In vitro cytotoxic rechallenge to model active CAR T and NK-cell cytotoxic activity, exhaustion, and persistence.

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ABSTRACT

Since their discovery in the late 1980s, chimeric antigen receptors (CARs) have allowed us to give immune effector cell activation antibody-like specificity (IECs). The FDA has so far approved five CAR T cell products for the treatment of B cell acute lymphocytic leukemia, diffuse large B cell lymphoma, and multiple myeloma. CAR T cells have shown great promise in the context of hematologic malignancies.

Chimeric Antigen Receptor (CAR) T cell therapy is arguably one of the most significant breakthroughs in cancer treatment. There are currently five FDA-approved products that are commercially available. However, despite their success, these CAR T-cell therapies cannot induce long-term durable responses in approximately 50% of leukemia or lymphoma-treated patients. Similarly, the results of CAR T-cells in solid tumors have been somewhat disappointing. Therefore, there is an urgent need to design and develop novel CAR T cells with improved efficacy in hematologic malignancies and solid tumors. ROR1 is a carcinoembryonic antigen expressed in different cancers and is associated with tumor stemness, proliferation, metastatic transformation, and treatment resistance. In this project, we optimize an anti-ROR1 CAR using a humanized single-chain variable fragment (scFv) with second (2G) or third-generation (3G) costimulatory domains.

NK cells have shown a great cytotoxic ability comparable and even greater than cytotoxic T lymphocytes. Further, they are crucial in the immune response against tumor cells as they can distinguish malignant cells from healthy ones through interactions between MHC class I antigens and Killer Immunoglobulin-like Receptors. Although, tumors have developed mechanisms to overcome this strategy of immune surveillance and thus grow and metastasize. CAR-bearing NK cells overcome these immunosuppressive mechanisms and direct these cytotoxic IECs towards the cancer cells. Of note, NK cells don't elicit graft versus host disease and therefore it constitutes an ideal platform for off-the-shelf CAR-driven cellular therapy.

Although most thoroughly described in T cells, exhaustion also occurs in NK-cell mediated cytotoxicity. Chronic infections and cancer has shown to hamper the NK-cell function through a variety of receptor-ligand interactions, microenvironment conditions, soluble inhibitory cytokines, and intercellular communication via extracellular vesicle trafficking.

OBJECTIVES

To evaluate a re-challenge platform that allows a rapid and multiplex evaluation of the cytotoxicity, exhaustion, and persistence of CAR-IECs in vitro.

METHODS

We generated lentivirus transduced CD19 CARs (FMC63-41BB-z) using T cells from healthy donors and NK-92 cells for comparison. T cells were expanded for 12 days, and a 41.9% CAR+ expression was achieved (CART19). Transduced NK-92 cells were sorted by FACS to obtain a population of 98.3 % CAR+ cells (CARNK19) and subsequently expanded for 12 days. JeKo-1 cells were used as CD19+ targets and BxPC3 cells as CD19 ^{neg} control (both cell types were GFP-Luc-PuroR).

Four separate ROR1 constructs using different costimulatory domains were designed and optimized in silico. We developed a luciferase-based rechallenge cytotoxicity assay. We diluted the effector to target (E/T) ratio to obtain a logarithmic trendline of the cells' cytotoxicity using Jeko-1 ROR1^{+} and K562 ROR1^{-}.

We developed a Luciferase-based rechallenge cytotoxicity assay. To do this, we adjusted the effector to target (E/T) ratio and calculated the cytotoxicity of the cells as a logarithmic trendline. E/T ratios to obtain 50% viability (IC50) at 4 hours for CARNK19 and 24 hours for CART19 were utilized as proxies for the product's potency. To achieve 70% cytotoxicity, both CAR Immune Effector Cells (IECs) were co-cultured with their targets at an E/T ratio. Using GFP exclusion in flow analysis, we calculated the number of IEC still present in the culture after 24 hours with the target (IEC cells/mL = total cells/mL x GFP neg%). Using this rechallenge method, we repeated the plating of E/T ratio dilutions to generate daily IC50 curves for a total of 5 days. Day 0 and Day 2 were used to evaluate CAR and PD1 expression.

