

**Engineering NKG2D ligand affinity transforms EGFR-targeted NK cell engagers into high-potency effectors against pancreatic cancer**

Seul-Gi Lee<sup>a†</sup>, Myungjee Lee<sup>a†</sup>, Hye-Min Lee<sup>b</sup>, Ga-Hyun Son<sup>a</sup>, Sang-Rok Yoon<sup>a</sup>, Byeong-Ho Chae<sup>a</sup>, Dae-Seong Kim<sup>a</sup>, Kyung-Mi Lee<sup>b</sup>, and Yong-Sung Kim<sup>a,c\*</sup>

<sup>†</sup> These authors contributed equally to this work

<sup>a</sup> Department of Molecular Science and Technology, Ajou University, Suwon 16499, Republic of Korea

<sup>b</sup> Department of Biochemistry and Molecular Biology, College of Medicine, Korea University, Seoul 02841, Republic of Korea

<sup>c</sup> College of Advanced Bio-convergence Engineering, Ajou University, Suwon 16499, Republic of Korea

\*To whom correspondence should be addressed: Yong-Sung Kim, Ph.D.

Department of Molecular Science and Technology, Ajou University

206 Worldcup-ro, Yeongtong-gu, Suwon 16499, Korea

Tel.: +82-31-219-2662; E-mail: kimys@ajou.ac.kr

ORCID: Yong-Sung Kim: 0000-0003-2673-1509

This supplementary data includes:

Supplementary Tables 1 to 2

Supplementary Figures 1 to 6

## Supplementary Information

**Supplementary Table 1.** Biochemical and biophysical properties of  $\alpha$ EGFR $\times$ NKG2DL.

| Molecule                        | MW (kDa) | Purification Yield (mg L <sup>-1</sup> ) <sup>a</sup> | apparent $K_D$ for NKG2D (nM) <sup>b</sup> | Target | $k_{on}$ (M <sup>-1</sup> s <sup>-1</sup> ) <sup>c</sup> | $k_{off}$ (s <sup>-1</sup> ) <sup>c</sup> | $K_D$ (nM) <sup>c</sup> | $R^2$ <sup>c</sup> |
|---------------------------------|----------|-------------------------------------------------------|--------------------------------------------|--------|----------------------------------------------------------|-------------------------------------------|-------------------------|--------------------|
| $\alpha$ EGFR $\times$ dummy    | 98.4     | 20.2 $\pm$ 1.3                                        | -                                          | EGFR   | (4.04 $\pm$ 0.21) $\times 10^5$                          | (2.19 $\pm$ 0.18) $\times 10^{-4}$        | 0.54 $\pm$ 0.05         | 0.99               |
| $\alpha$ EGFR $\times$ MICA     | 131.1    | 19.8 $\pm$ 1.3                                        | 11.6 $\pm$ 0.9                             | EGFR   | (5.07 $\pm$ 0.18) $\times 10^5$                          | (1.84 $\pm$ 0.15) $\times 10^{-4}$        | 0.36 $\pm$ 0.03         | 0.99               |
|                                 |          |                                                       |                                            | NKG2D  | (2.39 $\pm$ 0.12) $\times 10^4$                          | (5.49 $\pm$ 0.09) $\times 10^{-3}$        | 229 $\pm$ 12.4          | 0.98               |
| $\alpha$ EGFR $\times$ MICB     | 131.1    | 20.6 $\pm$ 1.1                                        | 99.1 $\pm$ 14.4                            | EGFR   | (3.61 $\pm$ 0.18) $\times 10^5$                          | (1.67 $\pm$ 0.16) $\times 10^{-4}$        | 0.47 $\pm$ 0.05         | 0.99               |
|                                 |          |                                                       |                                            | NKG2D  | N.Q. <sup>d</sup>                                        | N.Q. <sup>d</sup>                         | N.Q. <sup>d</sup>       | -                  |
| $\alpha$ EGFR $\times$ ULBP1    | 120.8    | 21.4 $\pm$ 2.7                                        | 119.2 $\pm$ 23.1                           | EGFR   | (5.12 $\pm$ 0.22) $\times 10^5$                          | (2.18 $\pm$ 0.19) $\times 10^{-4}$        | 0.42 $\pm$ 0.04         | 0.99               |
|                                 |          |                                                       |                                            | NKG2D  | N.Q. <sup>d</sup>                                        | N.Q. <sup>d</sup>                         | N.Q. <sup>d</sup>       | -                  |
| $\alpha$ EGFR $\times$ ULBP2    | 120.2    | 19.0 $\pm$ 2.4                                        | 95.0 $\pm$ 17.1                            | EGFR   | (4.24 $\pm$ 0.16) $\times 10^5$                          | (1.66 $\pm$ 0.13) $\times 10^{-4}$        | 0.39 $\pm$ 0.03         | 0.99               |
|                                 |          |                                                       |                                            | NKG2D  | N.Q. <sup>d</sup>                                        | N.Q. <sup>d</sup>                         | N.Q. <sup>d</sup>       | -                  |
| $\alpha$ EGFR $\times$ ULBP0601 | 119.8    | 17.2 $\pm$ 2.6                                        | 14.9 $\pm$ 0.6                             | EGFR   | (5.52 $\pm$ 0.26) $\times 10^5$                          | (3.13 $\pm$ 0.22) $\times 10^{-4}$        | 0.57 $\pm$ 0.05         | 0.99               |
|                                 |          |                                                       |                                            | NKG2D  | N.Q. <sup>d</sup>                                        | N.Q. <sup>d</sup>                         | N.Q. <sup>d</sup>       | -                  |
| $\alpha$ EGFR $\times$ ULBP0602 | 119.7    | 22.5 $\pm$ 1.7                                        | 2.4 $\pm$ 0.2                              | EGFR   | (5.81 $\pm$ 0.38) $\times 10^5$                          | (3.58 $\pm$ 0.33) $\times 10^{-4}$        | 0.62 $\pm$ 0.07         | 0.99               |
|                                 |          |                                                       |                                            | NKG2D  | (2.50 $\pm$ 0.06) $\times 10^4$                          | (7.03 $\pm$ 0.13) $\times 10^{-4}$        | 28.1 $\pm$ 0.8          | 0.99               |

