MODELING NKG2D-BASED CAR-T CELL THERAPY IN ANIMALS WITH ACUTE MYELOID LEUKEMIA

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BACKGROUND

The Natural Killer group 2D (NKG2D) receptor binds to 8 stress-induced ligands (NKG2DL): the MHC I chain-related A and B (MICA, MICB) and the UL16 binding protein family (ULBP1-6) (**Figure 1A**). These ligands are absent or very low expressed in normal tissues, but frequently expressed in large variety of tumors, rendering NKG2D a promising tool for cancer immunotherapy[1,2].
Celyad's lead CAR-T product, CYAD-01, is based on the full length human NKG2D fused to the intracellular domain of CD3ζ (**Figure 1B**). To broaden our understanding of CYAD-01 activity, in this study we examined in depth CYAD-01 efficacy and activity in an aggressive AML xenograft model. Specifically, we investigated distinct dosing and injection schemes and monitored the CYAD-01 persistence and biodistribution in and without the context of cancer.

METHODS

To assess the anti-tumor efficacy of CYAD-01 CAR-T cells against AML, we used an aggressive preclinical AML model, where THP-1-luc-GFP cell line (AML subtype M5) are injected in NOD SCID Gamma-c-/- mice (NSG). In this model, mouse survival is barely over 2 weeks without treatment. CYAD-01 cells, Mock T cells or vehicle were administered IV 1 week after the establishment of THP-1 xenografts. One or three weekly injections of one (10 million) or four different doses (0.3, 1, 3 and 10 million) of CYAD-01 cells were used, as indicated in the distinct experiments. Tumor burden was evaluated by *in vivo* bioluminescence imaging and persistency of CYAD-01 cells by flow cytometry on mouse blood detecting human CD45 positive cells.

RESULTS

Different doses of CYAD-01 CAR-T cells exhibit effective anti-tumor activity in an aggressive AML mouse model

To assess the effect of CYAD-01 dose in an aggressive AML model, THP-1 bearing mice received 3 weekly injections of 0.3, 1, 3 and 10 million T cells. All doses showed a trend to increase mouse survival and to reduce tumor burden, but this was significant for the 2 higher doses (**Figure 2A and B**). While the kinetics of persistency of the CAR-T cells were similar in all doses, with a peak after the second injection, dose dependent levels were observed (**Figure 2C**). The peak after the second injection indicates that a combination of the number of cells injected and the activation via the tumor load is important for T cell engraftment and persistence.

Multiple injections of CYAD-01 CAR-T cells induce a longer survival and better tumor control in a murine model of AML

Using the most effective dose of 10 million CYAD-01 cells, a comparison of the anti-tumor efficacy of a single or multiple injections was performed. After 3 injections, the tumor regression was maintained longer compared to mice that received a single injection (**Figure 3A** representative of the 3injections schedule). The tumor burden was maintained significantly lower for 2 more weeks and mouse survival was higher in the multiple injection scheme (**Figure 3B and C**). Moreover, a higher percentage of the CYAD-01 CAR-T cells was observed indicating that the persistency of the CAR-T cells can be improved by multiple injections (**Figure 3D**).

FIGURES

FIGURE 1: NKG2D receptor recognizes 8 different stress ligands expressed in a large variety of tumors.

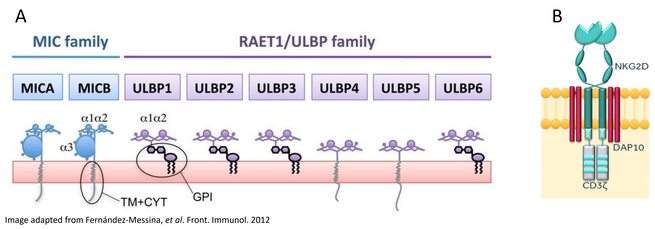


Image adapted from Fernández-Messina, *et al.* Front. Immunol. 2012

FIGURE 2: Assessment of tumor burden, survival and CAR-T cell persistency in a THP-1 based AML animal model treated with 3 weekly injections of CYAD-01 CAR-T cells.

