% of the maximum release.

**Cell conjugation assay**—HeLa target cells were stained with PKH67 (green), and effector NKL cells were stained with PKH26 (red). Both PKH67 and PKH26 were purchased from Sigma-Aldrich (USA) and used according to the manufacturer's specifications. The NKL cells and target HeLa cells were mixed at a ratio of 2:1. The NKL-HeLa cell mixture was centrifuged at 80 ×g for 1 min and then incubated at 37°C for 5, 10, 20 or 30 min. The cell mixture was then gently resuspended, fixed with 1% paraformaldehyde and analyzed by flow cytometry. The conjugate formation was

calculated according to a formula: Conjugate Formation = the portion of PKH26/PKH67 double-positive events  $\div$  (the portion of PKH26/PKH67 double-positive events + the portion of single PKH26-positive events).

*Surface plasmon resonance (SPR) measurements*--The binding affinity between ECD, ECD1 or TEV protease and the CD155 protein was analyzed at 25°C using a Biacore 3000 system and a CM5 chip (GE Healthcare). PBS buffer (pH 7.4) was used for the conjugation of the CD155 protein to the CM5 chip. Tris-NaCl buffer (300 mM NaCl, 50 mM Tris, pH 8.5) was used in the detection of the binding of rCD226-ECD and rCD226-ECD1 to the chip-conjugated CD155. After each cycle of data collection, the sensor surface was regenerated with 10 mM NaOH. Sensorgrams were fit globally with Biacore 3000 analysis software (BIAevaluation Version 4.1) using the 1:1 Langmuir binding mode.

*Intracellular cytokine detection by flow cytometry*--The protocol for intracellular cytokine detection was used as previously described (24). In brief, cells were stimulated with target cells or an activating antibody, and 1 h later, monensin (10  $\mu$ g/ml; Sigma) was added to prevent the secretion of the induced cytokines into the supernatant. After continued culture for 2 h at 37°C and 5% CO<sub>2</sub>, the cells were harvested and fixed. Next, we permeabilized the cells, added a PE-conjugated anti-granzyme B antibody and incubated the mixture for 1 h at room temperature. After washing twice with PBS (pH 7.2), the samples were analyzed by flow cytometry.

*Western blot analysis*--NK-92 and target cells were cultured in serum-free medium for 2 h to reduce the background phosphorylation of ERK1/2. The cells were stimulated and then boiled in Laemmli sample buffer. The proteins from 10<sup>6</sup> cells were resolved by SDS-PAGE electrophoresis (7.5-12.5% acrylamide) and then transferred to nitrocellulose membranes. Next, the membranes were blocked using 1×TBS with

5% (w/v) BSA and 0.1% Tween-20 and then probed with primary antibodies. The proteins were visualized using the appropriate HRP-conjugated secondary antibody and detected by enhanced chemiluminescence.

*Confocal microscopy*--NKL cells and HeLa cells were mixed at a 2:1 ratio for 15 min at 37°C in suspension. The cell mixture was gently resuspended and adhered to poly-L-lysine-coated glass slides for 15 min at 37°C. The cells were then fixed with 4% paraformaldehyde for 15 min at 37°C. The fixed cells were washed and incubated with primary antibodies against cell-surface CD226 markers for 30 min at 4°C. The slides were washed with PBS supplemented with 0.1% Tween-20 (PBST) and incubated with fluorescently labeled secondary antibodies for 30 min at 4°C to visualize CD226. The slides were then washed with PBST and incubated with a fluorescently labeled CD11a antibody for 30 min at 4°C. The slides were again washed with PBST and visualized using a Zeiss LSM 710 laser-scanning confocal microscope. Membrane protein clustering was scored when the fluorescence intensity at the effector-target conjugate interface was at least twice the sum of the fluorescence of the unconjugated membranes.

*Preparation of monoclonal antibody*--Recombinant CD226 extracellular domain 1 (ECD1) protein was used to prepare monoclonal antibody as antigen, the hybridoma was produced with a standard protocol. Hundreds of hybridomas specific to CD226 were screened by ELISA, among which hybridoma 2E6 and 3B9 exerted agonistic function and could bind CD226 ligands by FACS analysis. The characterizations of these two monoclonal antibodies were included Supplementary Table. 1.

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

be statistically significant.