<sup>a</sup> Purification yield was calculated from a 100 mL scale HEK293F culture and extrapolated to a 1 L scale. Data are represented as mean  $\pm$  SD (n = 2).

<sup>b</sup> Apparent  $K_D$  was determined by native ELISA using rhNKG2D ECD. Data are represented as mean  $\pm$  SD (n = 3).

<sup>c</sup> The binding kinetics of ICEs to rhNKG2D ECD and rhEGFR ECD were analyzed by BLI. Each value represents the mean  $\pm$  SD of two independent experiments. In each experiment, at least four datasets were used to determine kinetic constants. The association rate constant ( $k_{on}$ ), dissociation rate constant ( $k_{off}$ ), equilibrium dissociation constant ( $K_D$ ), and goodness of fit ( $R^2$ ) were calculated using Octet Data Analysis software (version 11.0 ForteBio).

<sup>d</sup> N.Q. indicates “not quantifiable”, although experiments were performed.

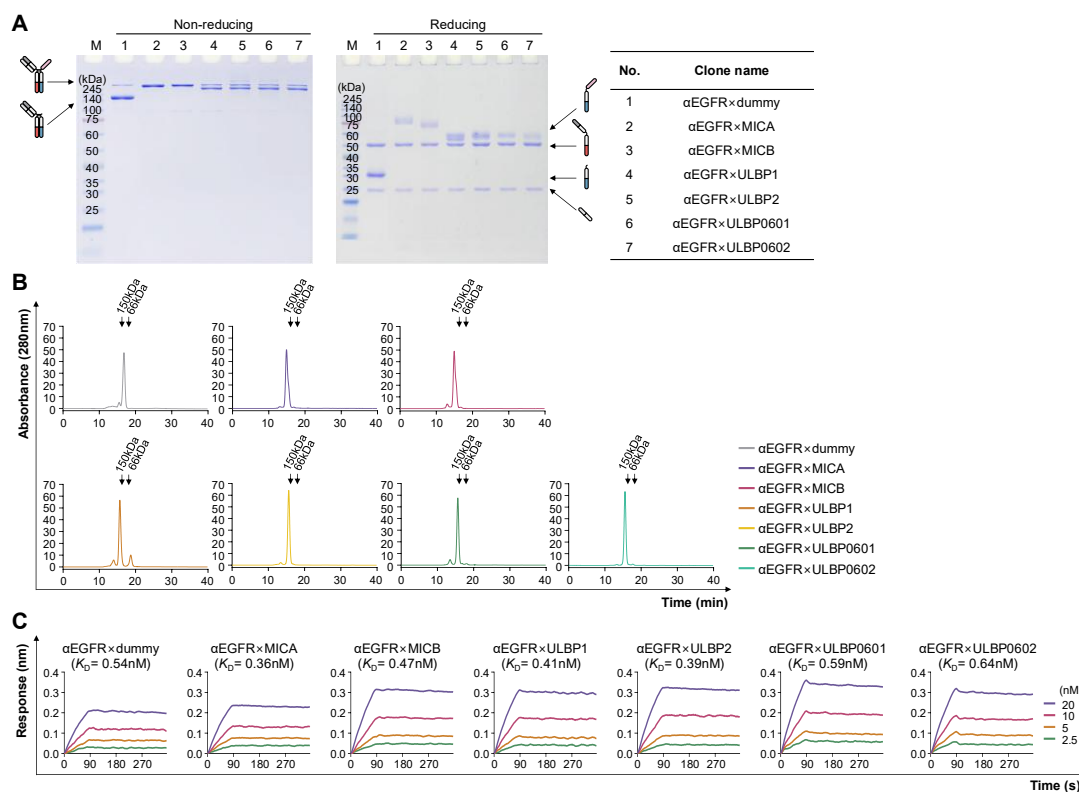
**Supplementary Table 2.** Biochemical and biophysical properties of affinity-improved ULBP6-fused  $\alpha$ EGFR $\times$ ULBP6 ICEs.

| Molecule                        | MW<br>(kDa) | Purification<br>Yield (mg L <sup>-1</sup> ) <sup>a</sup> | $k_{on}$ (M <sup>-1</sup> s <sup>-1</sup> ) <sup>b</sup> | $k_{off}$ (s <sup>-1</sup> ) <sup>b</sup> | $K_D$ (nM) <sup>b</sup> | $R^2$ <sup>b</sup> | Affinity<br>improvement<br>(fold) <sup>c</sup> |
|---------------------------------|-------------|----------------------------------------------------------|----------------------------------------------------------|-------------------------------------------|-------------------------|--------------------|------------------------------------------------|
