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703.CELLULAR IMMUNOTHERAPIES: BASIC AND TRANSLATIONAL

Heme Attenuates T Cell Exhaustion and Drives Effector Function: Implications for Immune and Adoptive T Cell Therapy

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Introduction: Though frontline agents have been effective in multiple myeloma (MM) therapy, a significant proportion of MM patients relapse and succumb to refractory disease. B-cell maturation antigen (BCMA)-directed chimeric antigen receptor (CAR) T therapy represents an exciting novel therapeutic strategy for MM but still suffers from limited *in vivo* T cell expansion and CAR T cytotoxicity. Altering the CAR T manufacturing process to produce CAR T cells with less exhausted phenotypes and robust killing is an active area of research. Mitochondrial function and energetics play a central role in regulating T cell fate and function, and heme (iron-protoporphyrin IX) is critical for maintaining electron transport chain activity and oxidative phosphorylation. Heme can be sourced from the extracellular milieu, diet, or intrinsic heme biosynthesis and has a multitude of cellular functions throughserving as a cofactor for enzymatic and metabolic reactions. Additionally, heme is catabolized by heme oxygenase 1, producing the antioxidants biliverdin and bilirubin critical to preserving cellular redox homeostasis. In the present study, we investigate the effects of heme on CAR T cell efficacy. Our results suggest that heme supplementation during *ex vivo* CAR T manufacturing or supplementation during CAR T-tumor cell engagement can lead to decreased T cell exhaustion and increased tumor killing.

Methods: Human healthy donor and MM patient T cells were activated and expanded with α-CD3/CD28 and IL-2 for 7 days +/- hemin, after which immunostaining and metabolic assays were conducted. For CAR T manufacture, T cells were transduced with CAR construct starting on day 3 and were co-cultured with antigen-expressing tumor cells *in vitro* on day 7-8. Cytotoxicity and cytokine production were assayed after 24 hours of co-culture.

Results: In a dose-dependent manner, heme supplementation significantly decreases proportions of LAG3 $^+$ and TIM3 $^+$ and decreases proportions of CD62L $^+$ and TCF1 $^+$ cells in both CD4 $^+$ and CD8 $^+$ subsets isolated from healthy donor and MM patient peripheral blood. Supportive of these observations, inhibition of *de novo* heme biosynthesis leads to the opposite effect with an increase in proportions of PD1 $^+$, LAG3 $^+$, TIM3 $^+$, and CD62-L $^+$ cells. CAR T cells manufactured with heme (heme-CAR T cells) have significantly reduced terminally exhausted phenotypes (PD1 $^+$/LAG3 $^+$/TIM3 $^+$) and increased enrichment of effector memory cells (CD62L $^-$CD45RA $^-$) in both CD4 $^+$ and CD8 $^+$ subsets. Inquiry of cellular energetics of heme supplemented CAR T cells reveals reduced reactive oxygen species (ROS) and increased mitochondrial membrane potential, consistent with previous reports showing increased membrane potential in effector T cells for greater production of cytokines such as IL-17A, IL-17F, and IFNγ. In co-culture with tumor cells *in vitro*, heme-CAR T cells have higher tumor-specific lysis and production of TNFα and IL-2. CAR T-tumor cell engagement in the presence of heme resulted in significantly decreased proportions of exhausted CAR T cells and increased production of TNFα. Heme also increases BCMA surface expression in several MM cell lines, highlighting a viable strategy for enhancing BCMA CAR T engagement with MM. Lastly, MM patient bone marrow-localized T cells demonstrate significantly decreased terminally exhausted phenotypes and increased differentiation into effector subsets when expanded with heme.

Conclusion: We show that heme increases effector T and CAR T cell function and decreases T cell exhaustion. Increased cytotoxicity of heme-supplemented CAR T cells was associated with reduced ROS and increased membrane potential. Ongoing *in vivo* studies aim to characterize the efficacy of heme-supplemented CAR T cells in MM xenograft models. Our studies have broader implications for investigating heme supplementation in *ex vivo* CAR T manufacturing for a wide range of hematological cancers and underscore the need for further investigation of heme uptake and metabolism for strategic enhancement of T-cell based immunotherapies.

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