NKG2D-based multispecific CAR T-cells to overcome antigen escape and improve anti-tumor efficacy

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BACKGROUND

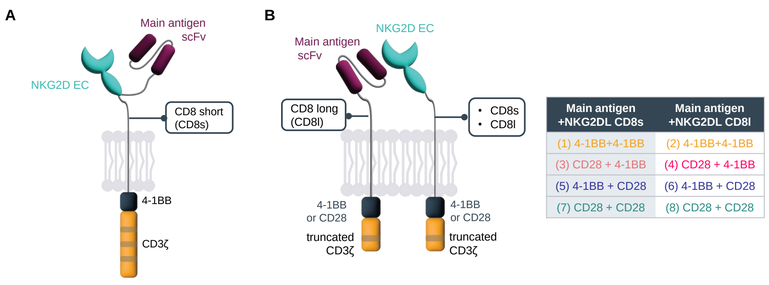
- NKG2D ligands (NKG2DL) are eight stress-induced ligands expressed by cancer cells but absent from healthy cells. Given their expression in a large range of cancer indications, their tumor specificity, and the low likelihood of complete loss of all ligands at the same time, NKG2DL are attractive targets for multispecific chimeric antigen receptor (CAR) T-cells.
- Anti-CD19 CAR T-cells represent a real therapeutic approach for B-cell malignancies. However, despite the impressive remission rate, patient's relapse occurs due to among others antigen loss. To tackle this short-lived efficacy, multispecific CAR T-cell therapies targeting several antigens were developed and are currently assessed clinically. Even more strikingly, in the context of solid tumors, antigen heterogeneity has been identified as one of the major challenges that has so far limited the success of CAR T-cells in other indications. Here, we describe our efforts to overcome these issues by developing multispecific CAR T-cells targeting NKG2DL and other antigens. We make the proof of concept of our NKG2D-based multispecific platform with three validated antigens, which are CD19, BCMA and PSMA.

METHODS

- We designed multispecific CAR utilizing tandem and dual NKG2D-based constructs that encompass the extracellular (EC) domain of the natural NKG2D receptor fused to or co-expressed with a CAR targeting a main antigen.
- In the tandem receptor, the main antigen scFv was placed in distal position while NKG2D EC was in proximal position and linked to the transmembrane domain via a short hinge (15 aa) derived from CD8. This receptor, as well as single CAR controls, contain 4-1BB and a full CD3ζ as co-stimulatory and stimulatory domains respectively (**Figure 1A**). In dual receptors, the main antigen-specific CAR contained a CD8 long hinge and was co-expressed with an anti-NKG2DL CAR encompassing a CD8 short or long hinge. These receptors contain 4-1BB or CD28 as co-stimulatory domain and a truncated CD3ζ as stimulatory domain (**Figure 1B**).
- PBMCs were activated on day 0 and incubated with the respective retroviral vector on day 2. Transduced T-cells were then selected with magnetic beads and expanded for 4 days.
- Cytokine secretion, cytotoxic activity and proliferation of multispecific CAR T-cells were evaluated *in vitro* against main antigen positive and negative cancer cells. In addition, for CD19/NKG2DL multispecific CAR T-cells, anti-tumor activity of lead candidates was also evaluated *in vitro* against primary B-ALL cells and *in vivo* in a B-ALL relapse model.

