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CMV epitope-specific CD4+ T cells are inflated in HIV+ CMV+ subjects

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Abstract

Select CMV epitopes drive life-long $CD8^+$ T cell memory inflation, but the extent of CD4 memory inflation is poorly studied. $CD4^+$ T cells specific for human CMV (HCMV) are elevated in HIV^+ $HCMV^+$ subjects. To determine whether HCMV epitope-specific $CD4^+$ T cell memory inflation occurs during HIV infection, we used HLA-DR7 tetramers loaded with the glycoprotein-B DYSNTHSTRYV (DYS) epitope to characterize circulating $CD4^+$ T cells in co-infected, HLA-$DR7^+$ long-term non-progressor HIV subjects with undetectable HCMV plasma viremia. DYS-specific $CD4^+$ T cells were inflated among these HIV^+ subjects compared to those from a HIV^- $HCMV^+$ HLA-$DR7^+$ cohort, or to HLA-DR7-restricted $CD4^+$ T cells from the HIV co-infected

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Contributions: COA, MAP, SG, CW, PK, EJP, DMK, SAK and SAM designed research studies. COA, CH, DKJ, BGE, MHJ, SAK and SAM acquired samples. COA, SG, AC, LB, RG, CH, and DKJ conducted experiments. COA, SG, AC, and LB acquired data. COA, SG, WJM, EJP, SK and SAM analyzed data. COA, MAP, PK, DMK, SAK and SAM wrote the manuscript, and all authors edited it.

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cohort that were specific for epitopes of HCMV phosphoprotein-65, tetanus toxoid precursor, Epstein-Barr virus nuclear antigen 2 or HIV gag protein. Inflated DYS-specific $CD4^+$ T cells comprised effector memory or effector memory-RA^+ subsets with restricted TCR-beta usage and nearly monoclonal CDR3 containing novel conserved amino acids. Expression of this near monoclonal TCR in a Jurkat cell transfection system validated fine DYS specificity. Inflated cells were polyfunctional, not senescent, and displayed high *ex vivo* levels of granzyme-B, CX_3CR1, CD38 or HLA-DR, but were less often $CD38^+HLA\text{-}DR^+$ co-expressing. The inflation mechanism did not involve apoptosis suppression, increased proliferation or HIV gag cross-reactivity. Instead, the findings suggest that intermittent or chronic expression of epitopes such as DYS drive inflation of activated $CD4^+$ T cells that home to endothelial cells and have the potential to mediate cytotoxicity and vascular disease.

Introduction

Classical $CD4^+$ and $CD8^+$ memory T cell responses against viruses expand during primary infection and contract to low magnitudes after infection resolution (1). However, $CD8^+$ T cell responses to select epitopes of human (HCMV) (2, 3), rhesus (4), and murine cytomegalovirus (MCMV) (5–9) persist for decades at very high magnitudes after primary infection or during latency. This phenomenon is termed “memory inflation” and has been best characterized among CMV-specific $CD8^+$ T cells that consist of mainly $CD45RO^+$ $CCR7^-$ $CD27^-$ T cells (effector memory/T_{EM}) and their $CD45RA^+$ revertants, $CD45RO^-$ $CCR7^-$ $CD27^-$ T cells (effector memory-RA^+/T_{EMRA}) (8–12). CMV-specific $CD8^+$ T cells express high levels of CX_3CR1 that bind CX_3CL1 (fractalkine), which is expressed on vascular endothelial cells (VECs), a major target of CMV latent infection (1).

Classical CMV-specific $CD8^+$ T cells display an IL-7-receptor-alpha/$CD127^+$ programmed cell death protein-1^-, PD-1^- phenotype (capable of homeostatic proliferation controlled by IL-7 and other cytokines), while inflated CMV-specific $CD8^+$ T cells are $CD127^-$ PD-1^- T cell immunoglobulin and ITIM domain/$TIGIT^-$ Granzyme B^+ CX_3CR1^+ with evidence suggesting they are maintained by low-level exposure to persistent antigen from stochastic CMV reactivation (1, 13–16). These data suggest inflated responses are maintained through recurrent stimulation by peptide-MHC (17–19) produced by persistent, stochastic expression of specific CMV transcripts (20–22). These epitopes are presented to CMV-specific T cells by latent HCMV-infected, non-hematopoietic reservoirs, including VECs, lymph node (LN) stroma cells, and cells in the bone marrow and lungs (1, 23–25). Maintenance of inflated CMV-specific T cell responses might also depend on their longer telomeres that positively correlate with persistence (26), or on epitope cleavage by constitutive proteasomes (6, 27).

CMV-specific $CD4^+$ T cells suppress HCMV lytic replication (28) and maintain $CD8^+$ T cell inflation (29). HCMV lysate-specific $CD4^+$ T cells persist at high magnitudes in HIV^+ $HCMV^+$ co-infection (30), which might be due to higher HCMV disease burden (31, 32). Yet it is not known whether $CD4^+$ T cells specific to individual HCMV epitopes undergo memory inflation in co-infected subjects. Glycoprotein B/gB has the highest population prevalence of CD4 responses of any HCMV protein (33). gB polyprotein colocalizes to endosomes that process and present its class II epitopes directly from infected endothelial

cells upon IFN-γ-induced HLA class II expression (28, 34, 35) without needing professional APCs. gB-loaded endosomes are also secreted as immunogenic exosomes that stimulate $CD4^+$ memory T cells (36, 37). In HLA-DRB1*07:01 ($DR7^+$) persons, the most immunogenic gB epitope is the extremely conserved DYSNTHSTRYV (DYS) epitope that is recognized by cytotoxic, CX_3CR1^+ $CD4^+$ T cells (11, 38).

HIV^+ $HCMV^+$ co-infection is implicated in the emerging higher incidence of HCMV-related, non-AIDS comorbidities of cardiovascular diseases including hypertension, coronary artery disease, and stroke despite suppressive antiretroviral therapy (ART) (31, 39–43). These disease risks are further increased in co-infected subjects with elevated $CD4^+$ T cell activation ($CD38^+HLA\text{-}DR^+$) (44), which are mostly CMV-reactive (45) and are reduced by anti-CMV therapy (46). Indeed, CMV-reactive $CD4^+$ CX_3CR1^+ T cells have been proposed as potential mediators of these comorbidities (36, 47, 48). Increased magnitudes of $CD4^+$ CX_3CR1^+ T cells positively correlate with arterial stiffness (49), and these populations significantly decrease in magnitude after anti-CMV therapy (50). However, the specific epitopes and activation phenotype of these CMV-reactive $CD4^+$ CX_3CR1^+ T cells remain unknown.

We propose a model where HIV^+ $HCMV^+$ co-infection increases stochastic, nonproductive HCMV reactivation that drives CD4 memory inflation. We hypothesized that HLA-DR7-restricted DYS-specific (DYS^+) $CD4^+$ T cells from HIV^+ $HCMV^+$ $DR7^+$ subjects undergo increased memory inflation compared to similar cells from HIV^- $HCMV^+$ $DR7^+$ subjects, and these cells upregulate CX_3CR1, CD38 and HLA-DR. To test this hypothesis, we studied the *ex vivo* frequencies among subjects, response magnitudes and properties of DYS^+ $CD4^+$ T cells both in HIV^+ $HCMV^+$ long-term non-progressors (to avoid confounding effects of HIV-induced subclinical HCMV expression) and in HIV^- $HCMV^+$ individuals using DR7-restricted DYS (DR7:DYS) tetramer because cytokine-based assays can underestimate actual T cell response magnitudes and the expression of phenotypic markers can change after re-stimulation (51). The threshold for inflation was arbitrarily set at 1% of circulating $CD4^+$ T cells as there is no standard minimum in the literature.

