

PHOSPHORYLATION OF CD28-Y218 MODULATES IL-2 SECRETION AND ANTITUMOR EFFECT OF CAR-T CELLS, IN A PRECLINICAL MODEL OF PANCREATIC CANCER

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Background CD28-derived co-stimulation modules are frequently used as part of the signaling domain of chimeric antigen receptors (CAR). These modules contain well characterized tyrosine-containing moieties (YMN and PYAP) that become phosphorylated upon CAR activation, resulting in IL-2 secretion and T cell expansion.^{1,2} Previous work by our group has shown that an additional tyrosine residue, located in the C-terminus of CD28 (Y218), was also phosphorylated upon CAR activation.³ In this work we sought to evaluate the functional relevance and molecular mechanism of CD28-Y218 phosphorylation in CAR-T cells.

Methods We first analyzed the kinetics of Y218 phosphorylation. To that end, prostate stem cell antigen (PSCA) specific CAR-T cells were stimulated through co-culture with HPAC pancreatic cancer cells for 0, 1, 10, 30 and 60 minutes. Phosphorylation of Y218 was analyzed by Western blotting using a phospho-specific antibody. To determine the functional relevance of this phosphorylation event, we generated mutant CARs where the tyrosine residue was replaced with a non-phosphorylatable amino acid (Y218F) and evaluated its effects *in vivo* and *in vitro*. CAR-T cell cytotoxicity was monitored using the xCELLigence Real Time Cytotoxicity Assay (RTCA). A CD19-specific CAR was used as an alternative model, where NALM6 cells expressing firefly luciferase served as target cells. Luciferase activity was used as surrogate for target cell viability. In both models, we monitored cytokine production following overnight co-culture with target cells using the ELLA system (Biotechne, CA, USA). To evaluate *in vivo* antitumor effect of PSCA-specific CAR-T cells, NSG mice were injected (s.c.) with 1 HPAC cells and infused 14 days later with 5 CAR-T cells (i.v.). Tumor growth was monitored for 35 days.

Results We observed an antigen-dependent phosphorylation of Y218 which reached maximum levels approximately 10 min post-stimulation. Mutation of the CD28-Y218 residue did not affect CAR expression, cytotoxicity *in vitro*, or INF γ production. However, this mutation was associated with reduced IL-2 and TNF α secretion. *In vivo*, this mutation completely abrogated the therapeutic effect of these cells in the pancreatic cancer model.

Conclusions These results show that CD28-Y218 plays a role in CAR-T cell function, with direct impact on IL-2 secretion and antitumor efficacy. Future experiments will determine the specific signaling events that are triggered by Y218 phosphorylation.

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Ethics Approval This study was approved by the Institutional Animal Care and Use Committee (IACUC); approval number R ISO00010727.

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