Single Cell Profiling Reveals Unique TCR Repertoire and Transcriptomic Signatures of Epstein-Barr Virus-Specific T cells (EBVSTs) Armoured with Chimeric Antigen Receptors (CARs)

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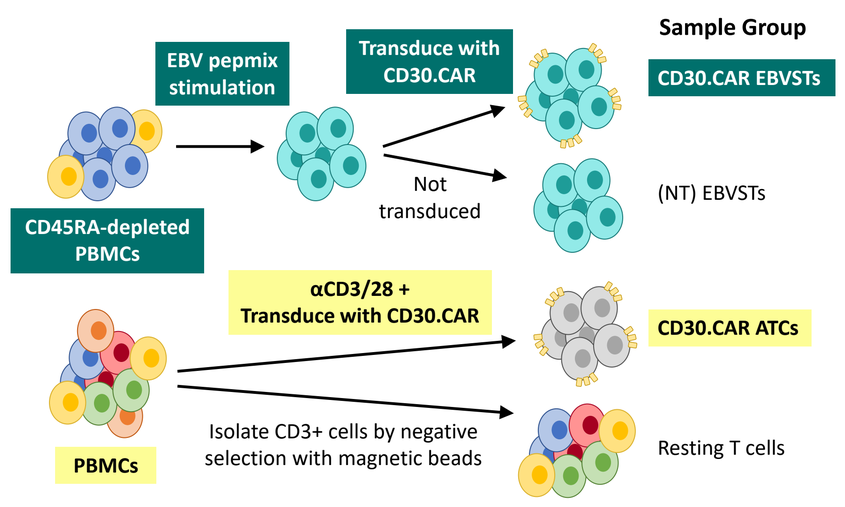
Abstract

Allogeneic Epstein-Barr Virus-specific T cells (EBVSTs) have thus far been a safe immunotherapy product in the clinic, with little graft-versus-host-disease reported in over 300 recipients to date[1,2]. Consequently, EBVSTs armoured with chimeric antigen receptors (CARs) have emerged as a promising platform for allogeneic T cell therapies[3]. Believing that a deep characterisation of CAR EBVST cell products can enhance insights into their clinical safety, we performed single cell T cell receptor (TCR) sequencing on CAR EBVSTs, taking CD30.CAR EBVSTs as a prototype.

Non-transduced (NT) EBVSTs and CD30.CAR EBVSTs generated from three healthy donors exhibited a narrower, more focused TCR repertoire by diversity indices, compared to resting T cells and CD30.CAR-transduced activated T cells (ATCs) from the same donors. EBVSTs harboured a lower frequency of unique TCR clonotypes and a higher proportion of expanded clones, indicating a tendency toward clonal dominance. Narrower TCR repertoires corresponded to greater IFNγ and TNFα secretion in response to stimulation with EBV peptides, with no cross-reactivity toward other common viruses, and were associated with lower reactivity to alloantigens in mixed lymphocyte reactions.

To investigate whether CAR EBVSTs might have better characteristics than CAR ATCs, we concurrently performed single cell transcriptomic sequencing on NT EBVSTs, CD30.CAR EBVSTs, and CD30.CAR ATCs. Transcriptomic signatures revealed that each sample type contained two major, distinct populations of CD4+ T cells: proliferative and activated. While the proliferative subsets appeared to be closely related, the activated populations had distinct transcriptional profiles. In particular, CD30.CAR EBVSTs appeared to be more polyfunctional and metabolically poised than CD30.CAR ATCs. The restricted TCR repertoire and inferred functional superiority of CD30.CAR EBVSTs may underlie their safety and efficacy observed in early-stage clinical trials and preclinical models, with the larger implication that these qualities extend to CAR EBVSTs targeting antigens beyond CD30.

