Unbiased multiparameter analyses reveal mechanisms of variable restimulation-induced cell death sensitivity across human T cell populations.

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Abstract

Restimulation-Induced Cell Death (RICD) is a crucial self-regulatory apoptosis pathway that constrains effector T cell expansion during an adaptive immune response. Our lab and others have implicated multiple genes involved in regulating RICD sensitivity, but molecular mechanisms that explain highly heterogeneous RICD susceptibility across healthy human donors remain nebulous. We hypothesized that relative RICD sensitivity could be explained by specific gene expression programs that correspond to functional differentiation states of multiple, distinct effector T cell subsets. We took an unbiased approach to this question by amassing extensive phenotypic data on T cells collected from >50 healthy human donors, employing spectral flow cytometry and scRNA-seq on effector T cells from the most highly resistant vs. sensitive individuals. Our results unveiled enrichment of CD8+ T cells expressing senescence (CD57, NKG2D) and exhaustion (TIGIT) markers in highly sensitive donors; indeed, FACS-based purification confirmed CD8+CD57+ T cells were markedly more susceptible to RICD compared to CD57- counterparts. Moreover, scRNA-seq revealed upregulation of PI-3K pathway genes in RICD sensitive donors, consistent with enhanced PI-3K signaling in senescent T cells. RICD sensitive donors also harbored more “aged” CD4+ T cells expressing ZEB2 and cytotoxicity genes (PRF1, GZMB/K, GNLY, NKG7). Conversely, RICD-resistant CD4+ and CD8+ effector T cells were enriched for genes/markers corresponding to more stem-like properties (IL7R, SELL, CD27, LRRN3, LEF1, BACH2). Indeed, more naïve/central memory T cells were observed in initial PBMC collected from RICD-resistant donors. Collectively, our work provides the first “atlas” of human RICD variability that can be used to elucidate and predict relative RICD susceptibility across human donors, informing new approaches for optimizing adoptive T cell immunotherapies and understanding capricious T cell responses to infection/immunization.

Background

- The adaptive immune system utilizes T lymphocytes to effectively control invading pathogens and preserve immunological memory. Once naïve T cells are activated via stimulation of the T cell receptor (TCR) and co-stimulation through CD28, they expand clonally and differentiate into distinct effector T cell subsets capable of combating specific pathogens.
- The relative strength of TCR restimulation, the presence of IL-2 and metabolic programming all help to determine the proportion of an effector T cell population that will undergo restimulation-induced cell death (RICD), which is thought to constrain excessive effector T cell proliferation during an immune response.
- RICD sensitivity across human donors is extremely variable, and never reaches 100%.
- The molecular mechanisms that tune relative RICD sensitivity in human effector T cells remain poorly understood; defining these pathways may help explain why different individuals mount robust, excessive or insufficient immune responses to antigen.
- **We hypothesize that heterogeneity in RICD sensitivity across healthy human donors is defined by differences in transcriptional and metabolic programs that govern T cell subset phenotypes.**

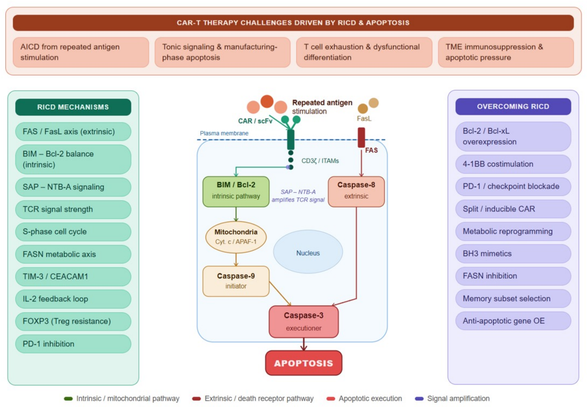


Figure 1. RICD in T Cells: Mechanisms, CAR-T Challenges, and Potential Therapeutic Targets. RICD takes place during CAR-T ex vivo proliferation. Targeting RICD sensitivity tuning could reduce CAR-T apoptosis, modify transfusion products, and improve patient prognosis. Defining molecular and cellular mechanisms that drive RICD sensitivity will accelerate development of therapeutic strategies for improving CAR-T persistence.