| $\alpha$ EGFR $\times$ ULBP0602 | 119.9       | 27.7 $\pm$ 4.0                                           | (2.77 $\pm$ 0.06) $\times 10^4$                          | (8.59 $\pm$ 0.12) $\times 10^{-4}$        | 31.0 $\pm$ 0.8          | 0.99               | -                                              |
| $\alpha$ EGFR $\times$ ULBP6#1  | 119.9       | 28.0 $\pm$ 3.6                                           | (2.07 $\pm$ 0.05) $\times 10^4$                          | (7.62 $\pm$ 0.76) $\times 10^{-5}$        | 3.67 $\pm$ 0.38         | 0.99               | 8.4                                            |
| $\alpha$ EGFR $\times$ ULBP6#2  | 119.9       | 26.6 $\pm$ 3.7                                           | (3.99 $\pm$ 0.05) $\times 10^4$                          | (9.71 $\pm$ 0.64) $\times 10^{-5}$        | 2.43 $\pm$ 0.16         | 0.99               | 12.8                                           |
| $\alpha$ EGFR $\times$ ULBP6#3  | 120.0       | 29.2 $\pm$ 4.4                                           | (3.26 $\pm$ 0.08) $\times 10^4$                          | (1.36 $\pm$ 0.10) $\times 10^{-4}$        | 4.17 $\pm$ 0.34         | 0.99               | 7.4                                            |
| $\alpha$ EGFR $\times$ ULBP6#4  | 120.0       | 29.5 $\pm$ 5.4                                           | (3.19 $\pm$ 0.08) $\times 10^4$                          | (2.06 $\pm$ 0.12) $\times 10^{-4}$        | 6.44 $\pm$ 0.41         | 0.99               | 4.8                                            |

<sup>a</sup> Purification yield was calculated from a 100 mL scale HEK293F culture and extrapolated to a 1 L scale. Data are represented as mean  $\pm$  SD (n = 2).