Materials and Methods

Subjects

HIV^+ and HIV^- subjects were randomly recruited through the Vanderbilt Comprehensive Care Clinic (IRB 030005), Vanderbilt Stem-Cell Clinic (IRB 061215), and the Australian Red Cross (IRB 2011/02) after they signed consent forms authorized by respective IRBs. $HCMV^+$ or $HCMV^-$ status was determined by CMV IgG serology. Class II HLA-typing was performed at the Institute for Immunology and Infectious Diseases (Perth, Western Australia), or at DCI Tissue Typing Laboratory (Nashville, TN). PBMCs were isolated, frozen and thawed as previously described (52).

Tetramers and peptides

The NIH Tetramer Core Facility (contract HHSN272201300006C) synthesized DRB1*07:01-restricted PE-, APC-, and BV421-conjugated HCMV $gB_{217–227}$ |

DYSNTHSTRYV; HCMV $pp65_{177–191}$ | EPDVYYTSAFVFPTK (EPD) (53); Human $CLIP_{87–101}$ | PVSKMRMATPLLMQA; tetanus toxoid (TT) $precursor_{586–605}$ | LINSTKIYSYFPSVISKVNQ (LIN) (54) and HIV $gag_{293–312}$ | FRDYVDRFYKTLRAEQASQE (FRD) (55) tetramers. DRB1*07:01:PRSPTVFYNIPPMPLPPSQL (DR7:PRS, EBV $EBNA2_{276–295}$) tetramer was synthesized by Beranoya Research Institute (Seattle, WA) (56). A2:NLVPMVATV (A2:NLV, CMV $pp65_{495–503}$) tetramer was synthesized as described (57). The NIH AIDS Reagent Program, Division of AIDS, NIAID, provided HCMV pp65 Peptide Pool (overlapping 15-mers; #11549), HIV-1 PTE Gag Peptide Pool (overlapping 15-mers; #12437), and CMV AD169 strain (#1910). Lyophilized DYS, EPD, FRD, PRS and LIN peptides, and 19 overlapping, high DR7-affinity HIV gag peptides from the HIV-1 PTE Gag Peptide Pool (predicted by NetMHCII 2.2 Server) were synthesized at ≥98% purity (GenScript).

Flow cytometry

Cryopreserved PBMCs were thawed, washed in PBS (Corning), and disentangled with Nuclease S7 (Roche). Depending on assay, PBMCs were left untouched or negatively-enriched for $CD4^+$ T cells (Miltenyi Biotec). Using our modified version of a class II tetramer stain protocol (56), we first stained for dead cells (Life Technologies), and washed with human Ab serum (Corning). Next, we stained with pre-titrated tetramer volumes at 37°C (1 h), anti-CCR7 Ab at 37°C (20 min), and room temperature surface protein stain (20 min) and, if necessary, intracellular Ab stain (20 min) at room temperature after fixation and permeabilization (BD). mAbs included CCR7-BV421 (150503), CD3-BV711 (UCHT1), CD4-PerCP-Cy5.5 (RPA-T4), CD45RO-PE-CF594 (UCHL1), CD27-PE-Cy7 (M-T271), CD14-V500 (M5E2), CD19-V500 (HIB19), IFN-γ-FITC (B27), TNF-α-PE-Cy7 (MAb11), CD4-FITC (SK3), Bcl-2-PE (Bcl-2/100), Granzyme-B-FITC (GB11), CX_3CR1-PE (2A9-1), Ki-67-PE-Cy7 (B56), CD57-FITC (NK-1), CD45RA-PE-Cy7 (L48), CD38-PE-Cy7 (HIT2), and HLA-DR-FITC (G46-6) that were ordered from BD; CD8-APC-AF750 (3B5) from Invitrogen; PD-1-PE (EH12.2H7) from BioLegend; TIGIT-PE-Cy7 (MBSA43) and CD28-PE-Cy7 (CD28.2) from eBiosciences; and custom CD127-PE-Cy5.5 (R34.34) from Beckman Coulter. Cells were sorted with FACSAria-IIIu (BD) or collected on LSR Fortessa (BD). FlowJo (v10.1r5; Tree Star) was used for analysis. Only background-subtracted, $tetramer^+$ response magnitudes 3× > the respective CLIP tetramer response magnitudes were considered positive. Median fluorescence intensity (MFI) analyses were done only on samples stained and collected the same day.

ELISpot

IFN-γ ELISpot was conducted on a BioMek FX^P high-throughput platform (Beckman Coulter) using the Human IFN-γ $ELISpot^{BASIC}$ (HRP) kit (Mabtech). Cryopreserved PBMCs were thawed and rested overnight in R10 media (52) before triplicate stimulations of 150,000–250,000 cells/well in MultiScreen-IP Filter Plates (Millipore) with no peptide, or with 0.001 μg/μl final concentrations of DYS, EPD, FRD, PRS or LIN epitope, or HCMV pp65 peptide pool, HIV-1 PTE Gag peptide pool, or anti-human CD3 (Mabtech). IFN-γ spot-forming units were developed using tetramethylbenzidine (Mabtech) and counted with ELISpot reader (AutoImmun Diagnostika) after drying. Positive spots = data > background mean + 3 times background SEM (38).

Bulk-cell TCR sequencing

TCRβ gene sequencing of DNA extracted (#DC6701; Promega) from bulk-sorted T_{EM} or T_{EMRA} DYS^+ and DYS^- $CD4^+$ T cells was completed and bioinformatically analyzed by Adaptive Biotechnologies. CDR3 proportions, productive template fractions and clonalities, and V(D)J segments were analyzed on ImmunoSEQ Analyzer v3.0. Circos plots were generated using VDJtools and *circlize* (58, 59). The TCR CDR3 data have been deposited in the NCBI Sequence Read Archive repository (https://trace.ncbi.nlm.nih.gov/Traces/study/?go=home) under study accession number SRP113337 and in VDJDB (https://vdjdb.cdr3.net).

TCR artificial expression and stimulation by LCL-pulsed epitopes

TCRα and TCRβ CDR3 sequences of Subject 10027's DYS^+ sorted single-cells were determined using a previously published technique (60). Briefly, single cells were sorted into separate wells in a 96-well plate containing RT-PCR buffer using FACSAria-IIIu cell sorter (BD) for three rounds of PCR amplification using nested, barcoded, TCR-specific and Illumina Paired-End primers (60). Purified PCR products were sequenced on Illumina MiSeq platform. After sequence analyses, TCRα and TCRβ products with identical barcodes were selected for full TCR gene completion using the international ImMunoGeneTics database. Following published methods (61), the DYS-specific TCRα and TCRβ genes were cloned into pSELECT-GFPzeo plasmids (InvivoGen) and expressed in Jurkat cells (clone E6-1, TIB-152; American Type Culture Collection) along with pNFAT-Luciferase (Affymetrix). These cells and lymphoblastoid cell lines (LCLs) were maintained in R10 media (52). DYS or control epitopes were pulsed with the LCLs to stimulate the DYS-specific TCRs expressed on the Jurkat cells. Luciferase absolute light units were measured using FilterMax F5 Multi-Mode Microplate Reader (Molecular Devices). The TCRα and TCRβ CDR3 data have been deposited in the NCBI Sequence Read Archive repository (https://trace.ncbi.nlm.nih.gov/Traces/study/?go=home) under study accession number SRP113337 and in VDJDB (https://vdjdb.cdr3.net).

