Dual-switch CAR-T cells: Orthogonal molecular switches to control activation and elimination of CAR-T cells to target $CD123^{+}$ cancer

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Background

Improvement of the efficacy and safety of chimeric antigen receptor (CAR)-T immunotherapies requires controlled activation and termination of the T cells when transfused into patients. Here we present two independently regulated molecular switches that can elicit specific and rapid induction of cellular responses upon exposure to their cognate ligands. T cell costimulation is controlled by the homodimerizer rimiducid that triggers signaling cascades downstream of MyD88 and CD40 via an engineered protein termed iMC. A rapamycin-controlled pro-apoptotic switch (iRC9) that induces dimerization of caspase-9 mitigates possible CAR-T cell toxicity. iRC9 is a chimeric protein comprisingan FKBP-rapamycin binding (FRB) domain in tandem with FKBP12 and caspase-9. This design permits rapamycin, a heterodimerizing ligand, to function as a homodimerizer. When combined with a first generation CD123-specific CAR, these molecular switches allow for controlled, robust expansion of engineered T cells to control acute myelogenous leukemia (AML) *in vitro* and *in vivo* combined with a rapid and efficient safety mechanism to block excessive cytokine release.

Methods & Results

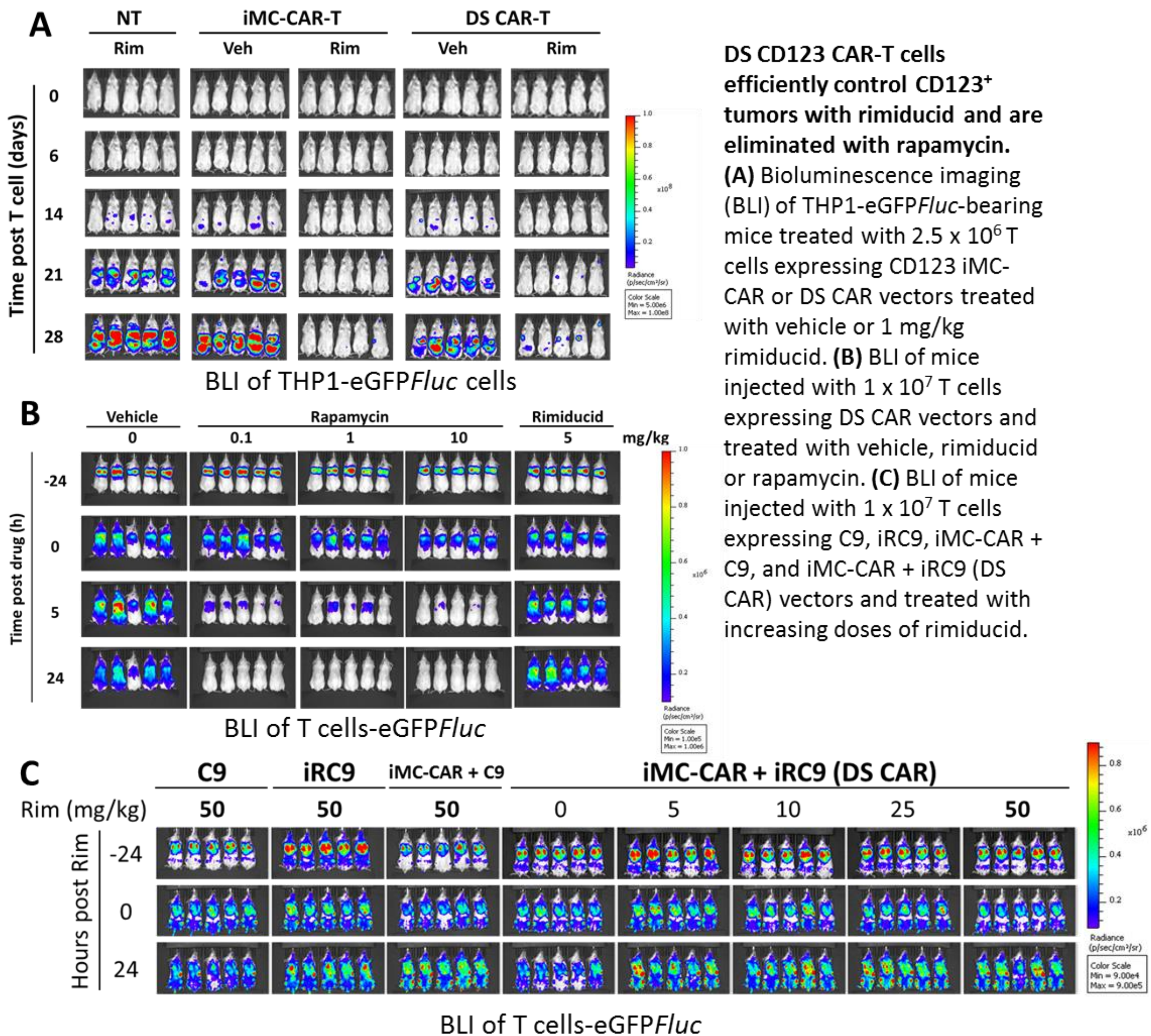
T cells were activated and co-transduced with pSFG-iMC.2A-CD123CAR.ζ and pSFG-iRC9.2A-ΔCD19 vectors to generate Dual-switch (DS) CAR-T cells. Combined transduction of iRC9 and iMC-CAR vectors produced CD123-directed CAR-T cells that eliminated $CD123^{+}$ THP1 and MOLM13 AML cells, but not $CD123^{-}$ HPAC tumor cells, in a co-culture assay. Cytokine secretion and target cell killing were dependent on the dose of rimiducid (EC_{50} < 0.5 nM) to activate iMC costimulation. When challenged in a THP1-eGFP*Fluc* tumor-bearing mouse xenograft model, activation of the on-switch by rimiducid (1 mg/kg) in DS CAR-T cells enhanced tumor killing measured by bioluminescence imaging and T-cell expansion determined by splenocyte flow cytometry and vector copy number analyses.

