Multispecific CD19 and NKG2D ligands CAR T-cells are effective against CD19 positive and CD19 negative B-cell malignancies

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BACKGROUND

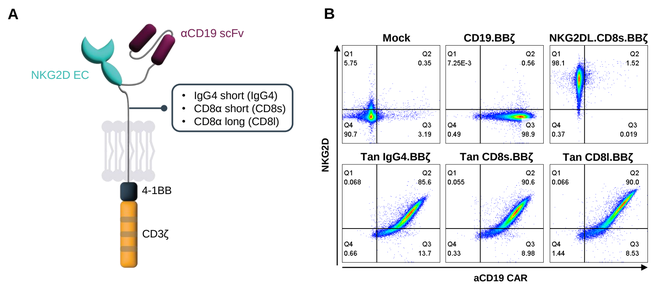
- Anti-CD19 chimeric antigen receptor (CAR) T-cells represent a highly promising strategy for B-cell malignancies. However, despite the inspiring remission, patient's relapse occurs due to among others antigen loss. To tackle this short-lived efficacy, multispecific CAR T-cell therapies targeting several B-cell antigens were developed and are currently assessed clinically.
- 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, including B-cell malignancies, their tumor specificity, and the low likelihood of complete loss of expression, NKG2DL are attractive targets for multispecific CAR T-cells, including CD19/NKG2DL multispecific CAR T-cells.

METHODS

- We designed different NKG2D/CD19 multispecific CAR, utilizing both tandem and dual NKG2D-based constructs that encompass the extracellular (EC) domain of the natural NKG2D receptor fused to, or co-expressed with an anti-CD19 CAR, respectively.
- In tandem receptors, anti-CD19 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 IgG4 or CD8 or a via long hinge (50 aa) derived from CD8. The tandem receptors, 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 anti-CD19 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 5A**).
- 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 the CD19/NKG2DL multispecific CAR T-cells were evaluated *in vitro* against both CD19+ and CD19- cancer cells. In addition, anti-tumor activity of selected candidates were also evaluated *in vitro* against primary B-ALL cells and *in vivo* in a B-ALL relapse model.

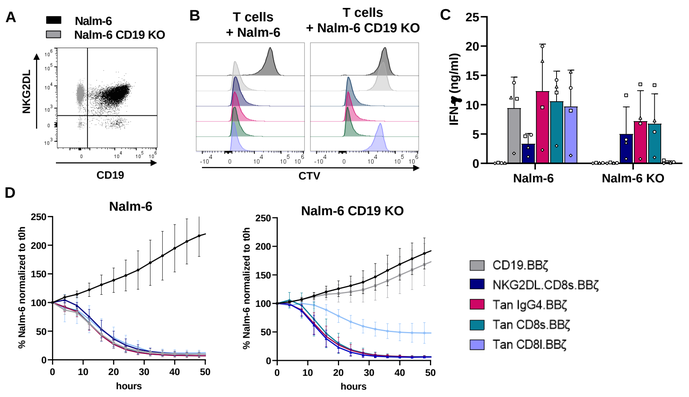
FIGURES

Figure 1: CD19/NKG2DL tandem CAR are highly expressed



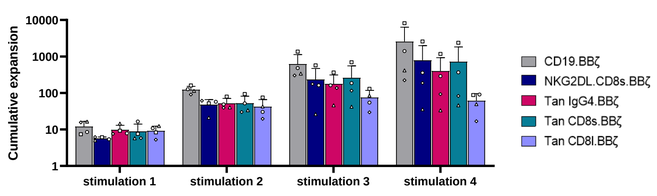
(A) Schematic representation of tandem receptors (**B**) NKG2D and CD19 CAR expression measured by FACS at harvest.

Figure 2: Tandem CAR T-cells with a short hinge are highly active even in the absence of CD19 antigen



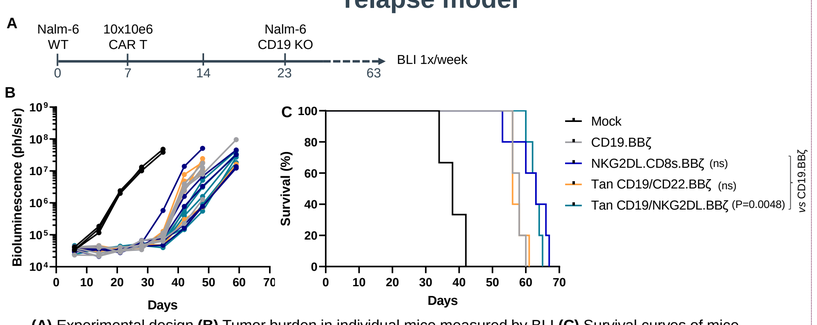
(A) CD19 and NKG2DL expression in Nalm-6 WT and CD19 KO. (**B**) T-cell proliferation after 96h of co-culture with Nalm-6 WT and CD19 KO cancer cells (representative experiment). (**C**) IFN-γ secretion after 24h of co-culture with Nalm-6 WT and CD19 KO (n=4, mean+SD). (**D**) Percentage of viable Nalm-6 WT and CD19 KO relative to t0h in co-culture with control and tandem CAR T-cells (n=4, mean±SD).

