Effects of IL-2 and IL-15 on the proliferative and antitumor capacities of allogeneic anti-CD20 CAR-engineered γδ T cells in a 3D B cell lymphoma spheroid assay

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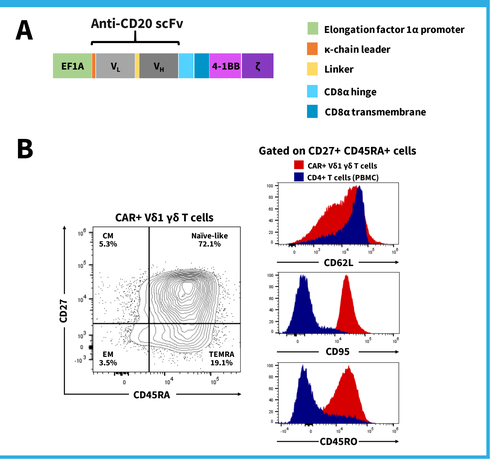
INTRODUCTION

- Autologous chimeric antigen receptor (CAR) T cells have been shown to be efficacious for the treatment of B cell malignancies; however, the widespread adoption and application of CAR T cell products to a broader range of malignancies still face multiple challenges (eg, safety and manufacturing issues, tumor antigen escape, and impaired T cell trafficking, infiltration and activation in tumors).[1,2]
- To overcome these challenges, Adicet Bio is developing an allogeneic γδ T cell-based CAR T cell platform, which capitalizes on the intrinsic abilities of Vδ1 γδ T cells to recognize and kill transformed cells in an MHC-unrestricted manner, to migrate to epithelial tissues, and to function in hypoxic conditions. [3,4]
- To gain a better understanding of the requirements for optimal intratumoral CAR Vδ1 γδ T cell activation, proliferation, and differentiation, we developed a three-dimensional (3D) tumor spheroid assay, in which tumor cells acquire the structural organization of a solid tumor and establish a microenvironment that has oxygen and nutrient gradients.[5] Importantly, through the addition of cytokines and/or tumor stromal cell types, the spheroid microenvironment can be modified to reflect hot or cold tumors.[5]
- Here, we report on the use of a 3D CD20+ Raji lymphoma spheroid assay to evaluate the effects of IL-2 and IL-15, positive regulators of T cell homeostasis and differentiation, on the proliferative and antitumor capacities of CD20 CAR+ Vδ1 γδ T cells.

Allogeneic CD20 CAR+ Vδ1 γδ T cells

- Healthy donor PBMC-derived Vδ1 γδ T cells are selectively activated with an agonistic anti-Vδ1 monoclonal antibody and then genetically modified by gamma-retroviral transduction to express a CD20-targeting chimeric antigen receptor (CAR).
- The CD20 CAR is comprised of a fully human anti-CD20 single chain variable fragment, CD8α hinge and transmembrane domains, and the 4-1BB and CD3ζ signaling domains (**panel A**).
- After transduction, γδ T cells are further expanded in culture and then enriched by αβ T cell depletion. The enriched γδ T cells are predominantly of the Vδ1 γδ T cell subset. The CD20 CAR+ Vδ1 γδ T cell lot used in these studies contains 93% Vδ1 γδ T cells, of which 82.5% are CD20 CAR+.
- Phenotypic analysis reveals that the vast majority of CD20 CAR+ Vδ1 γδ T cells possess a naïve-like or less differentiated T cell memory phenotype, in that they co-express markers associated with both naïve T cells (CD27, CD45RA, CD62L) and memory cells (CD95, CD45RO) (**panel B**).

