Development of an In Vitro Exhaustion Assay for Functional Screening of CAR T Cell Enhancements

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Background

Chimeric antigen receptor (CAR) T cells have emerged as a powerful tool in cancer therapy, by enabling targeted and potent antitumor immune response. However, sustained antigen exposure and immunosuppressive conditions within the tumor microenvironment often drive CAR T cell exhaustion, leading to impaired functionality. Here, we present several approaches to model persistent activation and induce exhaustion-like phenotypes in CAR T cells. Using multiple readout technologies, we established a robust and reproducible assay system for preclinical evaluation of next-generation CAR T cell therapies, focusing on mitigating CAR T cell exhaustion.

Method

γδ and αβ T cells were engineered to express a CAR targeting the CD19 antigen.

γδ and αβ CAR T cells were kept in culture for over 21 days, with or without the presence of stimuli (10 IU/ml IL-2, CD3/CD28 Dynabeads™, or Nalm-6 cells. Re-stimulation was performed every 3-4 days, concomintantly with medium change.

On Day 0, Day 10 and Day 17, Nalm-6_Luc (target) cells were co-cultured with CAR T cells (effector) at different E:T ratios (starting at 10:1). After 24 hours, cytoxicity was calculated by normalizing to high control (untreated Nalm-6_Luc in monoculture) after subtraction of low control (Staurosporine-treated Nalm-6_Luc in monoculture).

At indicated timepoints, phenotypical characterization was performed by surface staining for viability, CD19, Lag-3, PD-1, Tim-3, followed by fixation and intracellular staining for Ki-67.

Gating strategy consisted of sequential selection of cells, singlets, and viable cells.
In the Nalm-6 re-stimulation approach, CD19+ cells were additionally excluded.

Summary

- The nature of the stimulation affects the extent, kinetics, and expression pattern of exhaustion.
- Under the tested conditions, γδ T cells exhibited higher susceptibility to functional exhaustion compared with αβ T cells.
- The tested variants differ in their onset of exhaustion.
- Flow cytometry-based assessment of exhaustion marker expression, viability, and proliferation, combined with target cell killing assays, provides a powerful analytical approach.
- The treatment scheme, in combination with the presented analytical tools, enables the assessment of strategies aimed at delaying CAR T-cell exhaustion.

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Establishment of a robust and reliable assay system to model CAR T cell exhaustion, using multiple readout technologies.

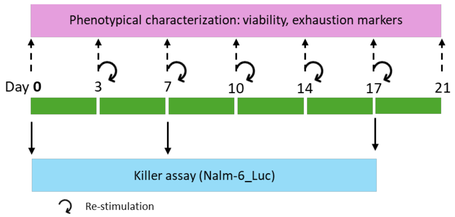


Figure 1: Establishment of a robust and reliable assay system to model CAR T cell exhaustion, using multiple readout technologies.