Figure 3: Tandem CAR T-cells display lower expansion compared to CD19 single CAR T in repeated antigen stimulation



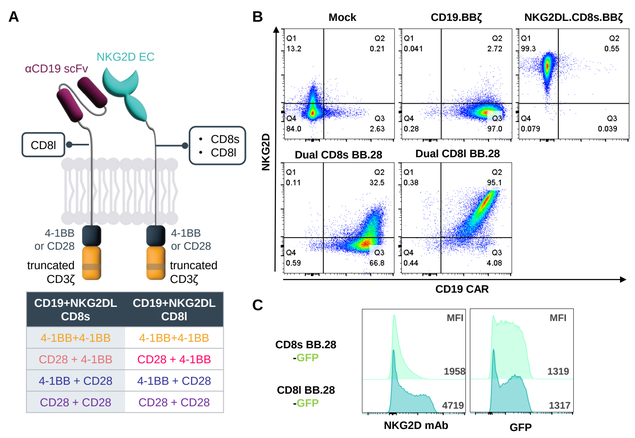
Cumulative expansion after each stimulation with HeLa overexpressing CD19 (n=4, mean+SD).

Figure 4: *in vivo* evaluation of tandem CAR T-cells in a B-ALL relapse model



(A) Experimental design **(B)** Tumor burden in individual mice measured by BLI **(C)** Survival curves of mice. Multiple comparisons performed with Log-Rank (Mantel-Cox) test (n=5 mice per group).

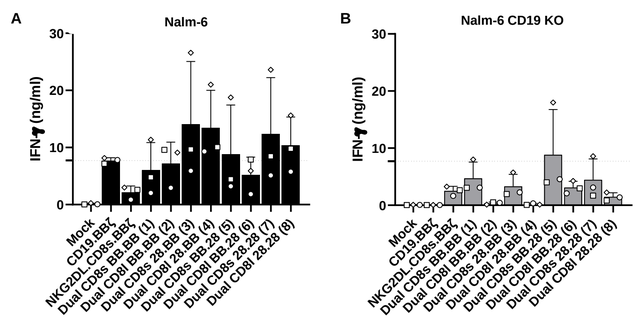
Figure 5: CD19/NKG2DL dual CAR are highly expressed



CD19+NKG2DL CD8s	CD19+NKG2DL CD8l
4-1BB+4-1BB	4-1BB+4-1BB
CD28 + 4-1BB	CD28 + 4-1BB
4-1BB + CD28	4-1BB + CD28
CD28 + CD28	CD28 + CD28

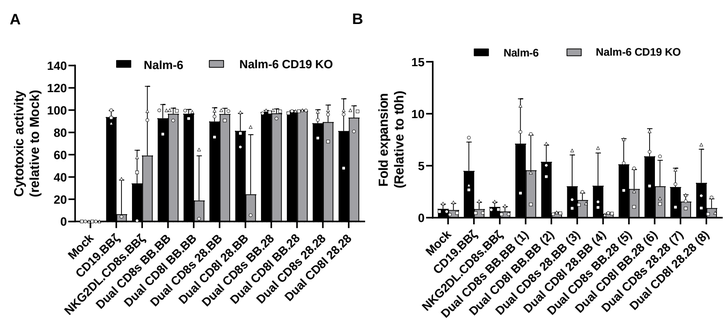
(A) Schematic representation of dual receptors (**B**) NKG2DL and CD19 CAR expression measured by FACS at harvest (representative experiment of n=5) (**C**) NKG2DL CAR detection with a monoclonal antibody and by GFP in GFP-tagged NKG2DL CAR dual receptors.

Figure 6: All but two dual CAR T-cell candidates secrete cytokines in absence of CD19 antigen



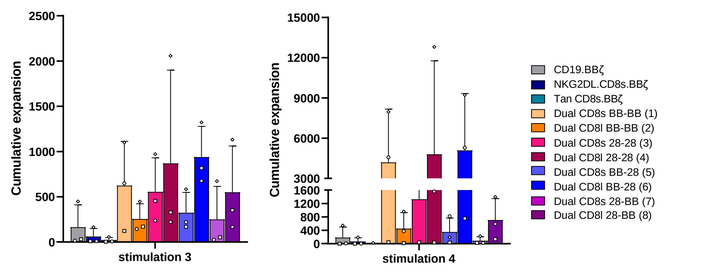
IFN-γ secretion after 24h of co-culture at 1:1 E:T ratio with Nalm-6 WT (**A**) and Nalm-6 CD19 KO (**B**) (n=3, mean+SD).

