# TurboCAR™ T Cells: CAR T Cells with Constitutive, Programmable Cytokine Signaling Outputs

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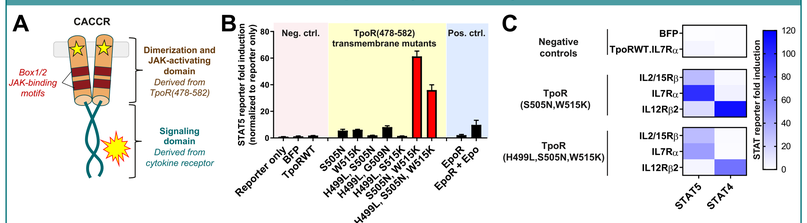
## Abstract

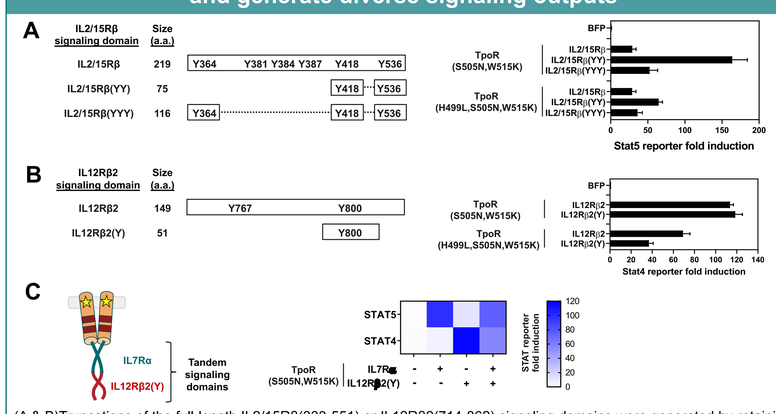
CAR T cell therapy has attained unprecedented success in the treatment of certain hematological malignancies in the autologous setting. However, having an allogeneic approach with a meaningful clinical benefit in hematological malignancies is desired. In addition, the clinical benefit in solid tumor indications has been limited, potentially in part due to suppressive solid tumor microenvironments (TME) that inhibit T cell effector function and persistence. While the provision of cytokine support can help CAR T cells overcome suppressive TME, combining CAR T therapy with systemically-administered cytokines/cytokine mimetics can result in toxicities and adverse events. Even cell therapies locally secreting cytokines can still activate neighboring cells and cause pleiotropic effects. To gain the benefit of cytokine stimuli without adverse effects, we designed TurboCAR™ T cells, which are CAR T cells co-expressing an inducible chimeric cytokine receptor or Constitutively Active Chimeric Cytokine Receptor (CACCR). CACCRs are homodimeric receptor chimeras that mimic cytokine signaling in a constitutive, CAR T cell-intrinsic fashion. For example, CACCRs may comprise (i) a membrane-tethered dimerization and JAK-binding domain derived from the thrombopoietin receptor (TpoR), fused to (ii) one or more intracellular signaling domains derived from the cytokine receptor(s) of interest. Mutations in the TpoR transmembrane domain result in constitutive receptor dimerization and activation of JAK2, which in turn phosphorylates the cytokine receptor signaling domains and cause STAT signaling. Each of these domains were individually optimized, and then combinatorially fused to generate CACCRs that mimicked downstream STAT signaling of cytokine receptors of interest. CACCRs mimicking signaling from multiple cytokines were generated. An EGFRvIII tool CAR was utilized to screen and select CACCRs based on TurboCAR™ T cell manufacturability and in vitro serial killing activity. Functional benefits conferred by CACCRs were further validated in the context of a CAR directed towards BCMA in a range of functional assays, demonstrating improved activity compared to the parental CAR. In conclusion, CACCRs are novel homodimeric cytokine receptor chimeras that can be tailored through fusion of dimerization and signaling domains to increase TurboCAR™ T cell activity, while circumventing safety risks associated with cytokine combination therapy.