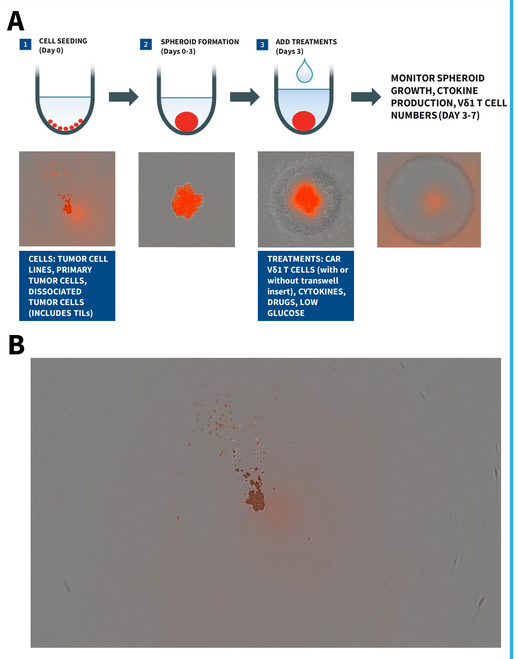


Experimental Set-Up of 3D Spheroid Culture System

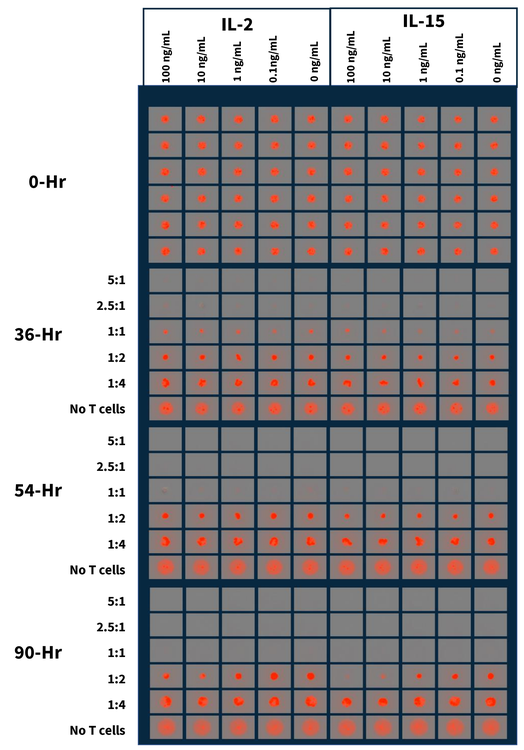
Experimental set-up (**panel A**)

- On Day 0, 1000 Raji cells, transduced with the Incucyte® NucLight Red lentivirus reagent, were seeded into the wells of 96-well ultra-low attachment (ULA) plates and cultured for 3 days in a 37°C 5% CO_2 incubator to form "spheroids" with diameters of ~500 µm. Formation and growth of spheroids were monitored using the spheroid module of the Incucyte® instrument.
- On Day 3, CD20 CAR+ Vδ1 γδ T cells (unlabeled) were plated (gently) onto spheroids at a single effector-to-target (E:T) ratio or at a range of E:T ratios in the presence of IL-2, IL-15, or no added cytokine.
- On Days 3 through 7, CD20 CAR+ Vδ1 γδ T cell-mediated Raji cell death was evaluated in real time by monitoring levels of red fluorescence using the Incucyte instrument.
- On Day 4 (ie, 24 hours after adding CAR T cells to the spheroids), culture medium samples were collected and analyzed for cytokine and chemokine levels.
- On Day 7, CAR T cell viability, numbers, and CAR expression were assessed by flow cytometric analysis and their gene expression profiles were evaluated by NanoString analysis.

Time-lapse movie of CD20 CAR+ Vδ1 γδ T cell-mediated Raji cell death in the 7-day spheroid assay (panel B). The clock starts right as the well is seeded with red Raji cells. On day 3, CAR Vδ1 T cells are added to the well at a 5:1 E:T with 1 ng/mL of IL-15. With time, there is a loss of red Raji cells. The non-fluorescent cells that ring the spheroid are the T cells. The size of the ring increases as the Vδ1 T cells proliferate. The total time elapsed is ~7 days.

