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Preservation of antigen-specific responses in cryopreserved CD4$^+$ and CD8$^+$ T cells expanded with IL-2 and IL-7

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ABSTRACT

Objectives: We sought to develop medium throughput standard operating procedures for screening cryopreserved human peripheral blood mononuclear cells (PBMCs) for CD4$^+$ and CD8$^+$ T cell responses to potential autoantigens.

Methods: Dendritic cells were loaded with a peptide cocktail from ubiquitous viruses or full-length viral protein antigens and cocultured with autologous T cells. We measured expression of surface activation markers on T cells by flow cytometry and cytometry by time of flight 24–72 h later. We tested responses among T cells freshly isolated from healthy control PBMCs, cryopreserved T cells, and T cells derived from a variety of T cell expansion protocols. We also compared the transcriptional profile of CD8$^+$ T cells rested with interleukin (IL)7 for 48 h after 1) initial thawing, 2) expansion, and 3) secondary cryopreservation/thawing of expanded cells. To generate competent antigen presenting cells from PBMCs, we promoted differentiation of PBMCs into dendritic cells with granulocyte macrophage colony stimulating factor and IL-4.

Results: We observed robust dendritic cell differentiation from human PBMCs treated with 50 ng/mL GM-CSF and 20 ng/mL IL-4 in as little as 3 days. Dendritic cell purity was substantially increased by magnetically enriching for CD14$^+$ monocytes prior to differentiation. We also measured antigen-dependent T cell activation in DC-T cell cocultures. However, polyclonal expansion of T cells with anti-CD3/antiCD28 abolished antigen-dependent upregulation of CD69 in our assay despite minimal transcriptional differences between rested CD8$^+$ T cells before and after expansion. Furthermore, resting these expanded T cells in IL-2, IL-7 or IL-15 did not restore the antigen dependent responses. In contrast, T cells that were initially expanded with IL-2 + IL-7 rather than plate bound anti-CD3 + anti-CD28 retained responsiveness to antigen stimulation and these responses strongly correlated with responses measured at initial thawing.

Significance: While screening techniques for potential pathological autoantibodies have come a long way, comparable full-length protein target assays for screening patient T cells at medium throughput are noticeably lacking due to technical hurdles. Here we advance techniques that should have broad applicability to translational studies investigating cell mediated immunity in infectious or autoimmune diseases. Future studies are aimed at investigating possible CD8$^+$ T cell autoantigens in MS and other CNS autoimmune diseases.

1. Introduction

The study of antigen specific T cell responses is important for autoimmune disease, cancers, and infectious disease. T cell mediated adaptive immune responses contribute vitally to the clearance of cancers and precancerous cells (reviewed in Refs. [1–6]), maintenance of tolerance, and induction of autoimmunity to self-antigens or transplant

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Abbreviations

CNS	Central nervous system
CSF	Cerebrospinal fluid
IL-2	Interleukin 2
IL-4	Interleukin 4
IL-7	Interleukin 7
IL-15	Interleukin 15
CEF	Cytomegalovirus, Epstein Barr Virus, Influenza pooled peptides
MVA	Mumps viral antigen
GM-CSF	Granulocyte macrophage colony stimulating factor
PBMC	Peripheral blood mononuclear cell
DC	Dendritic cell
HLA	Human leukocyte antigen
MHC	Major histocompatibility complex
LPS	lipopolysaccharide
PolyI:C	Polyinosinic:polycytidylic acid

antigens [7–15]. These cells also promote the clearance of infectious agents and infection-associated cancers, post-infectious autoimmune neurological disorders, and the maintenance of viral immunity or latency [16–27]. Therefore, it is important to develop robust methods for monitoring and characterizing the profile of circulating effector T cells from a variety of patient populations especially regarding how these T cells respond to key protein determinants. For example, in the case of autoimmune disease many patients present without a clearly identified autoantigen [10]. Other patients with autoimmunity including paraneoplastic CNS autoimmune diseases are diagnosed based upon serum autoantibody titers against a known antigen. Yet for many of these diseases the autoantibody disease-specificity is low and it is unclear whether these autoantibodies are indeed pathogenic or simply a surrogate marker for an associated cell-mediated autoimmune response in the T cell arm [28,29]. As such, more robust and versatile T cell assays for autoantigen responses may aid in the understanding of the pathogenesis of these autoimmune diseases in addition to improving diagnostic sensitivity and specificity when combined with existing autoantibody-based metrics. Likewise, rapidly measuring antigen-specific T cell responses would likely improve prognostication in patients with autoimmunity and/or neoplastic disease. This in turn could help target high-risk patients to aggressive therapies and could be used for immune monitoring to gauge therapeutic efficacy. In addition, widely utilizable antigen-specific T cell assays could improve risk stratification of patients during clinical trial enrollment and provide more sensitive and rapid readouts for trials investigating tolerance induction or immunosuppression in autoimmune disease or gauging immunogenic responses in cancer and vaccine trials.

Several obstacles have limited the development and utilization of assays measuring antigen-specific T cell responses. For example, the study of antigen-specific responses is simplified in syngeneic mice due to identical haplotypes within strains and thus conservation of immunodominant epitopes for individual proteins. Conversely, the extraordinary polymorphic nature of human MHC I and MHC II genes presents researchers with a considerable barrier to studying antigen specific T cell responses in human populations. This has meant that current methods for gauging antigen-specific T cell immunity in patients has largely relied on individualized HLA-haplotyping and ad hoc identification of immunodominant peptides followed by tetramer staining [30–35], stimulation with peptide libraries [34–37], or in silico analysis [38,39]. However, these approaches cannot be widely and rapidly utilized for screening antigen-specific T cell responses due to limitations in cost, time, and scalability. The study of antigen specific T cell responses is also limited by several factors including the low frequency of circulating blood T cells that are responsive to any given antigenic target, including ubiquitous viral antigens (<0.01%) [40–44], vaccine antigens (<0.1%) [45–49], and self and aberrant-self antigens associated with autoimmunity or cancer (<3%) [30,50–54]. Further limitations include artifacts from sample processing, time in culture, cryopreservation effects, and the confounding effects of in vitro T cell expansion [55–59].

We therefore sought to develop a simple, rigorous, highly reproducible assay that would overcome many of these limitations and could be used for initial screening of antigen-specific responses among T cells in a wide variety of clinical, paraclinical, and basic research settings. For this assay, we relied on patient-derived dendritic cells (DCs) for antigen presentation of protein determinants to autologous T cells. DCs can be readily differentiated from blood monocytes in vitro [60–64]. Importantly, DCs are potent professional antigen presenting cells that are uniquely capable of processing and presenting exogenous full length protein antigens on MHC I and MHC II molecules [65–73]. We could thus feed DCs full-length protein, and they would naturally process and present relevant immunodominant peptides to autologous T cells, which could be assessed for activation by flow cytometry. This allowed us to overcome the HLA-restrictions of peptide-based approaches while simultaneously increasing assay sensitivity due to the potent antigen presentation capacity of DCs. After optimizing this assay with peripheral blood mononuclear cells (PBMCs) isolated from fresh whole blood, we then sought to compare the extent to which antigen-specific T cell responses were preserved in samples following a variety of cryopreservation and T cell expansion protocols.

2. Materials and methods

2.1. Isolation and cryopreservation of peripheral blood mononuclear cells from patient blood

Blood from healthy donors was collected in EDTA tubes and PBMCs were isolated using Leucosep centrifuge tubes (VWR) following the manufacturer's instructions. Briefly, the bottom chamber was filled with Lymphoprep and top chamber overlaid with fresh patient whole blood. Tubes were spun at 1000g for 10 min, and the buffy coat was collected. Cells were washed with RPMI 1640 media containing 10% FBS, 100 U/mL *penicillin, 100 μg/mL streptomycin*, and 1% Glutamax. For later use, cells were stored in vapor phase liquid nitrogen in freezing media containing 10% DMSO.

2.2. Leukocyte magnetic-activated cell sorting

Monocytes were enriched from fresh or cryopreserved PBMCs by percoll gradient, using anti-CD11b/anti-CD14 biotinylated antibody cocktail and positive magnetic selection with Streptavidin Particles Plus (BD Biosciences), or by negative selection using human EasySep Monocyte Isolation kit (StemCell Technologies) following the manufacturer's instructions with EasySep magnet (StemCell Technologies). Briefly, for percoll-based enrichment PBMCs were resuspended in 2 mL RPMI 1640 media and mixed with 1 mL 90% Percoll (#17-0891-02, GE HealthCare, Chicago IL, USA), prepared by diluting Percoll 9:1 with PBS. The resulting 30% Percoll cell suspension was carefully overlaid on 2 mL of 70% Percoll solution to ensure that the interface between both layers stayed intact. After centrifugation at 560*g* for 15 min monocytes accumulated between the two gradients layers and were carefully transferred to a new tube and washed twice with PBS. For biotinylated antibody-based purification, 1 mL cryopreserved PBMCs were rested for 30 min in RPMI containing 10% heat inactivated human serum and then spun at 400g for 4 min. Cells were resuspended in 200 μL 1X iMAG buffer (BD Biosciences) and stained for 15 min on ice with saturating concentrations of biotinylated anti-human CD11b (ICRF44) and anti-human CD14 (M5E2, HCD14) and Human TruStain FcX Fc Receptor Blocking Solution (Biolegend). Stained cells were washed with iMAG buffer, spun at 400g

for 4min, and resuspended in 50 μL room temperature Streptavidin Particles plus for 30 min before magnetic isolation. $CD8^+$ T cells were isolated from cryopreserved PBMCs or T cells expanded from cryopreserved PBMCs using the human rapid $CD8^+$ T Cell isolation kit (StemCell Technologies), following the manufacturer's instructions with EasySep magnet (StemCell Technologies). Purity of monocytes, T cells, and B cells was measured by flow cytometry.

2.3. Leukocyte expansion and resting

For T cell expansion, tissue vessels were coated with 10 μg/mL anti-CD3 (OKT3) with or without anti-CD28 (9.3) in calcium magnesium free PBS for >1 h at 37 °C. Cryopreserved PBMCs were rapidly thawed in 37 °C water bath, washed once with 10% FBS RPMI and spun at 400g for 4 min. Cells were resuspended at 10^6/mL in complete media for leukocytes (CML) comprised of RPMI 1640 media containing 3% FBS, 1% non-essential amino acids, and 100 U/mL penicillin/streptomycin. Cells were plated at 2×10^6 cells per well of coated 6 well plate. Anti-CD3/anti-CD28 solution was aspirated just prior to plating cells. Cells were washed and replated at 2×10^6 cells per well every 2–3 days for 7–10 days. After expansion, T cells were rested for 48 h in 1 ng/mL IL-7 prior to CD8 T cell isolation and transcriptional analysis or immediately analyzed by flow cytometry and cryopreserved as before (2.1.1). In some experiments expanded T cells were rested in 10 ng/mL IL-7, IL-2, IL-15, or IL-21 for >5 days prior to flow cytometric analysis of surface marker expression or use in antigen recall assays. Alternative T cell expansion strategies included combinatorial treatment of PBMCs or purified T cells with IL-2, IL-7, IL-15, and/or IL-21 in uncoated or anti-CD3 coated tissue culture vessels. For monocyte expansion, PBMCs or purified monocytes were plated in CML variously supplemented with 50 ng/mL MCSF, 20 ng/mLIL-3, and/or 20 ng/mL IL-6 for 7–21 days.

