**Supplementary**

**Cryopreservation of Natural Killer Cells Pre-complexed with Innate Cell Engagers**

**Table S1.** Design of bispecific ICE® constructs.

| Bispecific ICE® constructs | Format | Target specificity | Target domain | Position of targeting domain | Effector specificity | Effector domain | Position of effector domain |
|---|---|---|---|---|---|---|---|
| EpCAM/NKp46 | scFv-IgAb | EpCAM | 42 | Fab | NKp46 | NKp46-1 | scFv |
| EpCAM/NKG2D | scFv-IgAb | EpCAM | 42 | Fab | NKG2D | KYK_2_0 | scFv |
| EpCAM/CD16A | scFv-IgAb | EpCAM | 42 | Fab | CD16A | P2C-47 | scFv |
| CD30/CD16A | scFv-IgAb | CD30 | HRS3 | Fab | CD16A | P2C-47 | scFv |
| Anti-EpCAM IgG1 | IgG | EpCAM | 42 | Fab | n.a. | n.a. | n.a. |
| Anti-EpCAM Fc-enhanced IgG1 | IgG | EpCAM | 42 | Fab | n.a. | n.a. | n.a. |

Fab, antigen-binding fragment; n.a., not applicable; scFv, single-chain variable fragment.

**Table S2.** Thermal stability of bispecific ICE® constructs evaluated by DSF.

| Construct | Additional Tm [°C] | Tm1 [°C] | Tm2 [°C] |
|---|---|---|---|
| EpCAM/NKp46 | 54.0 | 62.5 | 73.2 |
| EpCAM/NKG2D | n.a. | 61.7 | 72.5 |
| EpCAM/CD16A | n.a. | 62.0 | 71.5 |
| Anti-EpCAM IgG1 | n.a. | 63.5 | 75.0 |
| Anti-EpCAM Fc-enhanced IgG1 | n.a. | 47.5 | 74.3 |

The melting profile of ICE® constructs was assessed by recording the fluorescence signal following an increase in temperature by 1 Kelvin/minute and addition of SYPRO® Orange. DSF, differential scanning fluorometry; Tm, transition midpoint temperatures. n.a., not applicable

**Table S3**. ICE® construct stability

| Construct | Relative product content [%] | | | | | |
|---|---|---|---|---|---|---|
| | | 4–8°C | 25°C | 40°C | −80°C | pH 3.5 |
| | 0 days | 7 days | 7 days | 7 days | 3 freeze/thaw cycles | 90 min |
| EpCAM/NKp46 | 97.8 | 97.4 | 97.7 | 96.4 | 98.0 | 98.0 |
| Ep-CAM/NKG2D | 97.7 | 96.7 | 97.3 | 97.3 | 97.7 | 95.6 |
| EpCAM/CD16A | 98.2 | 98.4 | 97.9 | 94.9 | 97.3 | 98.1 |
| Anti-Ep-CAM Fc-en-hanced IgG1 | 96.4 | 95.6 | 95.7 | 94.1 | 95.5 | 96.0 |

ICE® constructs used at 1.5 mg/mL concentration were either incubated at 4–8°C, 25°C, or 40°C for 7 days, subjected to three freeze/thaw cycles at −80°C/ambient temperature or incubated at pH 3.5 for 90 min. The ICE® construct stability was measured by SE-HPLC. SE-HPLC, size exclusion-high-performance liquid chromatography; no data available for the CD30/CD16A ICE and anti-EpCAM IgG1.

**Table S4.** Cell-surface EpCAM expression levels on tumor cell lines

| Cell line | Mean SABC ± SD |
|---|---|
| Detroit 562 | 215240 ± 50255 |
| HCC-1954 | 184253 ± 5988 |
| HCC-1187 | 111931 ± 21910 |