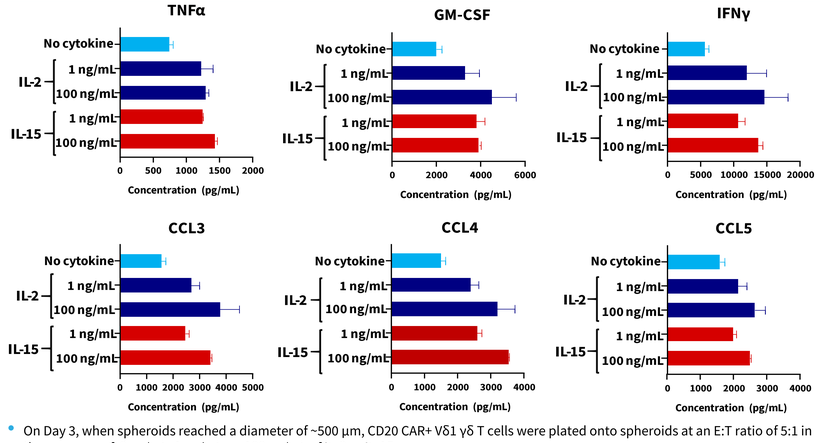


IL-2 and IL-15 potentiate CD20 CAR+ Vδ1 γδ T cell killing of Raji cells at low E:T ratios



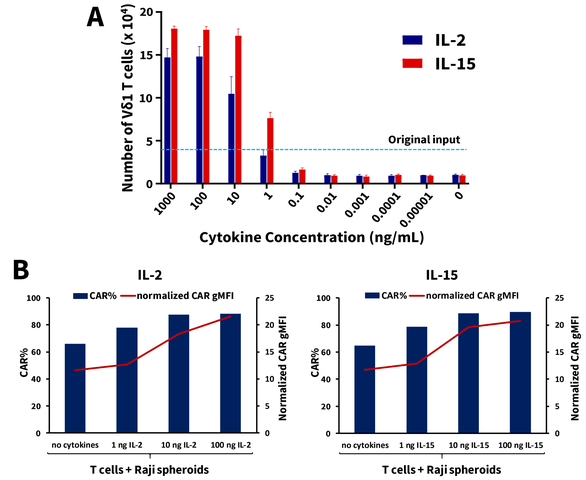
- On Day 3 (0-Hr), when spheroids reached a diameter of ~500 µm, CD20 CAR+ Vδ1 γδ T cells were plated onto spheroids at E:T ratios of 5:1, 2.5:1, 1:1, 1:2, and 1:4 in the presence of IL-2 or IL-15, with cytokine concentrations ranging from 0 ng/mL to 100 ng/mL. Raji spheroids that were cultured alone were also monitored to establish tumor growth in the presence or absence of IL-2 or IL-15. Data were collected over a 90-hour period after the addition of CD20 CAR+ Vδ1 γδ T cells.
- **At E:T ratios of 5:1, 2.5:1, and 1:1, the addition of IL-2 or IL-15, regardless of concentration, had no effect on the kinetics of Raji cell killing.**
- **At the low 1:2 E:T ratio, IL-2 or IL-15, at concentrations of 10 ng/mL and 100 ng/mL, enhances CD20 CAR+ Vδ1 γδ T cells-mediated Raji cell killing, with IL-15 appearing to be more potent than IL-2 at augmenting cytotoxic activity.**

IL-2 and IL-15 enhance cytokine and chemokine production by CD20 CAR+ Vδ1 γδ T cells 24 hours after CAR engagement



- On Day 3, when spheroids reached a diameter of ~500 µm, CD20 CAR+ Vδ1 γδ T cells were plated onto spheroids at an E:T ratio of 5:1 in the presence of 0 ng/mL, 1 ng/mL, or 100 ng/mL of IL-2 or IL-15.
- On Day 4 (ie, 24 hours after adding CAR T cells), culture medium samples were collected and analyzed for cytokine and chemokine levels using the MILLIPLEX MAP Human CD8+ T Cell Magnetic Bead Panel for the Luminex platform.
- **Addition of IL-2 or IL-15 to the co-culture of CD20 CAR+ Vδ1 γδ T cells and Raji spheroids enhances production of TNFα, GM-CSF and IFNγ, CCL3, CCL4, and CCL5 24 hours after CAR engagement (P<.05). No cytokines were detected in wells containing spheroids cultured alone, with and without cytokines (data not shown).**