Figure 7: All but two dual CAR T candidates display cytotoxicity and proliferate at low E:T in absence of CD19 antigen



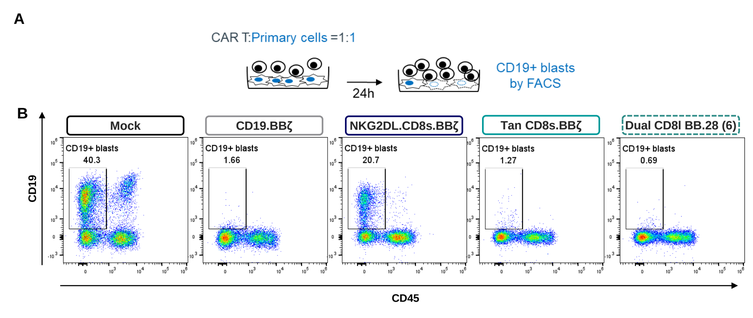
Relative cytotoxic activity **(A)** and proliferation **(B)** of T-cells after 6 days of co-culture at 1:10 E:T ratio with Nalm-6 WT and CD19 KO (n=3, mean+SD).

Figure 8: Most dual CAR T candidates expand better than CD19 single CAR T in repeated antigen stimulation



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

Figure 9: 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.

MAIN RESULTS

- Expression of tandem receptors was evaluated by staining T-cells with an anti-NKG2D antibody and a rhCD19-Fc + anti-IgG Fc. As shown in **Figure 1B**, all three tandem receptors were highly expressed.
- Functionality of tandem CAR T-cells was evaluated against the NKG2DL+ B-ALL cell line Nalm-6 and its CD19 KO derivative (**Figure 2A**). As expected, all T-cells, except Mock, proliferated and secreted cytokines when co-cultured with Nalm-6. In absence of the CD19 antigen, tandem CAR T-cells encompassing a short hinge conserved their functionality while tandem with CD8 long hinge displayed limited proliferation and cytokine secretion (**Figure 2B and 2C**). In a co-culture assay, tandem CAR T-cells with a short hinge rapidly eliminated both Nalm-6 WT and CD19 KO while tandem CAR T with a long hinge less efficiently controlled Nalm-6 CD19 KO growth (**Figure 2D**).
- 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 3**). However, for tandem candidates with a short hinge, this 2-fold difference only represents one cycle of cell division.
- Next, tandem CAR T-cells with the CD8 short hinge were evaluated *in vivo* in a B-ALL relapse model (**Figure 4A**) and compared to single CAR controls and to CD19/CD22 tandem CAR T-cells as a benchmark. Although in comparison to CD19 single CAR T-cells, the effect on tumor growth was not significant, CD19/NKG2DL tandem CAR T-cells increased mice survival while CD19/CD22 tandem CAR-T cells did not (**Figure 4B and 4C**).
- Both CD19 and NKG2DL CAR were were highly expressed in dual constructs (**Figure 5B**). Indeed, experiments with a GFP-tagged NKG2DL CAR confirmed that the lower NKG2DL 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 5C**).
- When co-cultured with Nalm-6 cells, all dual CAR T-cells secreted similar or higher levels of IFN-γ than CD19 single CAR T-cells (**Figure 6A**). All but two dual CAR T-cell candidates secreted IFN-γ in absence of CD19 antigen (**Figure 6B**).
- Similar results were observed for cytotoxic activity and proliferative capacity when CAR T-cells were challenged at 1:10 E:T ratio (**Figure 7A and 7B**).
- In stimulation 3 and 4 of a repeated stimulation assay with CD19+ HeLa cells, most dual CAR T-cells outperformed CD19 single CAR T-cells when assessing their proliferative capacity (**Figure 8**).
- Finally, cytotoxic activity of the selected tandem candidate and of one selected dual candidate was evaluated against CD19+ primary B-ALL cells. As shown in **Figure 9**, 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.

CONCLUSIONS

- **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*, tandem CAR T-cells outperformed CD19/CD22 CAR T-cells in an aggressive B-ALL relapse model. Two promising dual candidates are currently being assessed in a similar model.**
- **This further provides the proof-of-concept that NKG2DL are valuable targets in a multispecific CAR approach and are currently being explored in other 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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