Differential effects of NKG2D-based CAR architecture

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BACKGROUND

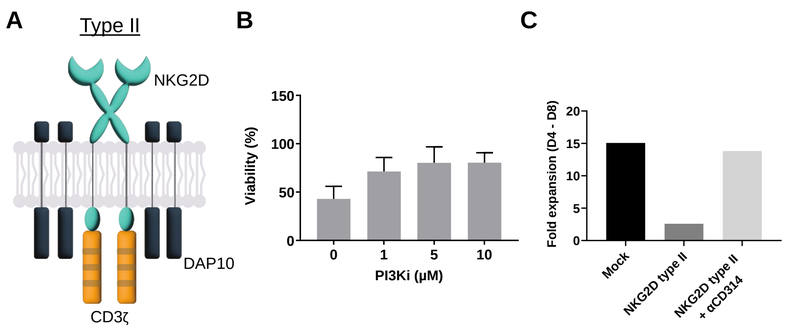
- The Natural Killer Group 2D (**NKG2D**) receptor binds to eight stress-induced ligands: the major histocompatibility complex class I chain-related A and B (MICA/B) and the UL16 binding protein family (ULBP1-6).
- NKG2D ligands (**NKG2DL**) are absent from most normal tissues, but frequently expressed in various types of tumors, making NKG2DL promising targets for cancer immunotherapy.
- The first generation of NKG2D-based CAR T-cells, **CYAD-01**, yielded encouraging results in a phase I study in difficult-to-treat AML patients. However, the cellular persistence of CYAD-01 CAR T-cells was limited.
- In addition, these first generation (in a **Type II** transmembrane protein configuration) NKG2D-based CAR T-cells suffered from fratricide during cell manufacturing or during the freeze thaw cycle prior to infusion in patients due to transient NKG2DL expression by activated T-cells. Fratricide was most likely also one of the reasons for the limited cellular persistence *in vivo*.
- Here, we present a second generation (in a **Type I** transmembrane protein configuration) NKG2D-based CAR T-cell, its production process optimization and demonstrate its superior activity in terms of cytotoxicity against tumor cell lines and proliferation upon stimulation over the first generation NKG2D-based CAR T-cells.

METHODS

- CAR T-cells were produced from healthy PMBCs, each configuration with its own optimized process. Briefly, PBMCs were activated on day 0 with TransAct and transduced with the respective retroviral vector in presence of an PI3K/AKT pathway inhibitor on day 2. Following 2 days of incubation, CAR T-cells were harvested and expanded further in presence of PI3K/AKT pathway inhibitor for an additional 2 days. On day 6, CD314 antibody was added for Type II NKG2D-based CAR T-cells whereas Type I NKG2D-based CAR T-cells were enriched with CD34 magnetic beads or as described in results. Both were expanded for a final 2 days in presence of PI3K/AKT inhibitor.
- Functional comparison between Type II and Type I was performed *in vitro* by assessing cytokine secretion and cytolytic activity in co-cultures with AML cell lines
- To further delineate the functional differences, Type II and Type I NKG2D-based CAR T-cells were serially co-cultured with PANC-1 cells to evaluate cytolytic activity, T-cell proliferation and immune checkpoint expression upon chronic antigen exposure.