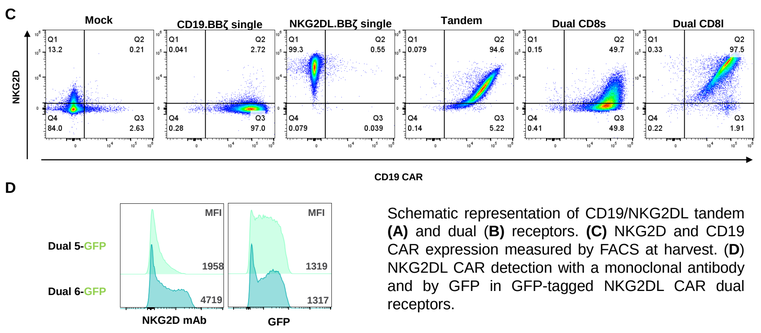
FIGURES

Figure 1: Tandem and dual CAR design



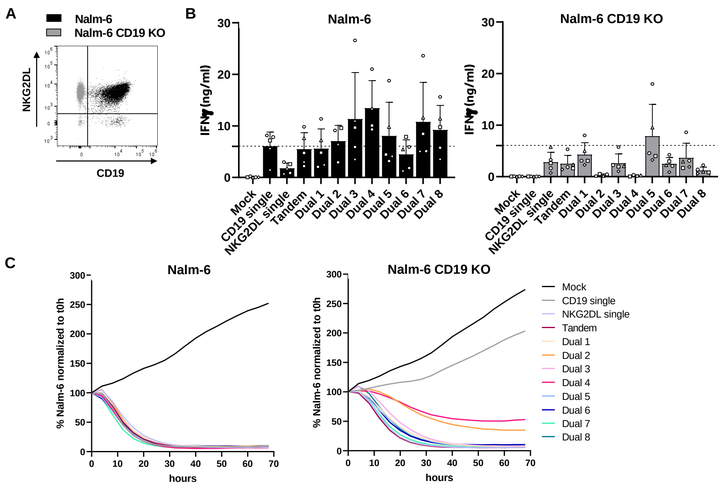
Main antigen +NKG2DL CD8s	Main antigen +NKG2DL CD8l
(1) 4-1BB+4-1BB	(2) 4-1BB+4-1BB
(3) CD28 + 4-1BB	(4) CD28 + 4-1BB
(5) 4-1BB + CD28	(6) 4-1BB + CD28
(7) CD28 + CD28	(8) CD28 + CD28

Figure 2: CD19/NKG2DL multispecific CAR are highly expressed



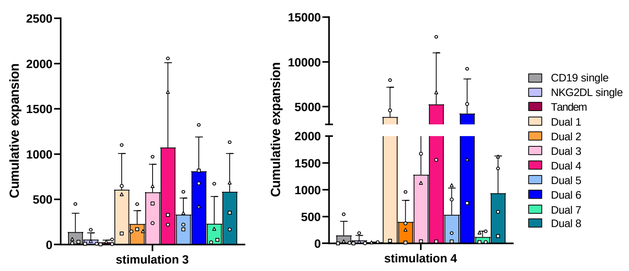
Schematic representation of CD19/NKG2DL tandem **(A)** and dual (**B**) receptors. **(C)** NKG2D and CD19 CAR expression measured by FACS at harvest. (**D**) NKG2DL CAR detection with a monoclonal antibody and by GFP in GFP-tagged NKG2DL CAR dual receptors.

Figure 3: All but two CD19/NKG2DL multispecific CAR T-cells are highly active even in the absence of CD19 antigen



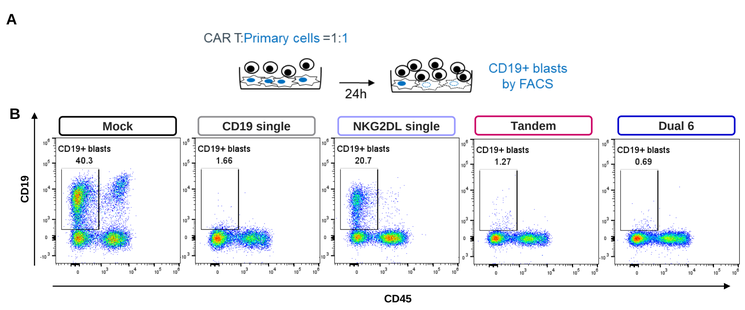
(A) CD19 and NKG2DL expression in Nalm-6 WT and CD19 KO. (**B**) IFN-γ secretion after 24h of co-culture with Nalm-6 WT and CD19 KO (n=4-5, mean+SD). (**D**) Percentage of viable cancer cells relative to t0h in co-culture with control and multispecific CAR T-cells (n=3, mean).

