Multifunctional natural killer cell engagers targeting NKp46 trigger protective tumor immunity

Poster #P776

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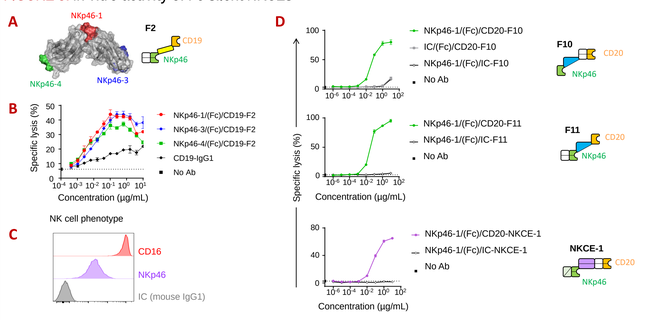
Background

Over the last decade, various new therapies have been developed to promote anti-tumor immunity. Despite interesting clinical results in hematological malignancies, the development of bispecific killer cell engager antibody formats directed against tumor cells and stimulating anti-tumor T-cell immunity has proved challenging, mostly due to toxicity problems.

We report here the generation of trifunctional natural killer (NK) cell engagers (NKCEs), targeting two activating receptors, NKp46 and CD16, on NK cells and a tumor antigen on cancer cells.

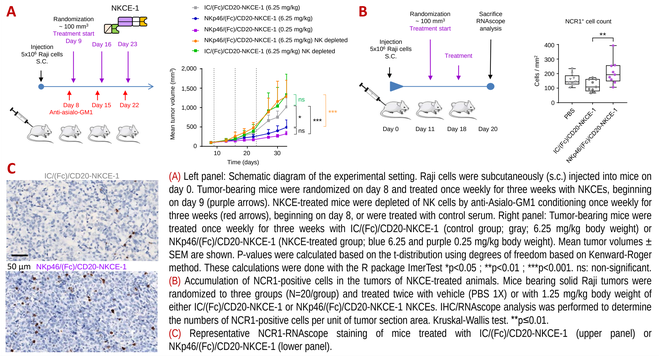
Trifunctional NKCEs were more potent *in vitro* than clinical therapeutic antibodies targeting the same tumor antigen. They had similar *in vivo* pharmacokinetics to full IgG antibodies, no off-target effects and efficiently controlled tumor growth in mouse models of solid and invasive tumors. Trifunctional NKCEs thus constitute a new generation of molecules for fighting cancer.

FIGURE 3: *In vitro* activity of Fc-silent NKCEs



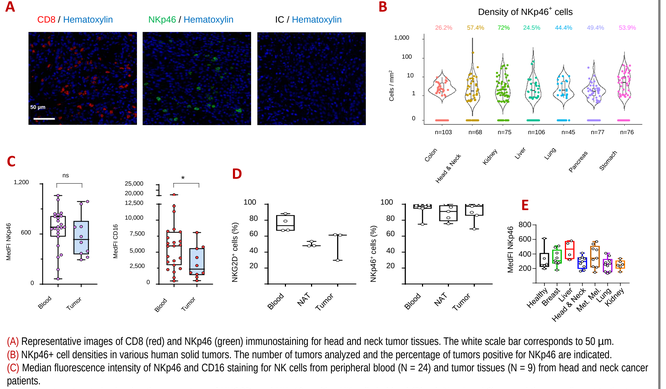
(A) Position of NKCE-1 (red), NKCE-4 (green) and NKCE-3 (blue) epitopes on NKp46 and molecular design of the F2 bispecific format.
(B) Comparison of the cytotoxicities of the different NKCEs with F2 format harboring diverse epitopes differing in specificity for NKp46 and the CD19 TA.
(C) Phenotype of the purified resting NK cells used in (B). Daudi cells were used as the target and purified resting NK cells as effectors. The data shown are representative of 3 independent experiments.
(D) Comparison of the cytotoxicities of the different NKCEs with NKp46-Fc silent formats harboring NKp46-1 epitope specificity and binding to the CD19 and CD20 TAs. Daudi cells were used as the target and purified resting NK cells as effectors. Data are representative of more than 10 independent experiments.