2.4. Dendritic cell differentiation

Fresh or cryopreserved PBMCs were washed in 10% FBS RPMI 1640 media, spun at 400g and plated at 2×10^6 cells per well of 6 well dish in CML containing 0–50 ng/mL each of GM-CSF (Peprotech) and/or IL-4 (Peprotech). After 3 days cells were analyzed by flow cytometry or given fresh media with GM-CSF/IL-4 for 4 additional days of differentiation. In some experiments, monocytes were enriched from PBMCs prior to differentiation. Monocyte and dendritic cell purity were assessed by flow cytometry. In some experiments DCs were similarly differentiated from purified monocytes or purified monocytes that had undergone 7 days in vitro expansion as described above.

2.5. Flow cytometry

Antibodies against human lymphocyte markers CD19 (FAB4877P), CD8a (HIT8a), CD4 (OKT4), CD3e (UCHT1); T cell activation markers CD25 (BC96), CD27 (O323, M-T271) CD44 (IM7), CD69 (FN50), CD11a (HI111); monocyte markers CD14 (M5E2), CD16 (B73.1), HLA-DR (L243); and DC markers CD11c (B-Ly6), CD1c (F10/21A3), CD141 (M80), CD80 (2D10), CD86 (BU63) were purchased from BD Biosciences or RND Systems. Prior to staining, cells were washed twice in FACS buffer comprised of calcium magnesium free PBS with 2 mM EDTA, 5% FBS, 1% BSA and 0.01% sodium azide. Cells were then stained with saturating concentrations of antibodies for 30 min on ice with agitation. Cells were washed 3 times with FACS buffer and acquired on an Attune NXT Flow cytometer (ThermoFisher). FCS files were exported and analyzed in FlowJo software version 10.4.2 (Tree Star Inc.).

2.6. Antigen recall assays

Cryopreserved PBMCs or monocytes were thawed and differentiated into DCs for 3 days. At the same time fresh or cryopreserved PBMCs containing >40% $CD3^+$ T cells were rested for 3 days in 1–10 ng/mL IL-7. After 3 days T cell purity generally exceeded 80%. DCs and T cell were then cocultured at 1:1 ratio in the presence or absence of 10 μg/mL mumps viral antigen (My BioSource); H3N2 influenza virus-A Wisconsin/67/05 recombinant protein (ProSpec), Epstein-Barr virus (HHV-4) EBV EBNA1 mosaic recombinant protein (ProSpec), cytomegalovirus mosaic recombinant protein (ProSpec), hepatitis B surface antigen (ProSpec), or peptides pooled from cytomegalovirus, Epstein- Barr virus, and influenza (CEF, Anaspec). In some experiments, cultures were co-treated with adjuvants including 500 ng/mL LPS (Sigma) or high molecular weight poly (I:C) (Calbiochem) for 24–72 h. In some experiments, after 48 h media was replaced with fresh CML supplemented with antigens. $CD4^+$ and $CD8^+$ T cell activation was assessed by flow cytometry using antibodies against activation markers including LFA-1, CD44, CD25, and CD69.

2.7. RNAseq analysis

$CD8^+$ T cells were isolated by magnetic activated cell sorting using human $CD8^+$ T Cell Isolation Kit (Miltenyi Biotec). For RNAseq analysis, mRNA was purified from sorted cells using RNeasy Micro plus kit (Qiagen). RNA integrity was quality tested using the Agilent Bioanalyzer. Samples with RIN scores >9 were used for library prep using TruSeq RNA Library Prep Kit v2 (Illumina) and subsequently submitted for non-targeted 100 bp sequencing reads on the Hi-Seq 4000 Illumina platform. Paired end reads were mapped to the human references sequence NCBI Build hg19. Expression levels of RefSeq-annotated genes that mapped to the human reference genome were calculated and reported in reads per kilobase of exon per million mapped fragments (RPKM). Genes with average log2 RPKM <1 were excluded from further analysis. For unbiased analyses, differentially expressed genes were identified by multiple t-tests using a false discovery rate of 0.2%. Targeted analysis of T cell marker gene expression was performed by two-way ANOVA.

2.8. Mass cytometry

Culture medium (CM) was prepared with sterile RPMI-1640 media (Gibco) supplemented with 10% fetal bovine serum (Atlanta Biologicals) and PenStrep (Gibco). Benzonase nuclease was purchased from Sigma-Aldrich. Maxpar reagents, including water, Cell Staining Buffer (CSB), Cell Acquisition Solution (CAS), Cell-ID Intercalator-Ir, Fix and Perm Buffer, Cell-ID 20-Plex Pd Barcoding Kit, and EQ Four Element Calibration Beads were purchased from Fluidigm. Paraformaldehyde (PFA) was purchased from EM Sciences and 10X PBS pH 7.2 was purchased from Rockland. Antibodies used for cell surface labeling and phenotyping were purchased from Fluidigm. According to the manufacturer's protocol, custom conjugated antibodies were generated in-house through the Mayo Clinic Hybridoma Core using Maxpar X8 Ab labeling kits (Fluidigm). The following antibodies were used: 089Y-CD45, 141Pr-CD196 (CCR6), 142Nd-IL-4, 143Nd-CD5, 145Nd-CD4, 147Sm-IL-6, 148Nd-CD278 (ICOS), 149Sm-CD25 (IL-2R), 151Eu-IL-5, 152Sm-TNFa, 153Eu-CD45RA, 154Sm-TIM-3, 155Gd-PD-1, 156Gd-CD183 (CXCR3), 158Gd-CD194 (CCR4), 159 Tb-CD197 (CCR7), 160Gd-CD28, 161Dy-CD274 (PD-L1), 163Dy-TGF-beta, 165Ho-CD45RO, 166Er-IL-10, 167Er-CD27, 168Er-IFNg, 169 Tm-CD19, 170Er-CD3, 172 Yb-IL-17a, 173 Yb-Granzyme B, 174 Yb-CD8a, 175Lu-Perforin, 176 Yb-CD127 (IL-7R). Cells were thawed and resuspended in CM containing 2.5 units/mL of benzonase nuclease. After washing, cells were rested for 1 h in CM at 37 °C before staining. After resting, 4×10^6 cells were resuspended in 1 mL of CSB. Each sample was incubated for 5 min with 0.5 μm cisplatin solution in PBS. Samples were then washed twice with CSB. An antibody cocktail of the entire phenotyping panel was prepared as a master mix before adding 50 μL of cocktail to samples resuspended in 50 μL of CSB. Samples were then incubated at room temperature for 45 min. After washing twice with CSB, samples were fixed with 2% PFA in PBS. After fixation and wash, samples were resuspended in 30 nM

intercalation solution. Afterward, 30 μL of unique barcoding reagent was added to each sample and incubated overnight at 4 °C. Cells were then washed with PBS and pooled prior to resuspension in a 1:10 solution of calibration beads and CAS at a concentration of 0.5 × 10^6 cells/mL. Prior to data acquisition, samples were filtered through a 35 μm blue cap tube (Falcon). Samples were loaded onto a Helios CyTOF® system (Fluidigm) using an attached autosampler and were acquired at a rate of 200–400 events per second. Data were collected as FCS files using the Cytof software (Version July 6, 1014). After acquisition intrafile signal drift was normalized to the acquired calibration bead signal using the Cytof software. Cleanup of cell debris, removal of doublets and dead cells was performed using FlowJo software version 10.5.3 (Ashland,