## RESULTS

### Recombinant expression and identification of the first ectodomain of human CD226

According to ExPASy predictions of CD226, we selected the first 243 amino acids as the whole extracellular domain (ECD) and the first 129 amino acids as the first N-terminal extracellular domain (ECD1) and used this information to construct expression vectors (Fig. 1A and 1B). The recombinant CD226 ECD and ECD1 proteins had correct molecular weights (28 kDa and 14 kDa, respectively) (Fig. 1C) and could be detected using an anti-CD226 mAb in western blot analysis (Fig. 1D and 1E). LC-MS also showed that the proteins were CD226 (Fig. 1F). We refolded the proteins from *E. coli* extracts, purified them using Ni-sepharose (Fig. 1G) and further confirmed their identities with SDS-PAGE (Fig. 1H).

### CD226 ligand binding requires its first ectodomain

To determine whether the CD226 ECD binds to its ligand on K562 cells, we used a competitive assay to measure the binding of rCD226-ECD or an anti-CD112 mAb with the ligand CD112 by flow cytometry. As shown in Fig. 2A, rCD226-ECD blocked the binding of the anti-CD112 mAb to CD112 on the cell surface, indicating that rCD226-ECD has a strong binding affinity for this protein. Next, we compared the binding of rCD226-ECD and rCD226-ECD1 with the ligand-positive cell lines K562 and HeLa or the ligand-negative cell line CHO-K1. We found the binding of these two proteins to HeLa cells could be blocked by the anti-CD226 mAb (DX11) (Fig. 2B), indicating that the binding of ECD1 is specific. In addition, we observed that rCD226-ECD and rCD226-ECD1 both specifically bound to the

ligand-positive cell lines (Fig. 2C). In the competitive binding experiment, both proteins were able to block the binding of the CD112 mAb to tumor cells (Fig. 2D). Importantly, using surface plasmon resonance (SPR), we determined that rCD226-ECD1, similar to rCD226-ECD, directly bound to its ligand CD155 on a protein chip (Fig. 3).

### rCD226-ECD1 reduces the cytotoxicity of NK-92 cells by blocking the binding of NK-92 cells and target cells

We then examined the function of rCD226-ECD1 in NK cell cytotoxicity. We showed that rCD226-ECD reduced the cytolysis of NK-92 cells (Fig. 4A). We compared the blocking ability of rCD226-ECD1 with that of rCD226-ECD and found that NK cell cytotoxicity against the CD226 ligand-positive cell lines K562 and HeLa was reduced similarly by both proteins (Fig. 4B). In a previous study, CD69 and granzyme B were shown to be markers of NK cell activation (25, 26). Here, we found that rCD226-ECD1 and rCD226-ECD reduced the expression of both CD69 and granzyme B on NK cells after co-culture (Fig. 4C and 4D), indicating that blocking with rCD226-ECD1 inhibits NK cell cytotoxicity via the CD226 activation receptor.

It was reported that CD226 is involved in the conjugation of CTL and NK cells to target cells (27). Using rCD226-ECD to block this conjugation, we found that the number of conjugates between PKH26 (red)-labeled NKL cells and PKH67 (green)-labeled HeLa cells were reduced using flow cytometric analysis (Fig. 5A and 5B). These experiments were repeated using rCD226-ECD1 to block conjugation, with similar results (Fig. 5C and 5D).

Binding of NK cells with target cells allows the formation of immune synapses before cytolysis, and CD226 may play a key role in immune synapse formation. We used a CD226

primary antibody and an Alexa Fluor 488 goat anti-mouse secondary antibody to stain CD226 (green) and used a PE-CD11a antibody to stain LFA-1 (red) on the surface of NK cells. Both proteins are reported to be critical in synapse formation. CD226 and LFA-1 and are highly expressed on NKL cells, and ICAM-1 is highly expressed on HeLa cells. NKL cells and HeLa cells were mixed at a 2:1 ratio for 15 min at 37°C in suspension, adhered to glass slides and stained by antibodies. By immunofluorescence microscopy, we observed significant aggregation of CD226 and LFA-1 in the immune synapse. When rCD226-ECD1 or rCD226-ECD was added to the NKL/HeLa co-culture system, the aggregation of CD226 and LFA-1, particularly at the immune synapse, was blocked (Fig. 6A and 6B). We observed that signaling via CD226 is critical for NK cell activation.

The ERK1/2 cascade is one of the important signaling pathways in NK cell cytotoxicity to tumor cells (28). Thus, we prepared two agonistic antibodies, 2E6 and 3B9. The characteristics and specific binding of 2E6 and 3B9 antibodies to the rCD226-ECD1 protein could be detected in western blot assay and FACS analysis (Table 1 and data not shown). We found that both antibodies could induce ERK1/2 signaling in NK-92 cells (Fig. 6C). This result suggested that the CD226 molecular could induce activation signaling via its first N-terminal extracellular domain.

