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Christopher Ronald Funk et al.

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Dual Inhibition of PI3KDelta/Gamma during Manufacturing Reprograms Metabolism of CAR T Cells to Enhance Expansion and Cytotoxicity Against CLL

Christopher Ronald Funk[1], *Shuhua Wang, MD*[1]*, *Alex Waller, BS*[2]*, *Claudia Edgar, BS*[1]*, *Aditi Sharma, PhD*[1]*, *Kevin Chen, BS*[1]*, *Vikas Gupta, MD, PhD*[3]*, *Shanmuganathan Chandrakasan, MD*[4]*, *Jaquelyn Zoine*[5,6]*, *Andrew Fedanov, BS*[5,6]*, *Sunil S. Raikar, MD*[7], *Jean L. Koff, MD*[1], *Christopher Flowers, MD, MS*[8], *Silvia Coma, PhD*[9]*, *Jonathan A. Pachter, PhD*[9]*, *Sruthi Ravindranathan, PhD*[1]*, *H. Trent Spencer, Ph.D.*[5,10]*, *Mala Shanmugam, PhD*[1] *and Edmund K. Waller, PhD, MD*[11]

[1]Department of Hematology and Medical Oncology, Emory University School of Medicine, Atlanta, GA
[2]Department of Lymphoma/Myeloma, Division of Cancer Medicine, The University of Texas MD Anderson Cancer Center, Houston, TX
[3]Emory University, Atlanta, GA
[4]Division of Bone Marrow Transplant, Department of Pediatrics, Emory University School of Medicine, Atlanta, GA
[5]Aflac Cancer and Blood Disorders Center, Children's Healthcare of Atlanta, Atlanta, GA
[6]Department of Pediatrics, Emory University School of Medicine, Atlanta
[7]Aflac Cancer and Blood Disorders Center, Emory University/Children's Healthcare of Atlanta, Atlanta, GA
[8]Department of Lymphoma and Myeloma, The University of Texas MD Anderson Cancer Center, Houston, TX
[9]Verastem Oncology, Inc., Needham, MA
[10]Department of Pediatrics, Emory University School of Medicine, Atlanta, GA
[11]Department of Hematology and Medical Oncology, Winship Cancer Institute, Emory University, Atlanta, GA

*signifies non-member of ASH

Chronic lymphocytic leukemia (CLL) creates an immune-suppressive microenvironment that induces terminal T-cell differentiation and T-cell exhaustion, thereby limiting the efficacy of chimeric antigen receptor (CAR) T cells. Indeed, analysis of CLL patients has shown that a high frequency of senescent T-cells (dashed line, Figure 1A) prior to CAR T-cell manufacturing was associated with disease progression and failure to benefit from CAR T (Fraietta *et al.* 2018). In a cohort of early stage untreated CLL patients, we show that 55% of patients had a predominant senescent phenotype (Figure 1A). This striking observation highlights the need for strategies to enrich non-senescent T cells for CAR T therapy in CLL patients. To address this need, we studied the effect of adding phosphoinositide 3-kinase inhibitors (PI3Ki) during *ex vivo* CAR T cell-manufacturing on T cell phenotype, metabolism, *in vivo* expansion, persistence and anti-CLL cytotoxicity in NOG mice bearing the human OSU-CLL compared with conventional CART.

To investigate the relative influence of PI3Kd-selective versus dual PI3K-d/g inhibition, we first cultured T cells from CLL patients activated with anti-CD3/CD28 beads across logarithmic dose scales of duvelisib or idelalisib. With dual-PI3K-d/g inhibition, CLL donor T cell expansion was 150% of conventionally expanded CART (Figure 1B) with dose-dependent decreases in expression of exhaustion markers TIM-3 and LAG-3 (Figure 1C). PI3K-d/g blockade increased frequencies of CD8+ CAR T cells, thus normalizing the ratio of CD4:CD8 CAR T cells (Figure 1D). To further characterize the effects of dual PI3K-d/g inhibition on T-cell phenotype, mass cytometry time-of-flight (CyTOF) analyzed the phenotype of CAR T cells cultured with or without duvelisib. Unsupervised clustering algorithms showed duvelisib increased frequencies and numbers of T-stem cell memory (T_{scm}), naïve, and central memory CD8+ CAR T cells (data not shown). Finally, unbiased clustering algorithms identified increases in frequencies of CD27+CD45RO- CD8+ CAR T cells which was confirmed in additional patients using flow cytometry (Figure 1E). In summary, duvelisib enriched populations of less differentiated, more potent CD8+ CAR T cells during manufacturing.