Droplet digital PCR (ddPCR)

ddPCRs were performed entirely using a QX200 AutoDG Droplet Digital PCR System (Bio-Rad). For both assays, plates of droplets of PCR mixture were automatically generated with an Auto-droplet generator/AutoDG and TaqMan oil for probes, heat-sealed and amplified with C1000 Touch thermal cycler. Droplets were read using a Droplet Reader. The magnitude of false-positive responses in no-template controls was 15% (data not shown). DNA concentrations±95% CI were determined from only wells with >12,000 droplets using QuantaSoft v1.7.4.0917 after manually setting the positive droplet threshold above the negative droplet signal of the no template controls in the same plate. Primers and probes concentrations: 900nM and 250nM, respectively. All PCRs were multiplexed with *RPP30* housekeeping gene. HCMV DNA quantitation: 20μl PCR mixture was prepared using DNA, ddPCR SuperMix for Probes, water and these CMV primers and probes: *IE1*-specific forward primer 5′-TGAAGCGCCGCATTGA, *IE2*-specific reverse primer 5′-TGGCCCGTAGGTCATCCA, and *IE1*-specific probe 5′-6FAM-TCTGCATGAAGGTCTTTGCCCAGTACATCC-TAMRA. Thermocycling conditions: 50°C

(2 min), 95°C (10 min), 40 cycles of 95°C (15 s) with 60°C (1 min) (2°C/s ramp rate). Modified HIV DNA quantitation (62): 20μl PCR mixture was prepared similarly, but using these HIV *LTR* primers and probes instead: forward primer 5′-AGCACTCAAGGCAAGCTTTA, reverse primer 5′-TGTACTGGGTCTCTCTGGTTAG, and probe 5′-FAM-GCAGTGGGTTCCCTAGTTAGCCAGAGAG-3IABkFQ. Thermocycling conditions: 95°C (10 min), 40 cycles of 94°C (30 s) with 60°C (1 min), and 98°C (10 min) (2°C/s ramp rate).

Statistics

GraphPad Prism v7.0a was used for non-parametric, two-tailed analyses of *Wilcoxon matched-pairs signed rank test* (paired), *Mann-Whitney U test* (non-paired), and *Spearman's rank correlation (ρ)* (linear regression). *$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$.

Results

HLA-DR7-restricted DYS$^+$ CD4$^+$ T cells are inflated in HIV$^+$ HCMV$^+$ DR7$^+$ compared to HIV$^-$ HCMV$^+$ DR7$^+$ subjects

Following the gating hierarchy in Supplemental Fig. 1, we verified tetramer specificity using DR7:CLIP tetramer stain of CD4-enriched PBMCs from time-point 1 (tp1; no ART) of 8 HIV$^+$ HCMV$^+$ DR7$^+$ subjects (Table I, and Fig. 1A). We also confirmed the HLA-DR7 restriction of the response by staining CD4-enriched PBMCs from 7 co-infected subjects lacking HLA-DR7 allele i.e. DR7$^-$ (Supplemental Fig. 2A). To determine the HLA-DR7$^+$ DYS$^+$ CD4$^+$ T cell response magnitude in the HIV$^+$ HCMV$^+$ DR7$^+$ subjects, the tp1 CD4-enriched or untouched PBMCs were stained with DR7:DYS tetramer. We detected high DYS$^+$ CD4$^+$ T cell magnitudes of response in 7 subjects (0.43–17.91%), with 6 of them displaying inflated responses (Fig. 1A). To determine if DYS$^+$ CD4$^+$ T cell inflation was abrogated after ART-induced HIV suppression, we stained aviremic PBMCs from later time-points (9.5 years median time lapse) of 4 co-infected individuals (time-point 2, tp2, in Table I) with the tetramer and detected values ranging from 0.11–26.34% (Fig. 1B). Inflation magnitude did not correlate with age (possibly due to small sample size, which could be increased in future studies), HIV infection duration, nadir CD4 count or HIV load (data not shown).

Importantly, we observed significantly lower magnitudes of DYS$^+$ CD4$^+$ T cells (0.01–1.32%) in 10 HIV$^-$ HCMV$^+$ DR7$^+$ subjects (Fig. 1C) compared to those of the HIV$^+$ HCMV$^+$ DR7$^+$ individuals (median 0.06% vs. 4.76%, P=0.004; Fig. 1D). Samples from tp1 of Subject 10013, and tp2 of Subjects 10004, 10027 and 10032 were used in this and all future experiments unless otherwise indicated. CD4 counts of the HIV$^-$ cohort were unavailable for absolute DYS$^+$ CD4 count comparison. To confirm that these cells are undergoing memory inflation, we analyzed DYS$^+$ CD4$^+$ T cells from five time-points of two HIV$^+$ DR7$^+$ subjects spanning a period of up to twelve years and detected stable magnitudes and absolute counts (Figs. 1E and 1F, respectively). Together, these findings identify inflated CD4$^+$ T cells against HLA-DR7-restricted DYS epitope of HCMV gB in HIV$^+$ HCMV$^+$ co-infected subjects.

HLA-DR7-restricted CD4+ T cells responses to other persistent and non-persistent epitopes are low in HIV+ HCMV+ DR7+ subjects

Tetramer stains of $CD4^+$ T cells specific for a DR7-restricted, highly conserved HCMV pp65 EPD epitope, which were absent in HIV^+ $HCMV^+$ $DR7^-$ subjects (Supplemental Fig. 2B), revealed significantly lower magnitude ranges in the HIV^+ $HCMV^+$ $DR7^+$ cohort (0.01–1.87%, Supplemental Fig. 2C) compared to inflated DYS^+ $CD4^+$ T cells in this cohort (median 0.04% vs. 4.76%, P=0.02; Fig. 1D). We did not detect any memory inflation of these EPD^+ $CD4^+$ T cells in four longitudinal samples obtained from the two subjects with the highest DYS-specific inflation over a twelve-year or less period (Figs. 1E and 1F). A similar trend was observed in the HIV^- $HCMV^+$ $DR7^+$ cohort between EPD^+ (0.003–0.04%, Supplemental Fig. 2D) and DYS^+ $CD4^+$ T cells (median 0.001% vs. 0.06%, P=0.03; Fig. 1D). As observed for DYS^+ $CD4^+$ T cells, EPD^+ CD4 response magnitudes were also significantly higher in HIV^+ $HCMV^+$ $DR7^+$ than in HIV^- $HCMV^+$ $DR7^+$ cohort (P=0.002; Fig. 1D), confirming a recent report using pp65 peptide pools instead (63). To determine whether the inflation could be due to generalized HIV-induced inflammation, we compared the magnitudes of $CD4^+$ T cells specific for DR7-restricted TT, EBV or HIV epitopes from the HIV^+ $DR7^+$ subjects to their DYS^+ $CD4^+$ T cell counterparts. We observed that the magnitudes of these other epitopes were undetectable or very low compared to the inflated DYS-specific response (P=0.0078 for each comparison; Supplemental Fig. 2E). To evaluate potential $CD8^+$ T cell inflation, we stained PBMCs from Subject 10027, who has the highest DYS^+ CD4 inflation (26.34%) and carries a HLA-A2:01 allele with A2:NLV tetramer, but detected a magnitude of only 0.75% to this epitope (Supplemental Fig. 2F). Collectively, these results indicate that other $DR7^+$ epitope-specific $CD4^+$ T cells in most co-infected subjects are present at lower magnitudes than DYS^+ $CD4^+$ T cells.

