2

THE INTEGRATION OF GENOMICS AND FUNCTIONAL CHARACTERIZATION OF "OFF-THE-SHELF" CD19-CAR-T CELLS ALLOWS THE IDENTIFICATION OF MULTIPLE T CELL SUBSETS WITH EFFICIENT ANTI-TUMOR ACTIVITY AND LOW PRO-INFLAMMATORY PROFILE

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Background: The principal goal of this study is to characterize the molecular and functional composition of "off-the-shelf" CD19-CAR-T cells generated from umbilical cord blood (UCB).

Methods: T cells were isolated from UBCs (N=40) and, upon activation *in vitro* using CD3 and CD28 mAbs, were transduced with lentiviral vectors encoding for CD19-CD28*z*-or CD19-4-1BB*z*-CARs. Engineered T cells produced from the peripheral blood lymphocytes (PBL; N=5) were used as reference. CD19-CAR-T cells were co-incubated or not with either $CD19^+$ or $CD19^-$ target cells to mimic the antigen-mediated engagement of the CARs, and then, multi-omics analyses, including metabolomics, transcriptomics, scRNA seq and *in vitro* functional assays, (Elispot, Luminex) were performed.

Results: Distinct molecular and functional profiles were observed according to the source of T cells used for the manufacturing of CD19-CAR-T cells. The transcriptomic profiling of these cells showed that chemokines involved in chemotaxis, adhesion, and activation, were up-regulated in UCB- vs. PBMC-CD19-CAR-T cells.

Other molecular pathways associated with pathways involved in allograft rejection, antigen processing and presentation, and pro-inflammatory functions were preferentially detected in PBMC-CD19-CAR-T cells. The multiplex analyses of the antigen-specific release of cytokines, chemokines, and growth factors highlighted that TH1-type cytokines or cytotoxic molecules were released by CD19-CAR-T cells isolated from both UCB or PBMCs. On the other hand, pro-inflammatory molecules, such as IL-9, IL-10, IL-13, IL-31, were preferentially associated with PBMC-CAR-T cells. scRNA sequencing data and long read-whole genomic methylation sequencing showed that different lymphocyte subsets could be identified within UCB-CAR-T cells and their deep phenotype and functional profile upon antigen-specific stimulation of the CARs. Differential antigen-specific metabolomic signatures were also found in association with the source of T lymphocytes expressing the CD19-CARs.

Conclusions: Although the integration of methylation and single cell transcriptomic profile of CAR-T cells is still ongoing, the data obtained from the multiplatform analyses showed that UCB-derived CAR-T cells are endowed with efficient anti-tumor “fitness” and negligible pro-inflammatory profile, with possible lower risk of inducing cytokine release syndrome.