Methods

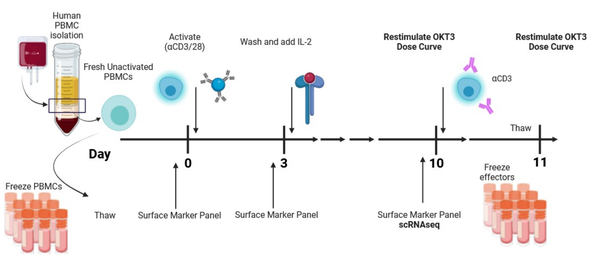
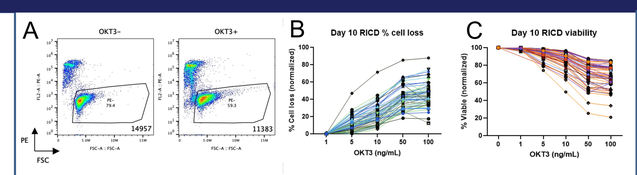


Figure 2. Approach for screening health human PBMC for RICD sensitivity: We banked PBMC from 50 healthy human donors and screened effectors from the 4-6 most sensitive and resistant donors for surface marker and transcriptional changes at rest. Donor information was deidentified except for age and sex.

Methods



D

Lineage	Activation	Memory/Naive	Senescence	Other Functional
CD3	CD25	CD45RA	CD57	CD73
CD19	CD69	CD45RO	KLRG1	FAS
CD56	CD40L	IL-7Ra		NKG2D
CD11b	ICOS		Homing/Chemokine	CRTH2
		Exhaustion	CCR7 CCR6	
Coreceptors	Costimulation	PD-1 TIM-3	CCR4 CCR10	Viability
CD4	CD28	CTLA-4 TIGIT	CCR5 CXCR3	Zombie
CD8	CD27	LAG-3		

Figure 3. Defining RICD resistant and sensitive human effector T cell donors. (A) 50 healthy human donors were screened for RICD sensitivity using Propidium Iodide staining. **(B-C)** RICD assay dose response curve shows highly variable interdonor and intradonor RICD sensitivity. **(D)** The 6 most resistant and sensitive donors were probed for surface marker expression using multiparameter flow cytometry.