Deployment of the off-switch induced fast (½ V_{max} ~ 8 hours) and efficient T cell elimination of in a caspase-3 activation assay with real-time monitoring by Incucyte microscopy as well as Annexin V detection by flow cytometry (DS CAR-T = 77.6% versus untransduced = 2.2% Annexin V^{+} when treated with 1 nM rapamycin). *In vivo* assessment of the suicide switch was performed with eGFP*Fluc*-labeled CD123 DS CAR-T cells in NSG mice. Rapamycin, but not rimiducid, treatment efficiently eliminated DS CAR-T cells within 24 hours in NSG mice, which is similar to the clinically validated rimiducid-regulated iC9 switch. Importantly, the off-switch was

insensitive to high rimiducid concentration, demonstrating that the on-switch regulator does not crosstalk with the safety switch.

Summary

Dual switch CAR-T, a novel platform comprising a first-generation CAR combined with regulated activation and apoptotic signaling elements, effectively controlled tumor growth and T cell expansion and elimination *in vitro* and *in vivo*. This dual switch technology provides a user-controlled system for managing persistence and safety of tumor antigen-specific CAR-T cells.



DS CD123 CAR-T cells efficiently control CD123$^+$ tumors with rimiducid and are eliminated with rapamycin. **(A)** Bioluminescence imaging (BLI) of THP1-eGFP*Fluc*-bearing mice treated with 2.5 x 10^6 T cells expressing CD123 iMC-CAR or DS CAR vectors treated with vehicle or 1 mg/kg rimiducid. **(B)** BLI of mice injected with 1 x 10^7 T cells expressing DS CAR vectors and treated with vehicle, rimiducid or rapamycin. **(C)** BLI of mice injected with 1 x 10^7 T cells expressing C9, iRC9, iMC-CAR + C9, and iMC-CAR + iRC9 (DS CAR) vectors and treated with increasing doses of rimiducid.