



## Background

CAR-T cells are generated in culture containing 18% oxygen, but oxygen levels in human tissues are lower, typically 5%. To understand how CAR-T cells function in 5% oxygen, we compared CD19 CAR-T cell expansion, differentiation, cytolytic activity and IFN- $\gamma$  production in 18% oxygen and 5% oxygen.

## Methods

Cryopreserved human normal PBMC were activated with CD3-CD28 beads, transduced with lentivirus encoding a tagged CD19-specific CAR, and cultured in 18% oxygen for 5 days. Half of the cells were transferred to a 5% oxygen chamber (Biospherix). After 8 more days of culture, the cells were analyzed by flow cytometry for CAR expression and T cell differentiation. CAR-T cells were added to HeLa-CD19 cells growing in 18% or 5% oxygen and cytotoxicity was measured by real-time cellular analysis (RTCA) using the XCelligence system (ACEA). The level of IFN- $\gamma$  secreted by the CAR-T cells was measured by ELISA.

## Results

CAR-T cells expanded as well or better in 5% oxygen than in 18% oxygen. CAR-T cells cultured in 5% oxygen exhibited a more differentiated phenotype, with a higher frequency of effector-memory T cells and effector T cells. In response to HeLa-CD19 cells, CAR-T cells cultured in 5% oxygen had slightly elevated cytolytic activity yet produced significantly lower levels of IFN- $\gamma$ . In response to Raji cells, which express CD19 endogenously, CAR-T cells cultured in 5% oxygen had unaltered cytolytic activity, yet produced significantly higher levels of IFN- $\gamma$ .

## Conclusions

These data show for the first time that CAR-T cells cultured in 5% oxygen are not impaired in cell expansion or effector functions. The effects of lowered oxygen levels differed depending on whether the target cell was a transfectant or expressed the target protein endogenously.

### Culture in 5% oxygen causes hypoxia.

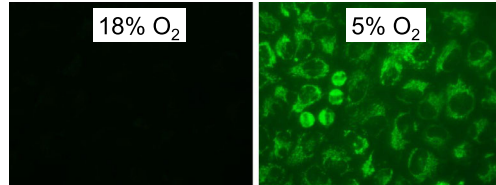


Figure 1. Cultures of HeLa-CD19 cells in 18% or 5% oxygen were incubated with a soluble green hypoxia reagent (Invitrogen).

### Culture in 5% oxygen does not affect CD19 expression on HeLa-CD19 cells.

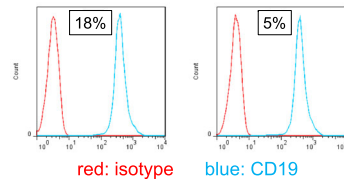


Figure 2. Cultures of HeLa-CD19 cells in 18% or 5% oxygen were stained with a CD19 antibody or an isotype control antibody, then analyzed by flow cytometry.

### Culture in 5% oxygen does not impair CAR-T cell expansion.

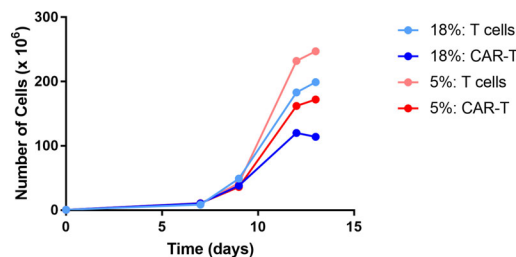


Figure 3. Cultures of CD19 CAR-T cells and non-transduced T cells in 18% oxygen or 5% oxygen were monitored for cell numbers over time.

### Culture in 5% oxygen increases T cell differentiation.

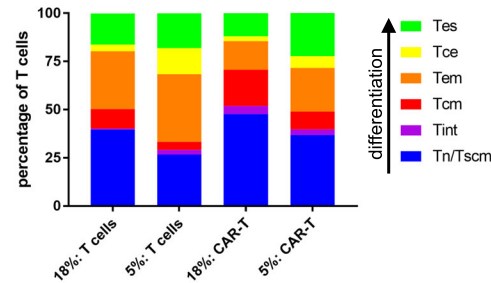


Figure 4. Cells were stained with antibodies against CD27, CD45RO and CD45RA, then analyzed by flow cytometry.

### Culture in 5% oxygen does not affect CAR expression.

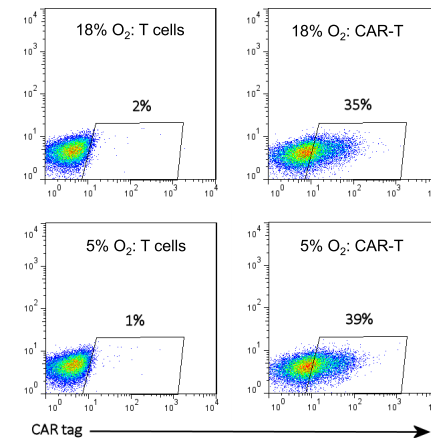


Figure 5. Cells were stained with an antibody against the tagged CAR (X-axis), then analyzed by flow cytometry.

### Culture in 5% oxygen increases CAR-T cytolytic activity for HeLa-CD19 cells but not for Raji cells.

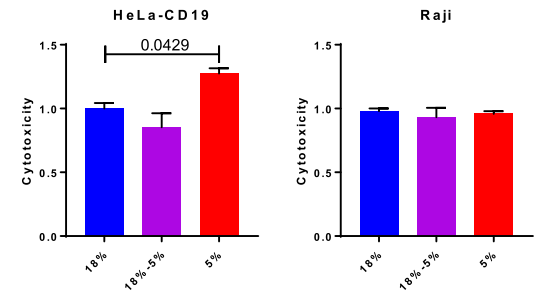
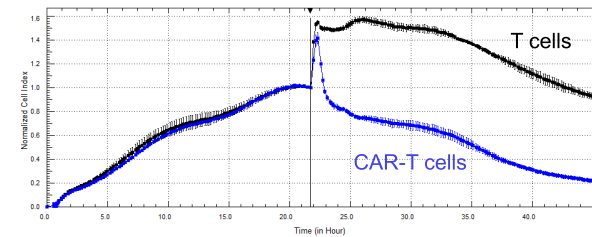


Figure 6. Cells were analyzed for cytolytic activity by RTCA assay. Cytotoxicity was normalized to 18% oxygen.

### Culture in 5% oxygen decreases IFN- $\gamma$ production in response to HeLa-CD19 cells but increases IFN- $\gamma$ production in response to Raji cells.

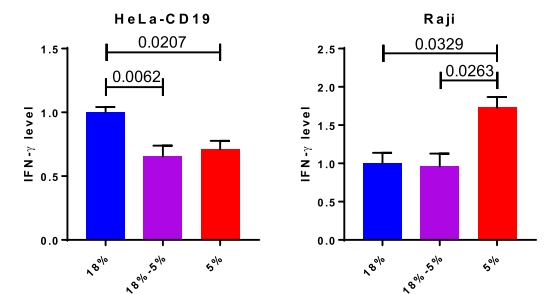


Figure 7. IFN- $\gamma$  secreted during the RTCA assay was quantitated by ELISA and normalized to 18% oxygen.