# Abstract # 2542

# CAR T with Temporally-Controlled, Programmable Cytokine Signaling Outputs

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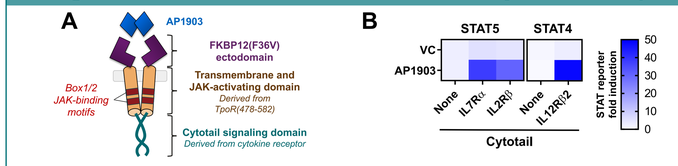
## Abstract

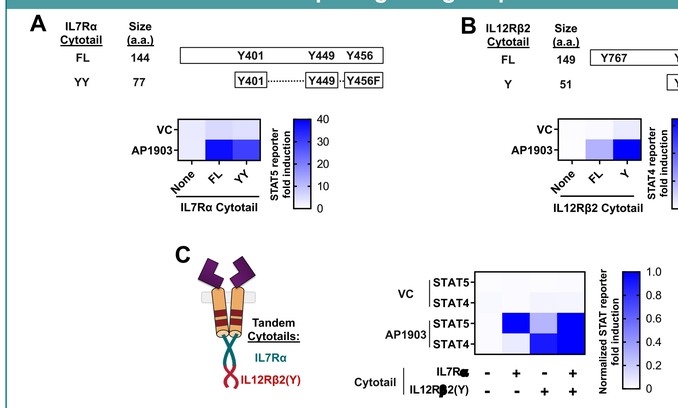
CAR T cell therapy has attained unprecedented success in the treatment of certain hematological malignancies in the autologous setting. However, having an allogeneic approach with a meaningful clinical benefit in hematological malignancies is desired. In addition, the clinical benefit in solid tumor indications has been limited in part due to suppressive solid tumor microenvironment (TME) that inhibits T cell effector function and persistence. While the provision of cytokine support can help CAR T cells overcome suppressive TMEs, conventional approaches, such as combining CAR T therapy with systemically-administered cytokines/cytokine mimetics, or engineering CAR T cells to constitutively secrete cytokines can result in toxicities and adverse events. To mitigate these safety concerns, we designed the inducible TurboCAR™ (iTurboCAR™), which is a CAR T cell coexpressing a homodimeric inducible chimeric cytokine receptor (iCCR) specifically activated by the clinically validated dimerizer drug, AP1903. iCCR comprises the FKBP(F36V) ectodomain, the transmembrane and JAK-activating domains of TpoR and one or more intracellular STAT-binding domains derived from the cytokine receptor (Cytotail) of interest. In response to AP1903, different iTurboCAR designs resulted in downstream STAT activation that mimicked signaling of the parental cytokine receptor. Furthermore, two Cytotails could be fused in tandem to generate dual signaling outputs from a single receptor. Using 2nd generation CARs directed towards solid tumor targets, we demonstrated that iTurboCAR T cells bearing different Cytotail fusions could be programmed towards divergent phenotypes, such as those promoting expansion (IL7Rα Cytotail) or effector function (IL12Rβ2 Cytotail). RNASeq analysis of iTurboCAR T cells bearing an IL7Rα.IL12Rβ tandem Cytotail (iTurboCAR.7.12) revealed near-identical gene expression profiles to control CAR T cells treated exogenously with IL-7 and IL-12, demonstrating that iTurboCARs closely mimicked the signaling of native cytokine receptors. In the absence of target cells, AP1903 treatment alone was sufficient to expand iTurboCAR.7.12 T cells more efficiently than exogenously supplemented IL-7 and IL-12, while preserving a juvenile memory phenotype. Compared to control CAR T cells, AP1903 treatment enhanced iTurboCAR T cell cytotoxicity in vitro, and promoted CAR T cell activity in a subcutaneous tumor model. In conclusion, iTurboCAR T cells coexpress a novel chimeric cytokine receptor that can improve the potency and persistence of CAR T cells, while minimizing safety risks associated with cytokine co-therapy or constitutive cytokine secretion. Furthermore, iTurboCAR T cells bearing different/combinatorial Cytotail fusions offer the flexibility for user-programmable signaling outputs, permitting control over CAR T cell phenotype and function.