To assess whether these phenotypic changes conferred functional benefit, the cytotoxicity of CAR T cells cultured with or without duvelisib was compared using the OSU-CLL cell line. Duvelisib-cultured CAR T cells (Duv-CAR T cells) had greater cytotoxicity against OSU-CLL cell line (Figure 1F). Subsequently, gene expression profiling of Duv-CAR T cells showed decreased glycolysis pathway scores, down-regulated glucose transporters, and up-regulated glutamine transporters relative to control CAR T cells, leading us to explore T cell metabolism. Elevated protein expression of PGC1-a, a critical regulator of mitochondrial biogenesis and autophagy, was associated with increased staining with NAO, a measure of mitochondrial mass in Duv-CAR T cells (data not shown). To directly measure mitochondrial content of cells, transmission electron microscopy of control CAR T and Duv-CAR T cells generated from CLL patients was performed after 14 days of culture with representative images shown in Figure 1H. A 1.45-fold increase in mitochondrial cross-sectional area was observed (representative images in Figure 1G), which imputes a 175% increase in mitochondrial volume for duvelisib cultured CAR T cells relative to control CAR T cells (p=0.0013; Figure 1G).

Following transfer to NOG mice engrafted with a human CLL cell line, Duv-CAR T cells demonstrated greater *in vivo* expansion (Figure 1H), faster elimination of CLL (data not shown), and improved mouse survival (Figure 1I) in a model of high disease burden OSU-CLL. In summary, dual PI3K-d/g inhibition during CLL patient-derived CAR T-cell manufacturing increased yields of T_{scm}, naïve, and central memory CD8+ Duv-CAR T cells with greater mitochondrial mass and enhanced efficacy in eliminating CLL in a mouse model.