FIGURE 4: Bispecific NKCEs promote tumor growth control *in vivo*



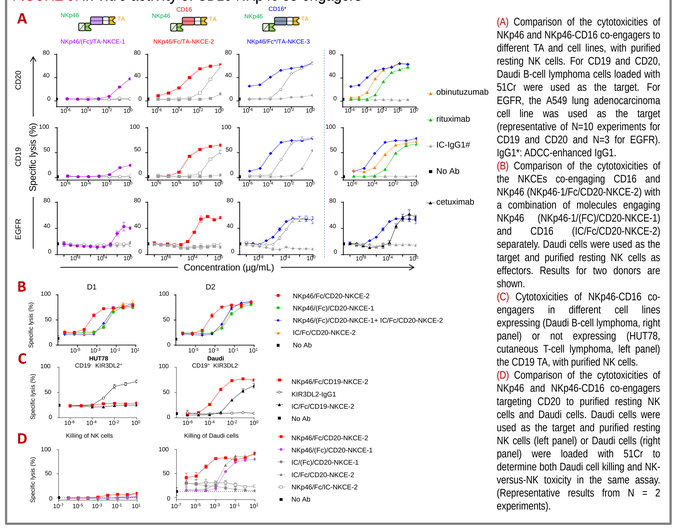
(A) Left panel: Schematic diagram of the experimental setting. Raji cells were subcutaneously (s.c.) injected into mice on day 0. Tumor-bearing mice were randomized on day 8 and treated once weekly for three weeks with NKCEs, beginning on day 9 (purple arrows). NKCE-treated mice were depleted of NK cells by anti-Asialo-GM1 conditioning once weekly for three weeks (red arrows), beginning on day 8, or were treated with control serum. Right panel: Tumor-bearing mice were treated once weekly for three weeks with IC/(Fc)/CD20-NKCE-1 (control group; gray; 6.25 mg/kg body weight) or NKp46/(Fc)/CD20-NKCE-1 (NKCE-treated group; blue 6.25 and purple 0.25 mg/kg body weight). Mean tumor volumes ± SEM are shown. P-values were calculated based on the t-distribution using degrees of freedom based on Kenward-Roger method. These calculations were done with the R package lmerTest *p<0.05 ; **p<0.01 ; ***p<0.001. ns: non-significant.
(B) Accumulation of NCR1-positive cells in the tumors of NKCE-treated animals. Mice bearing solid Raji tumors were randomized to three groups (N=20/group) and treated twice with vehicle (PBS 1X) or with 1.25 mg/kg body weight of either IC/(Fc)/CD20-NKCE-1 or NKp46/(Fc)/CD20-NKCE-1 NKCEs. IHC/RNAscope analysis was performed to determine the numbers of NCR1-positive cells per unit of tumor section area. Kruskal-Wallis test. **p≤0.01.
(C) Representative NCR1-RNAscope staining of mice treated with IC/(Fc)/CD20-NKCE-1 (upper panel) or NKp46/(Fc)/CD20-NKCE-1 (lower panel).