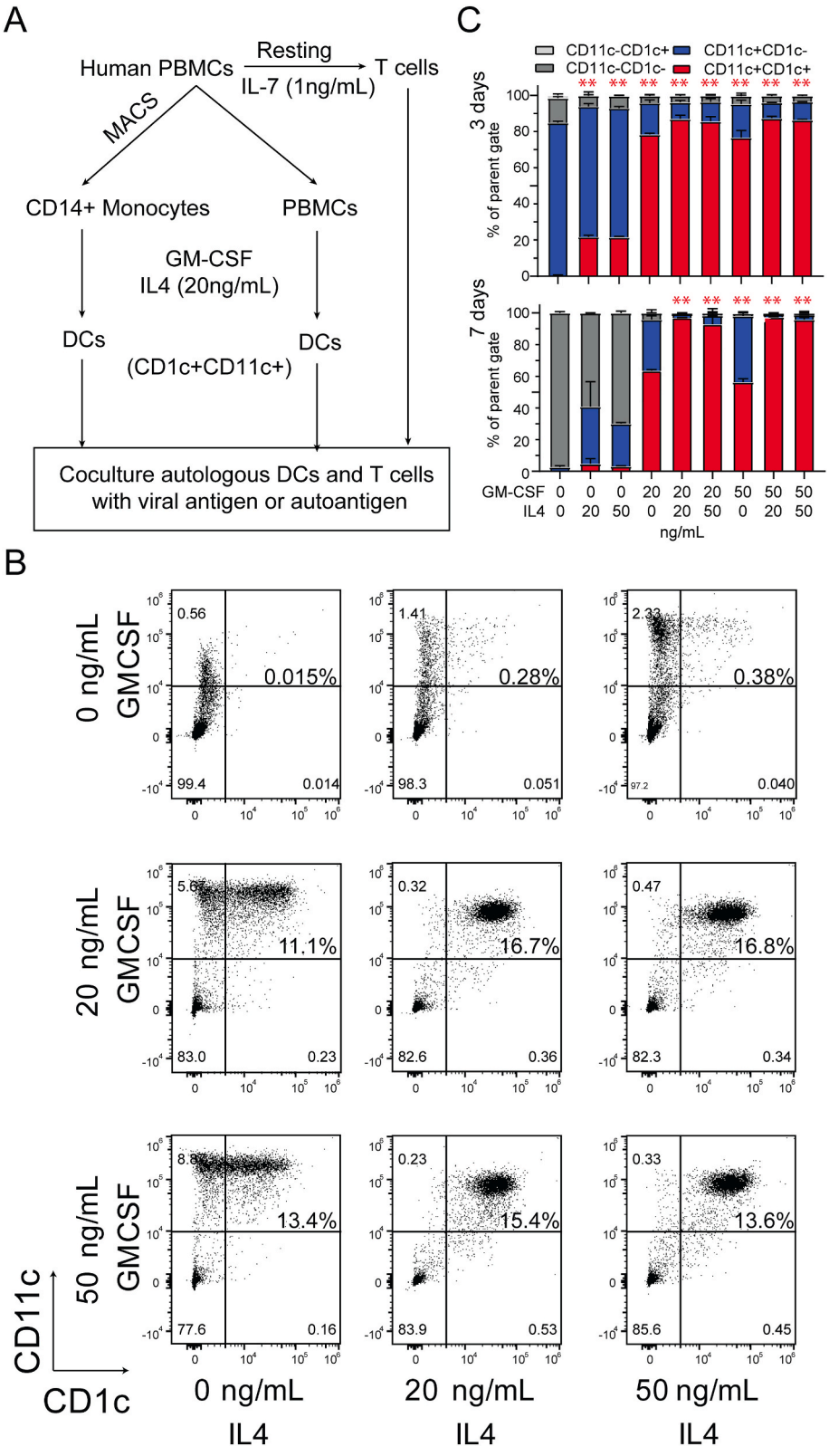

Fig. 1. A) Schematic of strategy for DC differentiation from PBMCs or monocyte-enriched PBMCs prior to co-culture with autologous T cells and antigen recall. B) Representative flow cytometry plots showing % PBMCs expressing DC markers CD11c and CD1c after 7 days in the indicated conditions. C) Graphs show percent of leukocytes (e.g. gated as in Fig. 2a) staining for CD11c and CD1c at 3 and 7 days in vitro. **P < .01 for $CD11c^+CD1c^+$ treated vs. $CD11c^+CD1c^+$ untreated (no GM-CSF IL-4).

OR). Cleaned FCS files were analyzed by the R-based tool Cytofkit version 3.8. Clustering and dimensionality reduction to 10,000 events per file was performed using the Rphenograph algorithm that included all 32 markers in the panel. Visualization of clusters was mapped onto a tSNE map. Relative marker intensities and cluster abundances per sample were visualized by a heatmap.

2.9. Statistical analyses

For multiple comparisons one-way or two-way analysis of variance (ANOVA) or non-parametric (Kruskal-Wallis) tests were performed as appropriate. Reported *P* values were corrected for multiple comparisons (Holm-Sidak correction for ANOVA; Dunn's correction for Kruskal-Wallis). Unpaired two-tailed Student's t-tests were used for comparisons made between two groups. Curran-Everett guidelines were followed [74].

2.10. Patient samples

All samples were obtained from consenting individuals, and all human sample collection protocols were approved by the Mayo Clinic Institutional Review Board (IRB# 17-004547). Whole blood samples were obtained from healthy donors for preparation of PBMCs. Cryopreserved PBMC samples from patients with anti-neuronal antibodies (Fig. 8C/D) were obtained from the Mayo Clinic Center for MS and Autoimmune Neurology Biorepository. Experimental manipulations and

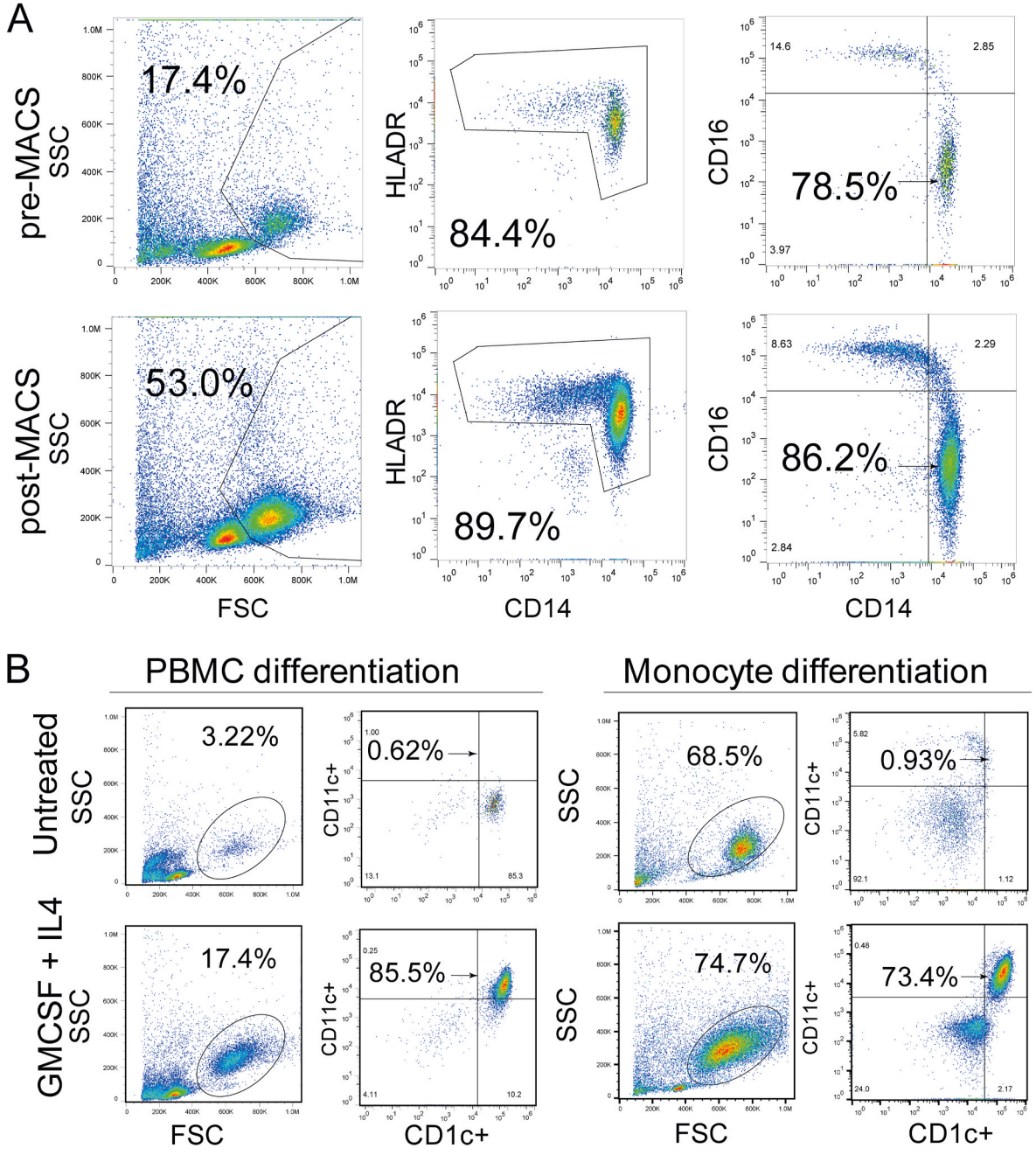

Fig. 2. Monocyte enrichment from PBMCs and differentiation into dendritic cells. Monocytes were enriched from PBMCs by magnetic activated cells sorting (MACS) using the Human Monocyte enrichment kit (BD Biosciences). Representative flow plots show percentage of cells exhibiting A) expression of monocyte markers before and after MACS or B) expression of dendritic cell markers among PBMCs or monocyte-enriched cells after 3 days differentiation in the indicated conditions.

analyses performed for each figure panel are outlined in Supplementary Table I.

3. Results

3.1. Assay design

We sought to devise a strategy to assay antigen specific T cell responses in healthy volunteers. We also sought to develop an assay that could be used for PBMCs freshly isolated from whole blood as well as cryopreserved PBMCs and cryopreserved T cells that had been expanded from cryopreserved PBMCs. As shown, (Fig. 1A schematic), to leverage the potent capacity of dendritic cells (DCs) to process and present full-length protein, our strategy relied on differentiation of DCs from blood monocytes. For this we freshly isolated PBMCs from whole blood and cultured them under DC-differentiation conditions for 3–7 days. We then pulsed these cells with either a cocktail of immunodominant peptides from CMV, EBV and influenza (CEF) or full-length protein antigens and co-cultured these DCs with autologous T cells. Prior to co-culture, T cells were rested in 1–10 ng/mL IL-7 or expanded by polyclonal stimulation or high dose treatment with IL-7 and IL-2 family cytokines. T cell activation was analyzed in cocultures 24–72 h after antigen treatment