**TABLE 1.** Features of the agonistic 2E6 mAb and 3B9 mAb

Recombinant CD226 extracellular domain 1 (ECD1) protein was used to prepare monoclonal antibody as antigen and hybridoma was produced with a standard protocol. Hundreds of hybridomas specific to CD226 were screened by ELISA. Both of 2E6 and 3B9 monoclonal antibodies were proved to specifically recognize CD226 ECD1 protein in western blot assay and activate NK92 cells.

| Clone                                                          | 2E6 mAb               | 3B9 mAb               |
|----------------------------------------------------------------|-----------------------|-----------------------|
| Isotype                                                        | IgG1κ                 | IgG1κ                 |
| Titer (culture supernatant)                                    | $8.13 \times 10^{-5}$ | $7.88 \times 10^{-5}$ |
| Titer (ascitic fluid)                                          | $9.12 \times 10^{-8}$ | $9.62 \times 10^{-8}$ |
| Affinity constant (L/M)                                        | $8.24 \times 10^7$    | $7.94 \times 10^7$    |
| Western blot                                                   | Yes                   | Yes                   |
| Flowcytometry                                                  | Yes                   | Yes                   |
| Activation of NK92 (Synthesis of IFN $\gamma$ + TNF $\alpha$ ) | Increased             | Increased             |

## DISCUSSION

NK cells provide the first line of defense against infectious pathogens and tumors by recognizing target cells via multiple surface receptors without the need for prior sensitization (4, 29). Of these receptors, CD226 has been found to be a co-stimulatory molecule in various systems (30-33) and has been characterized as an activating receptor for NK-mediated cytotoxicity (8, 11). CD226 is a member of the Ig superfamily along with a large group of other proteins (18, 19). Members of this protein family contain at least one Ig or Ig-like domain as a common structural feature. CD226 contains two Ig V-like functional domains within its extracellular domain (34). Two ligands have been identified: the poliovirus receptor (PVR or CD155) and nectin-2 (CD112), which belongs to the nectin family (18). Recent studies on CD96, another member of the Ig superfamily that shares the same ligand, CD155, as CD226, showed that the first Ig-like domain of CD96 might play a particularly important role in its interaction with

CD155 (35). A study of murine NK and T cells also showed that a naturally occurring CD226 splice variant lacking the outermost V-like domain is generated and suggested that CD226 may adhere to CD155 via the first CD226 domain (36). T cell immunoreceptor with Ig and ITIM domains (TIGIT) is a recently discovered NK inhibitory receptor, which has one Ig domain in its extracellular region (37). TIGIT shares the same ligands, CD155 and CD112, as CD226. The crystal structure of TIGIT-CD155 has been reported and shows that TIGIT interacts with CD155 via the first N-terminal domain of CD155 and that TIGIT assembles in cis-homodimers on the cell surface (38). Because TIGIT, CD155 and CD226 all belong to the Ig superfamily, CD226 ECD1 may work in the same manner as the extracellular domains of CD155 and TIGIT to recognize ligands. Recent studies have also revealed the crystal structure of the CD112 first N-terminal IgV domain and established two hypothetical models for the interaction between CD112 and CD226 (39). The results of this study support the hypothesis that CD226 interacts with CD112 via its first IgV domain.

NK cell-target cell adhesion is important for NK-mediated cytotoxicity. Our results demonstrate that when target cells are preincubated with rCD226-ECD1 or rCD226-ECD, NK cell-mediated cytotoxicity against tumor cells is significantly reduced by inhibiting cell-cell conjugation, suggesting that CD226 is important in the formation of the immunological synapse of NK cells. These findings are in accordance

with previous reports comparing CD226 with LFA-1 in terms of adhesion and signaling functions (15, 16). We also observed that low rates of positive signals remain in the cell cytotoxicity and cell adhesion assays after blocking with antibodies (Fig. 4C and Fig. 5D). This finding indicates that other activating receptors play a role in cytotoxicity independent of CD226-ligand binding.

In this study, we compared rCD226 ECD1 and rCD226-ECD and found that both have the same function, i.e., they were both able to bind to the CD155 protein or to ligands on target cells. This reduced NK cell cytotoxicity against CD226 ligand-positive target cells, inhibiting the activation of NK cells in our cell activity assay and inhibiting NK cell conjugation and immune synapse formation. There may be another issue in terms of measuring the function of the second extracellular domain of CD226 (ECD2). Based on these results, we cannot rule out the idea that ECD2 has no function. Even if ECD2 has a function in binding, it cannot avoid steric hindrance from ECD1. Thus, we explored the functions of the CD226 ECD1 and ECD proteins rather than that of ECD2.