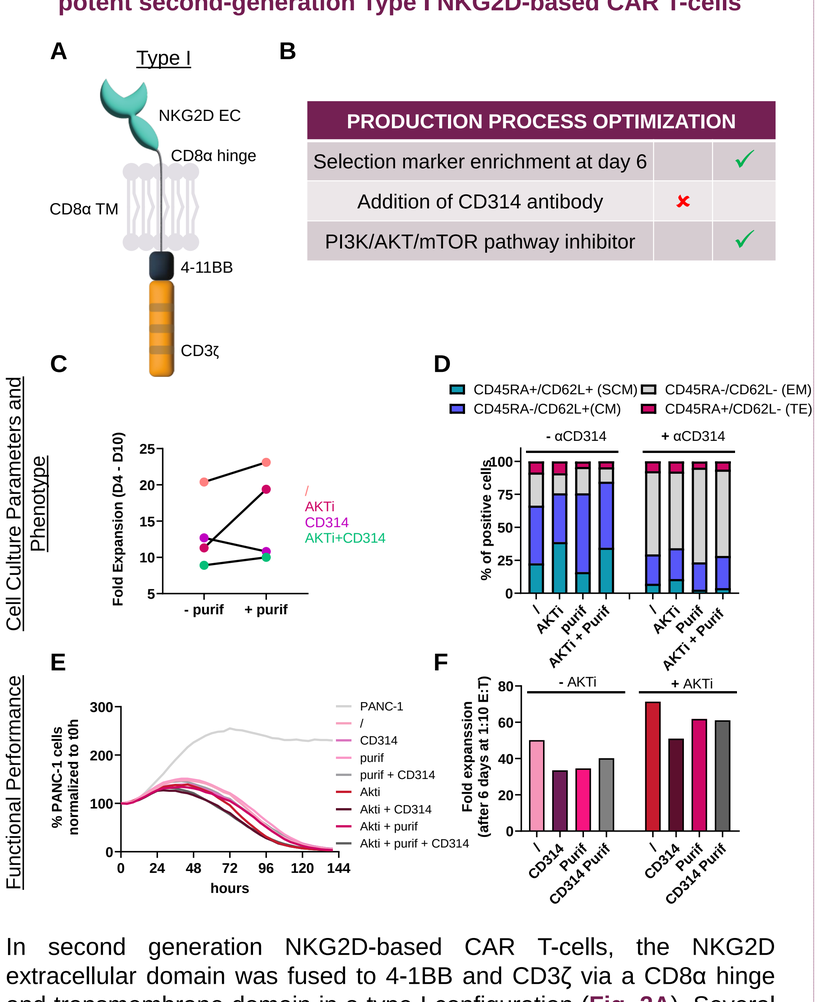
RESULTS

Figure 1: PI3K/AKT pathway blockade and CD314 Ab are essential for production of first-generation Type II NKG2D-based CAR T-cells



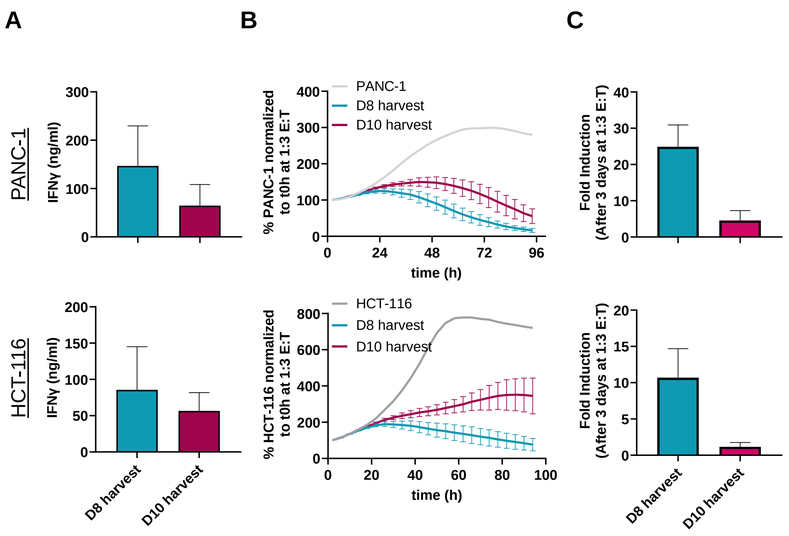
Type II NKG2D-based CAR T-cells consisted of full-length human NKG2D receptor fused with the human CD3ζ signaling domain, which interacts with the endogenous adaptor molecule DNAX-activating protein of 10 kDa (DAP10) (**Fig. 1A**). The addition of a PI3K inhibitor during the production process resulted in increased cell viability upon storage at 4°C for 48 hours (**Fig. 1B**). Introducing in culture an antibody that specifically blocked NKG2D binding to target ligands (αCD314) prevented fratricide and resulted thereby in an increased cell yield (**Fig. 1C**). (Breman *et al*. 2018).

Figure 2: Addition of AKTi and selection marker enrichment generate potent second-generation Type I NKG2D-based CAR T-cells



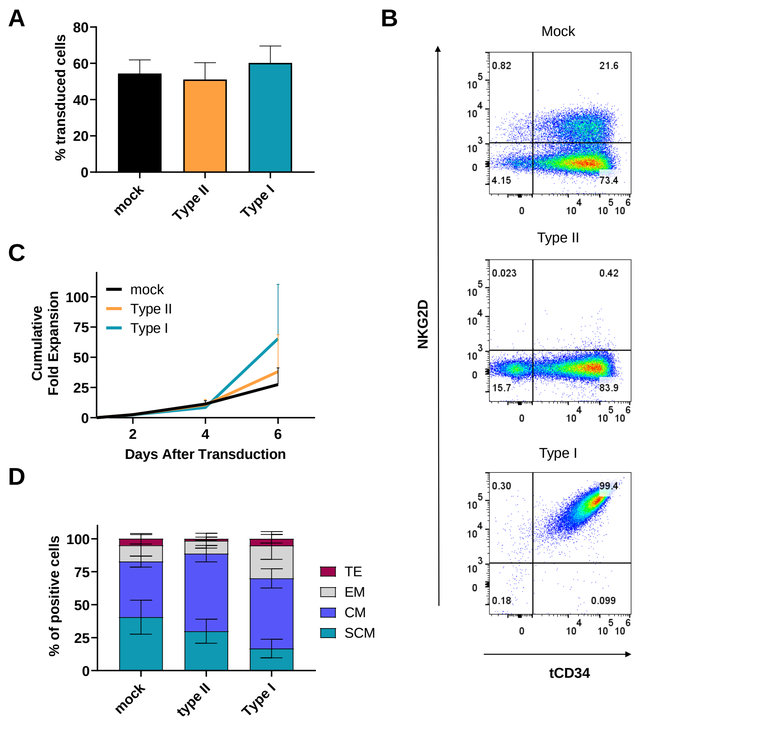
In second generation NKG2D-based CAR T-cells, the NKG2D extracellular domain was fused to 4-1BB and CD3ζ via a CD8α hinge and transmembrane domain in a type I configuration (**Fig. 2A**). Several parameters to optimize the production process were assessed (**Fig. 2B**). CD34 enrichment on Day 6 in the production process resulted in higher cellular yields, except when CD314 antibody was added (**Fig. 2C**). Addition of CD314 antibody additionally resulted in a more differentiated effector phenotype based on CD45RA/CD62L expression (**Fig. 2D**). Addition of an AKT inhibitor yields CAR T-cells with higher cytolytic (**Fig. 2E**) and proliferative capacity (**Fig. 2F**)

