4–BB AND OPTIMIZED CD28 CO-STIMULATION ENHANCES FUNCTION OF HUMAN MONO- AND Bi-
SPECIFIC THIRD-GENERATION CAR T CELLS

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Background Co-stimulatory signals regulate the expansion, persistence, and function of chimeric antigen receptor (CAR) T cells. Most studies have focused on the co-stimulatory domains CD28 or 4–BB. CAR T cell persistence is enhanced by 4–BB co-stimulation leading to NF-kB signaling, while resistance to exhaustion is enhanced by mutations of the CD28 co-stimulatory domain.

Methods We hypothesized that a third-generation CAR containing 4–BB and CD28 with only PYAP signaling motif (mut06) would provide beneficial aspects of both. We designed CD19-specific CAR T cells with 4–BB or mut06 together with the combination of both (BB06). We evaluated their immune-phenotype, cytokine secretion, real-time cytotoxic ability and polyfunctionality against CD19-expressing cells. We analyzed LCK recruitment by the different constructs by immunoblotting. We further determined their ability to control growth of Raji cells in NSG mice. Additionally, we engineered bi-specific CARs against CD20/CD19 combining 4–BB and mut06 and performed repeated in vitro antigenic stimulation experiments to evaluate their expansion, memory phenotype and phenotypic (PD1+CD39+) and functional exhaustion. Bi-specific CAR T cells were transferred into Raji or Nalm6-bearing mice to study their ability to eradicate CD20/CD19-expressing tumors.

Results Co-stimulatory domains combining 4–BB and mut06 confers CAR T cells with an increased polyfunctionality and LCK recruitment to the CAR (figure 1A), after repeated-antigen stimulation these cells expanded significantly better than second-generation CAR T cells (figure 1B). A bi-specific CAR targeting CD20/CD19, incorporating 4–BB and mut06 co-stimulation, showed enhanced antigen-dependent in vitro expansion with lower exhaustion-associated markers (figure 1C). Bi-specific CAR T cells exhibited improved in vivo anti-tumor activity with increased persistence and decreased exhaustion (figure 1D).

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Conclusions These results demonstrate that co-stimulation combining 4-1BB with an optimized form of CD28 is a valid approach to optimize CAR T cell function. Cells with both mono- and bi-specific versions of this design showed enhanced in vitro and in vivo features such as expansion, persistence and resistance to exhaustion. Our observations validate the approach and justify clinical studies to test the efficacy and safety of this CAR in patients.

http://dx.doi.org/10.1136/jitc-2021-SITC2021.105