In conclusion, CD226 triggers NK cell-tumor cell recognition and conjugation via its first extracellular Ig-like domain. NK cell-tumor cell conjugation results in NK cell activation through the surface receptor ECD1, which triggers the ERK1/2 signaling pathway, resulting in granule release and cytokine secretion, the combination of which is able to eliminate tumor cells.

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## FOOTNOTES

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<sup>3</sup>Abbreviations used in this paper: ECD, extracellular domain; SPR, Surface plasmon resonance; TIGIT, T cell immunoreceptor with Ig and ITIM domains.

## FIGURES and LEGENDS

**FIGURE 1.** Expression, purification and identification of recombinant CD226-ECD1. A. Tertiary structure prediction of the CD226 protein using the ExPASy/Smart tool. B. Design of the vector insert for the recombinant expression of the CD226 ECD1 and ECD proteins. C. SDS-PAGE analysis of rCD226-ECD1 expressed in *E. coli*. D. Western blot analysis of rCD226-ECD1 using an anti-His-tag mAb. E. Western blot analysis of rCD226-ECD1 using an anti-CD226 mAb. F. LC-MS analysis of rCD226-ECD and rCD226-ECD1 expressed in *E. coli*. G. Purification of rCD226-ECD1 with Ni-sepharose using a concentration gradient of imidazole for elution. H. SDS-PAGE analysis of rCD226-ECD and rCD226-ECD1 purified with Ni-sepharose.

**FIGURE 2.** Flow cytometry demonstrating the binding of CD226 ECD1 to its ligands on tumor cells. A. Binding of CD112 by rCD226-ECD. K562 cells were incubated with buffer (control), rCD226-ECD or BSA. The cells were then harvested, fixed and stained with a PE-conjugated CD112 mAb (R2.525). B. Blocking of anti-CD226 mAb binding to anti-rCD226-ECD1 and rCD226-ECD on tumor cells. HeLa cells were incubated with IgG (isotype control), rCD226-ECD, rCD226-ECD1, rCD226-ECD + anti-CD226 mAb (DX11) or rCD226-ECD1 + anti-CD226 mAb (DX11). The cells were then harvested, fixed and stained with an Alexa Fluor 488-conjugated anti-penta-His mAb. C. Binding of rCD226-ECD1 and rCD226-ECD to ligand-positive tumor cell lines. K562, HeLa and CHO-K1 (ligand-negative control) cells were incubated with buffer (control), rCD226-ECD1 or rCD226-ECD. The cells were harvested, fixed and stained with an Alexa Fluor 488-conjugated anti-penta-His mAb. D. Competitive binding of rCD226-ECD1 and rCD226-ECD with CD112 mAb on tumor cells. K562, HeLa and CHO-K1 (ligand-negative control) cells were incubated with buffer (control), rCD226-ECD1 or rCD226-ECD. The cells were harvested, fixed and stained with a PE-conjugated CD112 mAb and analyzed by flow cytometry.

**FIGURE 3.** Binding of rCD226-ECD1 with CD155, as detected by surface plasmon resonance (SPR). The CD155 extracellular segment-Fc fusion protein was conjugated to the chip, and rCD226-ECD1, rCD226-ECD, TEV or buffer (control) were flowed across the chip. The RU value of the chip was then detected. The CD155 extracellular segment-Fc fusion

protein was purchased from Sino Biological Inc.

**FIGURE 4.** Cytotoxicity of NK-92 cells against tumor cells was reduced by rCD226-ECD1. A. The cytotoxicity of NK-92 cells against K562 cells was detected using a 4-h  $^{51}\text{Cr}$  release assay with E:T ratios of 10:1 and 5:1. The target cells were blocked with buffer (control), BSA (5  $\mu\text{g/mL}$ ) or rCD226-ECD (5  $\mu\text{g/mL}$ ) at 37°C. Data were collected from at least three independent experiments and analyzed with a Student's t test, \*  $p < 0.05$ . B. The cytotoxicity of NK-92 cells against K562, HeLa or CHO-K1 cells was detected using a 4-h  $^{51}\text{Cr}$  release assay with an E:T ratio of 4:1. The target cells were blocked with buffer (control), rCD226-ECD1 (5  $\mu\text{g/mL}$ ) and rCD226-ECD (5  $\mu\text{g/mL}$ ) at 37°C. Data were collected from at least three independent experiments and analyzed with a Student's t test, \*  $p < 0.05$ . C-D. NK-92 cells were stimulated by K562 target cells (E:T ratio of 2:1) with or without blocking by rCD226-ECD or rCD226-ECD1 at 37°C. The expression of CD69 (C) and granzyme B (D) was detected by flow cytometry.