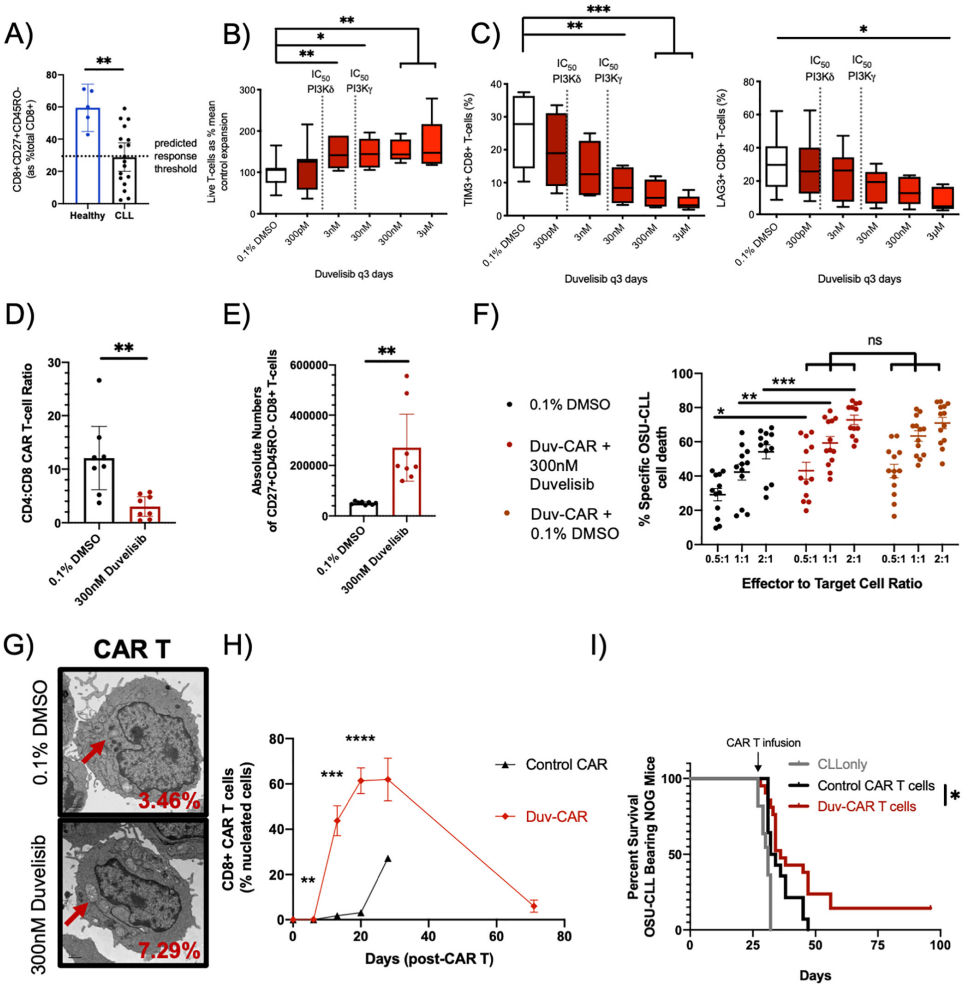

Figure 1. **Dual PI3K-δ/γ inhibition promotes increased numbers of naïve CD8+ T cells during CAR T-cell manufacturing, with greater mitochondrial mass, leading to enhanced *in vitro* cytotoxicity and *in vivo* expansion, and survival of OSU-CLL bearing NOG mice. (A)** CLL patients have lower frequencies of CD8+CD27+CD45RO- T cells than healthy controls. **(B)** T cells from CLL patients cultured with duvelisib show greatest fold-expansion at IC_{50} values sufficient to inhibit both PI3Kδ and PI3Kγ and **(C)** also resulted in decreased expression of immune checkpoint molecules TIM3 and LAG3 on the CD8+ subset. **(D)** PI3Kδ and PI3Kγ inhibition with duvelisib increased the frequency of CD8+ CAR T cells, resulting in decreased CD4:CD8 T cell ratios. **(E)** Accordingly, duvelisib increased absolute numbers of naive CD27+CD45RO- CD8+ CAR T cells correlated with remissions (Fraietta *et al.* 2018). **(F)** Control CAR T cells and Duv-CAR T cells ("effectors") from 4 of the analyzed CLL patients were incubated with OSU-CLL cells ("targets") at effector-to-target ratios of 0.5:1, 1:1, and 2:1 to show Duv-CAR T cells exhibit superior cytotoxicity against CD19+ OSU-CLL that occurred whether duvelisib was present in the reaction mixture (red) or had been washed out (orange). **(G)** Representative transmission electron microscopy (TEM) images from greater than 240 acquired images indicate duvelisib increased mitochondrial size, listed as a cross-sectional area that is a percentage of total cell area. Red arrows point to mitochondria. **(H)** Duv-CAR T cells cultured with duvelisib again had significantly enhanced *in vivo* expansion of CD8+ CAR T cells. **(I)** Duv-CAR treated mice exhibit a survival advantage relative to control CAR treated mice, with all control CAR mice meeting IACUC endpoints by day 47 and Duv-CAR living up to the termination of the experiment on day 96.
For all panels, * denotes p<0.05, ** denotes p<0.01, *** denotes p<0.001, **** denotes p<0.0001.

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Springer Healthcare Ibérica S.L.
Rosario Pino, 14 - 4ª Planta. 28020 Madrid. Spain
Tel.: +34 91 555 40 62. Fax: +34 91 555 76 89
E-mail: Miguel.Quesada@springer.com
www.springerhealthcare.com
www.springernature.com

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