Figure 4: Most CD19/NKG2DL dual but not tandem CAR T-cells display expand better than CD19 single CAR T-cells in repeated antigen stimulation



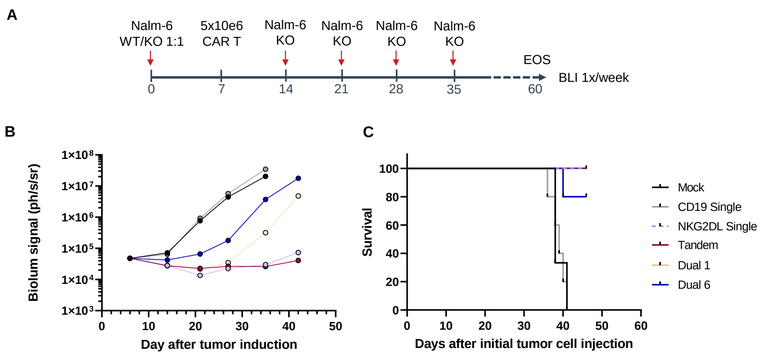
Cumulative expansion after stimulation 3 and 4 with HeLa overexpressing CD19 (n=4, mean+SD).

Figure 5: CD19/NKG2DL multispecific CAR T-cells are effective against CD19+ primary B-ALL cells



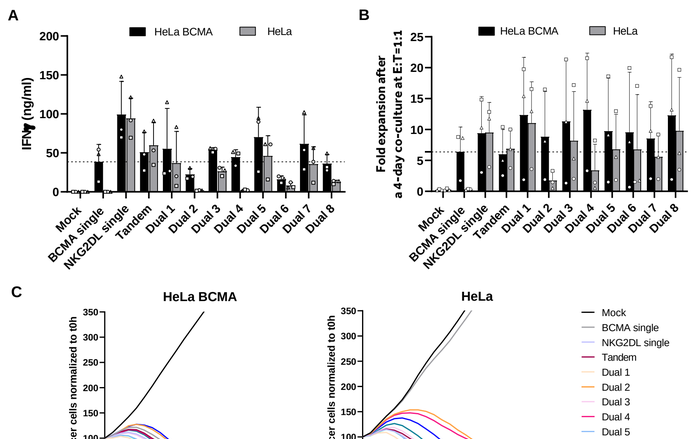
(A) Experimental design (**B**) Percentage of CD19+ living blasts after co-culture with CAR T-cells.

Figure 6: CD19/NKG2DL tandem CAR T-cells efficiently control tumor cells in a B-ALL relapse *in vivo* model



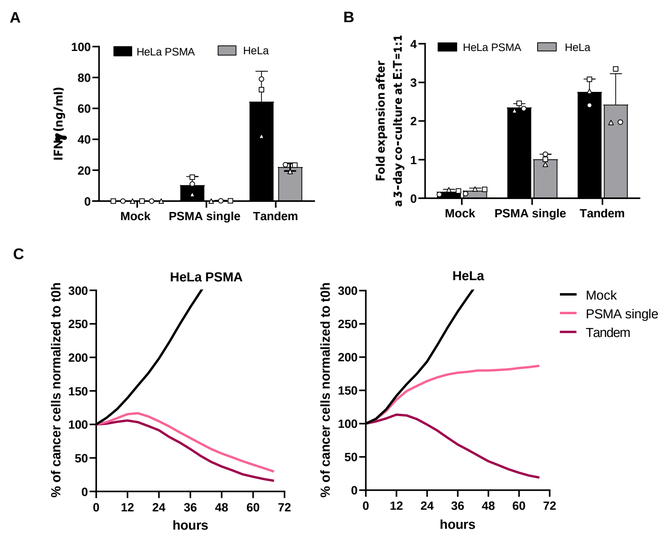
(A) Experimental design **(B)** Tumor burden measured by BLI (geometric mean +/- error) and **(C)** survival. 5 mice per group except in Mock, 3 mice.

Figure 7: BCMA/NKG2DL multispecific CAR T-cells are efficient even in absence of BCMA



(**A**) IFN-γ secretion after 24h of co-culture with HeLa BCMA and HeLa WT (n=3, mean+SD). (**B**) Fold expansion after 4 days of co-culture with HeLa BCMA and HeLa WT(**C**) Percentage of viable cancer cells relative to t0h in co-culture with control and multispecific CAR T-cells (n=3, mean).

Figure 8: PSMA/NKG2DL tandem CAR T-cells are efficient against PSMA+ and PSMA- cancer cells



(**A**) IFN-γ secretion after 24h of co-culture with HeLa PSMA and HeLa WT (n=3, mean+SD). (**B**) Fold expansion after 3 days of co-culture with HeLa PSMA and HeLa WT. (**C**) Percentage of viable cancer cells relative to t0h in co-culture with control and tandem CAR T-cells (n=3, mean).