## Design of homodimeric inducible chimeric cytokine receptors (iCCRs)



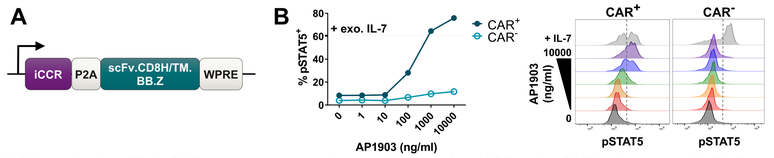

(A) Inducible chimeric cytokine receptors (iCCRs) are comprised of three primary domains: (1) an FKBP12(F36V) ectodomain that can be dimerized by the homodimeric small molecule AP1903, (2) a cell membrane-bound dimerization and JAK-activating domain derived from TpoR, coupled to (3) an intracellular signaling domain containing phosphorylable tyrosine residues derived from a cytokine receptor of interest (Cytotail). (B) iTurboCARs bearing various Cytotail signaling domains were evaluated in HEK293T cell luciferase reporter assays. AP1903-induced iTurboCAR activation elicited downstream STAT signaling expected of the parental cytokine receptor. Cytotails shown are derived from IL7Rα(316-459), IL2/15Rβ(333-551) and IL12Rβ2(714-862). VC: vehicle control.

## Cytotails can be tailored to reduce vector cargo size and generate multiple signaling outputs



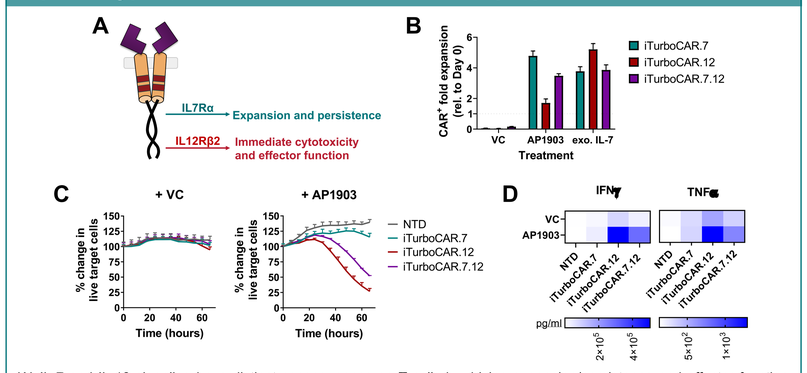

(A-C)Truncations of the full-length (FL) IL7Rα(316-459) or IL12Rβ2(714-862) signaling domains were generated by retaining regions bearing only the indicated tyrosine residues. Signaling activity was assessed in a STAT reporter assay. (A) Signal strength of the full-length IL7Rα(316-459) was recapitulated by the IL7Rα(YY) variant. (B) The significantly smaller IL12Rβ2(Y) signaling domain activated STAT4 more efficiently than the full-length IL12Rβ2(714-862). (C) Signaling domains derived from distinct cytokine receptors can be fused in tandem to inducibly activate multiple STAT pathways from a single iTurboCAR.

## iCCR signaling is CAR T cell-intrinsic and tunable



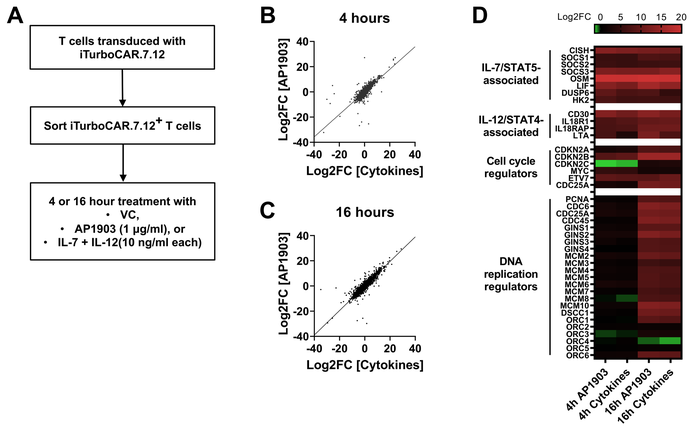

(A) Schematic of an iTurboCAR construct that permits co-expression of an iCCR and a CAR in the same cell. (B) Dose-dependent, AP1903-induced STAT5 activation was detected only in CAR+ T cells coexpressing an IL7Rα-bearing iCCR, but not in the CAR- population.

