Chronic anti-CD3/CD28 stimulation during cell expansion generates exhausted T cells with a distinct metabolic and cytotoxic phenotype

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Introduction

T cell exhaustion is one of the major causes of cancer immune evasion and contributes to the failure of immunotherapies targeted to solid tumors. Reversing T cell exhaustion is a promising strategy in cancer immunotherapy, as it can improve individuals' responses to immune checkpoint inhibitors and adoptive T cell therapies. Recent publications showed that impairment of mitochondria function plays a critical role in developing and maintaining T cell exhaustion. This prompted us to explore the possibility of using metabolic profiling of T cells to assess relative T cell exhaustion as a tool to optimize T cell therapy product development. In our study, we induced exhaustion in memory T cells via chronic stimulation with anti-CD3/CD28 Dynabeads and used briefly stimulated (24-hour) counterparts as controls. Across all three donors tested, chronically stimulated T cells exhibited reduced proliferation, increased percentage of PD1^{+}Tim3^{+} population, and decreased IL-2 secretion upon restimulation, while TNFα and IFNγ secretion varied by donor. We performed **Agilent xCELLigence Real-Time Cell Analysis (RTCA)** impedance-based, acute killing assays using EpCAM-BiTEs to engage T cells to the EpCAM expressing T47D cells. Surprisingly, we found that despite some donors secrete significantly less cytokines upon antigen restimulation, cells chronically exposed to anti-CD3/CD28 Dynabeads display similar acute cytotoxicity compared to its non-exhausted counterparts. Importantly, when assessed using **Agilent Seahorse XF assays**, these exhausted T cells displayed a very distinctive metabolic phenotype, including decreased maximal respiration and spare respiratory capacity (SRC), increased glycolytic activity, and reduced capacity to further increase glycolytic activity upon acute reactivation. These findings suggest that metabolic profiling can represent a valuable tool for identifying metabolically fit T cells and is a great addition to the current matrix of parameters for assessing T cell exhaustion.

Experimental

XF T Cell Metabolic Profiling Kit

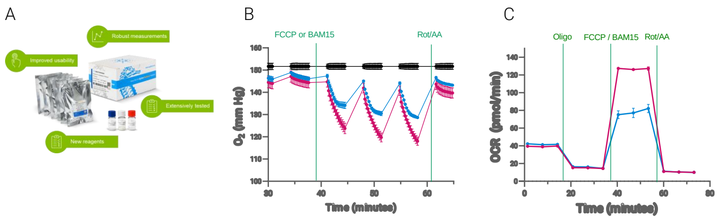

Figure 1: (A) The Agilent Seahorse XF T Cell Metabolic Profiling kit contains new reagents optimized to allow for complete measurement of T cell metabolism along with a dedicated analysis tool, Agilent Seahorse Analytics. (B-C) BAM15 and optimized concentration of FCCP were compared in mouse naïve CD8 T cells. (B) Changes in extracellular oxygen levels after uncoupler addition, highlighting the more consistent rate during the three minutes of instrument measurement obtained when adding BAM15. (C) OCR kinetic profile, illustrating underestimation of Max respiration obtained when FCCP is used as uncoupler. Similar results were obtained using mouse NK cells, human T and NK cells.

Workflow to generated exhausted T cells in vitro

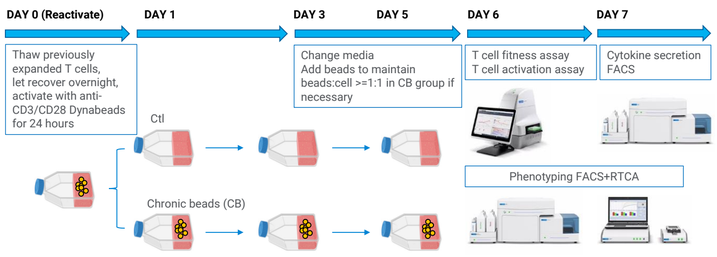

Figure 2. Frozen vials of human peripheral blood pan-T cells previously activated and expanded for 14 days were thawed in RPMI-1640 media supplemented with 10% FBS, 0.05 mM beta-mercaptoethanol and 300 U/mL human IL-2. After overnight recovery, T cells were reactivated with anti-CD3/CD28 Dynabeads. 24 hours after reactivation, cells were separated into control group and chronic beads (CB) group. For control group, beads were removed, for CB group, beads were retained during the whole expansion. Samples from three different donors were used for the study. For donor two, cytokine secretion FACS was performed six days instead of seven after reactivation. Agilent xCELLigence Real-Time Cell Analysis (RTCA) was performed only in with samples of donor one.