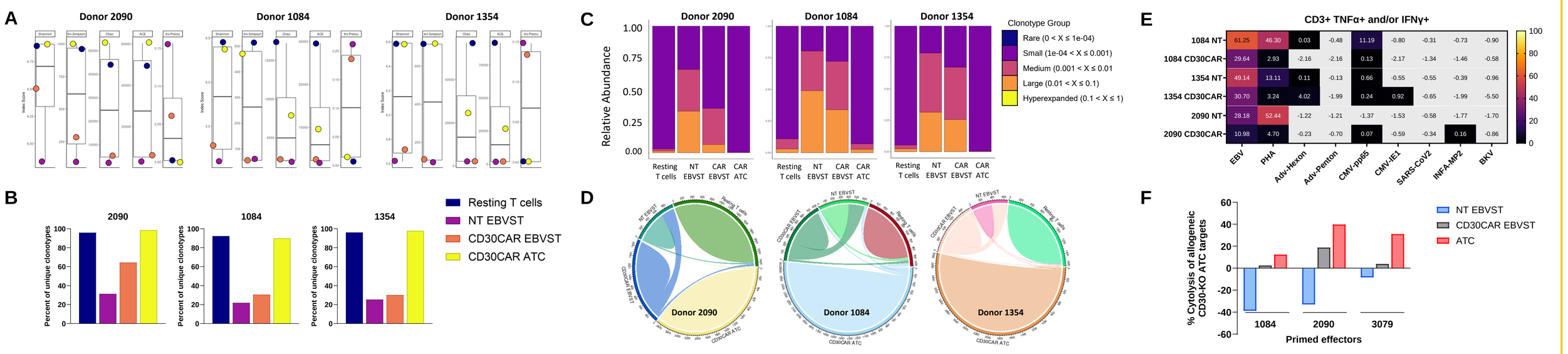
Study Approach



Primary goal: compare CAR EBVSTs and CAR ATCs, using CD30.CAR as a model

- Cells were generated from 3 healthy donors
- Each sample was labelled with a unique TotalSeq™-C hashing antibody
- Single cell data was generated using the 10x Genomics Chromium Next GEM Single Cell 5' + V(D)J sequencing
- Data analysis performed using the R packages Seurat and scRepertoire

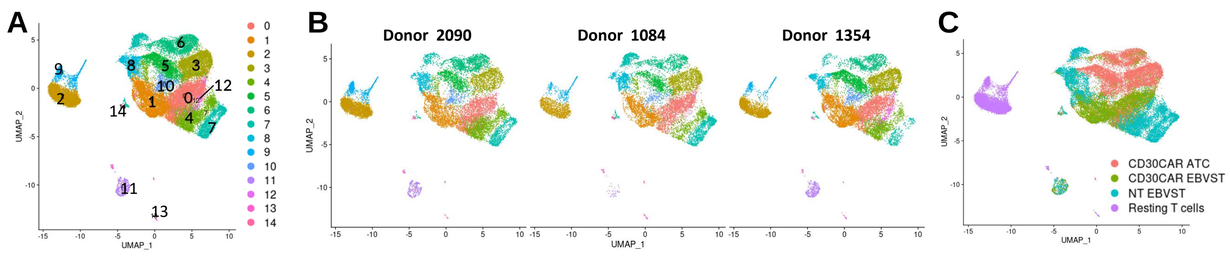
TCR Repertoire Analysis: CAR EBVSTs have a narrow repertoire compared to CAR ATCs, and exhibit reduced non-specific cross reactivity and alloreactivity

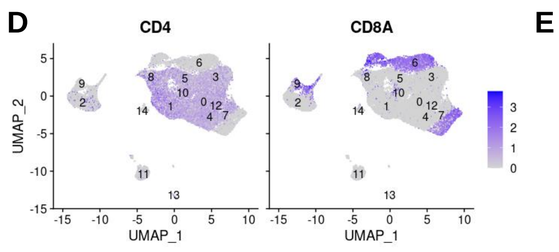


A) NT and CAR EBVSTs generally score lower on sample richness and evenness than CAR ATCs and resting T cells, indicating a more restricted repertoire. Diversity indices were calculated by down-sampling with 100 bootstraps using the minimum number of unique clonotypes (left to right): Shannon, Inverse Simpson, Chao, Abundance-Based Coverage Estimator (ACE), and Inverse Pielou. B) NT and CAR EBVSTs have smaller proportions of unique clonotypes, calculated for each sample by dividing the number of unique clonotypes by the total number of cells, with clonotype defined as TCRs sharing the same β chain CDR3 amino acid sequence. C) Clonal distribution revealed by binning clonotypes according to their relative abundance in each sample. NT and CAR EBVSTs harbor greater frequencies of expanded clones, indicating some driving of clonal dominance.

D) Chord diagrams indicate that there is a relatively large repertoire overlap between NT and CAR EBVSTs within donors, but not with CAR ATCs or resting T cells. E) NT and CAR EBVSTs respond only to peptides derived from EBV and not other viruses. EBVSTs were stimulated with PHA as positive control or virus peptides, then stained intracellularly for IFNγ and TNFα. Values shown are frequencies of TNFα and/or IFNγ-producing CD3+ T cells in the presence of stimulants minus their respective media-alone frequencies. F) CAR EBVSTs kill allogeneic target cells less than ATCs but more than NT EBVSTs in mixed lymphocyte reactions set up to evaluate the potential for graft-versus-host reactivity. Effectors (graft) were primed and boosted with irradiated allogeneic CD30-KO ATCs (host), then co-cultured with the same, non-irradiated allogeneic CD30-KO ATCs for 24h at a 1:1 ratio. Cytolysis was measured relative to target-alone condition.