## Design of homodimeric Constitutively Active Chimeric Cytokine Receptors (CACCRs)



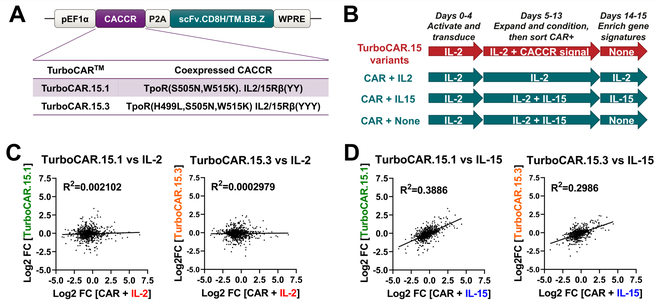

(A) CACCRs are comprised of two primary domains: (1) a cell membrane-bound dimerization and JAK-activating domain derived from TpoR, coupled to (2) an intracellular signaling domain containing phosphorylable tyrosine residues derived from a cytokine receptor of interest. (B) Using an IL7Rα-based CACCR, TpoR transmembrane domain mutants were screened to identify those capable of enforcing strong, constitutive receptor dimerization and signaling. (C) CACCRs bearing full-length signaling domains derived from the indicated cytokine receptors induced downstream STAT signaling expected of the parental cytokine receptor. Full-length signaling domains are IL2/15Rβ(333-551), IL7Rα(316-459) and IL12Rβ2(714-862).

## CACCR signaling domains can be tailored to reduce vector cargo size and generate diverse signaling outputs



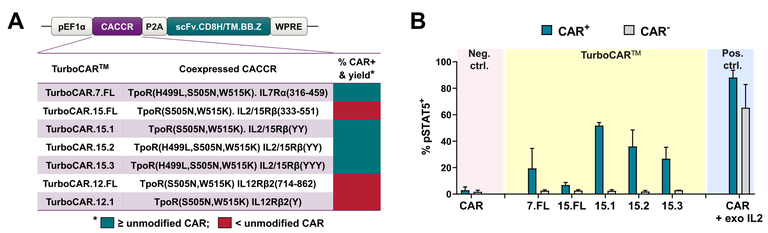

(A & B)Truncations of the full-length IL2/15Rβ(333-551) or IL12Rβ2(714-862) signaling domains were generated by retaining regions bearing only the indicated tyrosine residues. Signaling activity was assessed in a STAT reporter assay. (A) Signal strength of the full-length IL2/15Rβ(333-551) was recapitulated by the IL2/15Rβ(YYY) variant, and surpassed by the IL2/15Rβ(YY) variant. (B) The significantly smaller IL12Rβ2(Y) signaling domain activated STAT4 efficiently, with the potential to recapitulate the signal strength of full-length IL12Rβ2(714-862). (C) Signaling domains derived from distinct cytokine receptors can be fused in tandem to simultaneously activate multiple STAT pathways from a single CACCR.