DYS+ CD4+ T cells consist of T_{EM} and/or T_{EMRA} subsets

We determined the memory phenotype of DYS^+ $CD4^+$ T cells by measuring surface expression of memory markers CD45RO, CCR7 and CD27 to define T_{EM}, T_{EMRA}, central (T_{CM}; $CD45RO^+$ $CCR7^+$ $CD27^+$), transitional (T_{TM}; $CD45RO^+$ $CCR7^-$ $CD27^+$), naïve (T_{Nai}; $CD45RO^-$ $CCR7^+$ $CD27^+$), and intermediate (T_{Int}; $CD45RO^-$ $CCR7^-$ $CD27^+$) subsets (Supplemental Fig. 1) (10). Compared to the non-DYS^+ (DYS^-) $CD4^+$ T cells, DYS^+ $CD4^+$ T cells from HIV^+ $HCMV^+$ $DR7^+$ subjects were biased toward T_{EM} (46.6–97.97% vs. 6.1–51.9%, P=0.0156) and T_{EMRA} (0.03–48.1% vs. 0.4–16.7%, P=0.0781) (Fig. 2A), and similar observations were made in the HIV^- $HCMV^+$ $DR7^+$ cohort (Fig. 2B). Most $CD45RO^-$ DYS^+ and DYS^- $CD4^+$ T cells were $CD45RA^+$ as shown in Subject 10027 (Fig. 2C).

DYS-stimulated CD4+ T cells secrete IFN-γ and TNF-α

Most HIV^+ $HCMV^+$ $DR7^+$ PBMC samples stimulated with DYS produced IFN-γ in high-throughput ELISpot, and only Subject 10013 responded to EPD (Fig. 2D), indicating that tetramer staining was more sensitive or that the cells were dysfunctional. Subject 10004 did not respond to either epitope possibly due to dysfunction or anergy. 1 of 3 screened HIV^- $HCMV^+$ $DR7^+$ subjects responded to DYS stimulation (Fig. 2E). Responses to HIV FRD epitope, EBV PRS epitope or TT LIN epitope were relatively diminished in HIV^+ $DR7^+$ subjects compared to DYS-induced responses and not detected in HIV^- $DR7^+$ subjects

(Figs. 2D and 2E, respectively). Dual IFN-γ and TNF-α intracellular cytokine staining of Subject 10027's PBMCs, which produced the largest IFN-γ ELISpot response to DYS, confirmed that the ELISpot responses originated from CD4s and not CD8s, and suggested that these inflated cells were likely polyfunctional (Fig. 2F), as previously reported (11, 35).

Inflated DR7$^+$ DYS$^+$ CD4$^+$ T cells have highly restricted TCRβ repertoires

TCR analyses were conducted only on subjects with adequate DYS$^+$ CD4 magnitudes of response for bulk cell sorting and sequencing: HIV$^+$ Subjects 10027, 10040, 10069 and 10032 (26.34%, 8.95%, 17.91% and 7.52%, respectively) and HIV$^-$ Subject 20 (1.32%) as a control. We observed highly restricted TCR-beta-variable (TCRβV) and -joining (TCRβJ) gene pairing in bulk-sorted T_{EM} and T_{EMRA} subsets of DYS$^+$ CD4$^+$ T cells compared to the more diverse DYS$^-$ counterparts in all subjects (Fig. 3, A – E). The dominant TCRβV and TCRβJ gene families of each individual's DYS$^+$ CD4$^+$ T cells comprised 69.5% to 99.7% of the DYS$^+$ CD4$^+$ T cell repertoire, and were identical between their T_{EM} and T_{EMRA} subsets (Supplemental Fig. 3, A – E). However, the dominant TCRβV and TCRβJ gene families of DYS$^-$ CD4$^+$ T cells were lower (10.42–56.46%) and different between T_{EM} and T_{EMRA} subsets (Supplemental Fig. 3, A – E). These findings indicate a strong TCRβ conservation among inflated DYS$^+$ CD4$^+$ T cells.

Inflated, DR7$^+$ DYS$^+$ CD4$^+$ T cells utilize nearly monoclonal CDR3s

We analyzed the CDR3 repertoires of productive V(D)J rearrangements (in-frame and without stop codons) of the bulk-sorted DYS$^+$ CD4$^+$ T cells and observed that they were dominated by specific clones with unique V(D)J rearrangements (69.41–99.64%, median=91.37%) (Fig. 4, A – E). Interestingly, we discovered that 97.29% and 7.6% of the productive DYS$^+$ CD4$^+$ T_{EM} CDR3 repertoires of Subjects 20 (HIV$^-$) and 10069 (HIV$^+$), respectively, were identical. T_{EM} CDR3 analysis of a HIV$^-$ HCMV$^-$ subject showed no clonal expansion (data not shown), suggesting that clonal expansion among DYS$^-$ T_{EM} CDR3s might be tied to HCMV$^+$ status. The DYS$^+$ and DYS$^-$ CDR3 clonal dominance reflected their respective TCRβ gene-family distributions. DYS$^+$ T_{EM} and T_{EMRA} dominant clones within each subject were identical, and this is likely a reflection of the reversible T cell differentiation from T_{EM} (CD45RO$^+$ CD45RA$^-$) to T_{EMRA} (CD45RO$^-$ CD45RA$^+$) (11).