MAIN RESULTS

- Expression of CD19/NKG2DL multispecific receptors was evaluated by staining T- cells with an anti-NKG2D antibody and a rhCD19-Fc + anti-IgG Fc. As shown in **Figure 2C**, all were highly expressed. Indeed, experiments with a GFP-tagged NKG2DL CAR confirmed that the lower NKG2DL CAR level observed for constructs with a short hinge NKG2DL CAR was due to a lower detection by the monoclonal antibody rather than to a lower expression (**Figure 2D**).
- Functionality of CD19/NKG2DL multispecific CAR T-cells was evaluated against the NKG2DL+ B-ALL cell line Nalm-6 and its CD19 KO derivative (**Figure 3A**). All multispecific CAR T-cells secreted similar or higher levels of IFN-γ than CD19 single CAR T-cells. However, in absence of CD19, dual 2 and 4 did not secrete IFN-γ (**Figure 3B**). In a co-culture assay, all CAR T-cells rapidly eliminated both Nalm-6 WT and CD19 KO, except dual 2 and 4 that less efficiently controlled Nalm-6 CD19 KO growth (**Figure 3C**).
- In a repeated stimulation assay with HeLa expressing CD19, we observed that tandem CAR T-cell expansion was systematically lower in comparison to CD19 single CAR T-cells (**Figure 4**). However, for tandem candidates with a short hinge, this 2-fold difference only represents one cycle of cell division.
- Next, cytotoxic activity of the tandem and of one selected dual candidate was evaluated against CD19+ primary B-ALL cells. As shown in **Figure 5**, cancer cells were rapidly eliminated by CD19/NKG2DL tandem and dual CAR T-cells. NKG2DL single CAR T-cells cells lysed about 50% of blasts while CD19 single and CD19/NKG2DL multispecific CAR T-cells lysed nearly all blasts, showing the relevance of targeting NKG2DL in B-ALL.
- Next, tandem and two selected dual candidates were evaluated *in vivo* in a B-ALL relapse model (**Figure 6A**) and compared to single CAR controls. Bioluminescence data show a strong tumor control in NKG2DL single and tandem CAR T-cells (**Figure 6B**) and an increased survival in NKG2DL single and multispecific CAR T-cells injected mice (**Figure 6C**).
- Functionality of BCMA/NKG2DL multispecific CAR T-cells was evaluated against HeLa or HeLa overexpressing BCMA. Several multispecific CAR T-cells secreted similar or higher levels of IFN-γ than BCMA single CAR T-cells. However, in absence of BCMA dual 2 and 4 did not secrete IFN-γ, as observed for CD19/NKG2DL dual CAR T-cells (**Figure 7A**). This absence of cytokine secretion was accompanied by a lower expansion (**Figure 7B**) and a lower cytotoxic activity (**Figure 7C**) in absence of BCMA while other candidates conserved functionality.
- Finally, we evaluated PSMA/NKG2DL tandem CAR T-cells and showed that this candidate secreted more IFN-γ than PSMA single CAR T-cells in presence and in absence of PSMA (**Figure 8A**). Regarding proliferation and cytotoxic activity, single and tandem CAR T-cells performed similarly (**Figure 8B**). When co-cultured with HeLa WT cells, only PSMA/NKG2DL tandem CAR T-cells proliferated and were able to control tumor cell growth (**Figure 8C**).

CONCLUSIONS

- **This data provides the proof-of-concept that NKG2DL are valuable targets in a multispecific CAR approach.**
- **Specifically, we showed that CD19/NKG2DL multispecific CAR T-cells, and in particular dual receptors, are highly effective *in vitro* against CD19+ and CD19- cell lines and against CD19+ primary B-ALL cells. *In vivo*, CD19/NKG2DL tandem CAR T-cells outperforms dual CAR T-cells in controlling tumor growth in an aggressive B-ALL relapse model.**
- ***In vitro* data generated with BCMA/NKG2DL and PSMA/NKG2DL multispecific CAR T-cells further validate this approach and its application in other hematological and solid indications.**

AFFILIATIONS, DISCLOSURES & AKNOWLEDGMENTS

J.B., B.V., C.J.H., V.V. and E.B. are employed by Celyad Oncology SA. This poster is published for information only.

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