205 SPECIFIC AMPK AGONISM DURING CART *IN VITRO* EXPANSION ENHANCES OXIDATIVE METABOLISM AND IMPROVES *IN VIVO* LEUKEMIA CLEARANCE

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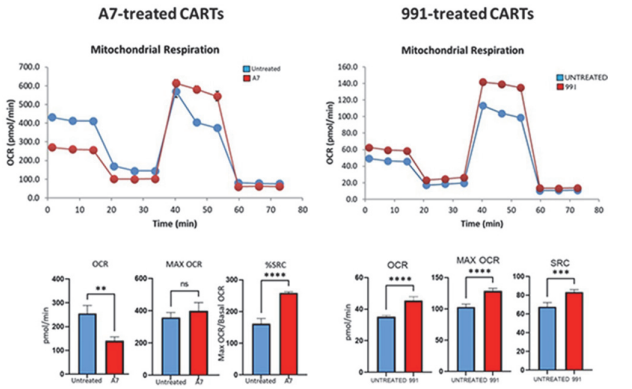
Background While chimeric antigen receptor (CAR)T therapy has seen great success in pediatric leukemia, relapses continue to occur in up to 1/3 of patients.[1] CART persistence *in vivo* correlates with ongoing remission, and promoting oxidative metabolism and mitochondrial health during *in vitro* expansion creates more functional and persistent CARTs *in vivo*.[2] AMP-activated protein kinase (AMPK) is a cellular energy sensor with prominent roles in mitochondrial health and biogenesis.[3] We hypothesized that promoting AMPK activity during CART expansion would enhance mitochondrial metabolism, leading to improved anti-leukemia clearance *in vivo*.

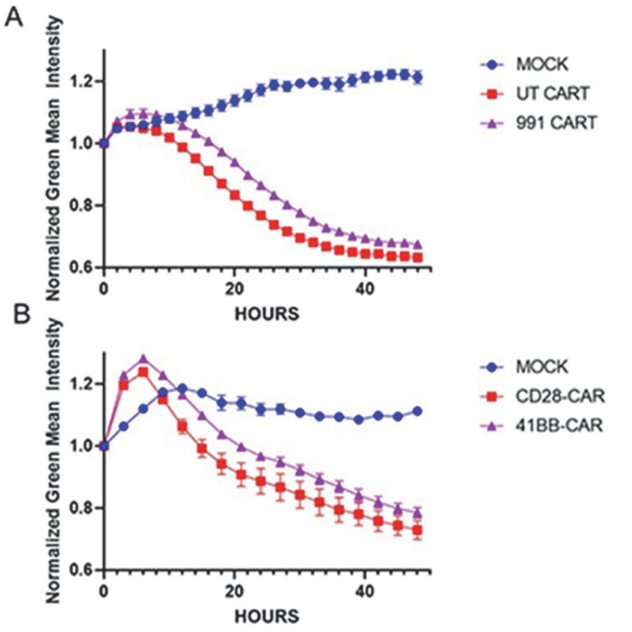
Methods Healthy human T cells underwent lentiviral transduction with a CD19-targeting CAR containing the CD28 costimulatory domain. Transduced CARTs were expanded in complete media supplemented with IL2 and either AMPK agonist Compound 991 (991), AMPK agonist A769662 (A7), or DMSO (control). After expansion, cells were assessed for metabolic function *in vitro* using the Seahorse Metabolic Analyzer. *In vitro* cytotoxicity against NALM6 targets expressing zzGreen was measured by monitoring mean fluorescence intensity using the Incucyte. For *in vivo* studies, NSG mice were injected on Day -7 with luciferase+ NALM6 leukemia cells followed by CARTs +/- agonist on Day 0, with luminescence followed weekly by IVIS imaging.

Results 991 treatment enhanced CART oxidative metabolism over DMSO-treated controls, while A7 treatment significantly reduced initial oxygen consumption, leading to 991 treatment being chosen for further study (figure 1). Despite this potential metabolic advantage, 991-treated CARTs showed reduced *in vitro* cytotoxicity against NALM6 targets compared to DMSO-treated controls, which interestingly mimicked the cytotoxicity advantage of CD28-costimulated CARTs compared to 41BB-costimulated CARTs (figure 2). Given 41BB-CARTs show enhanced persistence over CD28-CARTs *in vivo* (2), we pursued further *in vivo* studies with our agonist-treated CARTs. Indeed, despite the slower killing *in vitro*, 991-treated CARTs outperformed the DMSO-treated control CARTs *in vivo*, with significantly reduced luminescence by IVIS imaging and improved overall survival (figure 3).

Conclusions AMPK agonism during *in vitro* expansion of CARTs with 991, but not A7, creates metabolically desirable CARTs for immunotherapy. This was demonstrated by increased oxidative metabolism after expansion *in vitro* and improved leukemia clearance *in vivo*. However, anti-leukemia activity appeared decreased with *in vitro* assessments. These studies identify AMPK as an attractive target in immunotherapy, with attention paid to how this pathway is activated, and also suggest the potentially limited utility in using *in vitro* cytotoxicity as a predictor of *in vivo* function in leukemia.



Abstract 205 Figure 1 991 treatment, but not A7 treatment, enhances CART cell oxidative metabolism. Human CART cells were generated from healthy human donor T cells through lentiviral transduction, and then expanded in either DMSO (untreated), A769662 (A7), or Compound 991 (991). CARTs then underwent restimulation overnight before assessment utilizing the mitostress kit for the Seahorse Metabolic Analyzer. ** p<0.01, *** p<0.001, **** p<0.0001



Abstract 205 Figure 2 AMPK-agonist treated CARTs show decreased cytotoxicity vs untreated CARTs in vitro, which is mimicked by the cytotoxicity of 41BB vs CD28 CARTs. (A) CD28-costimulated CARTs were generated and expanded with or without 991. After expansion, cells were placed in the Incucyte against zzGreen+NALM6 targets, with green fluorescence followed to measure cell killing over 48 hours. (B) The experiment was repeated using CD28-costimulated and 41BB-costimulated CARTs. In both experiments, mock transduced human T cells were plated against NALM6 targets as a control.

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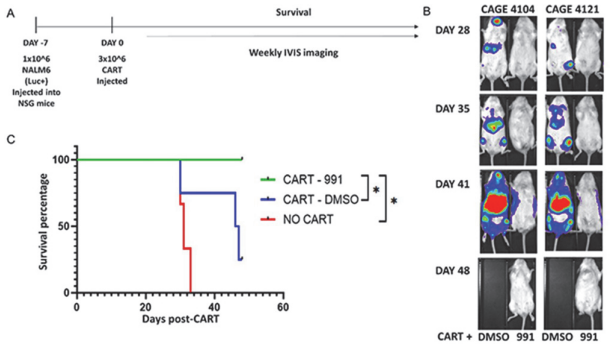

Abstract 205 Figure 3 991-treated CART cells outperform standard CARTs in vivo. Luciferase+ NALM6 cells were injected into NSG mice on Day -7, followed by injection with either 991-treated CARTs or DMSO-treated control CARTs on Day 0 (A). Tumor burden was followed weekly with IVIS imaging (B), and overall survival was determined (C). N= 3-4 mice per group. * $p < 0.05$.

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