**FIGURE 5.** Blocking of NK cell binding to tumor target cells by rCD226-ECD1. A. Flow cytometric analysis of NK-HeLa cell conjugates at 5, 10, 20 and 30 min. NK cells were stained with PKH26 (red) and HeLa cells were stained with PKH67 (green) at an E:T ratio of 2:1 at 37°C. B. Statistical analysis of E:T conjugate formation in A. C. NK cells were mixed with PKH67-labeled K562 cells (E:T ratio of 2:1) and incubated at 37°C for 5 min or 20 min and analyzed by flow cytometry. The incubation was performed with buffer (control) or rCD226-ECD1 (5  $\mu\text{g/mL}$ ). D. Statistical analysis of E:T conjugate formation in C.

**FIGURE 6.** Inhibition of ERK1/2 activation in NK cells by rCD226-ECD1. A. Confocal analysis of CD226 and CD11a in the immune synapse. Incubation between NK-92 effector cells and K562 target cells was performed with buffer (control), rCD226-ECD1 (5  $\mu\text{g/mL}$ ) or rCD226-ECD (5  $\mu\text{g/mL}$ ) at an E:T ratio at 2:1 for approximately 20 min at 37°C. B. Statistical analysis of the rates of immune synapse formation. Data were collected from at least three independent experiments and analyzed with a Student's t test, \*\*\*  $p < 0.0001$ . C. NK-92 cells were stimulated with the 2E6 or 3B9 antibody or RPMI-1640 medium (control), and the phosphorylation of ERK1/2 was detected by western blot analysis.