Transcriptomic Profiling: EBVSTs, CAR EBVSTs, and CAR ATCs are composed of two major, distinct populations of CD4+ T cells



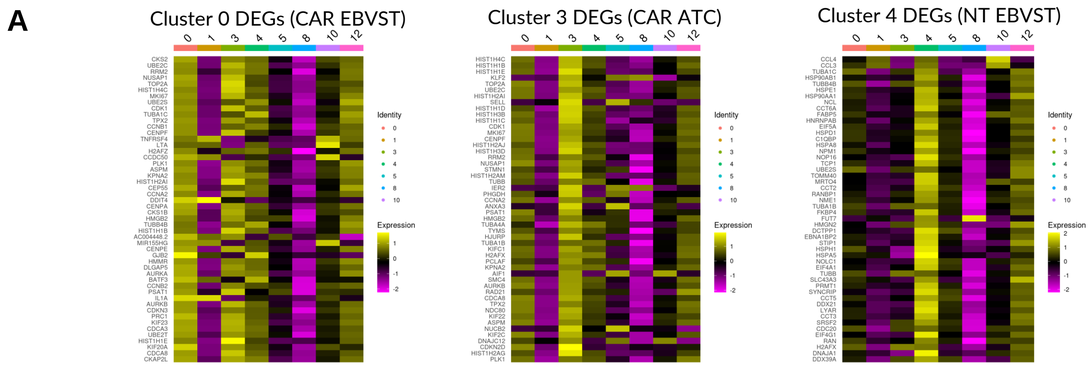


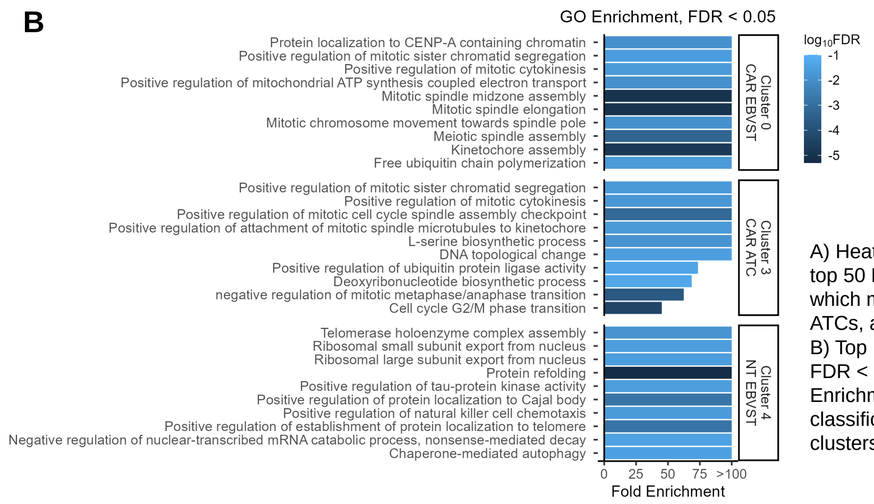
Group	NT EBVST				CAR EBVST		CAR ATC				Resting T	
Cluster	4	7	8	12	0	1	3	5	6	10	2	9
CD4/CD8	CD4	CD8	CD4	CD4	CD4	CD4	CD4	CD4	CD8	CD4	CD4	CD8
Frequency within group	32.02	28.95	16.09	2.53	40.74	39.41	33.57	25.01	22.53	4.24	74.78	20.82
Frequency within cluster	88.64	97.62	90.57	63.92	74.41	73.03	93.54	92.51	83.40	60.74	99.69	99.78

A) Donor-integrated UMAP projection of single cell transcriptomics data clustered using the default Seurat parameters. Fourteen cell clusters were identified. B) UMAP plots segregated by donor. C) UMAP plots coloured by sample group. Distribution of clusters was similar among donors but distinctly segregated between samples.