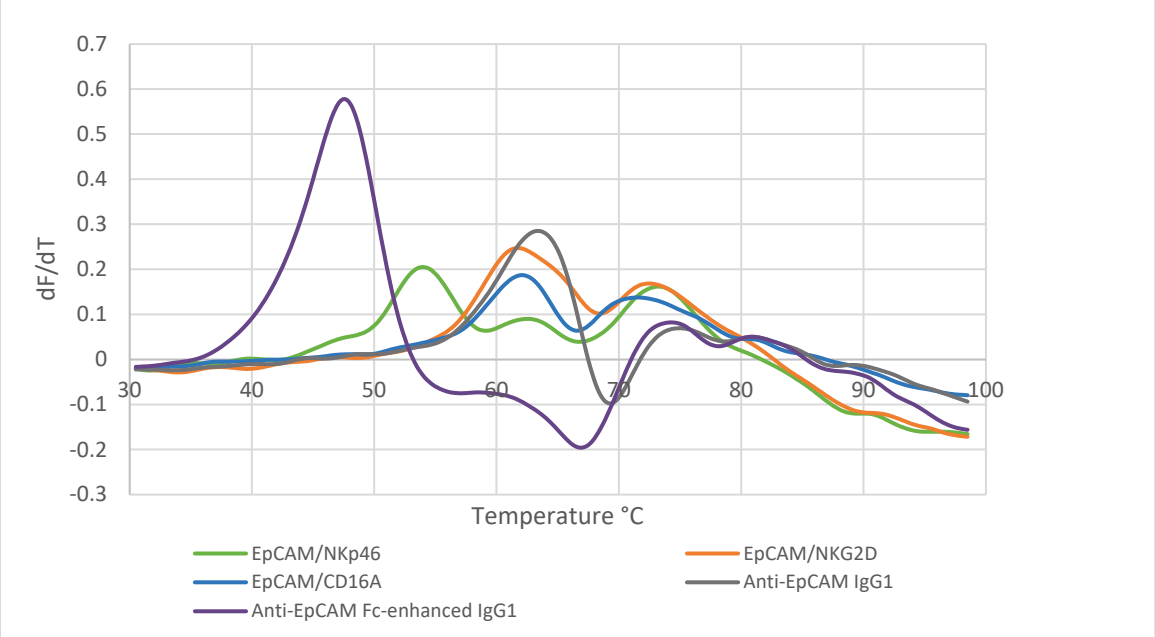
SABC per cell was determined by calibrated flow cytometry using anti-EpCAM mAb 323/A3. Mean values ± standard deviation of three independent assays are shown. mAb, monoclonal antibody; SABC, specific antibody binding capacity.

**Table S5**. Retention of ICE® constructs and control antibodies on the surface of NK cells at different dissociation phases

| Construct | Relative amount of remaining antibodies after dissociation [%] | | |
|---|---|---|---|
| | 6 hours<br>Mean ± SD | 24 hours<br>Mean ± SD | 48 hours<br>Mean ± SD |
| EpCAM/NKp46 | 52.0 ± 11.6 | 15.9 ± 16 | 9.0 ± 0.1 |
| EpCAM/NKG2D | 4.9 ± n.a. | 2.2 ± n.a. | n.a. |
| EpCAM/CD16A | 44.3 ± 9.3 | 17.3 ± 13.3 | 8.2 ± 12.3 |
| Anti-EpCAM IgG1 | −2.7 ± 2.2 | −3.0 ± 2.1 | n.t. |
| Anti-EpCAM Fc-enhanced IgG1 | −1.0 ± 1.2 | −1.0 ± 1.2 | n.t. |
| CD30/CD16A | 48.4 ± 9.8 | 21.6 ± 12.7 | 10.4 ± 12.5 |

Aliquots of enriched primary human NK cells were pre-complexed with 100 µg/mL of the indicated antibody constructs on ICE®, washed, and then incubated at 37°C in a large volume of medium for various time periods to allow dissociation. The retained antibodies were detected by FITC-conjugated goat anti-human IgG and the flow cytometric analysis. MFI determined for each antibody at the time point 0 was set to 100%, MFI of NK cells stained with secondary reagent alone was set to 0, and the relative amount of retained antibodies was calculated. MFI for some samples were lower than MFI for staining with a secondary reagent alone, leading to negative values; values <-10% were excluded. Mean values ± standard deviation from three independent experiments are shown. FITC, fluorescein isothiocyanate; ICE®, innate cell MFI, median fluorescence intensity; n.a., not applicable; n.t., not tested.

**Figure S1.** Thermal stability of bispecific ICE® constructs evaluated by DSF




The melting profile of ICE® constructs was assessed by recording the fluorescence signal (change of fluorescence signal over change of temperature dF/dT) after an increase in temperature by 1 Kelvin/minute and addition of SYPRO® Orange. dF/dT, fluorescence intensity/temperature; DSF, differential scanning fluorometry.