## IL2/15Rβ-derived CACCRs more closely mimic IL-15 signaling



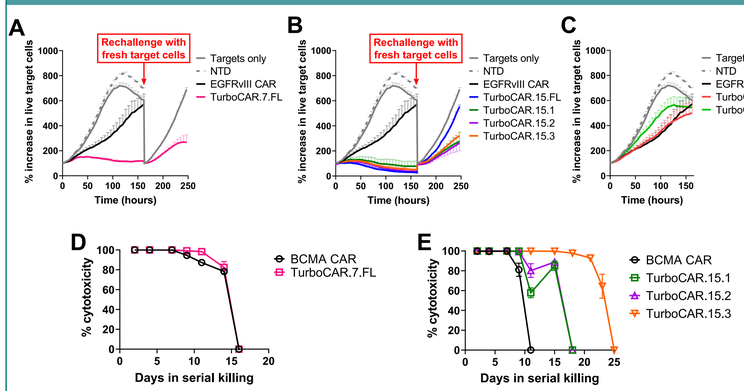

TurboCAR.15 variants derived from the full-length IL-2/15Rβ may mimic the signaling effects of IL-2, IL-15, or a combination of both. (A) Schematic of TurboCAR construct containing the indicated IL-2/15Rβ-derived CACCRs. (B) Experimental design for gene expression analysis using the Nanostring nCounter Human CAR T panel. TurboCAR.15 T cells were generated by expansion in IL-2 as per standard protocol. To mimic the aggregate of cytokine signaling received by TurboCAR.15 T cells, unmodified CAR T cells were conditioned in either IL-2 alone, or a combination of IL-2 and IL-15. (C & D) Scatter plots showing gene expression changes in TurboCAR.15 variants compared to (C) IL-2 conditioned and (D) IL-15 conditioned CAR T cells. Log2 FC was calculated by normalization to the "CAR + None" control.

## TurboCAR™ cytokine signaling is T cell-intrinsic



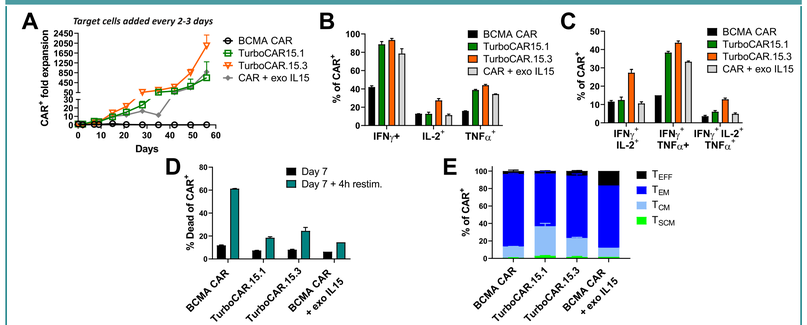

(A) Schematic of TurboCAR construct and list of selected TurboCAR abbreviations coexpressing the indicated CACCR formats. Manufacturability of each TurboCAR relative to the unmodified CAR is shown. (B) CACCR signaling activity was assessed in human TurboCAR T cells bearing the EGFRvIII-specific 2173 scFv by intracellular flow cytometry staining for pSTAT5. STAT5 activation was detected only in the CAR+, but not CAR- fraction of TurboCAR-transduced T cells, demonstrating TurboCAR T cell-intrinsic cytokine signaling. By contrast, CAR T cells that had been treated with exogenous IL2 showed STAT5 activation in both CAR+ and CAR- fractions.

## TurboCAR™ signaling domains enhance CAR T cell cytotoxicity



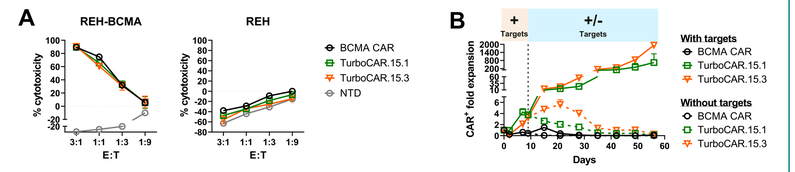

(A-C) Cytotoxicity of TurboCARs bearing the EGFRvIII-specific 2173 scFv and the indicated CACCRs were evaluated using U87-EGFRvIII-nucGFP target cells at an E:T of 1:3. Where indicated, EGFRvIII TurboCAR T cells were rechallenged with fresh target cells. Live target counts were monitored using the Incucyte Live Cell Imaging System. (A) TurboCAR.7.FL and (B) TurboCAR.15 variants showed enhanced initial potency, as well as long-term durability of cytotoxic responses. (C) TurboCAR.12 variants did not enhance the activity of CAR T cells in vitro, and were therefore excluded from further testing. (D & E) Long-term cytotoxicity of TurboCARs directed towards BCMA was assessed using MM1.S-Luc-GFP target cells at an E:T of 10:1. Fresh target cells were added every 2-3 days and target cell killing was determined by luminescence readout. (D) While TurboCAR.7.FL did not enhance activity, (E) TurboCAR.15 variants showed enhanced long-term cytotoxicity.