D) UMAP feature plots of CD4 and CD8α gene expression. CD4+ T cells constitute the majority of cells in all samples. E) Summary table of sample groups and clusters for CD4+ and CD8α+ cells by gene expression. NT EBVSTs, CAR EBVSTs, and CAR ATCs are each composed of two major, distinct populations of CD4+ T cells. *Analyses for CD8+ T cells are still underway.*

A) Proliferative clusters, which are similar across sample groups

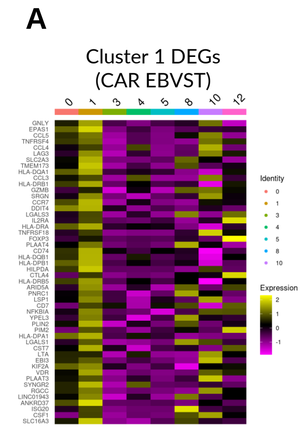


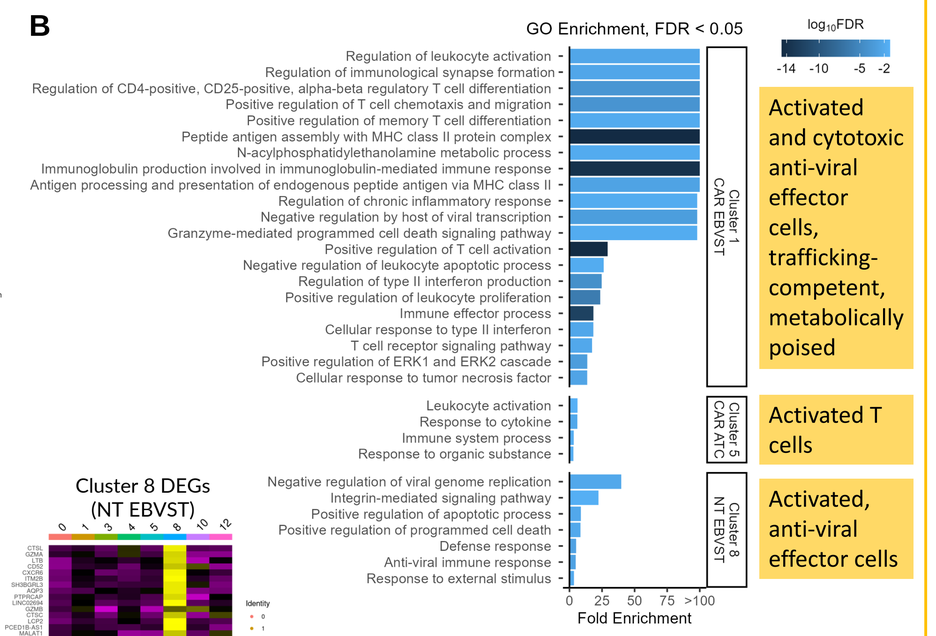


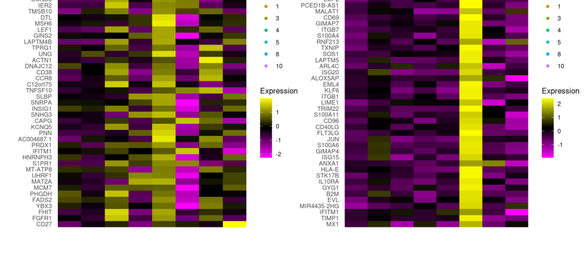
Proliferative clusters are involved in overlapping processes of cell cycle, proliferation, and cytoskeletal remodeling

A) Heatmap of average cluster expression of top 50 DEGs for CD4+ clusters 0, 3, and 4, which majorly comprise CAR EBVSTs, CAR ATCs, and NT EBVSTs respectively.
B) Top 10 Gene Ontology (GO) pathways with FDR < 0.05 as determined by Gene Ontology Enrichment Analysis powered by the PANTHER classification system on the top 50 DEGs of clusters 0, 3, and 4.

B) Activated clusters, which have unique signatures in each sample group







A) Heatmap of average cluster expression of top 50 DEGs for CD4+ clusters 1, 5, and 8, which majorly comprise CAR EBVSTs, CAR ATCs, and NT EBVSTs respectively. B) Gene Ontology (GO) pathways with FDR < 0.05 based on Gene Ontology Enrichment Analysis powered by the PANTHER classification system on the top 50 DEGs of clusters 1, 5, and 8.

Conclusions

- Like EBVSTs, CAR EBVSTs have a more focused repertoire than CAR ATCs
- Like EBVSTs, CAR EBVSTs recognize epitopes derived from EBV but not other common viruses, and exhibit low to no allogeneic targeting, making EBVSTs suitable universal donor T cells to be engineered with CARs as off-the-shelf therapy for various malignancies
- The proliferative CD4+ T cell populations in the different sample groups appear to overlap in function, while the activated populations have more divergent transcriptional profiles
- CAR EBVSTs appear to be more polyfunctional and metabolically poised than CAR ATCs

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