Figure 3: Optimized 8-day production process generates more potent Type I NKG2D-based CAR T-cells



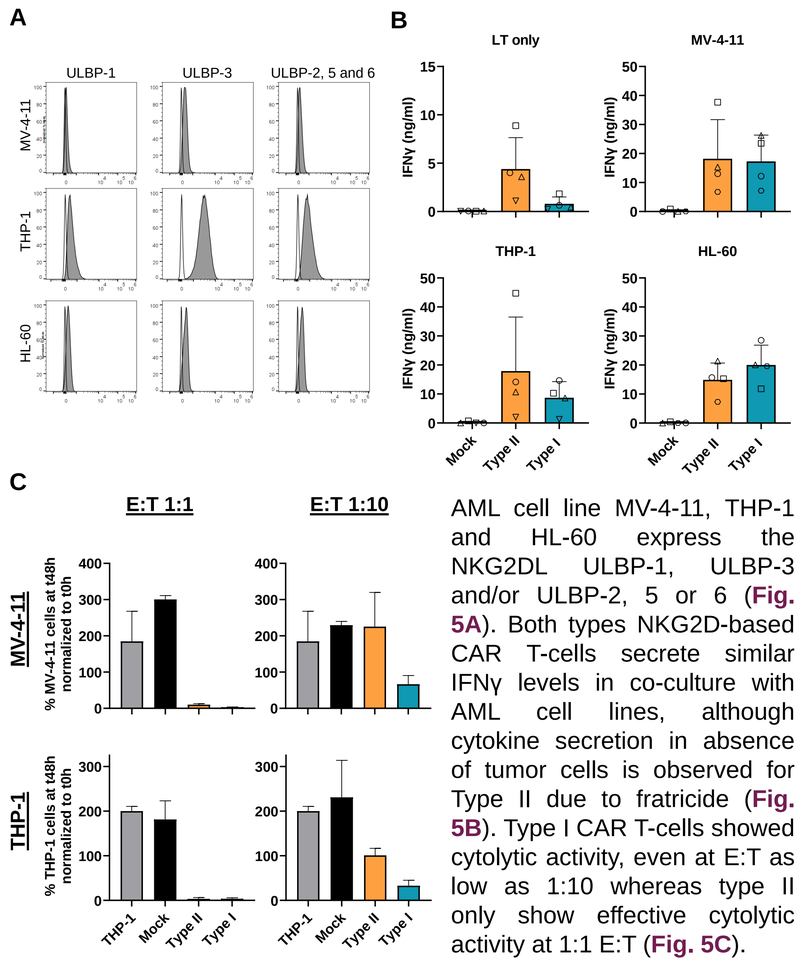
Finally, Type I NKG2D-based CAR T-cells were produced following the optimized production process and harvested after either 8 or 10 days of production. CAR T-cells produced in the 8-day production process secrete higher levels of IFNγ (**Fig. 3A**) and show higher cytolytic (**Fig. 3B**) and proliferative capacity (**Fig. 3C**) upon co-culture with PANC-1 and HCT-116 cells.