<sup>b</sup> The binding kinetics of ICEs to rhNKG2D ECD were analyzed by BLI. Each value represents the mean  $\pm$  SD of two independent experiments. In each experiment, at least four datasets were used to determine kinetic constants. The association rate constant ( $k_{on}$ ), dissociation rate constant ( $k_{off}$ ), equilibrium dissociation constant ( $K_D$ ), and goodness of fit ( $R^2$ ) were calculated using Octet Data Analysis software (version 11.0 ForteBio).

<sup>c</sup> Affinity improvement values represent the fold reduction in the  $K_D$  for rhNKG2D ECD binding relative to  $\alpha$ EGFR $\times$ ULBP0602 ICE.

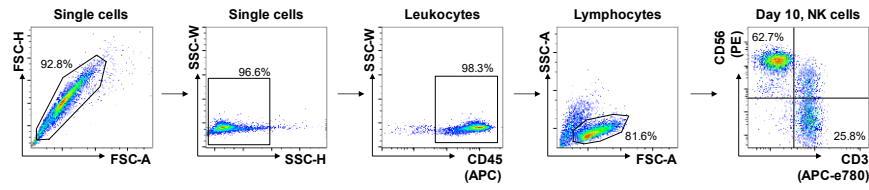


**Supplementary Figure 1. Biophysical characterization and EGFR-binding kinetics of  $\alpha$ EGFR×NKG2DL ICEs.**

(A) SDS-PAGE of purified  $\alpha$ EGFR×NKG2DL ICEs (4  $\mu$ g per lane) under non-reducing and reducing conditions on 12% polyacrylamide gels.

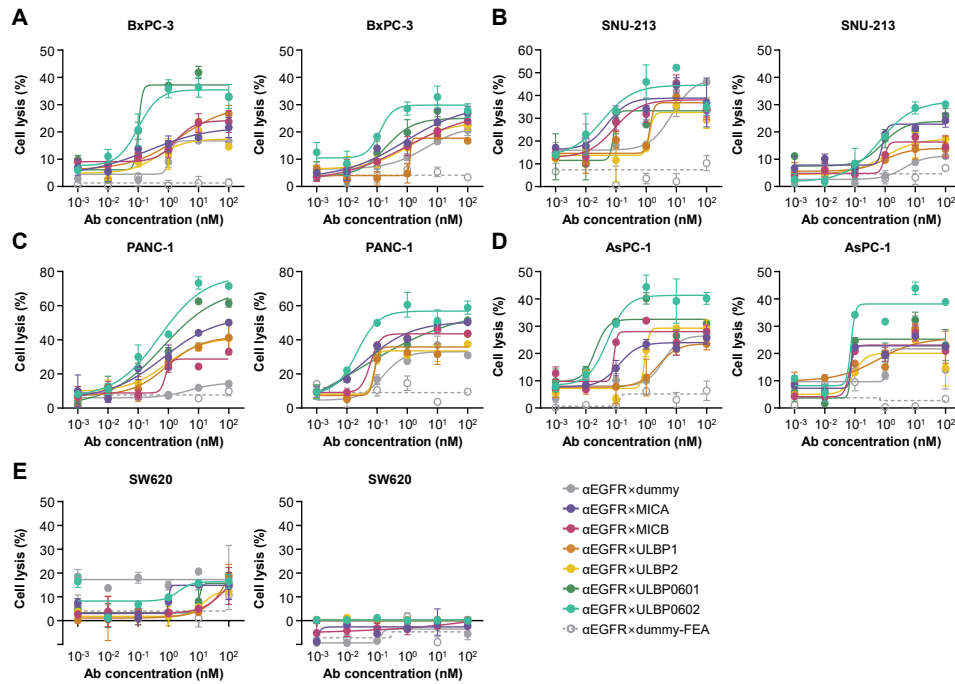
(B) Analytical SEC profiles of  $\alpha$ EGFR×NKG2DL ICEs (30  $\mu$ g per injection) on a Superdex 200 Increase 10/300 column with UV detection at 280 nm. Arrows indicate elution positions of molecular-mass standards (150 kDa, trastuzumab; 66 kDa, bovine serum albumin).

(C) Kinetic analysis of rhEGFR ECD binding by BLI. Representative sensorgrams are shown with increasing antigen concentrations indicated by color; kinetic parameters ( $k_{on}$  and  $k_{off}$ ) and the corresponding kinetic  $K_D$  values are provided in Supplementary Table 1.