FIGURE 1: NKp46 is expressed on NK cells in the tumor bed



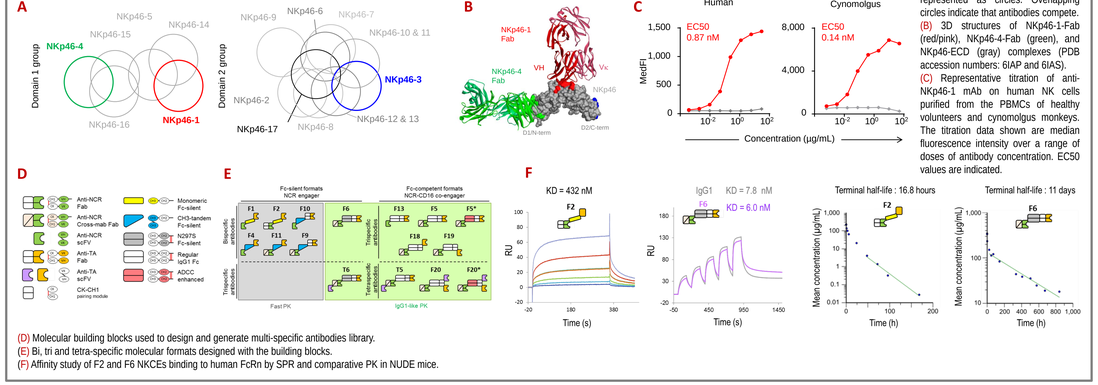
(A) Representative images of CD8 (red) and NKp46 (green) immunostaining for head and neck tumor tissues. The white scale bar corresponds to 50 µm.
(B) NKp46+ cell densities in various human solid tumors. The number of tumors analyzed and the percentage of tumors positive for NKp46 are indicated.
(C) Median fluorescence intensity of NKp46 and CD16 staining for NK cells from peripheral blood (N = 24) and tumor tissues (N = 9) from head and neck cancer patients.
(D) Flow cytometry study showing the percentage of NKG2D- and NKp46-positive NK cells in blood (Blood; N = 4 and 8), normal tissues adjacent to the tumor (NAT; N = 3 and 5) and tumors (Tumor; N = 3 and 7) from lung cancer patients.
(E) Median fluorescence intensity for NKp46 staining, on the flow cytometry of peripheral NK cells from cancer patients and healthy donors. Healthy donors (healthy; N = 6). Breast (N = 9). Liver (N = 4). Head and Neck (N = 9). Metastatic melanoma (Met. Mel; N = 9). Lung (N = 10). Kidney (N = 6).

FIGURE 5: *In vitro* activity of CD16-NKp46 co-engagers



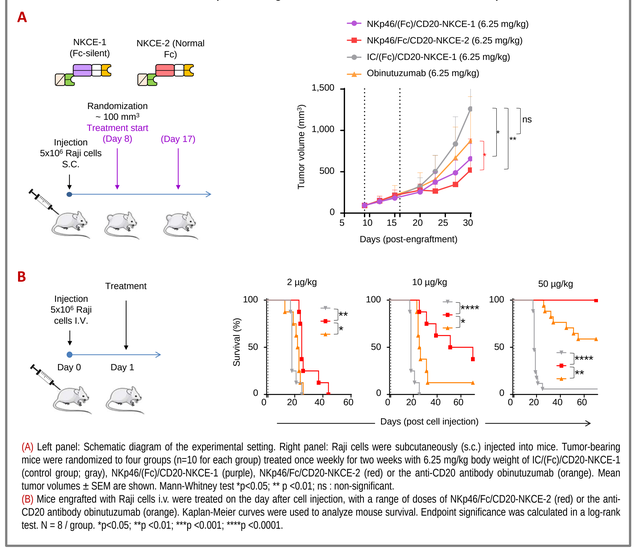
(A) Comparison of the cytotoxicities of NKp46 and NKp46-CD16 co-engagers to different TA and cell lines, with purified resting NK cells. For CD19 and CD20, Daudi B-cell lymphoma cells loaded with 51Cr were used as the target. For EGFR, the A549 lung adenocarcinoma cell line was used as the target (representative of N=10 experiments for CD19 and CD20 and N=3 for EGFR). IgG1*: ADCC-enhanced IgG1.
(B) Comparison of the cytotoxicities of the NKCEs co-engaging CD16 and NKp46 (NKp46-1/Fc/CD20-NKCE-2) with a combination of molecules engaging NKp46 (NKp46-1/(FC)/CD20-NKCE-1) and CD16 ((IC/Fc/CD20-NKCE-2) separately. Daudi cells were used as the target and purified resting NK cells as effectors. Results for two donors are shown.
(C) Cytotoxicities of NKp46-CD16 co-engagers in different cell lines expressing (Daudi B-cell lymphoma, right panel) or not expressing (HUT78, cutaneous T-cell lymphoma, left panel) the CD19 TA, with purified NK cells.
(D) Comparison of the cytotoxicities of NKp46 and NKp46-CD16 co-engagers targeting CD20 to purified resting NK cells and Daudi cells. Daudi cells were used as the target and purified resting NK cells (left panel) or Daudi cells (right panel) were loaded with 51Cr to determine both Daudi cell killing and NK-versus-NK toxicity in the same assay. (Representative results from N = 2 experiments).