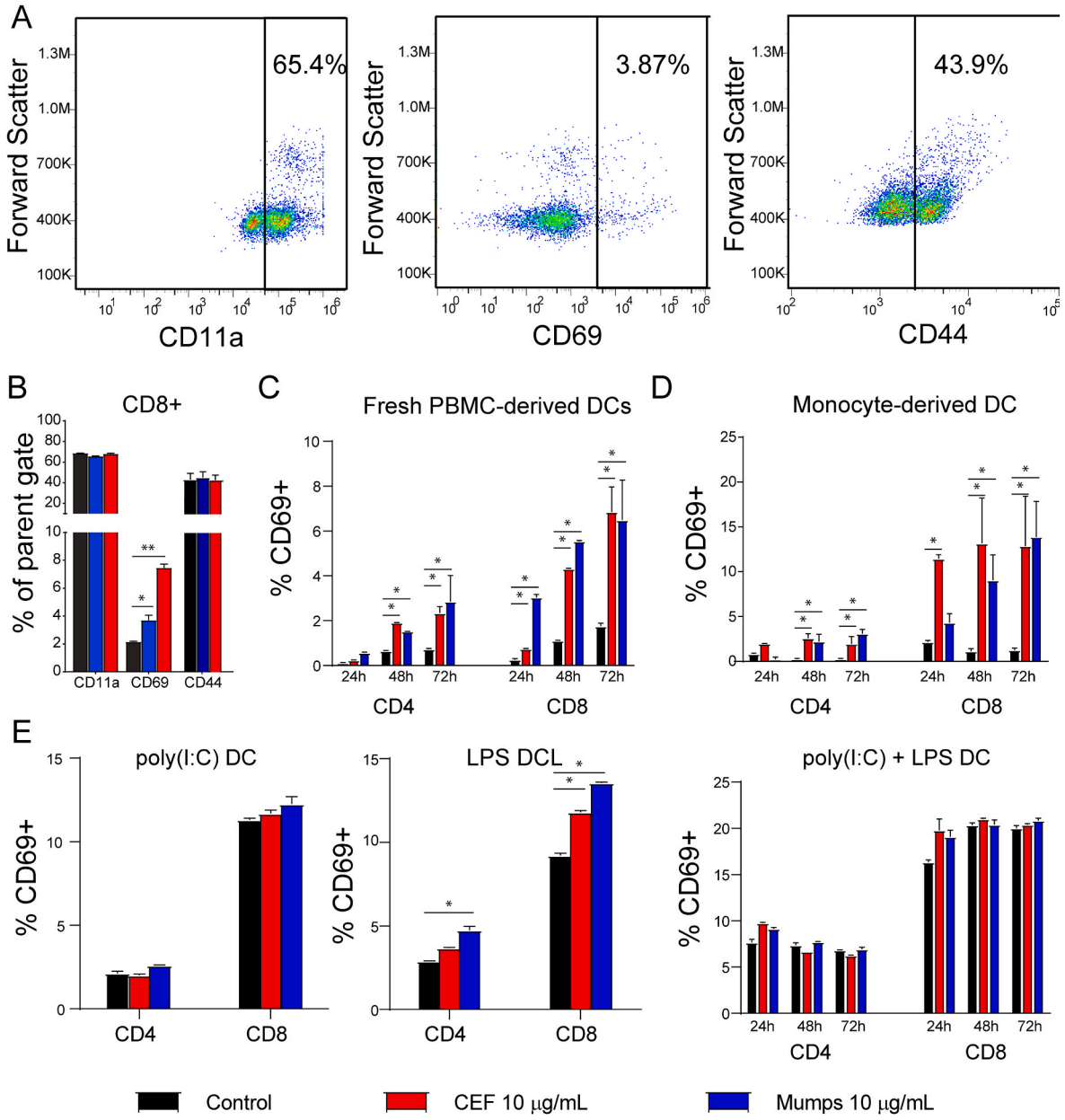

Fig. 3. Upregulation of CD69 on T cells upon coculture with dendritic cells presenting ubiquitous viral antigens. Patient T cells were rested and co-cultured with autologous DCs and control peptides from common pathogens (CEF = Cytomegalovirus, Epstein bar virus, influenza) or full-length viral antigens (Mumps = mumps viral protein extract) at the indicated concentration for 24–72 h. Activation was assessed by flow cytometry staining for CD69 as shown as well as CD44 and LFA-1 (representative flow plots shown in A; quantified in B). In some experiments, cells were given a second pulse of antigen (Ag boost) after 48 h and analyzed at 72 h. Bar graphs show percent of $CD8^+$ or $CD4^+$ T cells that were $CD69^+$. Enriching for monocytes prior to DC differentiation increased the percentage of $CD8^+$ T cells that upregulated CD69 in response to viral antigens (D) relative to DC generated from PBMCs (C). D) Treatment with TLR ligands poly (I:C) and LPS alone or in combination did not increase antigen-dependent T cell upregulation of CD69 as expected and caused elevated background levels of $CD69^+$ especially among $CD8^+$ T cells. poly (I:C) alone or in combination with LPS also abrogated antigen-dependent upregulation of CD69 in both $CD4^+$ and $CD8^+$ T cells. Error bars mean $^{\pm}$ SEM. *P < .05 vs control.

by flow cytometric assessment of canonical activation markers CD11a, CD25, CD44 and CD69.

3.2. Differentiation of dendritic cells from PBMC monocytes

As shown (Fig. 1B and C), treatment with 20–50 ng/mL GM-CSF for 3–7 days induced differentiation of $CD1c^+CD11c^+$ DCs with yield further increased by the addition of 20–50 ng/mL IL-4. The frequency of $CD4^+$ and $CD8^+$ T cells was markedly reduced at these times points as was surface expression of CD14 (data not shown). Furthermore, enriching for monocytes prior to growth factor treatment by magnetic isolation (Fig. 2A) or by Percoll gradient centrifugation (Supp. Fig. 1) increased the overall frequency of $CD1c^+CD11c^+$ DCs by 3 days differentiation (Fig. 2B; and data not shown). This strongly suggested that DCs were differentiating from $CD14^+$ monocytes as expected. Additionally, when $CD14^+CD11b^+$ monocytes were magnetically isolated from PBMCs these cells could be expanded by treatment with M-CSF,IL-3, and IL-6 for 7–21 days (Supp. Fig. 2A-C, 3), and these cells retained their capacity to differentiate into dendritic cells (Supp. Fig. 4).

3.3. T cell responses to ubiquitous viral antigens in DC-T cell cocultures derived from PBMCs

Next, we combined DCs with rested T cells derived from autologous PBMCs and incubated these cells with 10 μg/mL of immunodominant peptides from cytomegalovirus, Epstein Barr virus, and influenza, (CEF) or full-length mumps viral protein antigen (MVA). We then compared surface expression levels of the activation markers CD44, CD11a, and CD69 on T cells from these cocultures 24 h and 72 h after antigen addition (representative staining shown in Fig. 3A). Additionally, in some experiments cocultures were co-treated with LPS (500 ng/mL) or high molecular weight poly (I:C) (2 μg/mL) alone or in combination in an attempt to enhance antigen-specific T cell responses by driving upregulation of costimulatory molecules on DCs. We found that CD44, CD25, and CD11a were less sensitive as markers of T cell activation induced by CEF or MVA in $CD4^+$ and $CD8^+$ T cells or were expressed at higher baseline levels compared to CD69 (Fig. 3B). We also determined that MVA- and CEF-induced T cell activation as assessed by CD69 expression was elevated 48 and 72 h post treatment for $CD4^+$ T cells and at 24, 48, and 72 h post treatment for $CD8^+$ T cells. We noted that highest activation was observed at 72 h for both $CD4^+$ T and $CD8^+$ T cells (Fig. 3C) and that restimulating cells with antigen at 48 h post treatment did not further increase T cell activation at 72 h (data not shown). We also observed significant CE-F and MVA-induced CD69 upregulation on T cells when they were cocultured with DCs differentiated from magnetically purified monocytes (Fig. 3D) with somewhat improved signal to noise ratio at 48 and 72 h for $CD8^+$ T cells. To increase DC expression of costimulatory molecules and enhance antigen induced T cell activation we pretreated DCs with LPS or poly (I:C). However, neither LPS nor poly (I:C) treatment alone or in combination improved assay specificity as they both caused pronounced increases in CD69 surface expression in vehicle treated cells as well as CEF and MVA treated cells (Fig. 3E).

Next, we compared responses between T cells derived from freshly isolated PBMCs to the responses of T cells derived from cryopreserved PBMCs. Importantly, monocyte and lymphocyte levels remained consistent in fresh and cryopreserved PBMC samples from the same patient (Fig. 4A). We noted that antigen-specific T cell responses to CEF peptides and MVA protein were preserved among cocultures of T cells and DCs derived from cryopreserved PBMCs (Fig. 4B). Just as we had observed with fresh PBMCs, antigen-specific responses to CEF and MVA were enhanced when DCs were differentiated from magnetically purified monocytes (Fig. 4C). Similar results were obtained by treating cocultures with 10 μg/mL mosaic recombinant proteins from H3N2 Influenza Virus-A Wisconsin/67/05, EBV (HHV-4), or CMV (Supp. Fig. 5A).

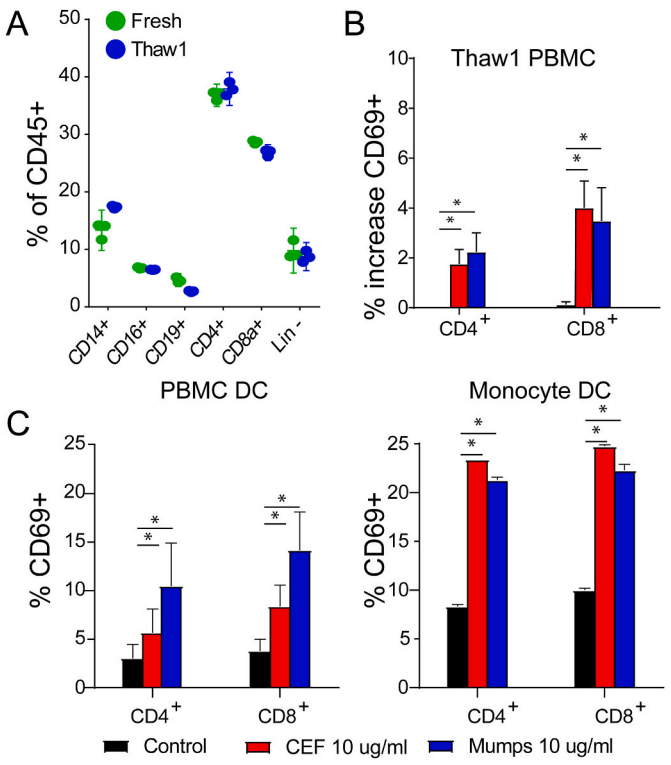

Fig. 4. T cells from cryopreserved human PBMCs retain responsiveness to ubiquitous viral antigens. A) PBMCs from healthy volunteers were stained for the indicated surface markers before or after cryopreservation. Results indicate little change in the frequency of monocytes, B cells, and T cells. B) Cryopreserved PBMCs were rested in IL-7 or differentiated into DCs over 3 days and subsequently cocultured with the indicated antigens for 72 h as in Fig. 3. Percent increase in frequency of $CD69^+$ cells among $CD4^+$ and $CD8^+$ T cells is shown relative to media treated controls. C) Enriching for monocytes prior to DC differentiation increased the percentage of $CD4^+$ and $CD8^+$ T cells that upregulated CD69 in response to viral antigens. Error bars mean ± SEM. *P < .05 vs control.