In vivo stimulation of inflated cells involves NFAT-mediated cellular activation and proliferation upon TCR ligation by peptide-MHC. To confirm this activity and the accuracy of the clonal CDR3 sequence, we simulated the antigen presentation conditions for Subject 10027 using autologous B cell-derived LCLs and the DYS epitope to stimulate autologous DYS$^+$ α:β TCR expressed on Jurkat cells with an NFAT-mediated luciferase reporter. Using single-cell sorting and TCR sequencing, we first determined the paired α:β TCR CDR3 sequences of autologous DYS$^+$ CD4$^+$ T cells to be TCRα CAGRSSNTGKLIF CDR3 (TCRαV25 and TCRαJ37), and TCRβ CASIHQGSTEAFF CDR3 (TCRβV6-5 and TCRβJ1-1) that matched Subject 10027's nearly monoclonal CDR3 sequence (Fig. 5A). After TCR expression and stimulation with autologous, DYS-pulsed DR7$^+$ LCLs, we detected a dose-dependent luciferase luminescence that was not present with no epitope, a different epitope (EPD) or a DR7$^-$ LCL (Fig. 5A). Subject 10069's LCL (DR7, DR8) confirmed that DYS was presented by DR7 and not DR3, which was the other DRB1 allele

of Subject 10027 (Fig. 5A). Clonality comparison revealed that DYS^+ $CD4^+$ T_{EM} and T_{EMRA} cells were significantly more clonal compared to DYS^- counterparts, and almost monoclonal in Subject 10032 (P=0.0078; Fig. 5B). We verified the expectation of more productive V(D)J rearrangements within DYS^+ compared to DYS^- $CD4^+$ T cells since the former were sorted based on specific HLA-restricted epitope recognition (P=0.0078; Fig. 5C). These results suggest that DYS^+ $CD4^+$ T cells are inflated via a highly clonal mechanism that likely involves DYS stimulation.

Different DYS^+ CDR3 clones share conserved amino acid

We assessed whether potential amino acid conservation among the different, dominant DYS^+ CDR3s of all subjects could explain their common recognition of DYS. Remarkably, our V(D)J alignments revealed two new conserved amino acids (serine (S), and threonine (T)) within the D-segments in addition to the published glutamine (Q) (64), all of which have polar, neutral side chains (Table II). We did not see a similar conservation among DYS^- CDR3 clones (Table III). These findings indicate that amino acids with polar and neutral side chains might be critical in DYS recognition.

Inflated $DR7^+$ DYS^+ $CD4^+$ T cells are $CD127^-$ $TIGIT^-$ and Granzyme B^+

We measured plasma HCMV DNA load of all subjects but detected no viral DNA despite being $HCMV^+$, suggesting that inflation of these circulating cells was not due to ongoing HCMV replication in the blood. Other samples such as saliva and semen in which active HCMV replication has been reported were unavailable for testing. We next determined whether these cells displayed similar $CD127^-$ $PD\text{-}1^-$ $TIGIT^-$ granzyme B^+ phenotype of inflated CMV-specific $CD8^+$ T cells (1, 14, 16). For all onward comparison experiments of inflated DYS^+ $CD4^+$ T cells (n=7), we used HIV^- $HCMV^-$ controls (n=10) to provide contrast with classical T_{EM} cells and avoid other potential inflationary HCMV epitope-specific responses, and also because HIV^+ $HCMV^-$ subjects are extremely rare. We focused on only T_{EM} because DYS^+ T_{EMRA} was present in only 5 HIV^+ $HCMV^+$ $DR7^+$ subjects. We compared CD127, PD-1, and TIGIT expressions on DYS^+ $CD4^+$ T_{EM} to $CD4^+$ T_{EM} from HIV^- $HCMV^-$ controls, and detected significantly lower CD127 (P=0.025), no difference in PD-1 (P=0.364), and significantly lower TIGIT (P=0.0001) among the inflated cells (Fig. 6, A – C). The dual IFN-γ and TNF-α secretions in Fig. 2F also suggest these cells are not exhausted. To further determine polyfunctionality, we compared *ex vivo* intracellular granzyme B levels of DYS^+ $CD4^+$ T_{EM} from the HIV^+ $HCMV^+$ $DR7^+$ subjects to controls, and detected significantly higher levels with DYS specificity (P=0.0001; Fig. 6D), confirming previous cytotoxicity (11, 28, 35, 38, 64) and polyfunctionality (11, 35) reports for DYS^+ $CD4^+$ T cells. B-cell lymphoma-2 (Bcl-2) protein MFI of inflated DYS^+ $CD4^+$ T cells were not different compared to controls (P=0.536; Fig. 6E). None of these protein levels correlated with the magnitude of DYS^+ CD4 inflation (data not shown). These findings reveal that inflated DYS^+ $CD4^+$ T cells are $CD127^-$ $PD\text{-}1^{+/-}$ $TIGIT^-$ and Granzyme B^+.

Inflated DR7^{+} DYS^{+} CD4^{+} T cells are CX_3CR1^{high} and are not undergoing higher proliferation

Latent HCMV reservoirs present endogenous DYS epitopes to DYS^{+} CD4^{+} T cells (28, 35, 65). Therefore we hypothesized that such reservoirs expand with HIV co-infection to cause memory inflation. While we could not directly measure HCMV reservoir size, we further hypothesized that expanded HCMV latent reservoirs, including VECs, would express more CX_3CL1 and consequently correlate with higher expression of CX_3CR1 on inflated DYS^{+} CD4^{+} T cells. Indeed, these cells had significantly higher CX_3CR1 MFI compared to controls (P=0.0007; Fig. 7A), confirming previous reports (11). To determine whether effectual TCR stimulation by DYS-presenting latent reservoirs caused ongoing proliferation *in vivo* and by extension memory inflation, we measured *ex vivo* Ki-67^{+} levels within inflated DYS^{+} CD4 T_{EM} and detected a slightly wider range of, but not significantly higher, magnitudes compared to controls (P=0.474; Fig. 7B), confirming studies on MCMV epitope-specific CD8^{+} T cell inflation (5, 9). Using CD57 and CD28 dual staining of Subject 10027's tp2 PBMCs, we observed that <2% of CD4^{+} DYS^{+} T cells displayed the CD57^{+} CD28$^{+/-}$ phenotype for replicative senescence (Fig. 7C) (66). CX_3CR1 and Ki-67 levels did not correlate with DYS^{+} CD4 inflation magnitudes (data not shown). These findings suggest that inflated DYS^{+} CD4 T cells might interact with HCMV reservoirs that express CX_3CL1 and their inflation is not linked to increased ongoing proliferation.

Inflation is not caused by DR7-restricted HIV gag epitope cross-reactivity

TCR cross-reactivity is ubiquitous and can occur between unrelated pathogens including HIV (gag) and influenza A virus (67). To assess cross-reactive TCR role in inflation, we repeated the HLA-epitope-TCR simulation experiment using 19 high-affinity DR7-restricted HIV gag epitopes instead, but detected no response (Fig. 7D). Also, HIV viremia was not associated with a significant increase in inflation compared to aviremia (P=0.625; Fig. 7E). These findings suggest that the inflation is not likely caused by cross-reactive HIV gag epitopes.

We also investigated whether HCMV might latently infect the inflated CMV-specific memory CD4^{+} T cells it induces. We optimized ddPCR quantitation of HCMV DNA using HCMV AD169 strain, but did not detect any HCMV DNA in DYS^{+} CD4^{+} T cells (Supplemental Figs. 4A and 4B).

