# Enhancing γδ CAR T Cell Function Through Alternative CD3 Cytoplasmic Domains

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## Background

Gamma delta (γδ) T cells are a unique subset of lymphocytes that possess innate and adaptive immune functions. Unlike conventional αβ T cells, γδ T cells recognize antigens in an MHC-independent manner. This allows them to detect a wide range of stress-induced ligands that are commonly expressed on tumor cells. This property makes γδ T cells particularly attractive for cancer immunotherapy, especially in cases where tumors evade immune surveillance by downregulating MHC molecules. Recent advances in cellular engineering have made it possible to generate γδ T cells that express chimeric antigen receptors (CARs), which combines their natural tumor-recognition capabilities with the targeted specificity of CARs. γδ CAR T cells offer several advantages over αβ CAR T cells, including lower graft-versus-host disease risk, suitability for allogeneic applications ("out-of-the-shelf"), and enhanced solid tumor infiltration due to tissue-homing properties. These properties provide a strong rationale for investigating γδ CAR T cells as a novel cancer therapy approach and underscore the importance of continued optimization to realize their full therapeutic potential.

## Method

**Generation and Characterization of γδ and αβ CAR T Cells**
T cells were isolated from healthy human donors and enriched for γδ T cells by selective depletion of αβ T cells. The enriched γδ T cells were activated, and two days after activation, the lentiviral CAR construct was added. Transduced γδ CAR T cells were subsequently expanded for an additional two weeks under standard culture conditions. In parallel, αβ CAR T cells were generated from the same donors using an analogous workflow, enabling direct donor-matched comparisons between γδ and αβ CAR T cell products.

The resulting CAR T cells were characterized by flow cytometry for surface CAR expression. Functional activity was assessed by measuring degranulation upon target engagement using CD107a staining. Cytotoxic activity was evaluated in vitro using a bioluminescence based-killing assay with luciferase-expressing NALM-6 target cells, and cytokine secretion was quantified by measuring IFN-γ and TNF levels following co-culture.

**In Vivo Efficacy Studies**
In vivo antitumor efficacy was assessed using a disseminated xenograft model with luciferase-expressing NALM-6 (NALM-6_Luc) cells. One million NALM-6_Luc cells were injected intravenously into NXG mice (Janvier). Mice received repeated supplementation with human IL-15 (1 µg per mouse) administered every 3–4 days to support CAR T cell persistence.
Tumor growth was monitored longitudinally by bioluminescence imaging using an IVIS® Lumina III imaging system (Revvity). The antitumor activity of standard γδ CAR T cells was directly compared with donor-matched αβ CAR T cells in this model.

## Summary

- Highly pure γδ and αβ CAR T cell products with robust and uniform CAR expression were successfully generated.
- Despite similar degranulation, αβ CAR T cells exhibited slower cytotoxic killing capacity, while producing higher levels of cytokines.
- Incorporation of the cytoplasmic CD3ε domain resulted in slightly reduced killing activity at shorter times, associated with lower cytokine secretion
- The NALM-6 model proved well-suited for CAR T cell evaluation due to its high engraftment rate and low tumor heterogeneity.
- *In vivo* efficacy studies revealed superior antitumor activity of αβ CAR T cells compared with γδ CAR T cells. Still, γδ CAR T cells might be an attractive out-of-the-shelf alternative for patients with limited options.

## Generation and characterization of CAR T cells

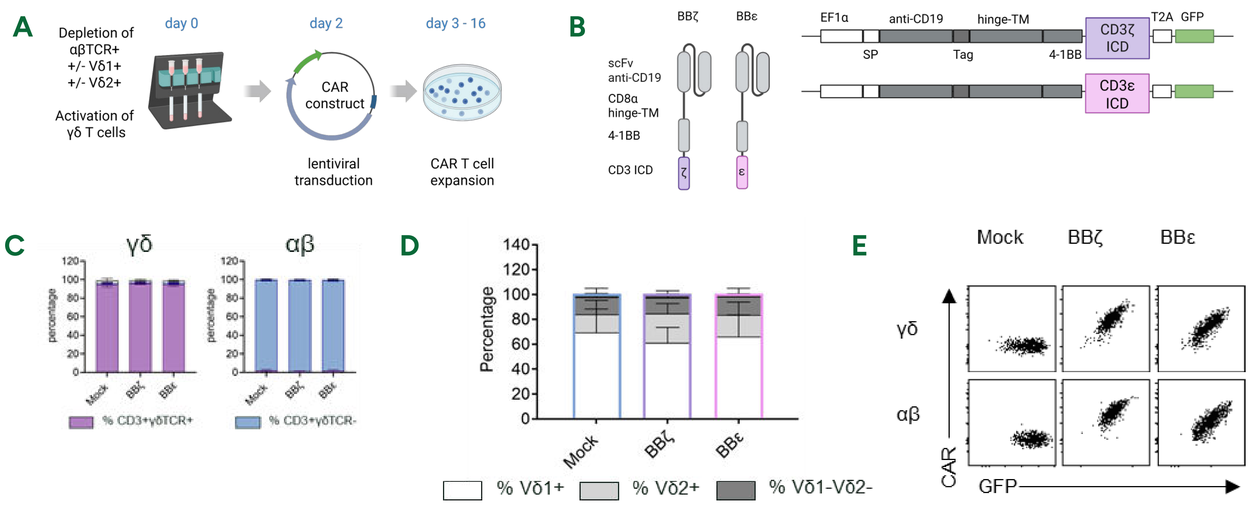


**Figure 1:** **(A)** Schematic overview of γδ CAR T cell expansion protocols. **(B)** Schematic representation of the chimeric antigen receptor (CAR) constructs used in this study. **(C)** Proportions of CD3⁺ and γδTCR⁺ cells after sorting, assessed by flow cytometry (n = 3-5). **(D)** Frequencies of Vδ1⁺, Vδ2⁺, and Vδ1⁻Vδ2⁻ γδ T cell subsets determined by flow cytometry; data pooled from 3-5 independent experiments. **(E)** Representative flow cytometry plots from a single donor showing GFP versus CAR expression for each CAR construct.