Spectral Flow Cytometric Profiling

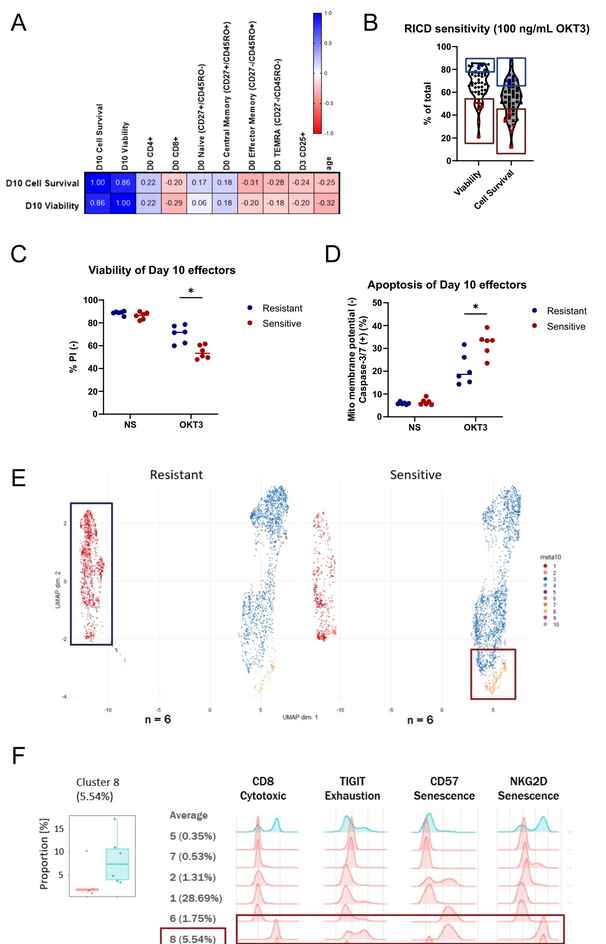


Figure 4. Surface marker expression analysis reveal subpopulations enriched in RICD resistant vs. sensitive donors before and after initial activation. (A) Pre-activation marker screening shows RICD resistance is directly associated with donor age, % central memory/naïve T cells, and inversely associated with effector memory and TEMRA T cells. **(B-D)** The 6 most resistant and 6 most sensitive donor samples were restimulated to validate RICD sensitivity based on viability (B-C) and MOMP/active caspase 3/7 (D). **(E)** UMAP clustering of RICD sensitive and resistant effector T cells (CD4+=red, CD8+=blue). **(F)** Cluster analysis shows enrichment of senescence (CD57, NKG2D) and TIGIT markers in RICD sensitive CD8+ T cell effectors (cluster 8), and naïve/central memory markers (IL-7Ra, CD28) in RICD resistant CD4+ T cell effectors (cluster 1).

Single Cell RNA-seq (scRNA-seq)

