INVENTION DISCLOSURE

Description of Technology

Phenotyping long-lived RA^{int}RO^{int} CAR T cells

In recent years, immunotherapy using Chimeric Antigen Receptor (CAR) T cells against tumor specific antigens has proven beneficial for cancer treatment and tumor eradication. Patient-to-patient variability, driven by changes in tumor microenvironment and intrinsic diversity of personalized CAR T cells, has been a key reason for incomplete efficacy of this highly promising therapy. We have identified a subset of CD4 and CD8 CAR T cells that have phenotypic and molecular attributes of long-lived pluripotent stem T cells. This subset is primarily characterized by intermediate co-expression of CD45RA and CD45RO (RA^{int}RO^{int}). This RA^{int}RO^{int} population homogenously expresses CD95 (Fas; a marker that distinguishes T_{scm} from naïve cells), CD127 (IL7R; essential for maintaining homeostatic proliferation) and CD27 (marker of central memory T cells) **(Figure 1)**. In line with a stem-central memory T cell (T_{scm}) like phenotype, protein levels of key T helper 1 and T helper 2 transcription factors (T-bet and GATA-3) are absent in this subset **(Figure 2)**, confirming the lack of commitment of this cell subset. Furthermore, glycolytic enzymes (typically associated with an effector T cell phenotype) are down-regulated in these cells **(Figure 2)**.

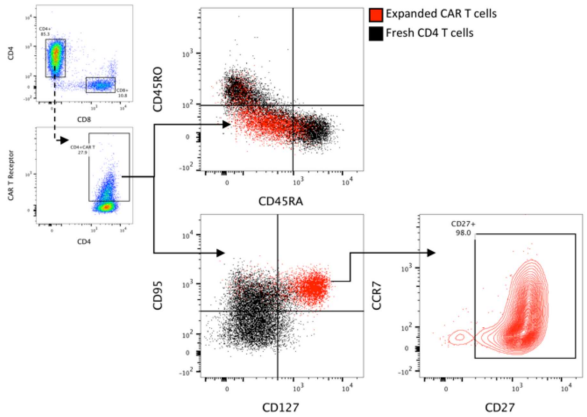

Figure 1. Expanded CAR T cells have a long-lived pluripotent stem T cell like phenotype. Expanded CAR+ CD4 T cells when contrasted against fresh CD4 T cells, show intermediate expression of CD45RA and CD45RO. This population can be further identified as expressing CD95, CD127 and CD27.

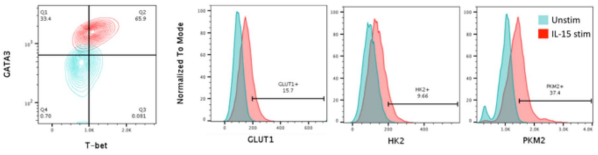

Figure 2. Expanded CAR T cells show low glycolytic and effector machinery. These cells do not express master transcription factors of T cell differentiation (GATA3 and T-bet), and lack the expression of glycolytic enzymes like GLUT1, HK2 and PKM2. The effector phenotype can be rescued after stimulation with IL-15 for 48 hours.

We have previously observed this phenotype in total CD4 T cells, but was never specifically attributed to CAR T cells. This uncommitted phenotype was supported by a gene-expression prolife akin to a quiescent phenotype (upregulation of fatty acid metabolism and oxidative phosphorylation, and downregulation of cell cycling pathways) **(Figure 3)**. Finally, and most remarkably, these cells retain the capacity to differentiate into all T cell subsets, including the effector subset. These data imply that the RA^{int}RO^{int} subset is a long-lived T cell subset that is capable of self-renewing and re-populating effector compartments. Such unique cell type can prove to be crucial for effective, long-lasting

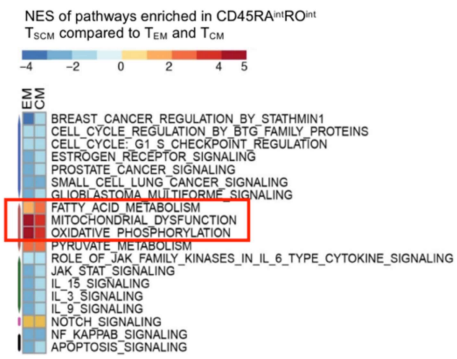

Figure 3. Cells expressing RA^{int}RO^{int} phenotype have a quiescent gene expression profile. When compared to central and effector memory subsets, the RA^{int}RO^{int} cell subset had lower expression of cell cycling pathways and higher expression of fatty acid metabolism (associated with senescence).

immune responses against the tumor. Below, we have devised a platform for the development of a long-lived and pluripotent CAR T population, with a capacity of effector differentiation.