## Functional *in vitro* evaluation of CAR T cells

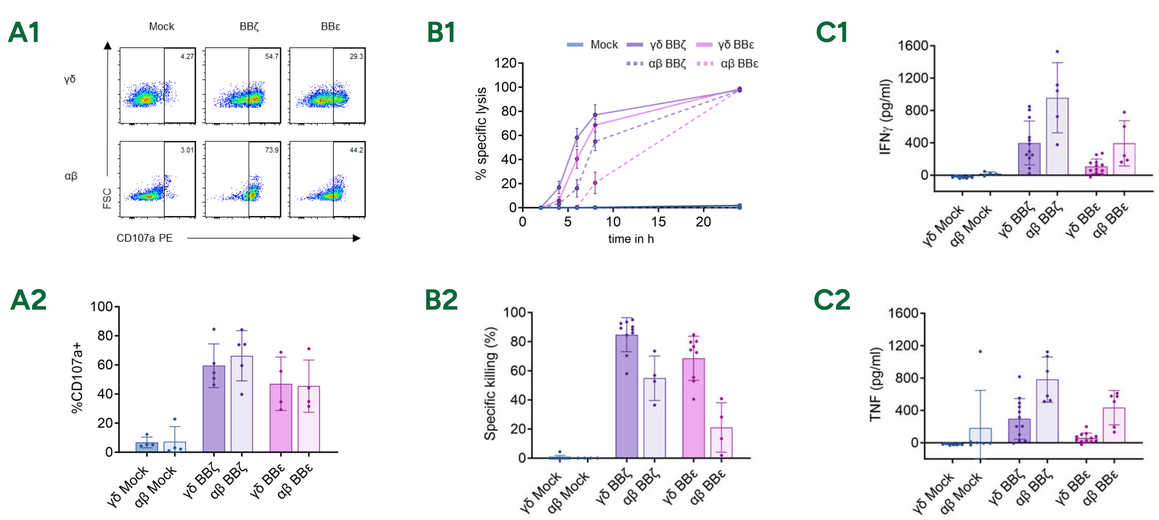


**Figure 2:** **(A1)** Representative flow cytometry plots of CD107a expression on CAR T cells after a 4-h co-culture with CD19⁺ NALM-6 cells at an E:T ratio of 1:1. **(A2)** Frequency of CD107a⁺ CAR T cells. (mean ± SD, n = 3–4 donors; each dot represents one donor) **(B1)** Specific lysis of NALM-6_Luc target cells over time. **(B2)** Quantification of specific lysis after 8 h of co-culture. Data are presented as mean ± SD from n = 3–4 independent donors. **(C)** CAR T cells were co-cultured with CD19⁺ NALM-6 tumor cells at an effector-to-target (E:T) ratio of 1:1 for 24h. Secretion of IFN-γ **(C1)** and TNF **(C2)** was quantified by ELISA. Data are shown as mean ± SD from 4–5 healthy donors, with each dot representing one donor.

## *In vivo* efficacy study

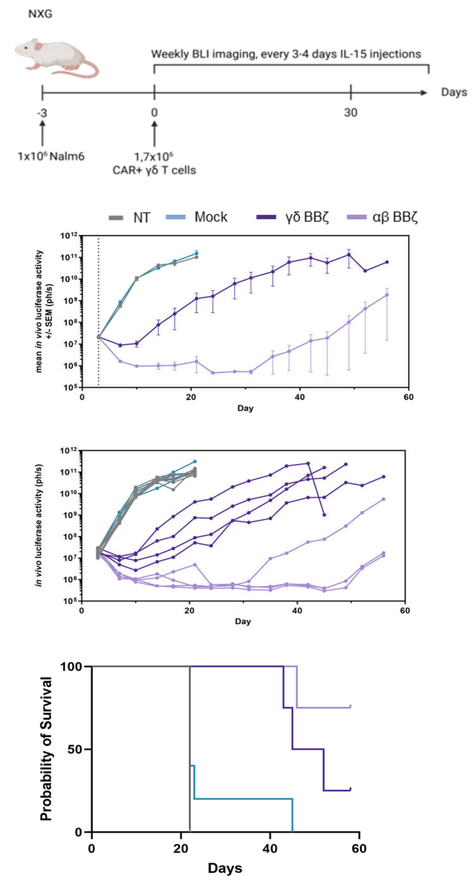


**Figure 3:** **(A)** Schematic overview of the in vivo efficacy study. One million NALM-6_Luc cells were injected intravenously into NXG mice (Janvier) on day -3. CAR T cells (1.7 million/mouse) were administered on day 0. To support CAR T cell persistence, mice received human IL-15 (1 µg per mouse) every 3–4 days. **(B)** Tumor progression in untreated control mice, mock-transduced γδ T cell–treated mice, γδ BBζ CAR T cell–treated mice, and αβ BBζ CAR T cell–treated mice was monitored by bioluminescence imaging using an IVIS® Lumina III system (Revvity). **(B1)** Mean in vivo bioluminescence signal over time, shown as mean ± SEM for each treatment group. **(B2)** Individual bioluminescence kinetics of single mice, illustrating inter-animal variability in tumor progression. **(B3)** Kaplan–Meier survival curves comparing overall survival across the different treatment groups.

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