FIGURE 2: anti-NKp46 antibody library and multispecific ab formats



(A) Epitope binning. Epitopes are represented as circles. Overlapping circles indicate that antibodies compete.
(B) 3D structures of NKp46-1-Fab (red/pink), NKp46-4-Fab (green), and NKp46-ECD (gray) complexes (PDB accession numbers: 6IAP and 6IAS).
(C) Representative titration of anti-NKp46-1 mAb on human NK cells purified from the PBMCs of healthy volunteers and cynomolgus monkeys. The titration data shown are median fluorescence intensity over a range of doses of antibody concentration. EC50 values are indicated.
(D) Molecular building blocks used to design and generate multi-specific antibodies library.
(E) Bi, tri and tetra-specific molecular formats designed with the building blocks.
(F) Affinity study of F2 and F6 NKCEs binding to human FcRn by SPR and comparative PK in NUDE mice.

FIGURE 6: Trifunctional NKCEs promoting ADCC are more efficient than bispecific mAbs *in vivo*



(A) Left panel: Schematic diagram of the experimental setting. Right panel: Raji cells were subcutaneously (s.c.) injected into mice. Tumor-bearing mice were randomized to four groups (n=10 for each group) treated once weekly for two weeks with 6.25 mg/kg body weight of IC/(Fc)/CD20-NKCE-1 (control group; gray), NKp46/(Fc)/CD20-NKCE-1 (purple), NKp46/Fc/CD20-NKCE-2 (red) or the anti-CD20 antibody obinutuzumab (orange). Mean tumor volumes ± SEM are shown. Mann-Whitney test *p<0.05; ** p <0.01; ns : non-significant.
(B) Mice engrafted with Raji cells i.v. were treated on the day after cell injection, with a range of doses of NKp46/Fc/CD20-NKCE-2 (red) or the anti-CD20 antibody obinutuzumab (orange). Kaplan-Meier curves were used to analyze mouse survival. Endpoint significance was calculated in a log-rank test. N = 8 / group. *p<0.05; **p <0.01; ***p <0.001; ****p <0.0001.

Reference Paper: *Gauthier et al.*, Cell 2019 Jun 13;177(7):1701-1713

Conclusion

We report here the development of a multispecific antibody technology for engaging NK cells. Trifunctional NKCEs targeting CD19, CD20 or EGFR as tumor antigens triggered tumor killing by human primary NK cells *in vitro*. *In vivo*, they induced NK cell accumulation in tumors and promoted tumor clearance in preclinical mouse models of solid and invasive cancers. The approach used was based on innovative IgG-Fc multispecific antibody formats and first-in-class agonist anti-NKp46 mAbs activating NK cells only when cross-linked by tumor cells, with no off-target effects.

Our multispecific technology provides a versatile platform with many different format options and the potential to co-engage up to three activating receptors on NK cells and two different tumor antigens on cancer cells. With the trifunctional NKCEs reported here, the binding affinities for NKp46 (KD=15 nM) and CD16 (KD=1 µM and 29 nM for NKCE-2 and NKCE-3, respectively) should favor NK cell targeting to the tumor microenvironment, in which NKp46 expression levels remain high in many tumor conditions, by contrast to CD16, NKG2D, NKp30 and NKp44. Importantly, trifunctional NKCEs were found to be more potent than a mixture of the bispecific reagents activating NKp46 and CD16 separately. The co-targeting of NKp46 and CD16 thus led to full NK cell activation. Together with the stronger anti-tumor activity of these molecules in preclinical models than of gold standard mAbs, such rituximab, obinituzumab and cetuximab, these results support the clinical development of NKCEs for cancer immunotherapy, as a complement to existing immuno-oncology approaches.