Effector differentiation, enrichment and self-renewal of the $RA^{int}RO^{int}$ subset of CAR T cells

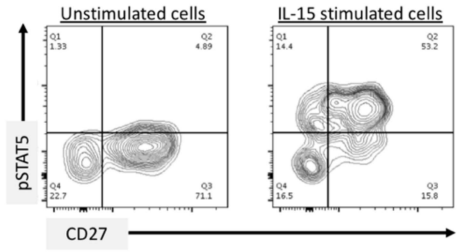

Figure 4. Stimulation of expanded CAR T cells for 48hours in the presence of IL-15 causes them to upregulate p-STAT5, a downstream target of IL-15 signaling; and results in an increase in the proportion of CD27- cells, associated with an increased effector phenotype.

As described above, the $RA^{int}RO^{int}$ CAR T population shows an uncommitted differentiation program which is highlighted by reduced glycolytic activity. We tested the change of lineage commitment within these cells upon the addition of an effector cytokine, IL-15. We observed that IL-15 stimulated CAR T cells swiftly upregulated phospho-STAT5 (a transcription factor directly regulated by IL-15 signal transduction), and demonstrated a shift towards the effector phenotype by downregulating CD27 **(Figure 4)**. In addition, $RA^{int}RO^{int}$ CAR T cells exposed to IL-15 had heightened metabolic activity and increased protein levels of the master transcription factors GATA-3 and T-bet **(Figure 2)**. Together, these results support the possibility that an effector phenotype deriving from the $RA^{int}RO^{int}$ population can be induced by IL-15 and other compounds which we will be screening in the near future.

Unlike stimulation with IL-15, addition of TGF-β and IL-1β led to the maintenance of the $RA^{int}RO^{int}$ phenotype. In addition to the maintenance of the uncommitted differentiation status of $RA^{int}RO^{int}$ cells, TGF-β and IL-1β downregulated the glycolytic machinery below the baseline levels **(Figure 5)**. The role of TGF-β as a sustainer of hematopoietic stem cell phenotype has previously been reported. The novelty of our findings highlights the role of these two cytokines and possibly others, in the maintenance of long-lived pluripotent CAR T cells.

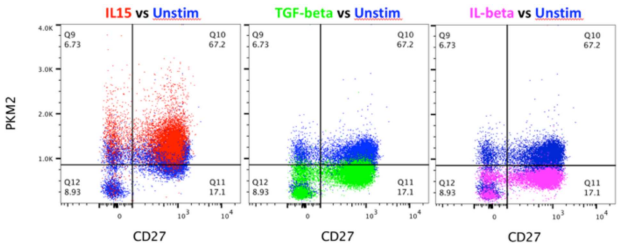

Figure 5. Protein levels of glycolytic enzymes, like PKM2, are lower in expanded CAR T cells and are maintained at a low level following stimulation with IL-1β and TGF-β (sustainers of the stem cell phenotype).

Implications

- Long-lived, self-renewing and pluripotent CART cells → Reduced cost of production
- Promoting effector differentiation → Increased efficiency of CAR T cell therapy
- Generation of iPSCs → Off-the bench universal platform
- Frequencies of these cells in the current available products can be used as a biomarker predictive of successful intervention

Next Steps

- Get pre and post-infusion samples and correlate its phenotype to outcome
- Identify cytokines/compounds/metabolites that augments stem-ness
- Identify cytokines/compounds/metabolites that augments effector differentiation
- Develop approaches to protect these cells from the immune suppressive tumor environment