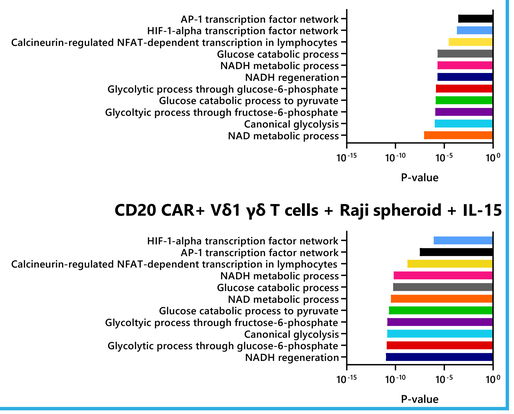
IL-2 and IL-15 promote proliferation and/or survival of Vδ1 γδ T cells expressing high CD20 CAR levels



- On Day 3, CD20 CAR+ Vδ1 γδ T cells were plated onto spheroids at a 5:1 E:T ratio (40,000 effectors to 8,000 target cells) in the presence of IL-2 or IL-15, with cytokine concentrations ranging from 0 ng/mL to 1000 ng/mL. On Day 7, the effects of IL-2 and IL-15 on CAR T cell viability, numbers, and CAR expression were assessed by flow cytometric analysis .
- **Panel A: Dose-dependent effects of IL-2 and IL-15 were observed on the number of viable Vδ1 γδ T cells recovered at the end of the 7-day co-culture period, with IL-15 being more potent than IL-2 at promoting proliferation and/or survival of Vδ1 T cells at concentrations ≥1 ng/mL (P<.01).**
- **Panel B: Flow cytometric analysis on Day 7 shows that IL-2 and IL-15 promote proliferation and/or survival of CD20 CAR+ Vδ1 γδ T cells, especially those expressing high CD20 CAR levels (normalized CAR gMFI = CAR+ gMFI/CAR− gMFI).**

Differential gene expression analysis confirms the positive effects of IL-2 or IL-15 on CAR-activated Vδ1 γδ T cells

- On Day 3, CD20 CAR+ Vδ1 γδ T cells were plated onto spheroids at a 5:1 E:T ratio in the presence of 10 ng/mL IL-2 or 10 ng/mL IL-15. On Day 7, the effects of IL-2 or IL-15 plus CAR activation on gene expression were assessed using NanoString's nCounter® CAR-T Characterization Panel by NanoString® Technologies.
- nSolver™ software was used to identify differentially expressed genes (ie, adjusted P value ≤.05 and $\log_2$-fold change ≥1) between CD20 CAR+ Vδ1 γδ T cells + Raji spheroids + IL-2/IL-15 and unstimulated (post-thaw) CD20 CAR+ Vδ1 γδ T cells. NDEx Integrated Query (v1.0) was used to identify relevant pathways that are associated with the significantly upregulated genes in the IL-2- and IL-15-treated co-cultures.
- **Addition of IL-2 or IL-15 to the co-culture of CD20 CAR+ Vδ1 γδ T cells and Raji spheroids leads to increases in the expression of genes involved in glycolysis, NAD metabolism (mitochondrial fitness), and NFAT/AP-1/HIF-1-α transcription networks (differentiation), which confirm the positive effects of IL-2 and IL-15 on CAR-activated CD20 CAR+ Vδ1 γδ T cells.**



SUMMARY AND CONCLUSIONS

- The addition of IL-2 or IL-15 to the co-culture of CD20 CAR+ Vδ1 γδ T cells and Raji spheroids leads to:
  - Increases in the number of viable Vδ1 γδ T cells recovered at the end of the 7-day co-culture period
  - Enhanced cytokine and chemokine production
  - Augmented cytotoxic activity at low E:T ratios
  - Selective maintenance of high CD20 CAR levels on Vδ1 γδ T cells and increased expression of genes involved in energy metabolism and T cell differentiation, which together may translate into enhanced functional capacity.
- Our results also highlight the utility of the 3D spheroid assay as a medium throughput *in vitro* method for assessing CAR Vδ1 γδ T cell activation, proliferation, survival, and differentiation in hot and cold tumors. Importantly, the assay can be adapted for different indications, different target antigens, and different CAR designs.

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