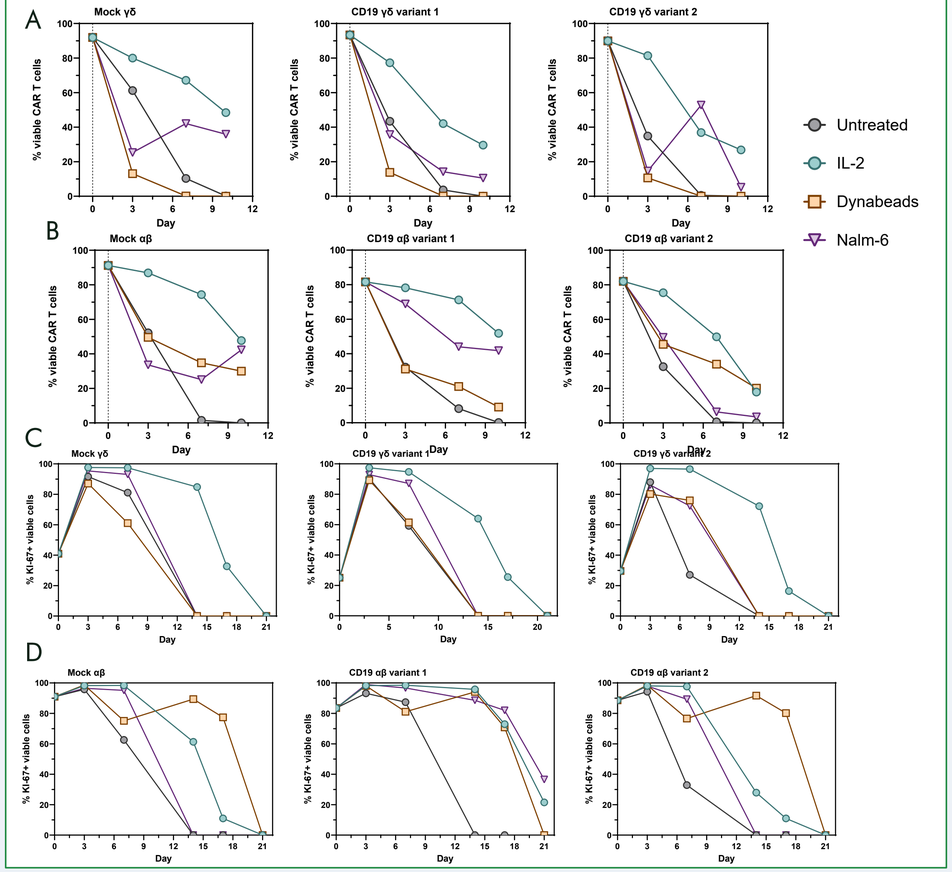
γδ and αβ CAR T cells were kept in culture for over 21 days, with or without the presence of stimuli (10 IU/ml IL-2, CD3/CD28 Dynabeads™, or Nalm-6 cells. Re-stimulation was performed every 3-4 days, concomitantly with phenotypical characterization of viability and key exhaustion markers via flow cytometry analysis. To evaluate how cytotoxicity has been affected by exhaustion, killer assays were performed at day 0, 10 and 17, using Nalm-6_Luc cell line as target cell line.

IL-2 enhances viability (A,B) and promotes proliferation (C,D) in vitro durability of both CD19 γδ and CD19 αβ CAR T cells.

Figure 2: IL-2 enhances viability (A,B) and promotes proliferation (C,D) in vitro durability of both CD19 γδ and CD19 αβ CAR T cells.

Frequency of viable cells significantly dropped over time. IL-2 enhanced viability rate in vitro of all tested CD19 γδ and αβ constructs, and particularly αβ CAR T cells. Frequency of Ki-67+ viable γδ CAR T cells was increased with IL-2 (A,B).

CD3/CD28 Dynabeads™ strongly affected γδ CAR T cells, by triggering activation-induced cell death (C). Contrary, while still having an impact on viability, Dynabeads™ boosted αβ CAR T cells expansion (D).



Expression pattern of Lag-3, PD-1 and Tim-3 exhaustion markers.

