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NKG2D as a chimeric antigen receptor – DAP10 provides optimal co-stimulation for NKG2D-based CARs

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BACKGROUND

- CYAD-01 (previously named NKR-2) are engineered T cells expressing a chimeric antigen receptor (CAR) composed of the full-length human natural killer group 2, member D receptor (NKG2D) fused to the CD3ζ cytoplasmic signaling domain (**Figure 1**)
  - NKG2D is a transmembrane receptor expressed by natural killer cells and some subsets of T cells that binds to 8 stress-inducible ligands frequently expressed on tumor cells: the MHC class I chain–related proteins A (MICA) and B (MICB) and the unique long 16 binding proteins (ULBP) 1–6 ligands [1,2]
  - The surface adaptor molecule DNAX-activating protein of 10kDa (DAP-10), which is endogenously expressed on T cells, associates with and stabilizes NKR-2 CAR expression
  - Ligand binding to NKR-2 triggers a primary signal via CD3ζ and a secondary signal via DAP-10, resulting in efficient T-cell co-stimulation and cytotoxicity
- NKR-2 is an autologous T-cell therapy that has shown promising results in multiple preclinical models and in the clinic [3]
- Preclinical results indicate that NKR-2 may also have anti-cancer effects beyond direct cancer cell killing [4]
  - Targeting neovasculature expressing NKG2D ligands
  - Cytotoxic killing of immunosuppressive cells within the tumor microenvironment (TME) such as regulatory T cells and myeloid-derived suppressor cells expressing NKG2D ligands
  - Recruiting and activating macrophages and myeloid cells within the stroma, causing a shift from an immunosuppressive to an immunostimulatory TME
  - Inducing a long-term memory immune response specific towards tumor antigens
- NKR-2 may be an effective treatment for solid and hematological tumor types that express NKG2D ligands and is currently being investigated in multiple clinical trials
- Addition of co-stimulatory domain in tandem with CD3ζ domain has been shown to improve *in vitro* T-cell activation and killing as well as *in vivo* anti-tumor efficacy and persistence [5].
- The objective of this study was to compare alternative NKG2D-based CARs constructs containing additional co-stimulatory proteins with our standard NKR-2 CAR (no co-stimulatory domain) in terms of expression, potency and cytokine secretion

METHODS

- In the NKR-2 + DAP10 construct, exogenous DAP10 (**Figure 1**) and NKR-2 are expressed from the same RNA transcript. Since DAP10 is necessary to stabilize and send NKG2D complex to the membrane, overexpression of DAP10 aims to increase NKR-2 levels at the cell surface
- NKR-2 + CD28 and NKR-2 + 4-1BB CARs were created by incorporating the CD28 or 4-1BB cytoplasmic domains , respectively, between the NKG2D and CD3ζ cytoplasmic domains of NKR-2, with the aim of providing additional signaling upon ligand binding and potentially improving proliferation, efficacy and persistence of NKR-2 (**Figure 1**)

All NKG2D-based CARs contained a truncated form of CD19 (tCD19) to allow the evaluation of transduction efficiency and purification of transduced cells.

A GALV-pseudotyped retroviral vector was used to transduce activated human PBMCs with the CAR constructs. As control (ctrl), PBMC were transduced with a mock vector coding only for tCD19. Two days after transduction, CD19+ cells were purified. This purification step abrogates differences in transduction efficiency between the NKR-2 CAR constructs. After purification, transduced T cells were expanded for 4 days in the presence of IL-2 and an NKG2D-blocking antibody. This antibody counteracts the fratricide effect otherwise observed among NKR-2-transduced T cells [6]

FIGURES

FIGURE 1: Schematic of NKG2D-based CARs

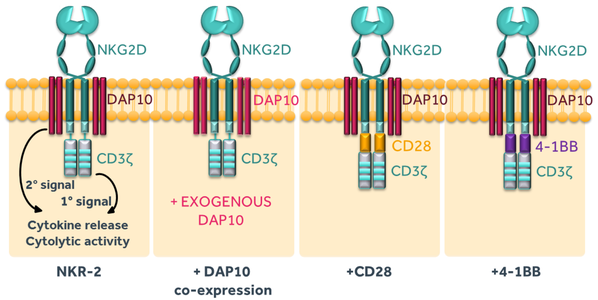


FIGURE 2: NKG2D expression in NKG2D-based CAR T cells

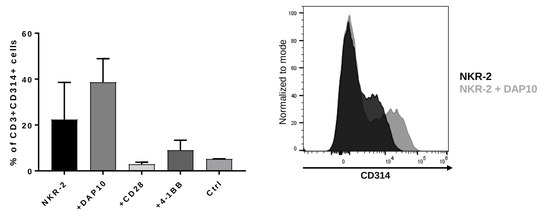


FIGURE 3: Cytotoxicity of NKG2D-based CAR T cells against PANC-1 cells

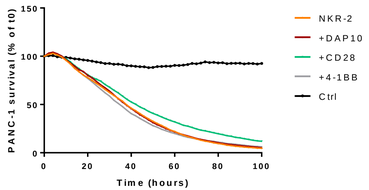


FIGURE 4: IFNγ release by NKG2D-based CAR T cells co-cultured with K562 cells or with coated MICA