Figure 1

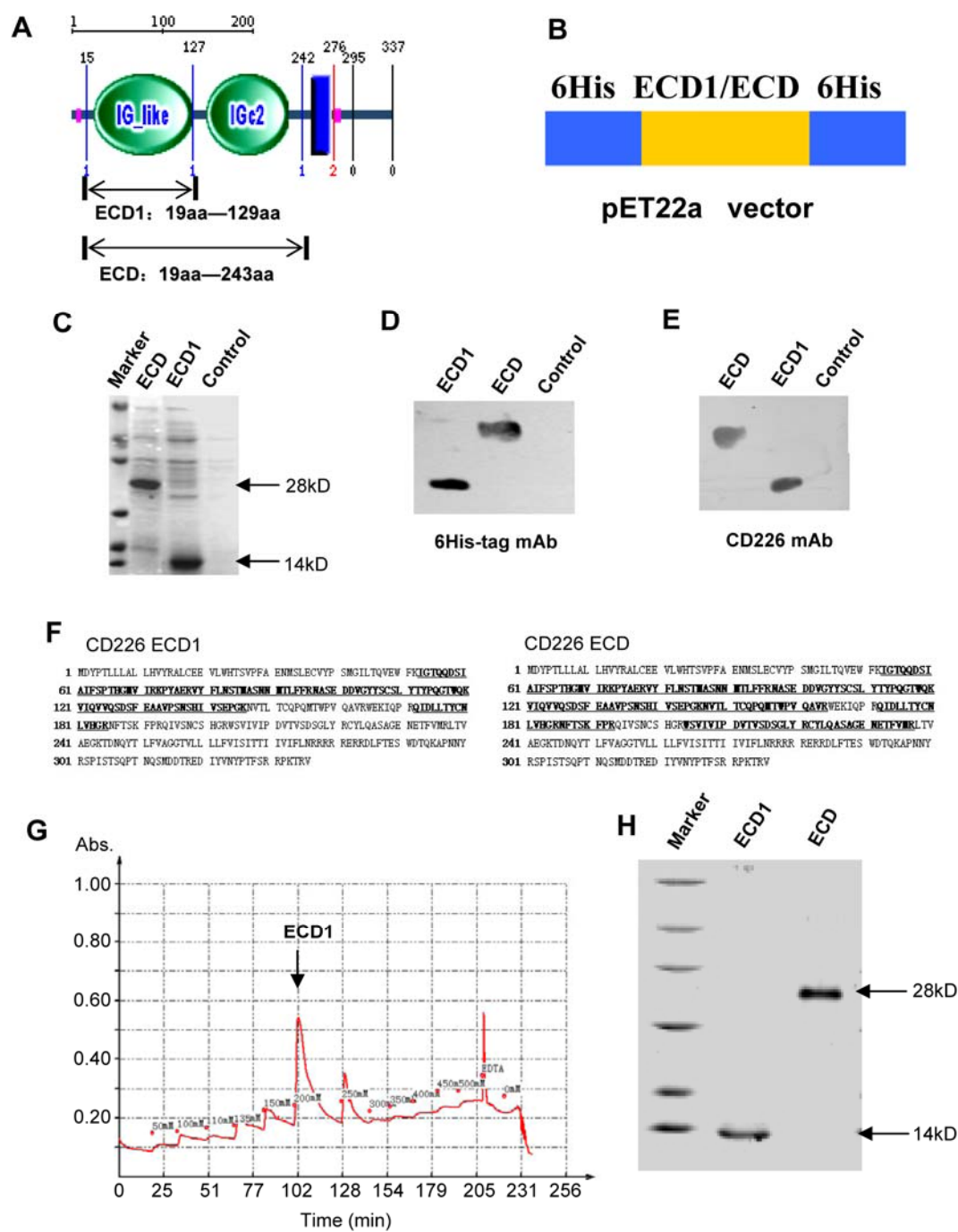


Figure 2

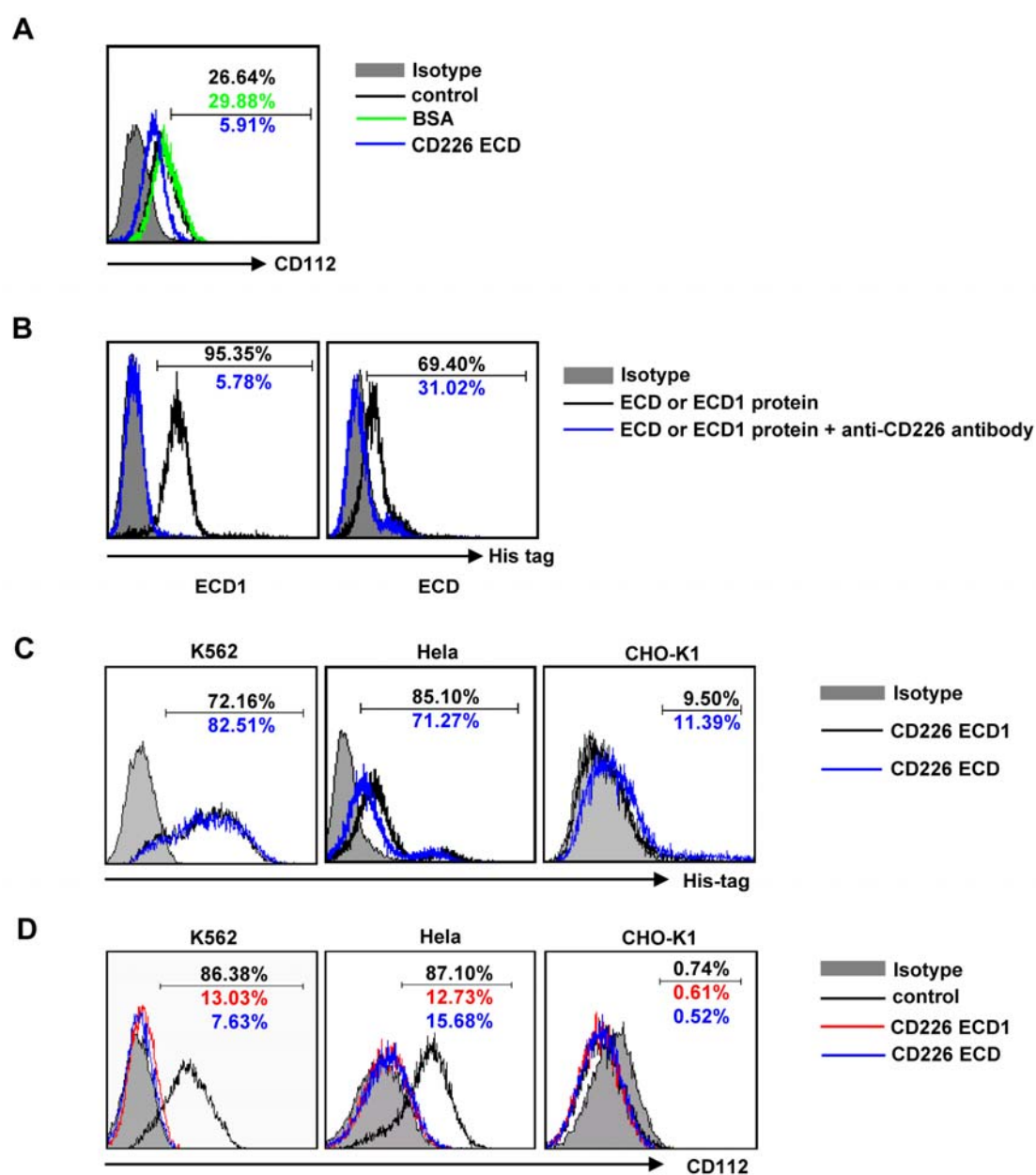