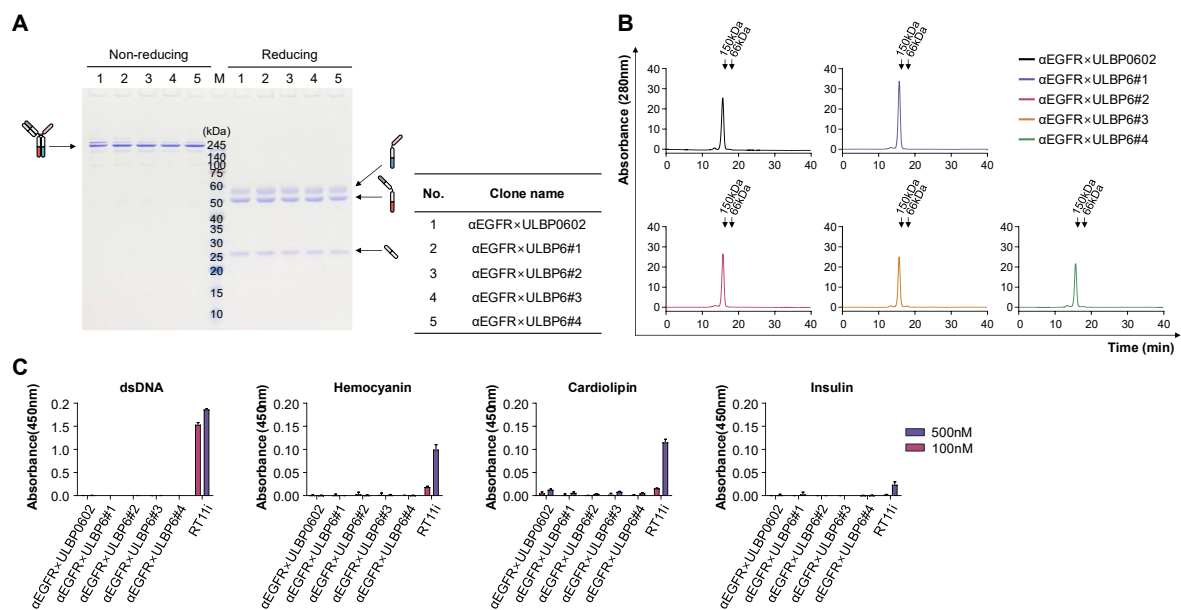
**Supplementary Figure 2. Flow-cytometry gating strategy for quantifying NK cells after *ex vivo* expansion of PBMCs.**

Representative gating scheme used to identify NK cells from expanded cells that were cryopreserved, thawed, and stimulated overnight with rhIL-2 (250 U/mL) prior to analysis. NK cells were defined as  $CD45^{+}CD3^{-}CD56^{+}$  cells stained with APC conjugated anti-CD45, APC-eFluor 780 conjugated anti-CD3, and PE conjugated anti-CD56. Unstained and isotype controls were included.



**Supplementary Figure 3. *In vitro* cytotoxicity of  $\alpha$ EGFR $\times$ NKG2DL ICEs using NK cells from additional donors.**

NK cell-mediated cytotoxicity assays were performed as in Fig. 2B using NK cells from two additional independent donors. Data are shown as mean  $\pm$  SEM of technical duplicates.