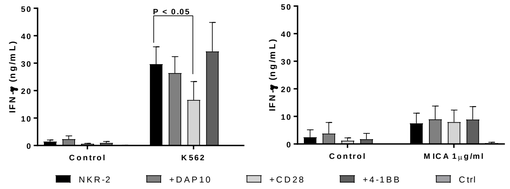
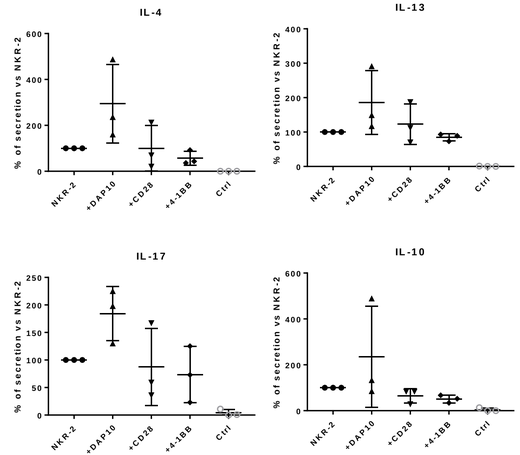


FIGURE 5: Cytokine release by NKG2D-based CAR T cells in the presence of MICA



RESULTS

NKG2D expression differs between NKR-2 constructs

NKG2D (CD314) and CD19 cell surface expression levels were measured by FACS before purification. Co-expression of DAP10 did not dramatically increase the percentage of NKG2D positive cells (**Figure 2, left**). However, it clearly increases the number of receptors at the cell surface when compared to NKR-2 alone (**Figure 2, right**). Surprisingly, expression of NKR-2 at the cell surface seems to be impaired by the addition of CD28 and 4-1BB co-stimulatory domains (**Figure 2, left**)

Kinetics of cytotoxicity are comparable between NKR-2 CAR T cells

T cells transduced with the different NKG2D-based constructs were co-cultured for 100 hours with a pancreatic cancer cell line (PANC-1) that stably expresses a red fluorescent protein. Cytotoxicity against PANC-1 cells was followed in real-time with the Incucyte live-cell imaging system. At an effector:target cell ratio of 1:1, the cytotoxicity kinetics of the different NKG2D-based CAR T cells was similar (**Figure 3**)

IFNγ secretion levels are not higher in alternative NKR-2 CAR T cells

To compare the ability of the different NKG2D-based CAR T cells to produce IFNγ in response to tumors cells, these cells were co-cultured for 48 hours with K562 cells, a CML cell line. As illustrated in **Figure 4 (left part)**, IFNγ release was observed only when NKG2D-based CAR T-cells were challenged with the tumor cells. The levels of IFNγ were comparable between constructs except in NKR-2 + CD28 T cells that secrete significantly lower levels. This difference was not observed when the NKG2D-based CAR T cells were incubated for 24 hours with MICA ligand coated on a plate (**Figure 4, right part**). In addition, the overall levels of cytokine secretion were lower, suggesting that other NKG2D ligands or other proteins could be involved in the response observed with K562

IL-4, IL-13 and IL-17 secretion levels are higher in NKR-2 + DAP10 transduced T cells

The levels of different cytokines secreted by T cells transduced with the different NKR-2 constructs in presence of MICA for 24 hours were measured by multiplex assay. Among these cytokines, increased levels of IL-4, IL-13 and IL-17 were detected for NKR-2 + DAP 10 T cells. On the contrary, T cells transduced with NKR-2 + CD28 or 4-1BB produced decreased levels of IL-10 (**Figure 5**). Interestingly, these trends were only observed when the CD4/CD8 ratio was above 1

CONCLUSIONS AND PERSPECTIVES

- Although NKG2D-based CAR with co-stimulatory domains showed very different T cell surface expression levels, there was no significant difference in either PANC-1 cytotoxicity or IFNγ secretion in presence of K562 cells or recombinant MICA. However, it is conceivable that at E:T ratio of 1:1 or at 1µg/ml MICA, the system is already saturated.
- Testing other E:T ratios and other concentrations of ligands would therefore be interesting to determine if new NKG2D-based CARs can confer higher sensibility of T cells to one or several targets.
- In T cells transduced with NKR-2 + DAP10, higher concentrations of IL-4, IL-13 and IL-17 were measured in the culture supernatant when coated MICA was used as ligand.
- In conclusion, while the addition of co-stimulatory domains CD28 or 4-1BB has been shown to confer interesting properties to classical scFV-based CAR T cells, it does not seem to provide advantages to NKR-2 CAR T cells *in vitro*. The overexpression of DAP10 allows T cells to express NKG2D-based CAR at their cell surface and tends to increase the secretion of pro-inflammatory cytokines. The increased secretion of these pro-inflammatory cytokines is of particular interest in the context of solid tumors and its significance needs to be assessed *in vivo*

References

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