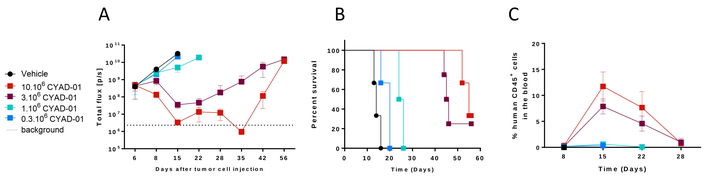


FIGURE 3: Assessment of tumor burden, survival and CAR-T cell persistency in a THP-1 based AML animal model treated with one or three injections of CYAD-01 CAR-T cells.

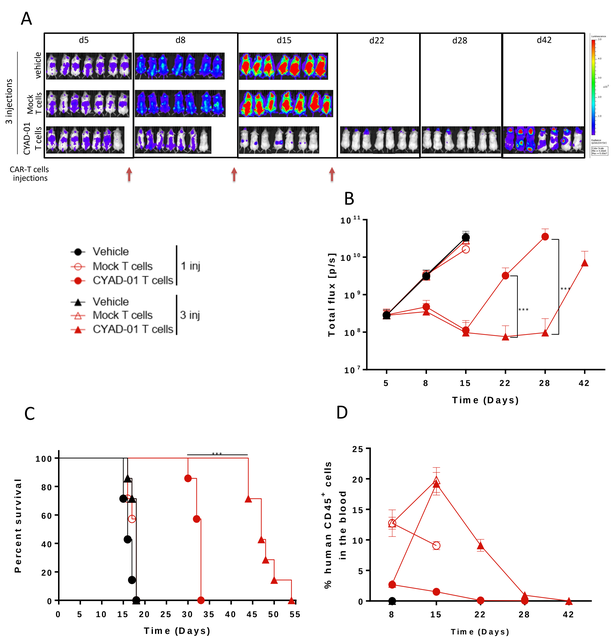


FIGURE 4: Biodistribution and persistency of CYAD-01 CAR-T cells in mice bearing or not THP-1 tumors.

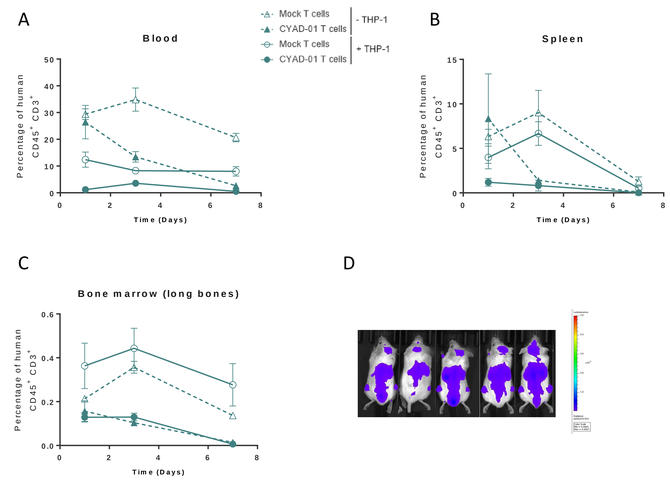
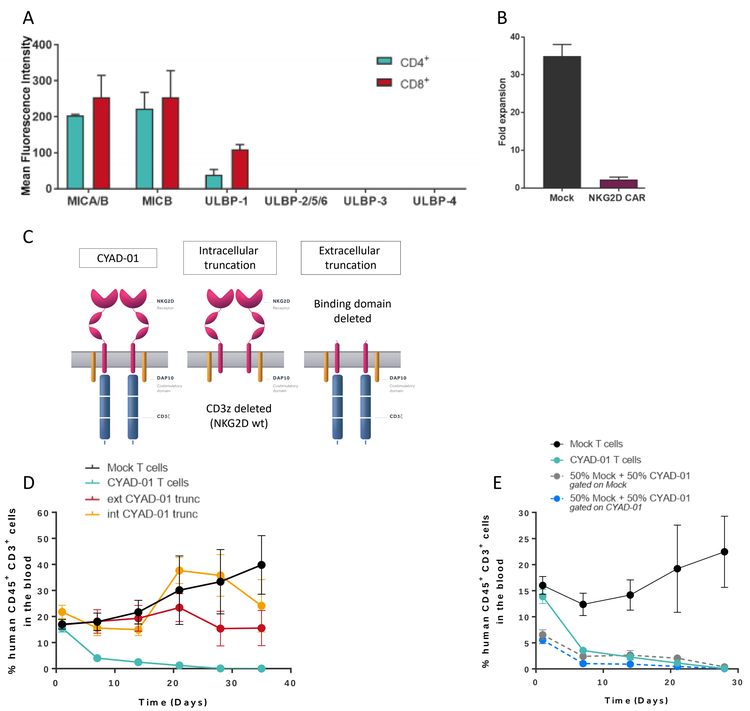