## TurboCAR™ T cells show superior expansion, survival and improved functional output following repeated target stimulation



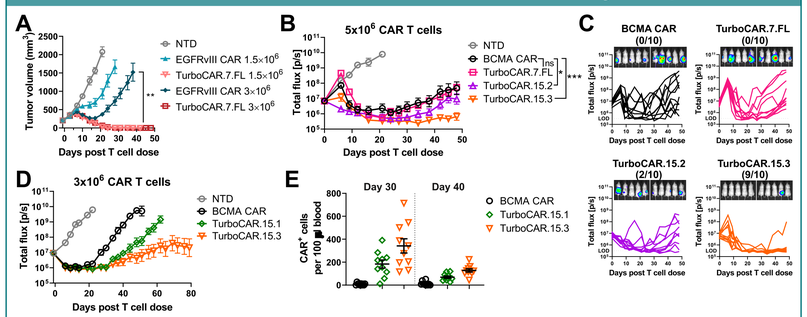

(A) On-target expansion of TRAC/CD52 dKO BCMA CAR T cells serially challenged with MM1.S-Luc-GFP targets. (B-D) On Day 7, CAR T cells were evaluated for intracellular cytokine expression and activation-induced cell death (AICD). TurboCAR T cells showed (B) improved expression of effector cytokines, (C) greater polyfunctionality and (D) were protected from AICD. (D) Analysis on Day 14 revealed an improved memory phenotype and delayed differentiation of TurboCAR T cells.

## Activity and survival of TurboCAR™ T cells is target-dependent




(A) TRAC/CD52 dKO BCMA TurboCAR T cells lyse BCMA-overexpressing REH target cells, but are inactive against the BCMA-negative parental line. (B) TRAC/CD52 dKO BCMA TurboCAR T cells were repeatedly exposed to MM1.S target cells every 2-3 days for the first 9 days to induce expansion. Following that, MM1.S target cells were either continually added or withdrawn from these cultures. Without targets, TurboCAR T cells ceased expansion and underwent a gradual decline.

## TurboCAR™ T cells show enhanced anti-tumor efficacy and persistence




(A) In vivo anti-tumor activity of EGFRvIII TurboCAR T cells was assessed in NSG mice bearing established subcutaneous LN229-EGFRvIII tumors, and intravenously infused with the indicated doses of CAR T cells. While unmodified CAR T cells delayed tumor progression, TurboCAR T cells completely eliminated tumors. (B-D) Efficacy of TRAC/CD52 dKO BCMA TurboCAR T cells was assessed in an orthotopic model of multiple myeloma using the MM1.S-Luc-GFP cell line. (B) At a dose of $5\times10^6$ CAR T cells, TurboCAR.15 variants prolonged the durability of anti-tumor responses, with (C) TurboCAR.15.3 preventing extramedullary relapse in 9/10 mice. Numbers in parenthesis represent the number of tumor-free mice. (D) A lower dose of $3\times10^6$ CAR T cells better differentiated the efficacy of TurboCAR.15 variants. (E) On the indicated days, CAR T cells in the peripheral blood of mice from (D) were enumerated. Increased persistence of TurboCAR.15 variants correlated with improved anti-tumor efficacy.

## Conclusions

- TurboCARs coexpress a homodimeric CACCR that confers constitutive, T cell-intrinsic cytokine signaling
- TurboCARs can be tailored for diverse, programmable and combinatorial signaling outputs
- TurboCAR T cells provide increased functional potency and persistence following chronic target exposure
- Despite improved expansion and persistence, TurboCAR T cell activity and survival is target-dependent
- In hematologic and solid tumor models, TurboCAR T cells enhanced anti-tumor activity in vivo



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