3.4. Expansion-induced gene expression changes in $CD8^+$ T cells predominantly do not persist following cryopreservation and recovery

Next, we expanded $CD4^+$ and $CD8^+$ T cells from cryopreserved PBMCs by culturing these cells in anti-CD3 and anti-CD28 coated tissue vessels for 7–10 days (Schematic Fig. 5A). This yielded an approximately 20-fold increase in viable cell number. As shown, more than 98% of cells remaining in these cultures after expansion were $CD4^+$ or $CD8^+$ (Fig. 5B and C). Expanded T cells were then cryopreserved for future use. We then gauged persistent transcriptional differences among $CD8^+$ T cells that were derived from PBMCs that had undergone one freeze thaw cycle (Thaw1), that had subsequently undergone polyclonal expansion in vitro (Expanded), or had been expanded and subsequently undergone a second freeze thaw cycle (Thaw2). For this we cultured the indicated cell populations in low dose IL-7 (1 ng/mL) for 48 h and then we magnetically purified $CD8^+$ T cells from these cells by negative selection. $CD8^+$ T cell purity was confirmed by flow cytometry (data not shown) and RNA was isolated for RNAseq analysis. As shown, principal component analysis of resulting gene expression data indicated that between group differences in gene expression far outweighed differences between cells from the same group that were isolated from different individuals (Fig. 5D). Furthermore, by cross-correlogram (Fig. 5E) Thaw1 $CD8^+$ T cells were more transcriptionally similar to Thaw2 $CD8^+$ T cells than to $CD8^+$ T cells isolated from Thaw1 PBMCs after expansion on anti-CD3/anti-CD28 coated plates. This may suggest that cryopreservation and recovery exerts a dominant effect on $CD8^+$ T

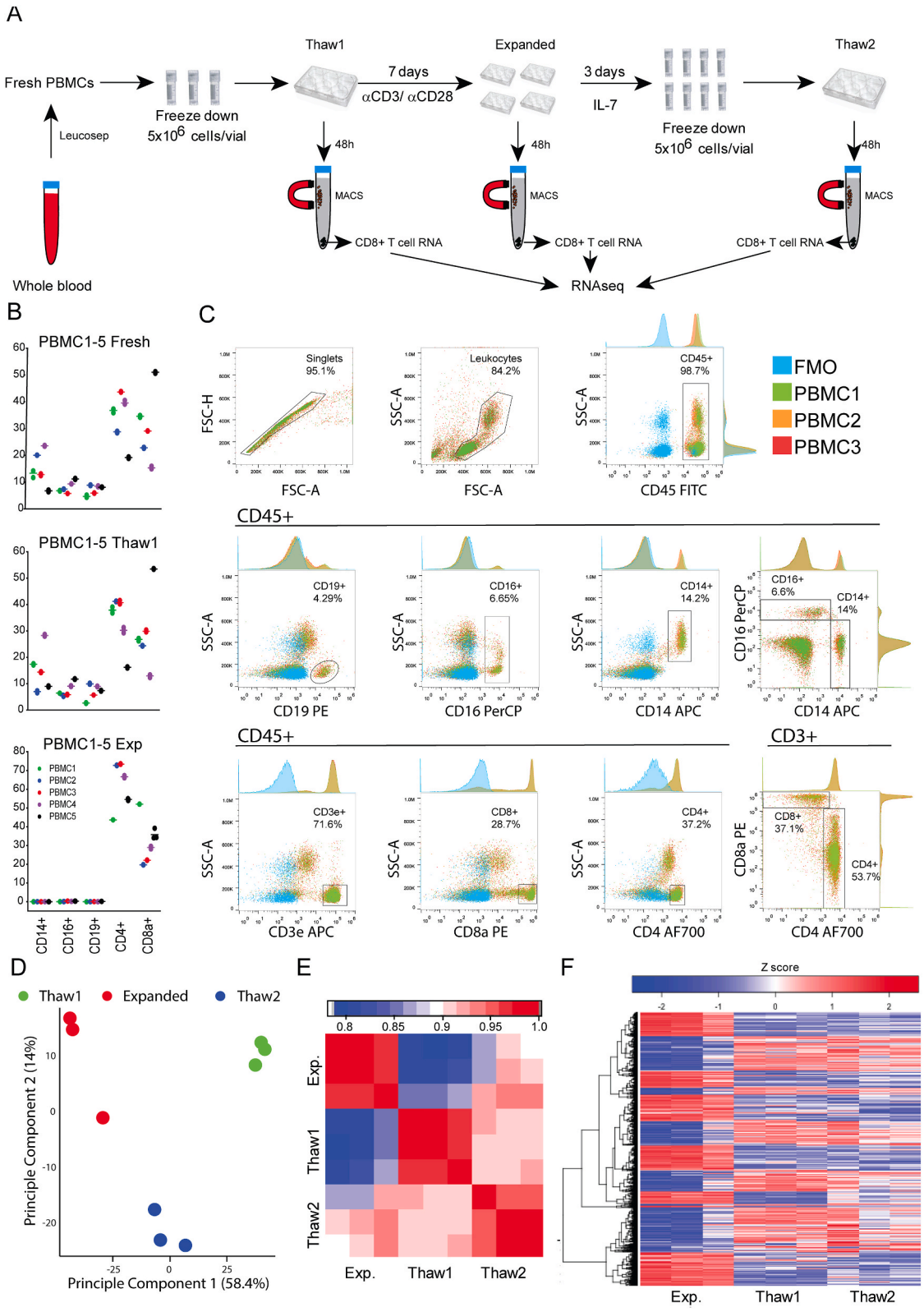

Fig. 5. Transcriptional profile of cryopreserved and anti-CD3/antiCD28 expanded human CD8$^+$ T cells. A) Standard operating procedure for collection, expansion, cryostorage, and transcriptional characterization of patient PBMC-derived CD8$^+$ T cells. B) Percent of CD45$^+$ PBMCs staining positive for the indicated markers in 5 control patients before cryopreservation, after cryopreservation, or after one that and subsequent T cell expansion with plate bound anti-CD3/antiCD28. C) Gating strategy for B cells (CD19), NK cells (CD16), monocytes (CD14), and T cells (CD3, CD8a, CD4) among CD45$^+$ cells. D) Principal component analysis of RNAseq data shows clustering of T cells from each treatment group. E) Correlogram shows >90% correlation in gene expression for CD8$^+$ T cells magnetically isolated from Thaw1 PBMCs and CD8$^+$ T cells isolated form Thaw2 PBMCs. F) Heat map showing genes differentially expressed in CD8$^+$ T cells magnetically purified from PBMCs after anti-CD3/anti-CD28 expansion. These genes showed similar expression in "Thaw1" and "Thaw2" CD8$^+$ T cells. Error bars mean $^{\pm}$ SEM.

cell transcriptional profile. Automated clustering of differentially expressed genes demonstrated that a majority of genes upregulated in post-expansion CD8$^+$ T cells relative to Thaw1 CD8$^+$ T cells were once again downregulated in Thaw2 CD8$^+$ T cells (Fig. 5E). Using a 0.2% false discovery rate, we identified protein coding genes that exhibited differential expression in expanded CD8$^+$T cells relative to Thaw1 CD8$^+$ T cells (Supp. Figure 6). Gene ontology analysis identified nuclear chromosome segregations, mitotic cell cycle process, and regulation of mitotic cell cycle phase transition as overrepresented Biological Process Gene Ontology terms (Supp. Table II). Of note, no protein coding genes exhibited differential expression in Thaw2 CD8$^+$ T cell relative to Thaw1 CD8$^+$ T cells using this method.

We also performed targeted analysis of T cell marker gene expression, including genes associated with regulatory T cells, memory T cells, resting memory T cells, T cell exhaustion, effector memory T cell activation, interferon gamma (IFNγ) response, T cell proliferation, and cytotoxicity (Supp. Fig. 7A-H). In these unadjusted analyses, expanded and Thaw2 CD8$^+$ T cells showed increased expression of regulatory T cell markers CD25 and IL-1RB2, while expanded CD8$^+$ T cells had reduced levels of PDCD1 (Supp. Fig. S7A). Expression of memory T cell markers, exhausted T cell markers and resting T cell markers showed little difference across groups apart from reduced IL-7R among expanded CD8$^+$ T cells (Supp. Fig. 7B), elevated CD52 among Thaw2 CD8$^+$ T cells (Supp. Fig. 7C), and elevated TIM3 among expanded CD8$^+$ T cells (Supp. Fig. 7D) relative to Thaw1 CD8$^+$ T cells. Similarly, CD8$^+$ T cells isolated from expanded or Thaw2 cells exhibited no significant differences in expression of memory T cell activation markers, aside from reduced CCL5 expression (Supp. Fig. 7E). More prominent differences were noted among proliferation and cytotoxicity markers. When compared to Thaw1 CD8$^+$ T cells, expanded CD8$^+$ T cells but not Thaw2 CD8$^+$ T cells exhibited increased expression of proliferation markers including LIF, NME1, FABP5, and ORC6 (Supp. Fig. 7F)—in agreement with our gene ontology analysis. Expanded CD8$^+$ T cells also exhibited increased expression of IFNγ response markers STAT1 and ISG15 (Supp. Fig. 7G), and they showed downregulation of cytotoxicity markers including GZMH, GZMK, and NKG7 with a trend toward decreased expression of PRF1, KLRB1, KLRD1, and KLRG1 (Supp. Fig. 7H). Reduced expression of GZMH, GZMK, and NKG7 was maintained in