## While iTurboCAR™.7 and iTurboCAR™.12 program divergent phenotypes, iTurboCAR™.7.12 manifests properties of both iCCRs



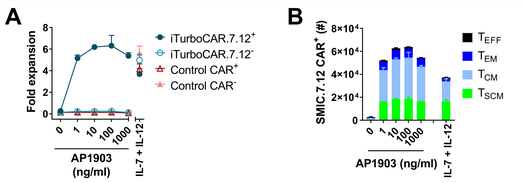

(A) IL-7 and IL-12 signaling have distinct consequences on T cells by driving expansion/persistence and effector function, respectively. To evaluate if iCCRs can program CAR T cell function, iCCRs bearing either the IL7Rα only, IL12Rβ2(Y) only, or tandem IL7Rα.IL12Rβ2(Y) were generated. (B) iTurboCAR T cells were cultured in the absence of target cells for 14 days in the presence of VC, AP1903 or exogenous IL-7. AP1903-induced iTurboCAR.7 activation promoted CAR T cell expansion, recapitulating the effects of exogenous IL-7 treatment. (C & D) Cytotoxicity of iTurboCAR T cells directed towards EGFRvIII was assessed using the Incucyte Live Cell Imaging System. iTurboCAR T cells were cocultured with EGFRvIII-overexpressing U87MG cells at an ineffective E:T ratio of 1:8 in the presence or absence of AP1903. AP1903-induced iTurboCAR.12 activation improved (C) CAR T cell cytotoxicity and (D) secretion of proinflammatory cytokines. While iTurboCAR.7 and iTurboCAR.12 preferentially promoted expansion and cytotoxicity, respectively, iTurboCAR.7.12 manifested the characteristics of the single iCCRs by simultaneously enhancing both aspects, albeit to intermediate extents.

## iCCRs closely mimics signaling of native cytokine receptors



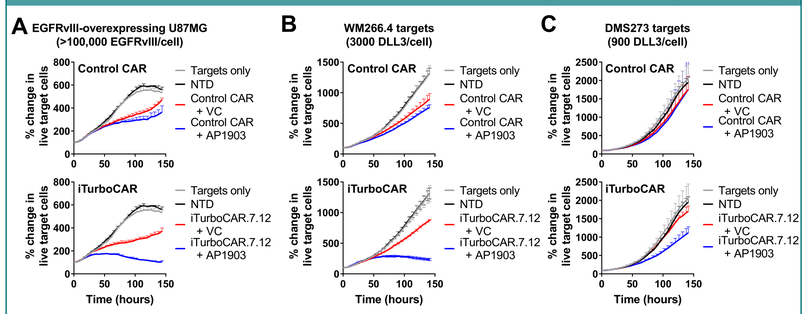

(A) Sorted iTurboCAR.7.12 CAR+ T cells were treated as indicated prior to RNASeq analysis. (B-C) Scatter plots of iTurboCAR.7.12 CAR T cells that had been treated with AP1903 or cytokines for (B) 4 hours or (C) 16 hours reveal near-identical gene expression profiles. Log2FC of each treatment group was calculated relative to VC-treated cells. (D) Heatmap of selected differentially expressed genes. iCCR and cytokine receptor signaling induced gene expression changes of similar magnitude in key pathways. Hallmarks of cytokine receptor signaling were induced after 4 hours, and this was followed by downstream transcriptional changes in cell cycle and DNA replication regulators after 16 hours.