Inflated DYS^{+} CD4^{+} T cells display elevated levels of CD38 or HLA-DR, but less often co-express CD38 and HLA-DR

We measured CD38 and HLA-DR dual and individual expression on inflated DYS^{+} CD4^{+} T cells. Although we observed no significant difference in their CD38^{+}HLA-DR^{+} co-expression magnitude compared to controls (P=0.091; Fig. 8A), we observed significantly higher levels of CD38^{+}HLA-DR^{+} co-expression on their DYS^{-} T_{EM} counterparts within the HIV^{+} HCMV^{+} cohort compared to controls (P=0.0001; Fig. 8A). Additionally, individual protein analyses revealed significantly higher levels (P=0.033 and P=0.033; Figs. 8B and 8C, respectively). Remarkably, there was a stepwise increase in the mean expressions from DYS^{-} T_{EM} of HIV^{-} HCMV^{-} to DYS^{-} T_{EM} of HIV^{-} HCMV^{+} to DYS^{-} and DYS^{+} T_{EM} of HIV^{+} HCMV^{+} subjects. These protein levels on DYS^{+} CD4^{+} T cells did not correlate with

the magnitudes of DYS^+ CD4 inflation (data not shown). CD38 was elevated on naïve T cells as expected (68). Finally, we quantified the latent HIV DNA in Subject 10027's DYS^+ $CD4^+$ T cells but did not detect any enrichment both without and with ART compared to the DYS^- counterparts, despite the inflation (P=0.25 and P=0.031, respectively; Supplemental Fig. 4C). HIV was not detected in enriched EPD^+ $CD4^+$ T cells, but low cell numbers might have limited the sensitivity. Overall, these findings indicate that inflated DYS^+ $CD4^+$ T cells do contribute to the increased T cell activation associated with higher risk of HCMV-related non-AIDS comorbidities in HIV^+ $HCMV^+$ subjects, but further studies are required to define the specific subsets of activated cells that correlate most closely with these adverse outcomes.

Discussion

Here, we show memory inflation of HLA-DR7 restricted, HCMV epitope-specific $CD4^+$ T cells in $HCMV^+$ long-term non-progressor HIV subjects that could potentially contribute to the higher T cell activation associated with elevated risks of HCMV-related non-AIDS cardiovascular comorbidities in such co-infected patients. *Ex vivo* DR7:DYS tetramer stains revealed persistent, inflated percentages of HCMV's DYS^+ $CD4^+$ T cells in our HIV^+ $HCMV^+$ $DR7^+$ subjects that consisted of mostly T_{EM} and T_{EMRA} subsets, and secreted IFN-γ and TNF-α upon *in vitro* DYS stimulation of their nearly monoclonal TCR repertoires. The 28.75% DYS^+ CD4 response magnitude of Subject 10027 measured in the fourth time-point of the longitudinal analyses is the largest reported CD4 response magnitude against DYS epitope to our knowledge, and is similar to the 24% magnitude of a DQ6-restricted $pp65_{41–55}$ LLQTGIHVRVSQPSL-specific CD4 response (11), although the HIV status of the subject was not specified. It is not clear why Subject 10030 had an extremely low DYS^+ $CD4^+$ T cell magnitude. We doubt this was due to CMV sequence variation because the DYS epitope and adjacent residues involved in proteosomal cleavage are known to be completely conserved.

Our findings represent the first *ex vivo* and tetramer-based evidence for CD4 memory inflation in HIV^+ subjects and it is striking in frequency and magnitude for the DYS epitope. It is quite remarkable that $DR7^+$ CD4 responses to other HCMV, TT, EBV or HIV epitopes analyzed in the same cohort were significantly lower. The reduced magnitude of IFN-γ responses to stimulations by these additional epitopes was reflected in the lower absolute counts among the stimulated cells in the ICS assay. Therefore, we believe that the inflation of DYS-specific $CD4^+$ T cells is more likely due to specific HLA-epitope-TCR interactions, and unlikely to be due to HIV-induced inflammation. This is also supported by the finding of a highly enriched CDR3 clonotype in HIV^- Subject 20. Also, it does not appear that this is an intrinsic feature of persistent viruses, as EBV epitope-specific response were of low magnitude or absent within the same individuals. Although we were unable to measure these responses among the HIV^- $DR7^+$ cohort due to IRB restrictions on re-inviting the subjects, we believe their magnitudes will similarly be low or undetectable. The EPD^+ $CD4^+$ T cell response was of unusually high magnitude in the HIV^+ Subject 10013 at both tp1 (1.87%) and tp2 (1.4%, data not shown). This individual was also the only subject in whom secreted IFN-γ was detected upon EPD stimulation. These findings suggest that the EPD epitope may drive a memory-inflated response in HIV, and we cannot exclude the possibility that

EPD might not also drive an inflated rarely response in HIV^- individuals. Interestingly, low-level DYS^+ $CD4^+$ T cell responses were detected in all HIV^- $HCMV^+$ $DR7^+$ subjects with the exception of one subject with 1.32% magnitude of response. The observation that one HIV^- subject had DYS^+ $CD4^+$ T cell inflation, which was predominantly T_{EM} phenotype, illustrates that memory inflation with this epitope can occur in HIV^- subjects. However, the prevalence and magnitude of memory inflation was substantially higher with HIV co-infection. Taken together the findings suggest that $CD4^+$ memory inflation can occur in HIV negative individuals but HIV acts to increase the prevalence and magnitude.

It is not fully understood how CMV, and why only CMV, induces chronic memory inflation and why this property has been conserved in mice (5–9), rhesus macaques (4) and man (2, 3). The inflated DYS and comparatively lower EPD responses in five HIV co-infected subjects parallel recent reports of different epitopes from the same protein inducing both high- and low-magnitude responses (5, 8). Potential explanations for DYS-specific inflation include the translation of gB mRNA without HCMV replication (69), gB colocalization to endosomes and endogenous presentation (28, 35), and the secretion of such gB epitope-loaded endosomes as immunogenic exosomes (36, 37). Endogenous epitope processing and presentation has been demonstrated recently to drive CD8 memory inflation (6, 27). However, the low pp65 EPD-specific responses might be due to pp65 polyprotein absence in immunogenic exosomes (37). Yet, this mechanism does not explain the published DQ6-restricted $pp65_{41–55}$ LLQTGIHVRVSQPSL epitope-induced inflation (11), suggesting that multiple factors underlie inflation. Differential gene expression patterns (70), and the presence of higher avidity TCRs specific for DYS might also play some role. It is important to note that cytotoxic $CD4^+$ T cells in general are elevated in HIV infection (71). This may be due to low CCR5 expression, especially by CMV-specific $CD4^+$ T cells, which protects such cells from HIV infection and might explain the lack of HIV DNA enrichment in our results (72).