FIGURE 5: Effect of *in vivo* fratricide in the persistency of CYAD-01 CAR-T cells.



Relevant litterature:
1 Lanier LL, *et al.* Cancer Immunol Res. 2015
2 Demoulin B, *et al.* Future Oncology. 2017

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RESULTS

Biodistribution of the CYAD-01 CAR-T cells differs between tumor-bearing and healthy mice

As CYAD-01 cells can be detected in the blood of THP-1-bearing mice only for 1 week after the last injection, we studied the biodistribution of the CAR-T cells. CYAD-01 cells quickly disappeared from the blood and the spleen of tumor-bearing mice (**Figure 4A and B**), suggesting potential homing where the cancer cells are located, such as the bone marrow. Interestingly, the percentage of the CYAD-01 cells remained low in the bone marrow of femurs and tibias (**Figure 4C**). As illustrated in **Figure 4D**, in a representative bioluminescence image of mice bearing THP-1 tumors, the bioluminescence is primarily observed in the bones around the spinal cord of the mice, potentially suggesting those as the main areas of CYAD-01 homing. This is currently under investigation.

Increased persistency of CYAD-01 CAR-T cells with different NKG2D truncations in mice

Our previous work has shown that T cell activation during manufacturing induces transient up-regulation of NKG2DL (mainly MICA and MICB), resulting in self-killing or fratricide, resulting in low cell yields (**Figure 5A and B**). While fratricide has been successfully tackled during manufacturing, *in vivo* fratricide could underlie the short persistence of CYAD-01 cells *in vivo*. To this end, we studied the persistence of CYAD-01 cells without functional CAR, bearing intracellular or extracellular truncation, thus unable to induce cell killing (**Figure 5C**).

CYAD-01 cells bearing intracellular or extracellular truncation, as well as control T cells, were detected with similar frequency until the end of the study, while CYAD-01 cells were undetectable after 2 weeks (**Figure 5D**). Moreover, the persistency of control cells when injected at 1:1 ratio with CYAD-01 cells was comparable to CYAD-01 T cells injected alone, suggesting CYAD-01-mediated killing of the control T cells (**Figure 5E**). These results unambiguously show that *in vivo* fratricide mediates short persistence of CYAD-01 cells and that inhibiting NKG2DL expression on CYAD-01 cells would be a means to increase persistence and thus efficacy.

CONCLUSION

- The multi-target specificity of the NKG2D-based CAR (CYAD-01) provides a strong potential to treat a broad range of cancer indications.
- Our study provides proof of principle that multiple injections of CYAD-01 cells exhibit effective anti-tumor activity in an aggressive animal model of AML. This has been confirmed by promising preliminary results from the Phase I THINK trial (NCT03018405) testing the potency of CYAD-01 T cells against hematological and solid tumors.
- Importantly, the biodistribution of CAR-T cells in the peripheral blood differs between healthy mice and AML models, probably due to CYAD-01 T cell homing to the areas where the cancer cells are located.
- *In vivo* experiments using truncated forms of the NKG2D CAR verified that fratricide drives the short persistency of CYAD-01 cells, suggesting that downregulating NKG2D ligand expression in CYAD-01 cells would be a means to increase CAR-T cell persistency and efficacy. This is currently investigated by the implementation of shRNAs targeting NKG2D ligands in the CYAD-01 vector.