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703.CELLULAR IMMUNOTHERAPIES: BASIC AND TRANSLATIONAL

CD19-CAR Cytokine Induced Killer Cells Armored with IL-18 Control Tumor Burden, Prolong Mouse Survival and Result in *In Vivo* Persistence of CAR-CIK Cells in a Model of B-Cell Acute Lymphoblastic Leukemia

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Rationale: Cytokine induced killer cells (CIK) are a promising cancer immunotherapy. Recently CIK cells modified to express chimeric antigen receptors (CAR) target CD19 expressing hematological malignancies including B-cell acute lymphoblastic leukemia (B-ALL). Despite encouraging clinical trial results, insufficient CAR-CIK anti-tumor activity and persistence pose major obstacles towards improved efficacy *in vivo*. Therefore, we optimized our CAR-CIK platform by introducing two modifications to the CD19-CAR construct. The first modification "armored" CAR-CIK cells by inserting the IL-18 gene in a bicistronic DNA plasmid enabling simultaneous surface expression of the CAR protein and secretion of IL-18, which demonstrated improved CAR-T cell function. The second modification attenuated the CD28 cytoplasmic signaling domain of the CAR molecule to enhance anti-tumor activity and *in vivo* persistence since recent findings demonstrate that persistent or chronic CAR signaling can impair anti-tumor activity and *in vivo* persistence.

Methods: CARCIK-1918 cells generated from donor-derived CIK cells are engineered to express CAR and IL-18 using the non-viral *Sleeping Beauty* (SB) transposon system. CIK cells were generated from donor PBMCs by the sequential addition of IFN-ϒ and stimulation with anti-CD3 antibody plus IL-2. After 2 days of stimulation CIK cells were co-electroporated with SB100X transposase mRNA and plasmid DNA containing a bicistronic transgene encoding both the anti-CD19 CAR and IL-18. This second-generation CAR contains a fusion of the CD28 transmembrane domain, intracellular CD28 and, TCR CD3ζ chain. In some instances, the cytoplasmic CD28 signaling domain is modified to attenuate CD28 signaling. This method of gene transfer results in stable surface expression of the CAR on CD3+ cells through a 17-day culture period. Day 17 cells are harvested, frozen and used for all subsequent studies.

CARCIK-1918 cells are stimulated *in vitro* with CD19+ REH tumor cells and the phenotype of activated cells was characterized by multi-color flow cytometry. IL-18 secretion was determined by cytokine bead array analysis of supernatants collected post stimulation. CARCIK-1918 cells with or without the attenuated CD28 signaling domain were tested *in vivo* in a xenograft model of B-ALL, using NSG mice engrafted with CD19+ Raji tumor cells. Tumor burden was assessed weekly by bioluminescence imaging, the *in vivo* persistence of CARCIK-1918 cells was measured by flow cytometry, and survival was monitored throughout the study.

Results: CARCIK-1918 cells express both IL-18 and CAR with or without attenuated CD28 signaling. Furthermore, stimulation of CARCIK-1918 cells with CD19+ REH tumor cells increases the frequency of CAR-CIK cells with a memory phenotype and concurrent upregulation of the high affinity IL-2 receptor, CD25, and the costimulatory receptor CD137. Expression of IL-18 leads to higher engraftment of CAR-CIK cells in tumor bearing NSG mice compared to unarmored controls. NSG mice engrafted with luciferase positive Raji cells demonstrated decreased bioluminescent signal following CARCIK-1918 treatment, consistent with CARCIK derived anti-leukemic activity. This anti-tumor activity corresponded to a statistically significant improvement in survival as mice treated with 2 nd generation CAR transgene CARCIK cells with or without attenuated CD28 signaling had a median survival of 37.5 (n=10; p=0.0001) and 38 (n=12; p =0.0001 days), respectively, compared to untreated mice that develop progressive highly disseminated tumor and succumb to disease by 3 weeks post tumor inoculation (n=11; median survival 18 days). Interestingly, mice treated with CARCIK-1918 cells with the CD28 attenuated signaling domain exhibited better tumor control and enhanced persistence *in vivo*, despite no significant improvement in survival compared to WT CD28 signaling CARCIK-1918 cells.

Summary: This study demonstrates that administration of CAR-CIK cells armored with IL-18 results in a significant survival advantage for tumor challenged animals versus untreated animals. Furthermore, modifying the cytoplasmic CD28 domain to attenuate signaling, results in better control of tumor burden, and persistence of CARCIK-1918 cells *in vivo*. Our study is the first report of IL-18 armored CAR-CIK cells which may present a promising, novel strategy for the treatment of B-ALL.

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