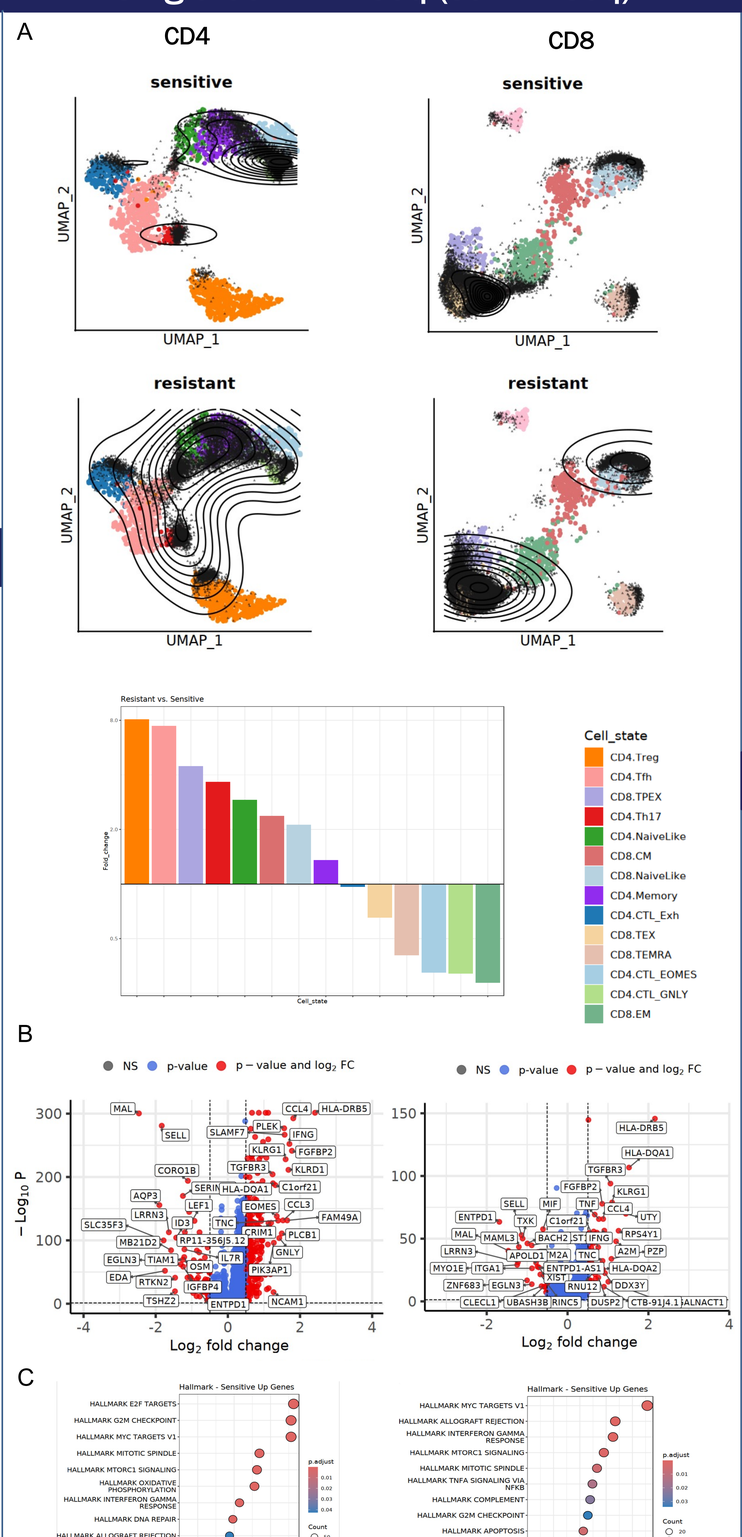


Figure 5. Projection of scRNA-seq query onto Tumor Infiltrating Lymphocyte (TIL) based reference atlas shows enrichment of distinct T cell subsets. (A) UMAPs generated by ProjecTILs package show differential enrichment of CD4 CTL and CD8 exhausted populations in the RICD sensitive donors, compared to CD4 stem-like and CD8 precursor exhausted populations in the RICD resistant donors. **(B)** Top DEGs for CD4+ and CD8+ T cells. Of note, RICD sensitivity in both CD4+ and CD8+ effector T cells is linked to accelerated T cell senescence/aging and exhaustion, driven in part by hyperactive PI-3K/mTOR signaling. RICD resistance in both CD4+ and CD8+ effector T cells is associated with more stem-like properties of naïve and central memory T cells, enforced by specific transcriptional enhancers such as LEF-1 or repressors such as BACH2. **(C)** Gene set enrichment analysis shows E2F signaling in resistant donors and glycolysis in sensitive donors.

terraFlow Analysis

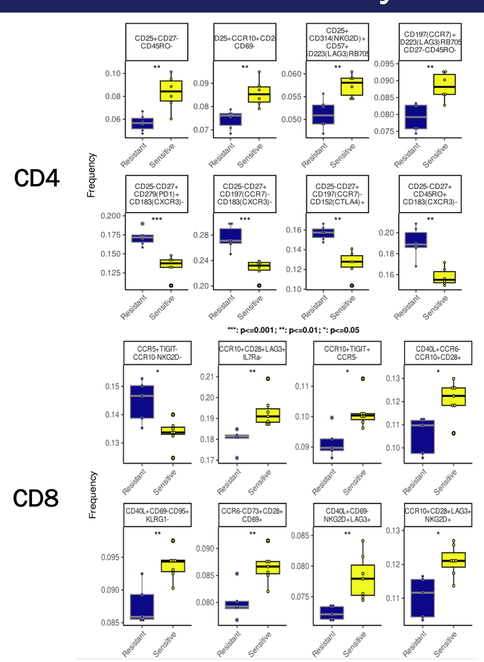


Figure 6: Terraflow Analysis validates surface marker findings. Terraflow is an automated, cloud-based flow cytometry analysis tool that identifies all possible cellular phenotypes that are differentially enriched in RICD-resistant vs. sensitive donors, validating CATALYST findings presented in Figure 4.

Conclusions

- Effector T cells from different healthy human donors can be screened and classified into “RICD resistant” vs. “RICD sensitive” populations, suggesting RICD susceptibility may be intrinsically determined and can be further interrogated.
- Differences in starting CD4 and CD8 T cell populations defined in freshly purified human donor PBMCs help to explain heterogeneity in human donor RICD sensitivity, with resistance correlating with higher proportions of naïve/central memory T cells and younger age.
- Multiparameter flow cytometry analysis revealed associations between cell surface markers of senescence and increased RICD sensitivity in CD8+ T cells as well as naïve/central memory CD4+ T cells and increased RICD resistance.
- RICD sensitivity in both CD4+ and CD8+ effector T cells is linked to accelerated T cell senescence/aging and exhaustion, driven in part by hyperactive PI-3K/mTOR signaling. This includes “aged” CD4+ T cells that upregulate ZEB2 and multiple genes associated with cytotoxic effector function (PRF1, GZMB, GNLY, NKG7, IFNG).
- RICD resistance in both CD4+ and CD8+ effector T cells is associated with more stem-like properties of naïve and central memory T cells, enforced by specific transcriptional enhancers (LEF1) or repressors (BACH2) and genes linked to elevated TGF-b signaling.
- Future work will develop targets to enrich RICD resistant populations to improve adoptive T cell therapy transfusion products. For example, the influence of BACH2 expression on RICD resistance may prove a potent target for therapeutic intervention. By aligning surface marker and transcriptional expression profiles with key targets for intervention, we will generate novel hypotheses about human T cell RICD sensitivity variations and create an “atlas” of human RICD variability.

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