RESULTS

FIGURE 1. *In vitro IC50 assessment* CAR T vs CAR NK

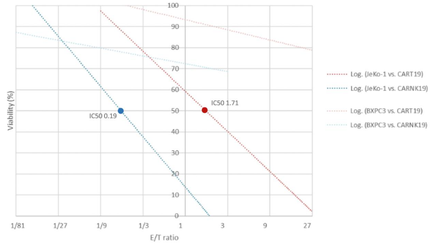


FIGURE 2. *In vitro* re-challenge CAR T vs CAR NK

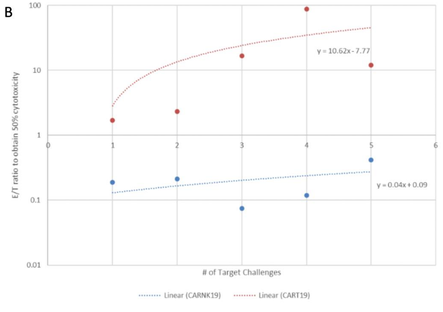


FIGURE 3. PD1 expression after 5 days of re-challenge

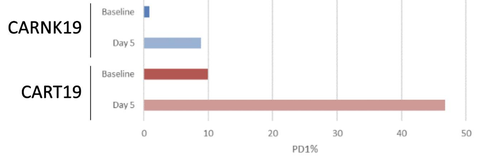


FIGURE 4: *In vitro* re-challenge of ROR1 CAR-T constructs

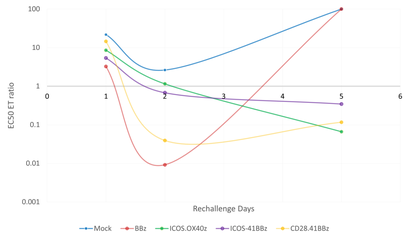


FIGURE 3: IFN-γ release after 5 days of re-challenge

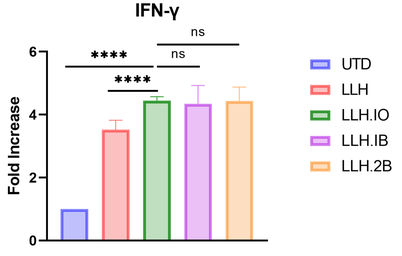
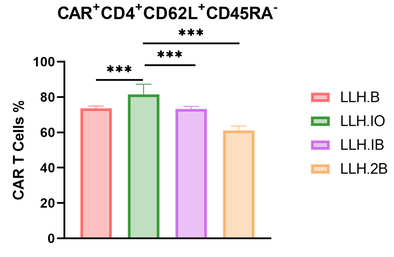


FIGURE 4: CAR T cell profile after 5 days of re-challenge



DISCUSSION

All the tested constructs showed specific ROR1 medicated cytotoxicity against ROR1 expressing cell lines. Third-generation (3G) constructs showed lower cytotoxicity levels after day 1 of repetitive rechallenge compared with second-generation (2G) CARs. However, the cytotoxic activity of 3G constructs gradually increased during the following rechallenges and excided by an order of magnitude the cytotoxicity observed by 2G-CARs at five days of the rechallenge. On day 5, there were no significant differences in IECs subsets among the constructs with a predominance of effector memory phenotype (CCR7-, CD45RA-), however in some cases we observed an increase expression of PD1, TIGIT, LAG-3, and TIM3 in the 2G constructs.

CONCLUSIONS

Our data shows the functionality of a repetitive target rechallenge in vitro platform to evaluate CAR-IEC. By performing repetitive cytotoxic challenges, we aim to mimic the dynamic in vivo interactions in the tumor microenvironment between IECs and target tumor cells. We propose to use the IEC50 calculated from this platform to evaluate cytotoxic functionality, exhaustion, and persistence of different CAR-IECs in an efficient and cost-effective manner, which is suited for multiplexing and robotic optimization.

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