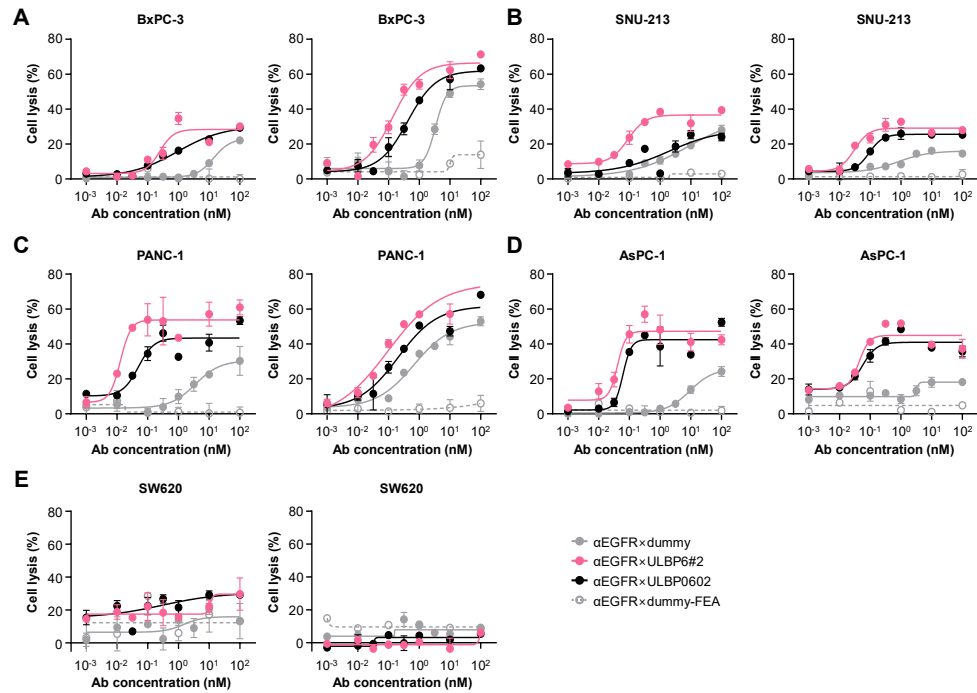


**Supplementary Figure 4. Biophysical characterization and nonspecific binding assessment of engineered αEGFR×ULBP6 ICEs.**

(A) SDS-PAGE of purified engineered αEGFR×ULBP6 ICEs (4 μg per lane) under non-reducing and reducing conditions on 12% polyacrylamide gels.

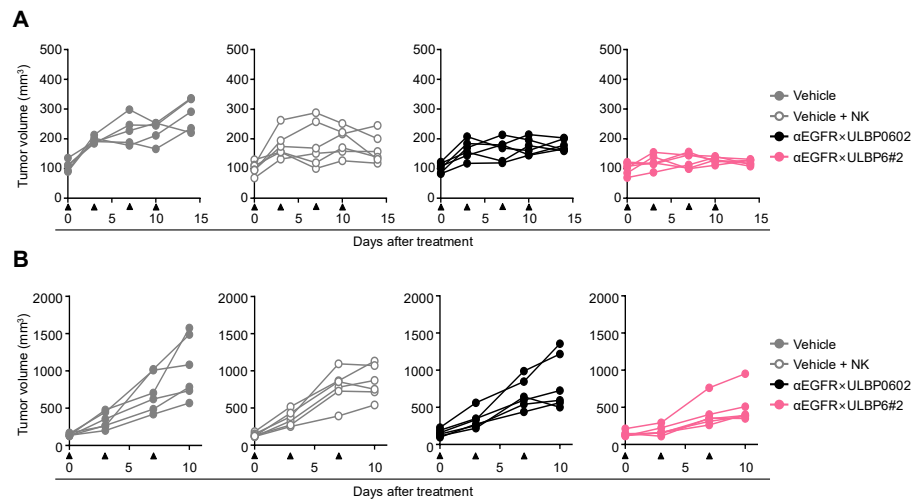
(B) Analytical SEC profiles of engineered αEGFR×ULBP6 ICEs (30 μg per injection) on a Superdex 200 Increase 10/300 column monitored at 280 nm. Molecular-mass standards are indicated (150 kDa, trastuzumab; 66 kDa, bovine serum albumin).

(C) Polyspecific binding to immobilized dsDNA, cardiolipin, hemocyanin, and insulin assessed by ELISA; RT11i was used as a positive control. Data are shown as mean ± SD (n = 3).



**Supplementary Figure 5. *In vitro* cytotoxicity of  $\alpha$ EGFR $\times$ ULBP6#2 versus  $\alpha$ EGFR $\times$ ULBP0602 using NK cells from additional donors.**

NK cell-mediated cytotoxicity assays were performed as in Fig. 4A and 4B using NK cells from two additional independent donors. Data are shown as mean  $\pm$  SEM of technical duplicates.



**Supplementary Figure 6. Individual tumor growth curves for NK-humanized PDAC xenograft studies.**

(A) Individual tumor growth profiles for the PANC-1 group corresponding to Fig. 5B.

(B) Individual tumor growth profiles for the BxPC-3 group corresponding to Fig. 5E.