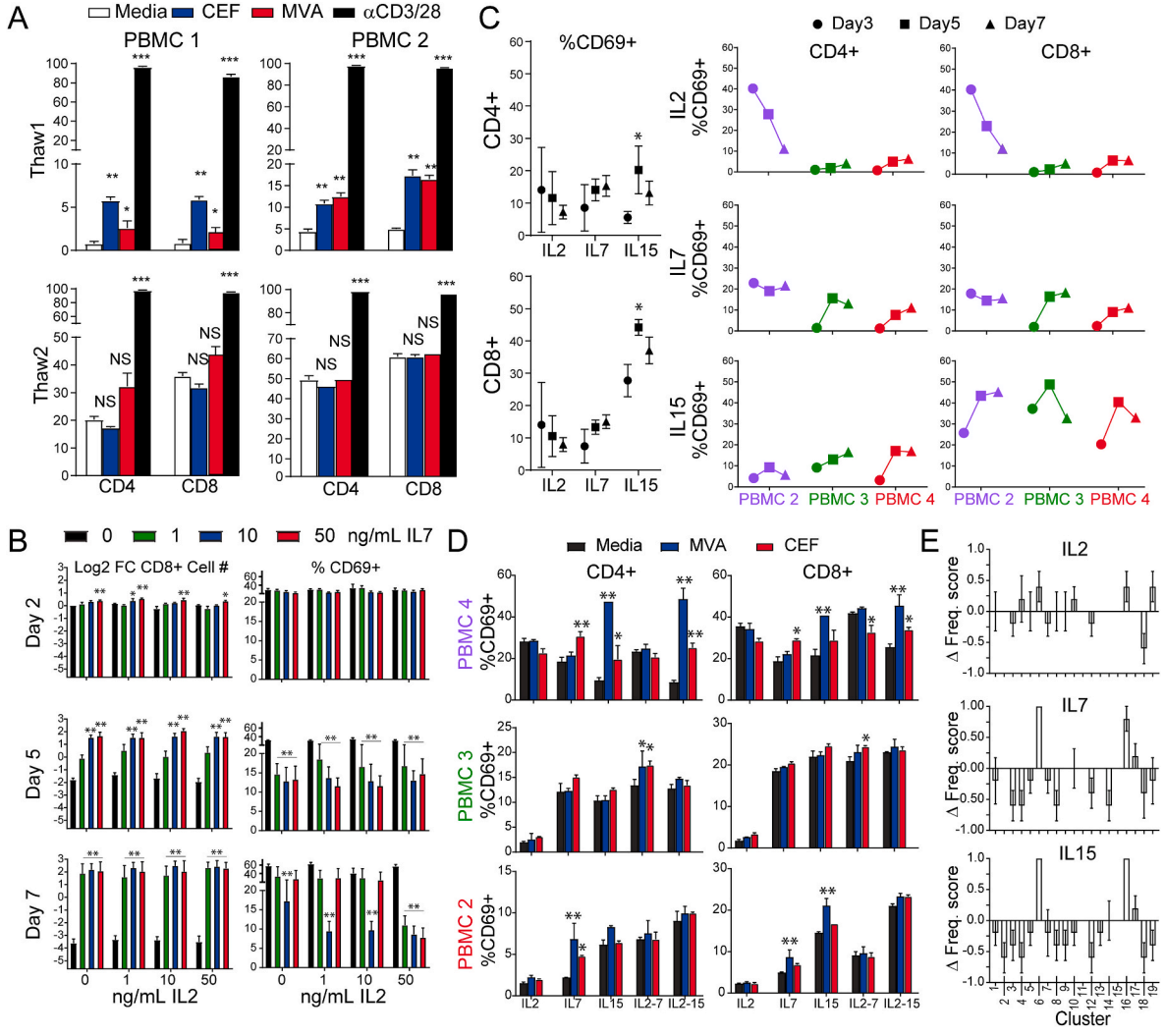

Fig. 6. Prolonged resting of anti-CD3/anti-CD28 human T cells in IL-2 family cytokines reduces baseline CD69$^+$ expression but fails to restore responsiveness to ubiquitous viral antigens. T cells were expanded from Thaw1 PBMCs with anti-CD3/anti-CD28 and subsequently underwent another freeze thaw cycle (Thaw2). A) Thaw2 CD4$^+$ and CD8$^+$ T cells show elevated baseline CD69 expression and reduced responsiveness to ubiquitous viral antigens relative to Thaw1 counterparts. B) Resting Thaw2 T cells in high concentrations of IL-2 and IL-7 causes further expansion and downregulation of CD69 over 7 days. C) Treatment of Thaw2 PBMCs with IL-2 causes downregulation of CD69 relative to IL-7 and IL-15 over 7 days. D) Thaw2 cells rested for 7 days in IL-2, IL-7, IL-15, IL-2 + IL-7, or IL-2 + IL-15 show variable responsiveness to ubiquitous viral antigens across patients. Cytometry by time of flight was performed on Thaw2 PBMCs rested as in (C). Cluster frequency changes for each treatment condition are summarized in (E). Error bars mean $^{\pm}$ SEM. *P < .05 **P < .01 vs media condition (A, D), vs 0 IL-2 0 IL-7 condition (B) or vs Day 3 (C).

Thaw2 CD8$^+$ T Cells along with trends for KLRB1 and KLRG1. Thus, while no mRNA expression differences were noted between Thaw1 and Thaw2, mass cytometric analysis identified differences including increased levels of CD25, IL-1RB2, CD52 and reduced levels of CCL5, GZMH, GZMK, NKG7 on Thaw2 cells compared to Thaw1.

3.5. Reduced capacity to assay Ag-specific T cell responses in cryopreserved T cells expanded from cryopreserved PBMCs

To ascertain functional differences between Thaw1 and Thaw2 CD8$^+$ T cells, we compared antigen-specific upregulation of CD69 in response to CEF peptides and MVA protein in Thaw2 and Thaw1 T cells co-cultured with antigen-pulsed autologous DCs derived from Thaw1 PBMCs (i.e. Thaw1 DCs + Thaw1 T cells vs Thaw1 DCs + Thaw2 T cells). Surprisingly, we found that despite their transcriptional similarity to Thaw1 T cells (apart from CD25, IL-1Rb2, CD52, GZMH, GZMK, and NKG7), Thaw2 T cells exhibited higher baseline expression levels of CD69 and failed to further upregulate CD69 expression in response to CEF or MVA treatment despite retaining responsiveness to anti-CD3/anti-CD28 treatment (Fig. 6A). Therefore, we sought to determine whether resting Thaw2 T cells in the presence of IL-2 family cytokines prior to antigen stimulation would return surface expression of CD69 on T cells to baseline levels and thereby improve dynamic range for sensitively detecting antigen-induced upregulation of CD69. As shown, (Fig. 6B and C), we determined that culturing these Thaw2 T cells in 10-ng/mL IL-7 with 1–50 ng/mL IL-2 for 7 days caused marked reduction in surface levels of CD69, while increasing total cell count 3-4-fold. However, these rested T cell did not retain responsiveness to cognate auto-antigens when co-cultured with autologous DCs (data not shown). Likewise, we tested how resting expanded T cells in IL-2, IL-7, or IL-15 affected CD69 levels. We found that unlike IL-2, treatment with IL-7 or IL-15 caused maintenance (IL-7) or an increase (IL-15) in the abundance of CD69$^+$ T cells in these cultures (Fig. 6C). Furthermore, resting in IL-2, IL-7, or IL-15 either alone or in combination did not restore antigen-induced T cell activation of anti-CD3/CD28 expanded T cells which failed to consistently upregulate CD69 in response to CEF and MVA antigen stimulation compared to their Thaw1 counterparts (Fig. 6D). Further characterization of Thaw2 cells rested for 7 days with IL-2, IL-7, or IL-15 using mass cytometry revealed that IL-7 and IL-15 rested cells exhibited an increase in the frequency of CD4$^+$CD27$^+$IFNγ$^+$ T cells (Cluster 6) and CD8$^+$CD27$^+$IFNγ$^+$ T cells (Cluster 16) compared to cells rested in media alone (Fig. 6E; Fig. 7A–C). This effect was also observed to a lesser extent in IL-2 treated cells relative to control treated cells.

3.6. Alternative methods of T cell expansion preserve capacity for detecting Ag specific T cell responses

We sought to compare alternative methods of T cell expansion to determine if these methods would better preserve antigen-specific T cell responses, which we had observed in Thaw1 cells. For this we compared IL-2 stimulation alone or with IL-7, IL-15 or IL-21 co-treatment. We found that for CD4$^+$ T cells IL-2 alone resulted in significantly less expansion than polyclonal stimulation with anti-CD3/antiCD28 by day 7 but significantly more expansion than cells treated with IL-2$^+$ IL-7, IL-2$^+$ IL-15, or IL-2 + IL-21 (Supp. Fig. 8A). In contrast, CD8$^+$ T cell expansion by IL-2$^+$ IL-7 and IL-2$^+$ IL-21 treatment did not differ significantly from IL-2 alone. IL-2 alone also resulted in lower levels of induction of CD69 in CD8$^+$ T cells compared to anti-CD3/anti-CD28 or combinatorial cytokine treatments. Likewise, IL-2 induced lower levels of CD69 compared to treatment with anti-CD3/anti-CD28, IL-2 + IL-7, or IL-2 + IL-15. IL-2 also resulted in significantly lower levels of CD11a induction for CD4$^+$ and CD8$^+$ T cells compared to IL-2 + IL-7 and IL-2 + IL-15 treated cells. In contrast, CD44 levels remained higher on CD4$^+$ T cells treated with IL-2 alone than for all other conditions as well as for IL-2 treated CD8$^+$ T cells compared to IL-2 + IL-15 treated CD8$^+$ T cells (Supp. Fig. 8B). This led us to predict that IL-2-mediated T cell expansion would better preserve an unactivated phenotype in CD4$^+$ and CD8$^+$ T cells and thus preserve CEF peptide responsiveness when these cells were co-cultured with expanded DCs and CEF antigen. Surprisingly, we

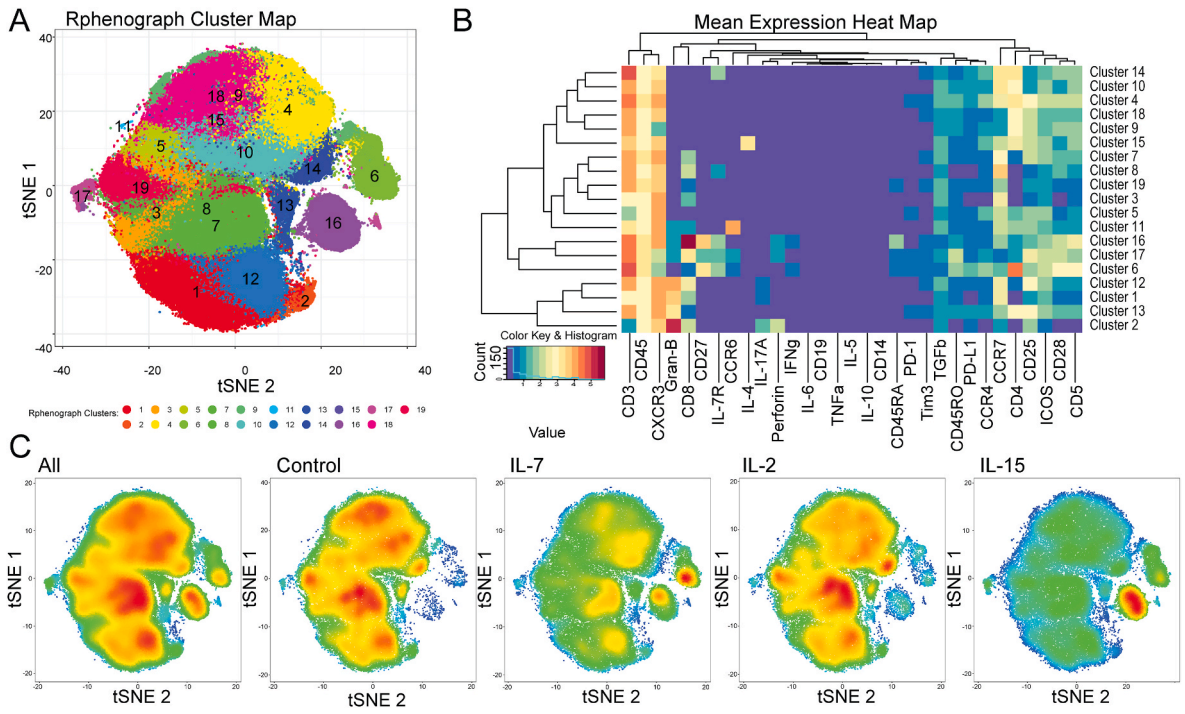

Fig. 7. Mass cytometric characterization of Thaw2 cells rested in IL-2, IL-7, or IL-15 T cells were expanded from Thaw1 PBMCs with anti-CD3/anti-CD28 and subsequently underwent another freeze thaw cycle (Thaw2). Cytometry by time of flight was performed on Thaw2 PBMCs rested for 7 days in IL-2, IL-7, or IL-15. A) R-phenograph cluster show the indicated clusters in tSNE plots. Heat maps show T cell marker expression for each cluster (B). Representative tSNE plots for each condition are shown (C).