Few studies have described the TCR repertoire of HLA class II-restricted epitope-specific CD4 responses based on tetramer sorted cells, and most were conducted *in vitro* or without TCR sequencing (55, 73–83). Notably, some of the repertoires of these single epitope-specific T cells are diverse with over six unique, dominant TCR gene families (55, 73, 80, 83). Therefore, to our knowledge, our work represents the first combination of *ex vivo*, class II tetramer-derived and deep sequencing-based identification of a nearly monoclonal TCR repertoire of inflated HLA-restricted epitope-specific $CD4^+$ T cells at the resolution of the CDR3. We discovered three new DYS-specific TCRβV gene families: TCRβV6-2, TCRβV5-6 and TCRβV28 in addition to the published TCRβV6-5 (64). CDR3 sequencing confirmed that the inflations were driven by nearly monoclonal expansions, especially in Subject 10032 where 99.4% of all DYS^+ T_{EM} were a single clonotype. HIV^- Subject 20's DYS^+ $CD4^+$ T cell clonality indicates that clonal expansion to DYS was not unique to HIV^+ subjects, a finding that again suggests that HIV co-infection is not necessary for, but rather increases the likelihood of, and amplifies DYS^+ $CD4^+$ T cell inflation. To determine if the inflation was unique to DYS, a comparison between bulk DYS^+ CDR3s and those of EPD, LIN, PRS, or FRD epitope would have been sufficient; however, magnitudes of cells specific to these additional epitopes were too low for that analysis. CDR3 sequences within DYS^- CD4 samples were generally polyclonal with a few exceptions. This noteworthy observation

might be driven by clonal expansions induced by epitopes that overlap with DYS as observed in HCMV IE1 epitopes (84), or to other inflation-inducing epitopes of HCMV or other pathogens. The presence of the dominant DYS^+ CDR3s within the DYS^- repertoire at relatively lower magnitudes reflected potential loss of tetramer binding, while the reverse could reflect non-specific binding to the DYS tetramer. The *in vitro* HLA-DR7-presented DYS stimulation of the inflated DYS^+ CD4 TCR in our Jurkat cell transfection system confirmed the specificity of the tetramer stain and accuracy of our bulk-cell and single-cell TCRα and TCRβ sequencing. We analyzed the dominant DYS^+ CDR3 sequences from different subjects for amino acid conservation as: (i) there are no reports of such conservation within inflationary $CD4^+$ T cell CDR3s, and (ii) even dominant clones of well-characterized, non-inflated HLA-A2:NLV CD8 responses from different individuals do not always contain conserved motifs (85). In addition to the published glutamine (64), we also discovered novel conservations of serine and threonine that preceded the germline glycine within the D-segment of the different, dominant DYS^+ CD4 CDR3 clones. These amino acids are polar with neutral side chains that might serve as TCR binding residue sites for hydrogen bond formation with DYS and HLA-DR7. Further verification by crystallographic reconstruction of the DR7-DYS-TCR complex is required.

Inflation may be due to intermittent, subclinical CMV reactivation or expression of specific transcripts. We detected no HCMV DNA in plasma samples from this cohort. Future studies could investigate this reactivation through monitoring of other specimens such as saliva, semen, etc. Although we were unable to determine the exact mechanism by which HCMV stimulates inflated responses in our human subjects, we analyzed *ex vivo* protein expressions of DYS^+ $CD4^+$ T cells and observed similarities ($CD127^-$ $PD\text{-}1^{+/-}$ $TIGIT^-$ Granzyme B^+) to those on inflated CMV-specific $CD8^+$ T cells reported to be maintained by low-level exposure to antigens from stochastic HCMV reactivation (1, 11, 16, 35). PD-1 might not be an appropriate co-inhibitory protein to evaluate on DYS^+ $CD4^+$ T cells due to their low levels of CD28 (64), which has been recently shown to mediate PD-1 suppression of T cells (86, 87). The normal expression levels of anti-apoptotic Bcl-2 suggest that DYS^+ $CD4^+$ T cell inflation is not due to apoptosis suppression, but might be due to other maintenance mechanisms such as longer telomeres (26) that could offset the normal rate of apoptosis. The persistence of inflated DYS^+ T_{EM} and T_{EMRA} subsets, despite the lack of $CCR7^+$ DYS^+ T cell thymic emigrants, is explained by reports that thymectomy does not affect memory T cell inflation or homeostasis (88).

Surprisingly, Ki-67 data suggests that the DYS^+ $CD4^+$ T cell inflations were not driven to significantly higher proliferation. While this observation might be due to cross-sectional sampling limitations, it does confirm findings in chronic MCMV models of inflation (5, 9). CD28 and CD57 analyses confirm that only a very limited number of the inflated cells are too senescent to replicate (66). The cause of inflation does not appear to involve DYS^+ $CD4^+$ TCR cross-reactivity with the $DR7^+$ HIV gag epitopes either. But, cross-reactivity with other $DR7^+$ HIV epitopes or to other inflated TCRs cannot be excluded. We observed increased inflation in two subjects when HIV replication was suppressed to undetectable levels with ART, suggesting that HIV replication is not required for maintenance of inflation.

The sites of HCMV latency are important (1) and HIV could alter the environment to help HCMV persist in long-lived non-hematopoietic cells in HCMV reservoir sites such as LNs and vascular endothelial cells (VECs). VECs can serve as latent HCMV reservoirs and also express the CX_3CR1 ligand—fractalkine. Vascular homing might bring CX_3CR1^{high} DYS^+ $CD4^+$ T cells in close contact with these potential HCMV reservoirs, resulting in re-stimulation and inflation. Although we did not detect HCMV DNA in the inflated cells, it is possible that they passively disseminate HCMV from LNs to vascular endothelium without getting infected, as LN DCs do for HIV. Herpes viruses such as CMV are species-specific and cause life-long infection. Therefore, it is also possible that the inflated responses provide a degree of protective immunity against other infections, making them mutually beneficial to CMV and its host.

Although $CD38^+$HLA-DR^+ co-expression on the inflated cells was not significantly elevated compared to controls, a wider distribution was observed with inflation. Remarkably, a similar comparison of $CD38^+$HLA-DR^+ co-expression on the DYS^- T_{EM} counterpart of the inflated cells to controls produced a significant difference. These DYS^- T_{EM}s consist of clonal CDR3 expansions (Fig. 4) that are potentially induced by other inflationary epitopes. Consequently, it is plausible that analyses of CD4 responses against a collection of inflationary epitopes or in a larger number of subjects might yield a difference. Both statistical trends are not due to generalized, HIV-induced activation because other pathogen/antigen specific $CD4^+$ T cells, including TT, are not necessarily more activated with HIV infection (45). This observation implies that inflated $CD4^+$ T cells in these subjects could potentially contribute to the increased T cell activation associated with greater risks of HCMV-related non-AIDS cardiovascular comorbidities that continue to plague HIV^+ subjects despite effective ART. The capacity of DYS^+ $CD4^+$ T cells to secrete granzyme B, IFN-γ and TNF-α might facilitate the development of these comorbidities (36, 47, 48). A larger cohort of HIV^+ $HCMV^+$ $DR7^+$ subjects with varying magnitudes of DYS^+ $CD4^+$ T cells is needed to directly evaluate the correlation of their activation with disease outcomes. The stepwise increments in $CD38^+$HLA-DR^+ levels indicate that HCMV infection without HIV co-infection increases $CD4^+$ T_{EM} activation in general, and HIV co-infection further synergizes such activation. This elevated activation might be tied to an HIV-induced latent HCMV reservoir expansion, presenting potential unintended negative consequences of HCMV vaccine candidates that contain inflation-inducing epitopes for all individuals, especially HIV^+ $DR7^+$ subjects. Although we studied HIV long-term non-progressors, a previous study found that CMV lysate induces high levels of CMV-specific $CD4^+$ T cells in HIV^+ subjects with ART-induced HIV aviremia (30), suggesting that our findings may generalize to a broader range of HIV-infected patients.