Results and Discussion

Chronic beads stimulation generates exhausted T cells

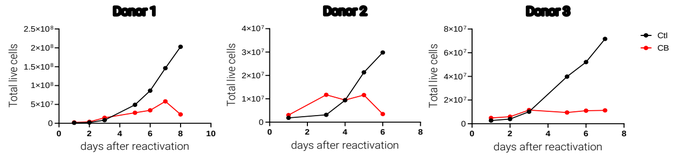

Figure 3. Chronic beads stimulation significantly impaired net T cell proliferation. Growth curve of control and chronic beads (CB) group for three different donors from day one to 6-8 days after reactivation.

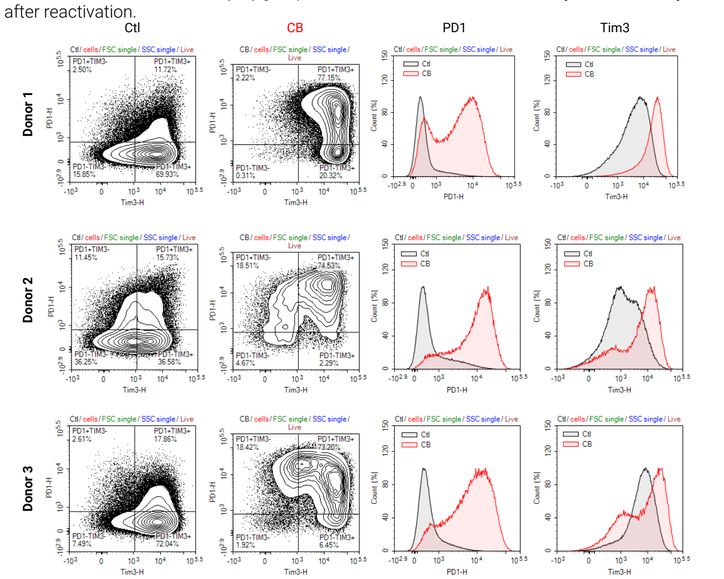

Figure 4. Chronic beads stimulation significantly increased exhaustion marker expression on T cells. Phenotyping FACS results for T cell exhaustion markers six days after reactivation.

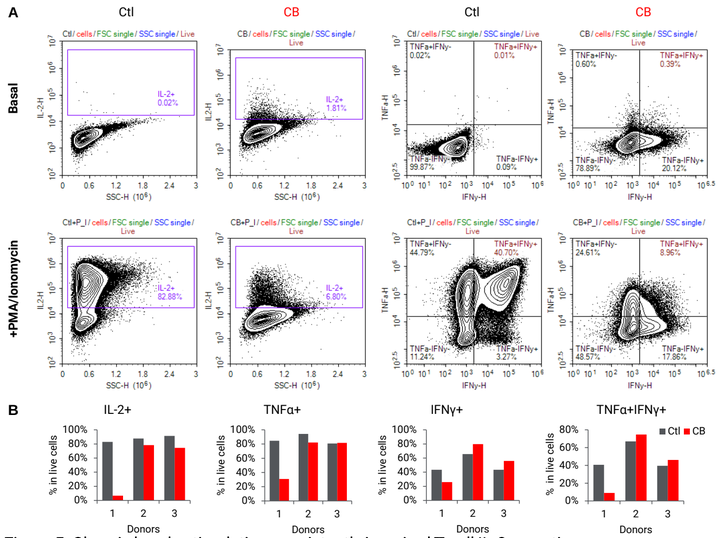

Figure 5. Chronic beads stimulation consistently impaired T cell IL-2 secretion upon restimulation. Six to seven days after reactivation, T cells from control and CB group were treated with vehicle or a combination of phorbol myristate acetate (PMA) and ionomycin for four hours for cytokine secretion analysis. (A) Gating for cytokine secretion seven days after T cell reactivation in donor one. (B) Percentage of cytokine positive cells after PMA/Ionomycin stimulation in each donor.