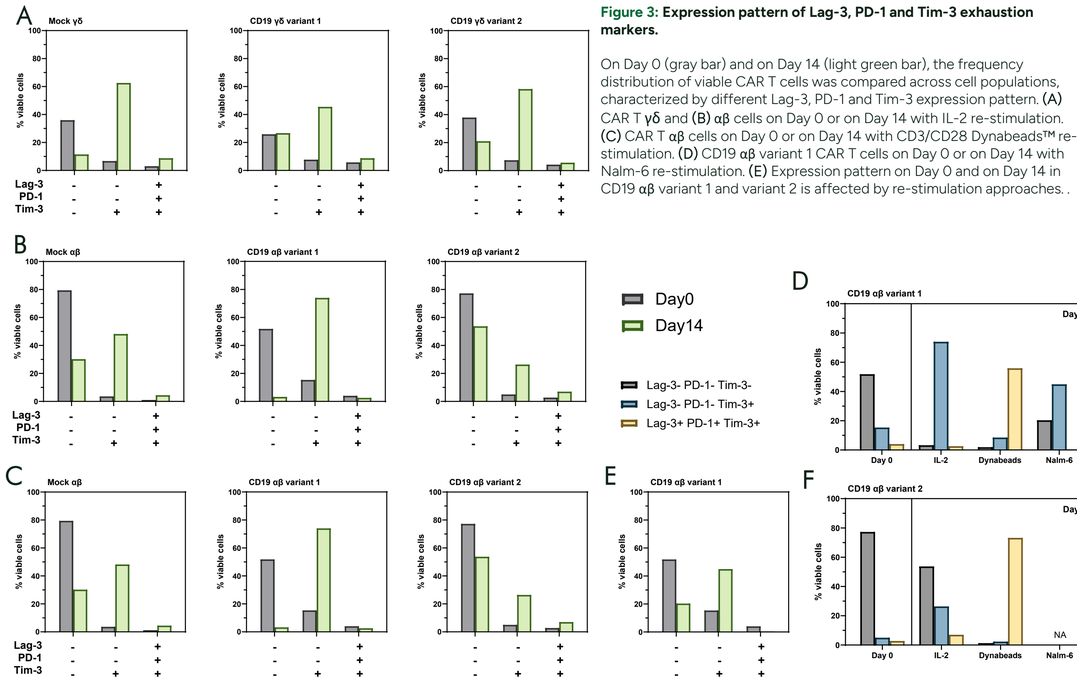


Figure 3: Expression pattern of Lag-3, PD-1 and Tim-3 exhaustion markers.

On Day 0 (gray bar) and on Day 14 (light green bar), the frequency distribution of viable CAR T cells was compared across cell populations, characterized by different Lag-3, PD-1 and Tim-3 expression pattern. (A) CAR T γδ and (B) αβ cells on Day 0 or on Day 14 with IL-2 re-stimulation. (C) CAR T αβ cells on Day 0 or on Day 14 with CD3/CD28 Dynabeads™ re-stimulation. (D) CD19 αβ variant 1 CAR T cells on Day 0 or on Day 14 with Nalm-6 re-stimulation. (E) Expression pattern on Day 0 and on Day 14 in CD19 αβ variant 1 and variant 2 is affected by re-stimulation approaches. .

CAR T killing functionality declines over time

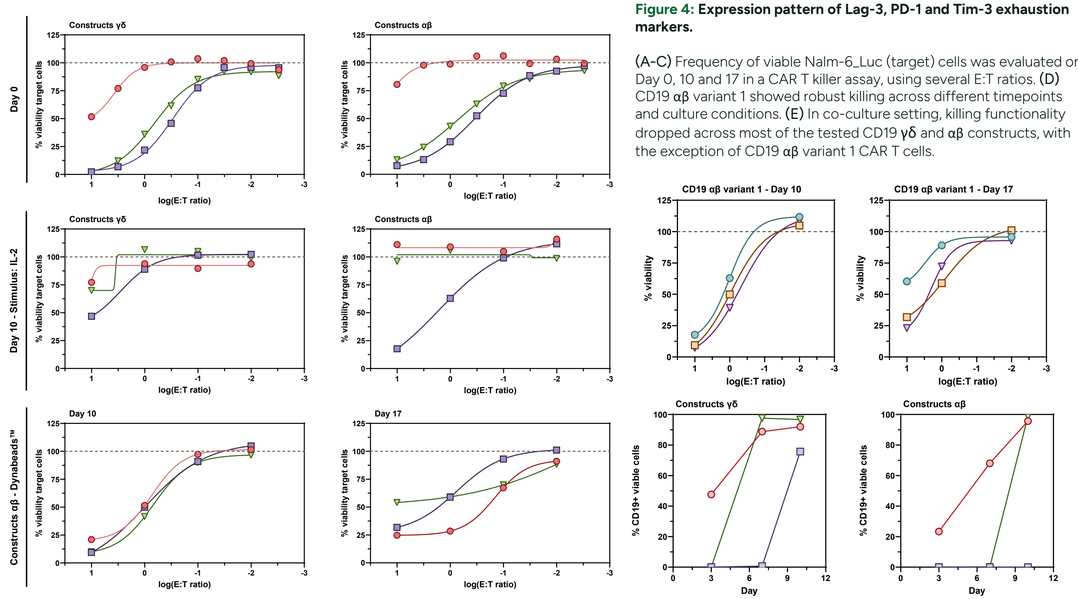


Figure 4: Expression pattern of Lag-3, PD-1 and Tim-3 exhaustion markers.

(A-C) Frequency of viable Nalm-6_Luc (target) cells was evaluated on Day 0, 10 and 17 in a CAR T killer assay, using several E:T ratios. (D) CD19 αβ variant 1 showed robust killing across different timepoints and culture conditions. (E) In co-culture setting, killing functionality dropped across most of the tested CD19 γδ and αβ constructs, with the exception of CD19 αβ variant 1 CAR T cells.