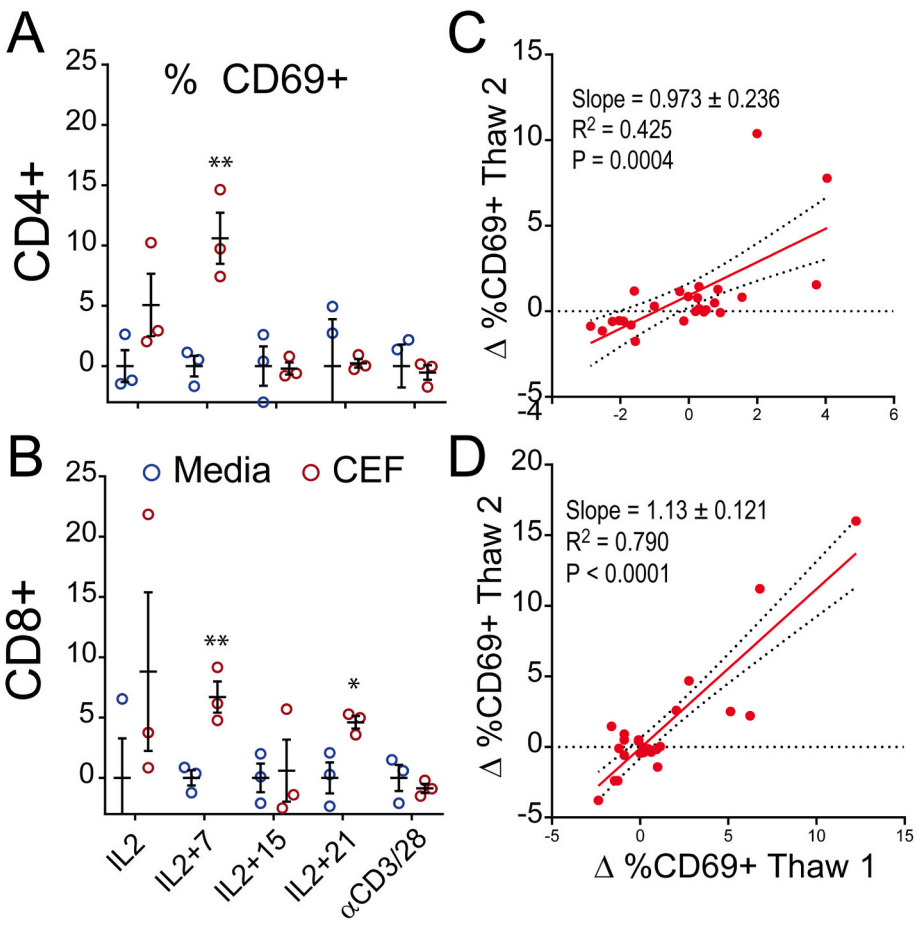

Fig. 8. Human T cells expanded with IL-2 and IL-7 retain responsiveness to ubiquitous viral antigens. Thaw1 PBMCs were expanded with 10 ng/mL of the indicated cytokines for 7 days. Subsequently, cells were stimulated with media or 10 μg/mL CEF for 72 h and % increase in $CD69^+$ on $CD4^+$ (A) and $CD8^+$ T cells (B) was determined by flow cytometry. Results indicated that $CD4^+$ and $CD8^+$ T cells from Thaw1 PBMCs that were expanded with IL-2 + IL-7 retained responsiveness to CEF peptides, whereas cells expanded with IL-2 + IL-21 only retained responsiveness among $CD8^+$ T cells. C-D) Correlation between antigenic responses in human T cells before and after expansion with IL-2 and IL-7. Unmanipulated cryopreserved PBMCs from healthy controls (n = 3) and patients with CNS autoantibodies (n = 3) were thawed (thaw1) and expanded with 10 ng/mL IL-2 and IL-7 for 7 days. In parallel treatments dendritic cells were differentiated from Thaw1 PBMCs using GMCSF (50 ng/mL) and IL-4 (20 ng/mL) for 7 days and then cryopreserved. These T cells and dendritic cells were cryopreserved for >7 days prior to thawing (thaw2). Antigen induced changes in $CD69^+$ T cell frequency were determined for thaw1 as described in Supp. Fig. 5 in response to media, CEF, or cognate CNS neural antigens in patients and controls. For thaw2, separate vials of dendritic cells and T cells thawed and rested for 1 h in complete media prior to being combined for antigen stimulation. Changes in $CD69^+$ T cell frequency were determined 72 h later by flow cytometry. Plots show correlation between Thaw1 and Thaw2 for antigen-induced change in $CD69^+$ T cell frequency across all antigen conditions relative to vehicle treatment. Slope, Pearson Correlation, and P values are shown for $CD4^+$ T cells (C) and $CD8^+$ T cells (D). Linear regression fit (red line) and 90% prediction range (dotted lines) are shown. *P < .05 **P < .01 vs media condition. Error bars SEM.

found that although IL-2 expanded T cells treated with CEF exhibited a trend toward higher frequency of $CD69^+$ cells among $CD4^+$ and $CD8^+$ T cells, only T cells expanded by co-treatment with IL-2 and IL-7 exhibited a statistically significant increase in $CD69^+$ cell frequency in response to antigen stimulation among both $CD4^+$ and $CD8^+$ T cell populations (Fig. 8A and B). Similar results were obtained using full-length protein antigens from CMV, EBV, influenza, and Mumps virus (Supp. Fig. 5B). Additionally, for both $CD4^+$ T cells and $CD8^+$ T cells we found a high degree of correlation between Thaw1 and Thaw2 (following 7 days IL-2/7 mediated expansion) in terms of $CD69^+$ T cell frequency changes measured in responses to a given antigenic stimulus (Fig. 8C and D). This indicated strong preservation of antigenic responsiveness using this expansion protocol. Subsequent mass cytometry (Fig. 9A–E) demonstrated that this method caused retention or expansion of several important T cell subsets including naïve T cells ($CCR7^+CD45RA^+CD45RO$-; $CD4^+$ = clusters 5, 16, 18, 9; $CD8^+$ = clusters 3, 10), central memory T cells ($CCR7^+CD45RA$-$CD45RO^+$; $CD4^+$ = clusters 1, 6, 14; $CD8^+$ = clusters 2, 15), and effector memory T cells ($CCR7^+CD45RA$-$CD45RO^+$; $CD4^+$ = clusters 10, 12; $CD8^+$ = cluster 13). For $CD8^+$ T cells we also observed maintenance of so called $CD45RA^+$ re-expressing T cells $CD45RA^+CD45RO$-$CCR7$- (T_{EMRA}; cluster 7). We also observed expansion of $CD3^+CD4^-CD8^-$non-classical T cells ($CD28^-$cluster 11; $CD28^+$ cluster 4). By paired analysis of human PBMC samples before and after expansion we further determined that expansion resulted in increased overall expression of CD5 and CD25 on $CD4^+$ T cells expanded with IL-2 + IL-7 (Supp. Fig. 9A). Furthermore, expansion with $IL\text{-}2^+$IL-7 cocktail caused both $CD4^+$ T cells and $CD8^+$ T cells to exhibit increased expression of CXCR3 and decreased surface expression of CCR7 and IL-7R (Supp. Fig. 9A and B).

4. Discussion

We report a simple, robust assay for quickly measuring antigen-dependent T cell activation using a coculture of autologous DCs and T cells. We demonstrate rapid generation of dendritic cells from PBMCs and monocytes, which serve as potent antigen presenting cells. We also demonstrate that CD69 is a sensitive indicator of antigen-dependent activation in human T cells. Moreover, these antigen-dependent T cell responses are retained in cryopreserved PBMCs relative to freshly prepared PBMCs. While polyclonal expansion of T cells with anti-CD3/anti-CD28 prevented subsequent antigen-dependent upregulation of CD69, expansion with an IL-2 + IL-7 cocktail preserved these responses. Critically, we also report that monocytes isolated from fresh whole blood or PBMCs or monocytes present among cryopreserved PBMCs can be expanded with M-CSF,IL-3, and IL-6 and that these monocytes can be subsequently cryopreserved and used to generated dendritic cells for antigen recall assays. Due to loss of cells, it is suggested that monocyte enrichment not be performed prior to monocyte expansion or DC differentiation since this substantially reduces overall DC yield and only modestly improves purity.

Prior studies have explored assays for measuring antigen-specific T cell responses against viral, bacterial, tumor-associated, autoimmune, or drug-associated antigens. Most of these assays do not utilize autologous DCs for antigen presentation and therefore have fewer potent antigen presenting cells capable of processing and presenting full-length protein antigens. Instead these assays utilize peptide libraries to stimulate T cells by displacing peptides present on surface MHC I complexes od other leukocytes [75–78]. This approach limits the number of proteins that can be investigated given limited patient cell numbers, in some cases