Acute cytotoxicity doesn't always positively correlate with cytokine release

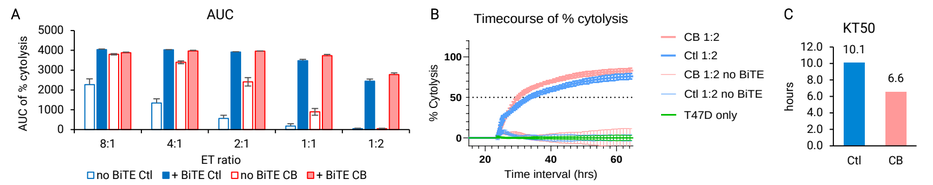

Figure 6. Agilent xCELLigence RTCA impedance-based, acute killing assay. Six days after reactivation, control and chronic beads group T cells from donor one were added to an E-Plate seeded with EpCAM expressing T47D cells at different E:T ratios, in the presence or absence of EpCAM-BiTEs. (A) Area under the curve for percent cytolysis 42 hours after adding effector. (B) Time course of percent cytolysis for control and CB group T cells at E:T ratio of 1:2, in the presence or absence of EpCAM-BiTE. (C) Hours needed to kill 50% of target cells (Kill time 50) at E:T ratio of 1:2.

Exhausted T cells generated through chronic beads stimulation have a distinct metabolic phenotype

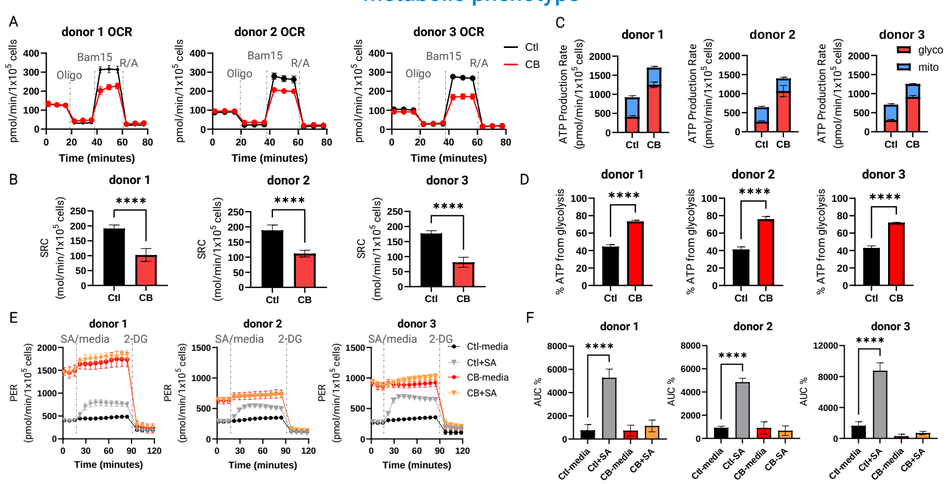

Figure 7. Distinctive metabolic phenotype of exhausted T cells in CB group. (A) OCR kinetic curve and (B) spare respiratory capacity (SRC) and (C) ATP production rate and (D) Percent ATP from glycolysis in T cell persistence assay six days after reactivation. Oligo: oligomycin-A. R/A: Rotenone/ antimycin. (E) Proton efflux rate (PER) in T cell activation assay.(F) Area under the curve (AUC) for PER normalized to baseline (measurement three). SA: anti-CD3/CD28 soluble T cell activator. 2-DG: 2-deoxy-glucose. Data are presented as mean ± SD, ****p<0.0001

Conclusions

- Our experimental model of T cells expanded under chronic bead stimulation result in a T cell population with distinctive immunophenotype (increased levels of Tim3 and PD1 expression) and impaired net proliferation.
- Exhausted T cells induced by chronic beads stimulation have a very distinctive and consistent metabolic phenotype with increased glycolytic activity and decreased mitochondrial bioenergetic capacity – a parameter associated with cell metabolic fitness.
- Despite the increased glycolytic activity, exhausted T cells showed reduced capacity to further increase glycolysis activity after acute reactivation.
- Cytokine release after PMA + ionomycn reactivation or acute cytotoxicity measured by real time cell killing may not always reflect the expected phenotype in exhausted T cell induced by chronic beads stimulation.
- Agilent Seahorse XF assays allow obtaining the complete metabolic phenotype of T cells and identifying cell populations that are metabolically fit, thus providing valuable information to improve cell therapy development.

References

1. Bispecific T Cell Engager (BiTE) Mediated Cancer Cell Lysis Assay https://www.agilent.com/cs/library/applications/an-bites-rtca-esight-5994-6004en-agilent.pdf
2. Assessing T cell Bioenergetic Poise and Spare Respiratory Capacity Using Extracellular Flux Analysis. https://www.agilent.com/cs/library/applications/an-xf-tcell-metabolic-profiling-kit-5994-4494en-agilent.pdf
3. Using the Agilent NovoCyte Flow Cytometer for Immune Suspension Normalization in Agilent Seahorse XF Assay. https://www.agilent.com/cs/library/applications/an-novocyte-seahorse-5994-6245en-agilent.pdf