Figure3

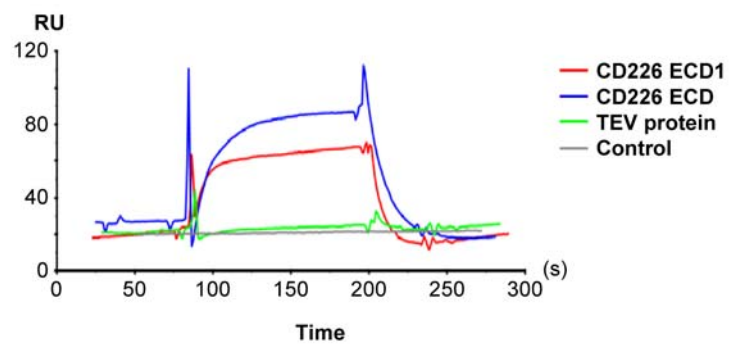


Figure 4

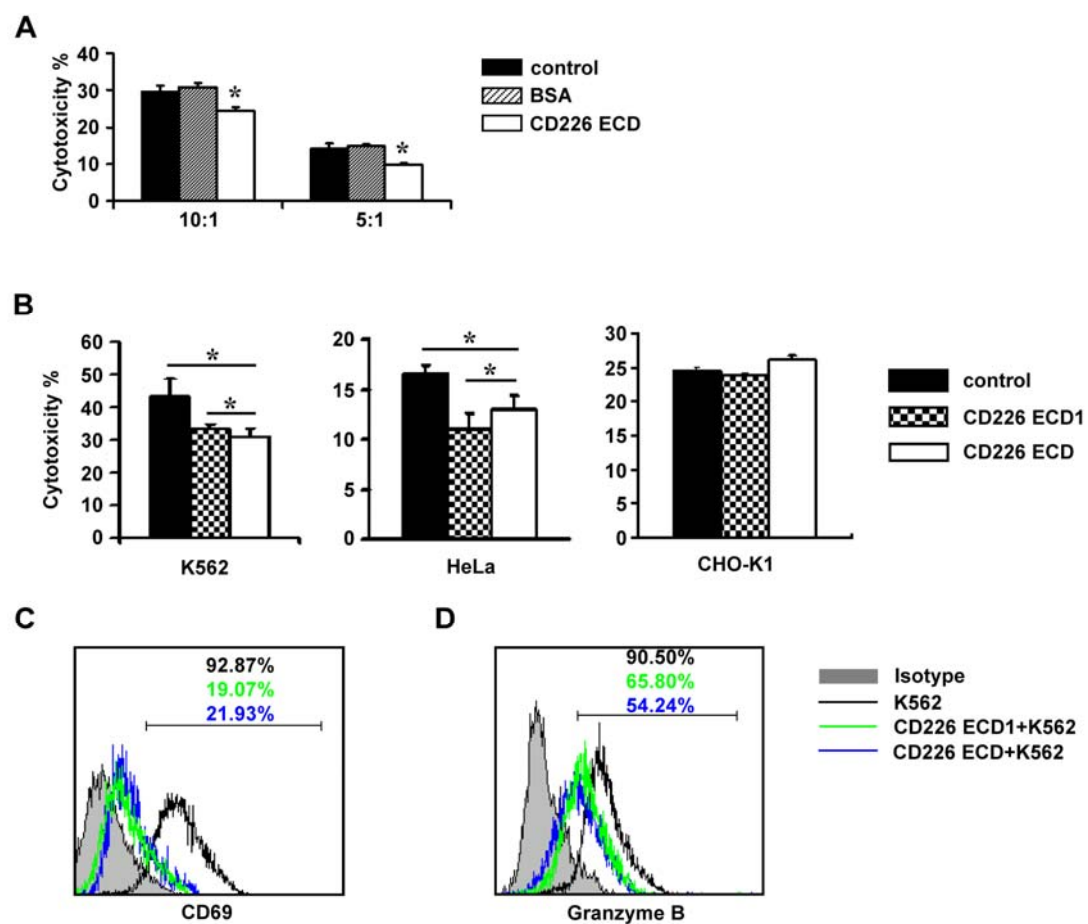


Figure 5

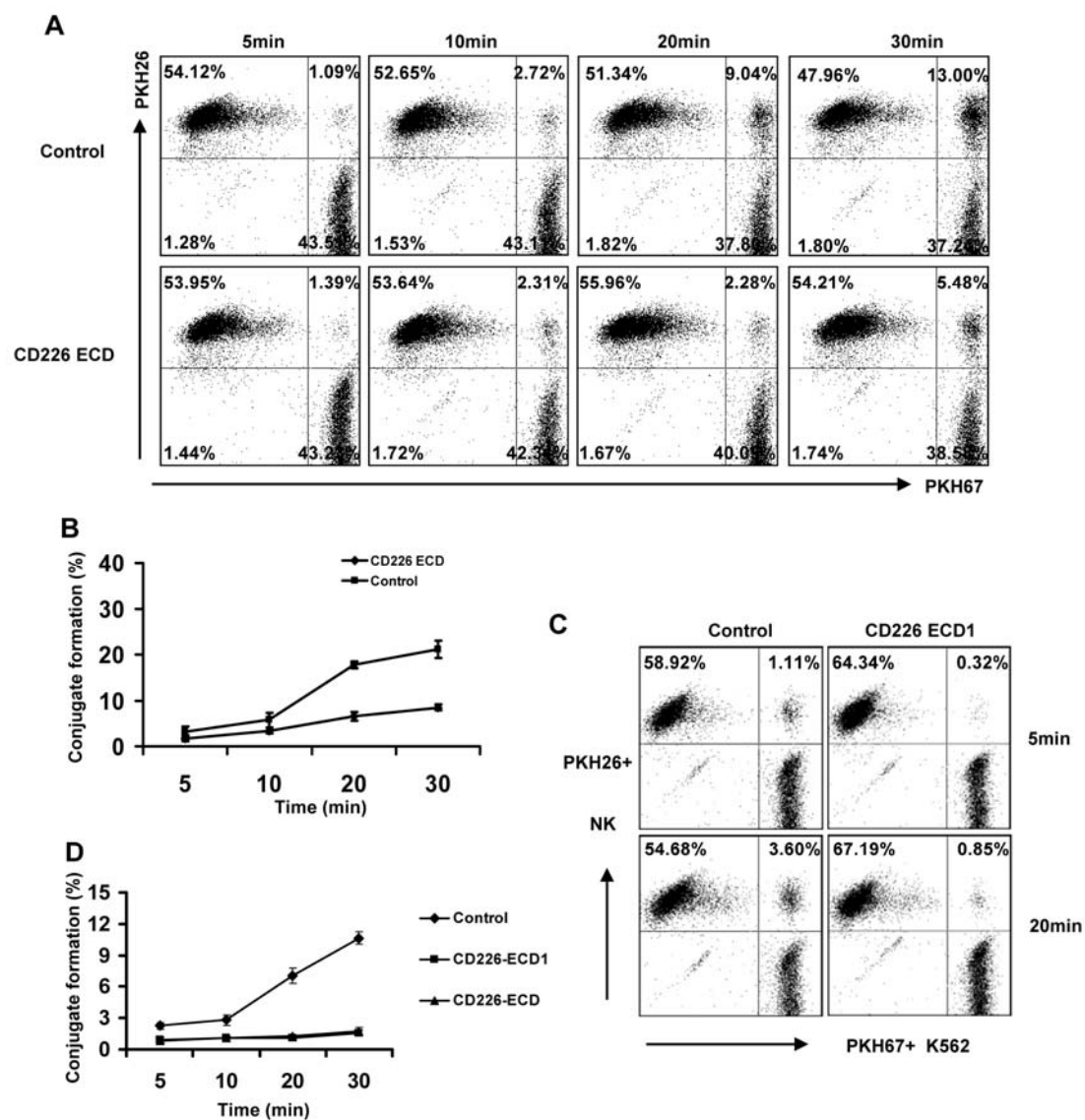


Figure 6