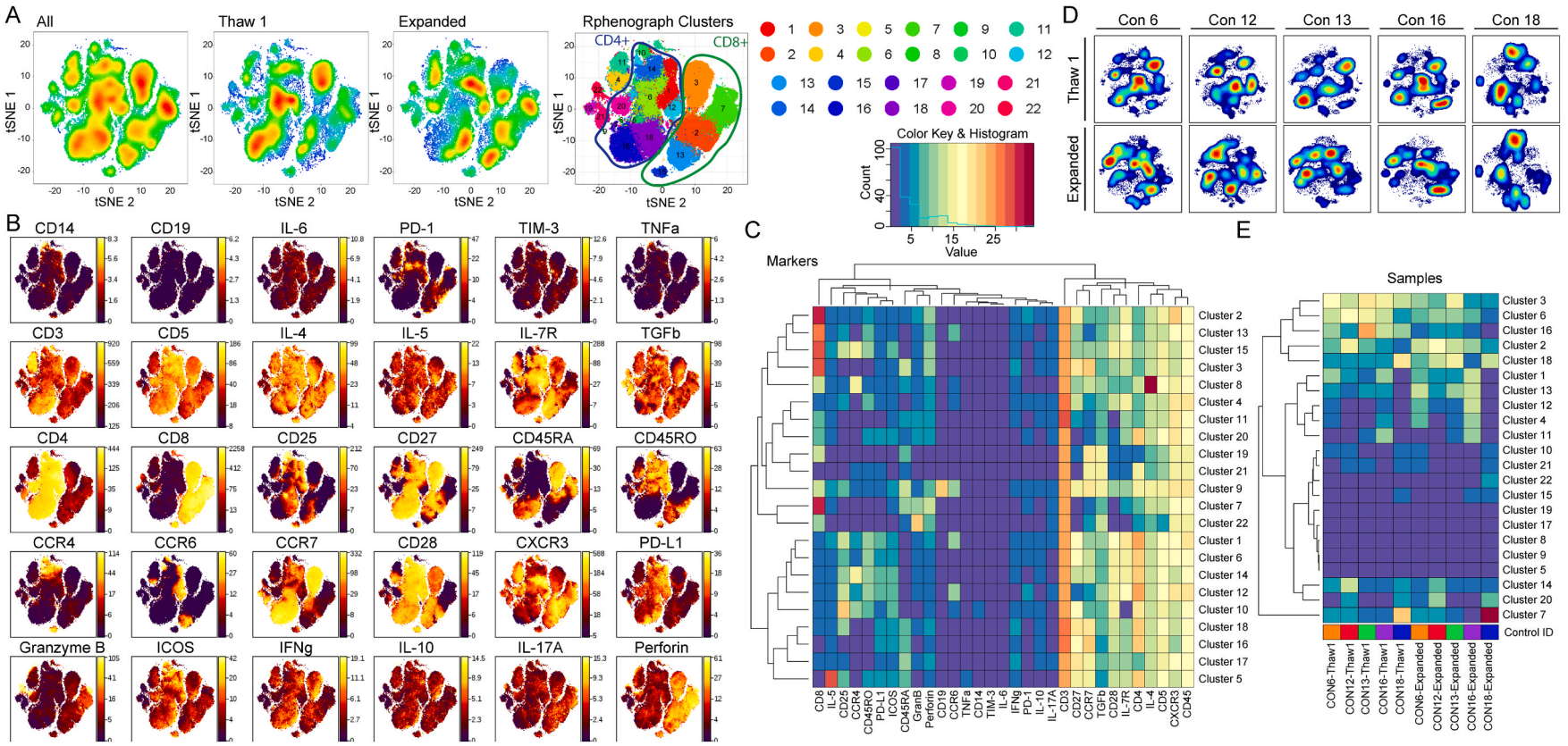

Fig. 9. Human T cells expanded with IL-2 and IL-7 retain responsiveness to ubiquitous viral antigens. We performed CyTOF on Thaw1 PBMCs from 5 healthy controls before and after they were expanded for 7 days by treatment with 10 ng/mL IL-2 + IL-7. A) Representative tSNE plots for total, Thaw1 and IL-2 + IL-7 expanded PBMCs are shown alongside R-phenograph cluster map showing expansion-induced enrichment of clusters 1, 2, 13, and 18. tSNE plots for each sample are shown in (B). C) Expression levels for each marker protein are overlaid on R-phenograph cluster map. As expected, we observed increased expression of IL-2R (CD25) and IL-7R among clusters enriched by expansion with IL-2 + IL-7 relative to related clusters 3, 6, 18, 16 that were unenriched. Other markers upregulated in these clusters included CCR6 and CD45RO and CXCR3. In contrast CCR7 and CD45RA were downregulated among these clusters. Heat maps show protein marker expression for each cluster (D) and cluster frequency for each sample (E).

necessitating the use of microtiter plates [76]. Recently, several groups have described using dendritic cells in T cell antigen stimulation assays [62,79–81]. For example, Carrio and colleagues have utilized a similar DC-based assay to investigate antigen specific responses against influenza and TB antigens in patient PBMCs. However, their assay focused on T cell cytokine production in ELISpot assays and identified fewer responding cells [79]. Indeed, many studies have focused on secreted factors or other methodologies that preclude the option for cell sorting or further transcriptional characterization of responding cells. For example, some have utilized intracellular cytokine staining for IFNγ to identify activated T cells in antigen stimulation assays [80,82]. Intracellular staining for IFNγ is a useful marker for identifying activated Th1 cells and Tc1 cells, which could be used for cell sorting and further transcriptional characterization. However, IFNγ as a marker is not a general marker of T cell activation and as such it will fail to identify T cells with alternative effector phenotypes. Other markers have also been proposed as more universal indicators of T cell activation or as more strongly correlated with T cell effector function or protection (e.g., in HIV elite controllers) including secretion of perforin, IL2, and TNFα. Moreover, the presence of polyfunctional T cells expressing all of these cytokines in combination with IFNγ in response to antigen stimulation have been shown to better correlate with protection [83–90] Moreover, it is likely that analysis of each of these factors alone would miss a subset of antigen-responsive T cells. For example, IFNg negative T cells have been demonstrated to secrete other molecular mediators associated with protection in response to viral antigen [90]. However, multiplexed analysis of these factors using assays with single cell resolution (e.g., intracellular cytokine staining flow cytometry or ELISPOT) can present technical challenges for broad utilization.

In addition to measuring secreted cytokines [75–77,79,81] others have used proliferation indicators, target killing assays, or surface activation markers on antigen presenting cells as indicators of T cell activation [80,81,91,92]. Several iterations of this latter technique have been explored including the evaluation of surface activation markers on B cells used as antigen presenting cells that are reliant on cognate CD4 T cells for activation [78]. However, this assay likely has limited utility for characterizing antigen specific $CD8^+$ T cell responses. Recently, Lee and Meyerson described a novel assay utilizing engineered antigen presenting cells expressing multiple HLA molecules, gene-encoded barcode-associated protein antigens, and surface expression of cytokine capture antibodies. They utilized this approach to determine the prevalence and effector phenotype of T cell responses against an array of protein antigens. However, again in this case the capture and further characterization of responding T cells is precluded by the nature of the assay. In summary, while the field is rife with assays for measuring antigen-specific human T cell responses some of which offer higher throughput for antigen testing, many of these assays suffer in terms of sensitivity or capacity for further characterization of responding T cells. These concerns are more thoroughly addressed by the assay we have described here, and which we have used previously for characterizing T cell responses in CNS autoimmune diseases in which at least one cognate antigen is known [93,94]. In our studies we identified CD69 as a sensitive marker of T cell activation, however it is unclear whether some of this activation is due to bystander T cell activation. Transient CD69 is known to contribute T cell entry into target tissues and is therefore likely to be upregulated acutely on a broad array of T cells undergoing activation regardless of the phenotype and cytokine secretion profile. However, we do not know the degree to which CD69 upregulation on T cells correlates with production of effector molecules in these cells. Therefore, to enhance specificity it may be necessary to perform follow up analyses in antigen responsive samples. These follow up analyses would help characterize the transcriptional profile, cytokine profile, T cell receptor clonality, and antigen-specificity of these cells.

T cell proliferation and polyclonal expansion has been studied by treatment with combinations of immobilized anti-CD3, anti-CD28, or IL-2 for over 30 years [95–98]. Importantly, long term anti-CD3/28 stimulation is known to cause high levels of activation induced cell death—with some arguing that transitioning to IL-2 stimulation may reduce this problem, though it may favor preferential expansion of EBV-specific T cells [99]. High dose IL-2 stimulation in conjunction with anti-CD3/28 has also been shown to promote expansion of regulatory T cells that maintain their phenotype in vitro [100] but the clonality of expanding cells is strongly influenced by media supplements [101]. Likewise, others have shown that treatment with anti-CD3 and IL-2 causes the emergence of suppressor cells that limit alloantigen responses [102]. Bere et al. compared T cell populations expanded by various protocols and found that treatment with IL-2 and plate bound anti-CD3 favored expansion of effector memory T cells whereas treatment with anti-CD3/28 beads and IL-2 favored expansion of central memory T cells—an effect that was further increased by the inclusion of IL-7 and IL-15 [103]. It is likely that the central or effector memory phenotype of the proliferating cells was also strongly influenced by the stimulus—a caveat that often goes unaddressed. A more recent study demonstrated expansion of central and effector memory T cells by treatment with blinatumomab (cocktail of anti-CD3/CD19) over 3 weeks and that CMV specific clones were detected at equivalent proportions before and after expansion [104]. Likewise, polyclonal expansion with anti-CD3 and IL-2 has been shown to preserve HIV-specific, CEF-specific and SARS-COV2-specific T cell responses as measured by IFNγ ELISpot and flow cytometry [105,106]. Meanwhile, selective expansion of populations of antigen-specific T cells has also been achieved by repeated treatment of autologous monocytes or dendritic cells with antigen or coculturing T cells with antigen-expressing autologous lymphoblastoid cells [107,108]. However, antigen stimulation ex vivo has been shown to cause loss of cytolytic T cell responses [107] and expansion of subdominant T cell clones at the expense of T cells specific for immunodominant antigens [109].

Our data suggest that polyclonal stimulation with anti-CD3/28 also causes upregulation of CD69, which slowly dissipates upon resting these expanded T cells in IL-2 or IL-7 for 7 days or more. However, these cells lose their capacity to upregulate CD69 in response to antigenic stimulation. In contrast, T cells expanded by treatment with a cocktail of IL-2 and IL-7 preserve antigen-dependent responses. Whether these cells retain responsiveness to other full-length protein antigens such as autoantigens or tumor associated antigens will be addressed in future studies. It is also notable that the degree of proliferation observed following IL-2 and IL-7 stimulation is significantly less than that observed by stimulation with anti-CD3 and anti-CD28. Additionally, we have not investigated whether continued proliferation is observed or indeed whether antigen-responsiveness is preserved following long-term treatment with IL-2 and IL-7. It would also be of interest to determine whether T cells upregulating CD69 in response to antigen stimulation could be sorted and expanded for cryopreservation, restimulated with antigen, or used for in vitro effector: target cytotoxicity assays. Future studies using these methods could rapidly screen at-risk patient populations for the presence of autoreactive T cells, monitor patients for the presence of protective T cell responses against key viral or bacterial proteins following vaccination or infection, or evaluate the presence of protective or deleterious T cell responses against tumor associated autoantigens in patients with neoplasms or paraneoplastic disease. This may also support follow up in-depth characterization of these responding T cells.

5. Conclusions

In summary, we outline a relatively simple but widely applicable strategy for quantifying antigen-dependent T cell responses in cryopreserved patient PBMCs using human monocyte-derived DCs as antigen presenting cells. Furthermore, we have identified CD69 as a sensitive indicator for human T cell activation in these assays, and we provide evidence that T cell expansion using an IL-2 + IL-7 cocktail preserves these antigen-specific responses.

Ethics approval and consent to participate

The Mayo Clinic institutional review board approved the use of all human materials and all experiments were conducted in compliance with the relevant regulations and guidelines. All subjects provided written informed voluntary consent after the nature and possible consequences of the study were explained. We confirm that we have read the Journal's position on issues involved in ethical publication and affirm that this report is consistent with those guidelines.

Consent for publication

Not applicable.

Availability of data and material

Data sharing is not applicable to this article. Please contact the author for data requests.

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Credit author statement

BDSC, CLH: Conceptualization. BDSC, CLH: Methodology. BDSC,: Validation. BDSC, CB, CL: Formal analysis. BDSC, RJ, CB, CL: Investigation. CLH: Resources. BDSC, CLH: Data curation. BDSC, RJ, CB, CL: Writing. BDSC, CLH, CB, CL, RJ: Writing – review & editing. BDSC, CL, CB: Visualization. BDSC, CLH: Supervision. CLH: Project administration. BDSC, CLH: Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jtauto.2022.100173.

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