Figure 4: Type I NKG2D-based CAR T-cells expand better during manufacturing than Type II while both have a similar phenotype



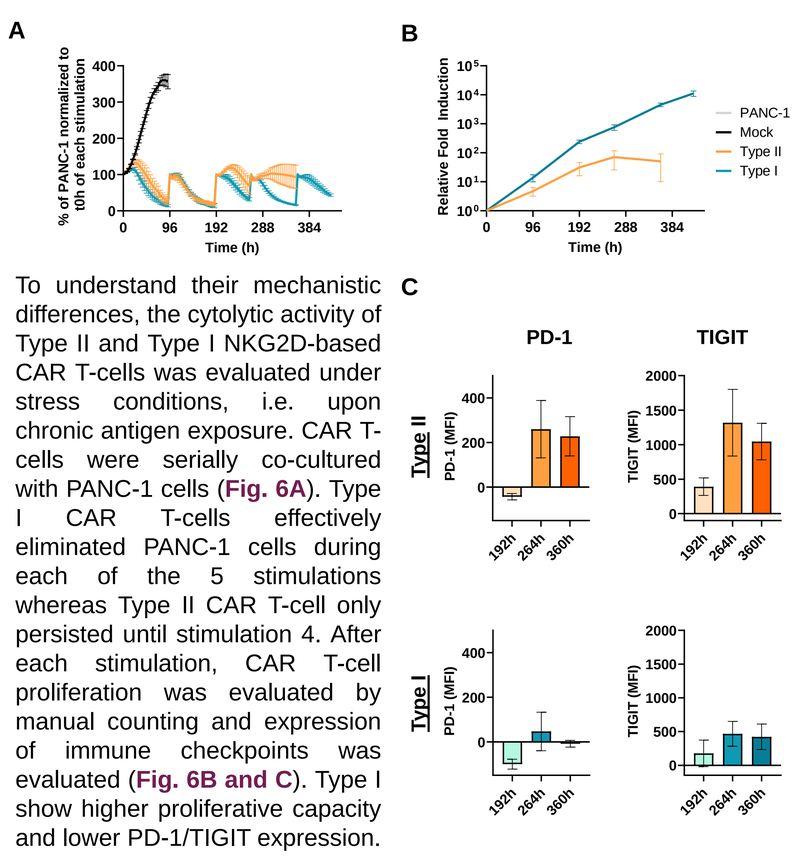
Type II and Type I NKG2D-based CAR T-cells as well as mock CAR T-cells expressing only the tCD34 selection marker were produced simultaneously. All constructs showed similar transduction levels (**Fig. 4A**). Endogenous NKG2D is observed in CD8+ mock CAR T-cells whereas the NKG2D receptor is internalized upon CD314 antibody binding for Type II CAR T-cells and hence not observed immediately after the production process (**Fig. 4B**). Higher cell yields were obtained for Type I NKG2D-based CAR T-cells compared to Type II (**Fig. 4C**) Based on CD45RA/CD62L expression, all CAR T-cells displayed a similar memory phenotype (**Fig. 4D**)

Figure 5: Type I NKG2D-based CAR T-cells show superior cytolytic activity against AML cell lines



AML cell line MV-4-11, THP-1 and HL-60 express the NKG2DL ULBP-1, ULBP-3 and/or ULBP-2, 5 or 6 (**Fig. 5A**). Both types NKG2D-based CAR T-cells secrete similar IFNγ levels in co-culture with AML cell lines, although cytokine secretion in absence of tumor cells is observed for Type II due to fratricide (**Fig. 5B**). Type I CAR T-cells showed cytolytic activity, even at E:T as low as 1:10 whereas type II only show effective cytolytic activity at 1:1 E:T (**Fig. 5C**).

Figure 6: Type I NKG2D-based CAR T-cells show higher proliferative capacity and lower levels of immune checkpoints compared to Type II



To understand their mechanistic differences, the cytolytic activity of Type II and Type I NKG2D-based CAR T-cells was evaluated under stress conditions, i.e. upon chronic antigen exposure. CAR T-cells were serially co-cultured with PANC-1 cells (**Fig. 6A**). Type I CAR T-cells effectively eliminated PANC-1 cells during each of the 5 stimulations whereas Type II CAR T-cell only persisted until stimulation 4. After each stimulation, CAR T-cell proliferation was evaluated by manual counting and expression of immune checkpoints was evaluated (**Fig. 6B and C**). Type I show higher proliferative capacity and lower PD-1/TIGIT expression.

CONCLUSIONS

- **Optimized Type I NKG2D-based CAR T-cells production process results in higher CAR T-cell yields compared to optimized Type II NKG2D-based CAR T-cell production process**
- **Type II NKG2D-based CAR T-cells suffer from fratricide, whereas Type I NKG2D-based CAR T-cells do not**
- **Compared to Type II, Type I NKG2D-based CAR T-cells:**
  - Secrete comparable or higher IFNγ levels upon co-culture with cancer cells
  - Demonstrate superior cytotoxic activity, especially at low E:T ratios
- **Serial co-culture with PANC-1 cells demonstrate a superior activity of Type I NKG2D-based CAR T-cells. This might be due to:**
  - Higher proliferative capacity
  - Lower expression of immune checkpoints PD-1 and TIGIT

AFFILIATIONS, DISCLOSURES & AKNOWLEDGMENTS

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