## AP1903-induced activation of iTurboCAR™.7.12 promotes CAR T cell expansion while preserving a favorable memory phenotype



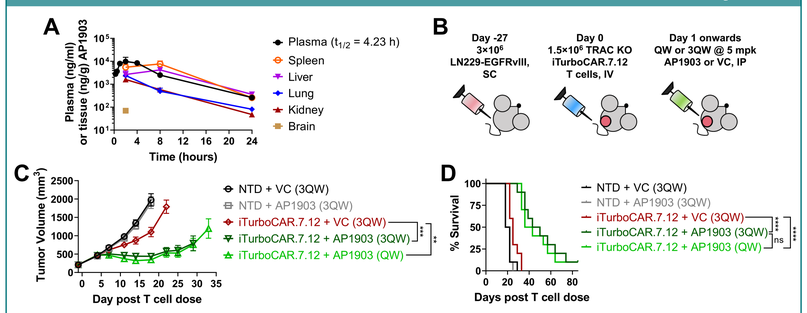

(A-B) CAR T cells were cultured in the absence of target cells for 14 days in the presence of the indicated doses of AP1903 or recombinant IL-7 and IL-12 (10 ng/ml each). (A) AP1903 specifically promoted the expansion of iTurboCAR+ T cells, but not iTurboCAR- T cells or control CAR T cells without an iCCR. (B) Despite enhanced expansion, iTurboCAR.7.12 T cells still preserved a favorable memory phenotype enriched in the stem cell memory ($T_{SCM}$) and central memory ($T_{CM}$) populations. Expansion and memory phenotype of iTurboCAR T cells were equal or better than that of exogenous cytokine treatment.

## AP1903-induced activation of iTurboCAR™.7.12 promotes cytotoxicity




In vitro cytotoxicity of iTurboCAR.7.12 T cells directed towards solid tumor targets was assessed using the Incucyte Live Cell Imaging System. (A) EGFRvIII CAR T cells bearing the 2173 scFv were cocultured with EGFRvIII-overexpressing U87MG at E:T=1:4 in the presence of VC or 10 ng/ml AP1903. (B-C) DLL3 CAR T cells bearing 26C8 scFv were cocultured with (B) DLL3high WM266.4 at E:T=1:9 in the presence of VC or 10 ng/ml AP1903, or (C) DLL3low DMS273 at E:T=1:1 in the presence of VC or 100 ng/ml AP1903. In the absence of AP1903, iTurboCAR.7.12 T cells showed similar activity as control CAR T cells. AP1903 treatment specifically enhanced the cytotoxicity of iTurboCAR.7.12 T cells.

## Activation of iTurboCAR™.7.12 enhances anti-tumor efficacy




(A) Pharmacokinetics of AP1903 intraperitoneally administered at 5 mpk in NSG mice. (B-D) Anti-tumor activity of EGFRvIII iTurboCAR.7.12 T cells was assessed in NSG mice bearing established subcutaneous LN229-EGFRvIII tumors using two AP1903 dosing regimens. (B) Experimental design. TRAC KO (TCRα constant knockout) T cells were generated by TALEN®-mediated gene editing. (C-D) AP1903-activated iTurboCAR.7.12 significantly improved (C) anti-tumor efficacy and (D) overall survival. Increasing AP1903 dosing from 1× to 3× per week did not further improve iTurboCAR.7.12 T cell efficacy, suggesting that despite its short half-life, weekly dosing of AP1903 was sufficient to support maximal iTurboCAR.7.12 activity.

## Conclusions

- iCCRs are homodimeric cytokine receptor chimeras that can be activated specifically by AP1903
- iCCRs can mimic the signaling of native cytokine receptors, and be tailored for diverse, programmable and combinatorial signaling outputs
- iTurboCAR™.7.12 CAR T cells show improved functional potency and persistence in the presence of AP1903
- iTurboCAR™ T cells provide the benefits of cytokine signaling while minimizing safety risks associated with cytokine co-therapy or constitutive cytokine secretion



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