In conclusion, we have shown that HIV^+ $HCMV^+$ co-infection boosts CD4 responses to HCMV gB's DYS and pp65's EPD epitopes, resulting in mostly memory-inflated DYS^+ $CD4^+$ T cells. To our knowledge, this is the first *ex vivo* evidence of both $CD4^+$ T cell memory inflation against the DYSNTHSTRYV epitope in HIV^+ subjects and nearly monoclonal CDR3 repertoire of inflated $CD4^+$ T cells that contain novel conserved motifs. Although the underlying mechanism may be multifactorial, we hypothesize that increased low-level exposure and subsequent clonal expansion targeting the DYS epitope from stochastic HCMV reactivation or expression largely contributes to our observation. Our

findings suggest that “memory inflation”-inducing epitopes might contribute to the immunopathogenesis of non-AIDS comorbidities and raise safety implications for CMV vaccines that contain inflation-inducing epitopes that should be considered in trials being planned in both HIV and non-HIV infected subjects. This work also suggests that the relative contributions of conventional and inflated CMV-specific T cell responses to protection of the host from infection or malignancy, vaccine responsiveness or co-morbidities of aging such as vascular disease should be considered separately.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

DYS	DYSNTHSTRYV
HCMV	human cytomegalovirus
MCMV	murine cytomegalovirus
LN	lymph node
gB	glycoprotein B
DR7$^+$	HLA-DRB1*07:01
EPD	EPDVYYTSAFVFPTK
TT	tetanus toxoid
LIN	LINSTKIYSYFPSVISKVNQ
FRD	FRDYVDRFYKTLRAEQASQE
PRS	PRSPTVFYNIPPMPLPPSQL
MFI	median fluorescence intensity
ddPCR	droplet digital PCR
LCLs	lymphoblastoid cell lines
T_{EM}	effector memory

T_{EMRA}	effector memory-RA^+
VECs	vascular endothelial cells
pp65	phosphoprotein-65
ART	antiretroviral therapy
tp1	time-point 1
tp2	time-point 2
TCRβV	TCR-beta-variable
TCRβJ	TCR-beta-joining

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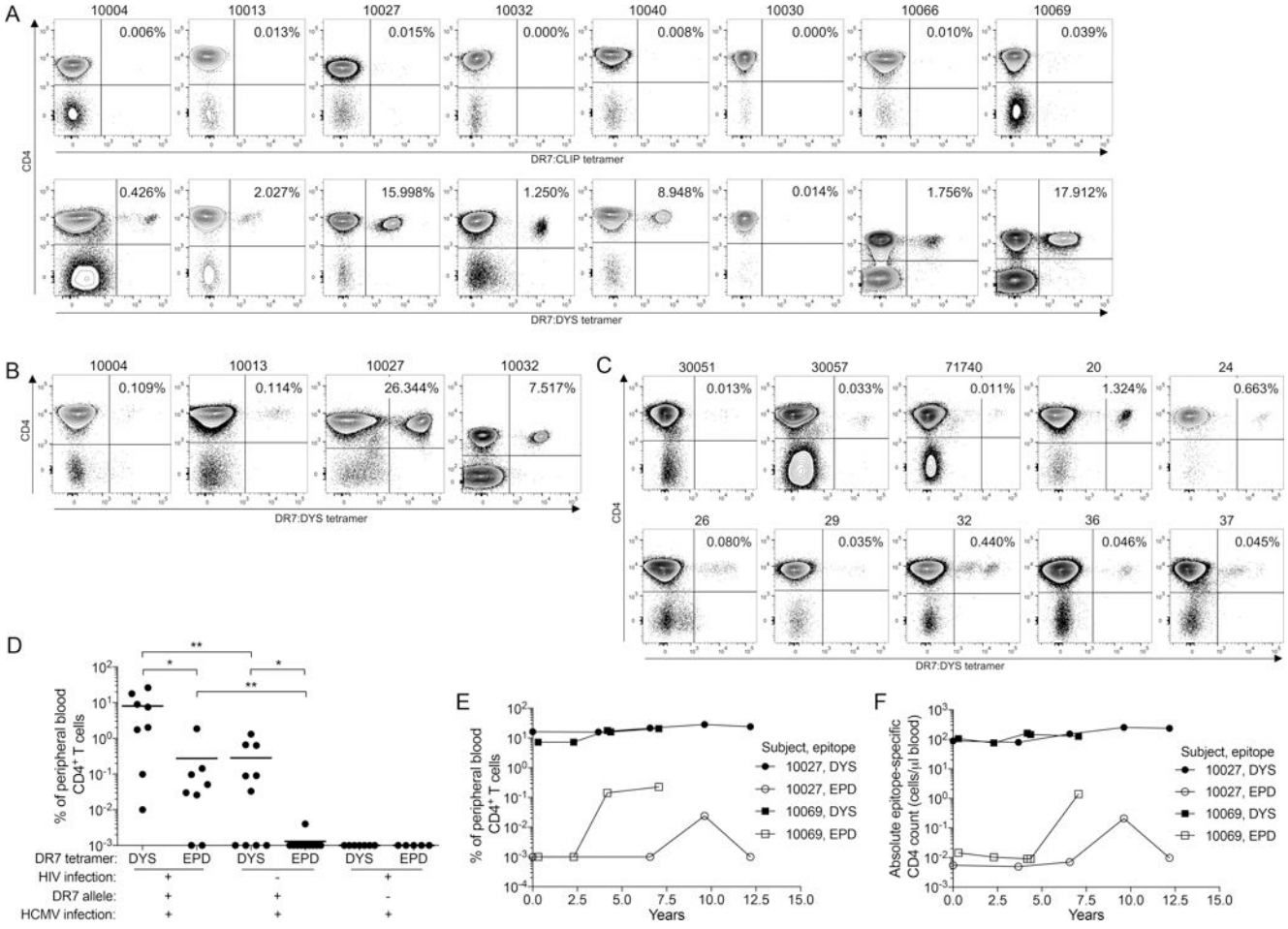

FIGURE 1.

HLA-DR7-restricted HCMV glycoprotein B DYS-epitope specific CD4$^+$ T cells undergo memory inflation in HIV$^+$ HLA-DR7$^+$ subjects. **A**–**B**: CD4-enriched or untouched peripheral blood mononuclear cells (PBMCs) from HIV$^+$ DR7$^+$ subjects were stained with DR7:CLIP or DR7:DYS tetramer for their (**A**) tp1 (no ART) (n=8), and (**B**) tp2 (on ART) samples (n=4). (**C**) DR7:DYS tetramer staining of HIV$^-$ DR7$^+$ subjects' PBMCs (n=10). (**D**) Response magnitude comparisons of DYS$^+$ and EPD$^+$ CD4$^+$ T cells from HIV$^+$ HCMV$^+$ DR7$^+$ (n=8; tp1 of Subject 10013, and tp2 of Subjects 10004, 10027 and 10032), HIV$^-$ HCMV$^+$ DR7$^+$ (n=10) and HIV$^+$ HCMV$^+$ DR7$^-$ (n=5–7) subjects determined simultaneously from the same samples per subject. **E**–**F**: Longitudinal (**E**) response magnitudes and (**F**) absolute counts of DYS$^+$ and EPD$^+$ CD4$^+$ T cells from Subjects 10027 and 10069. Values in (**D**) represent ≥3 biological replicates with means, except for the HIV$^+$ HCMV$^+$ DR7$^-$ cohort with no replicates.

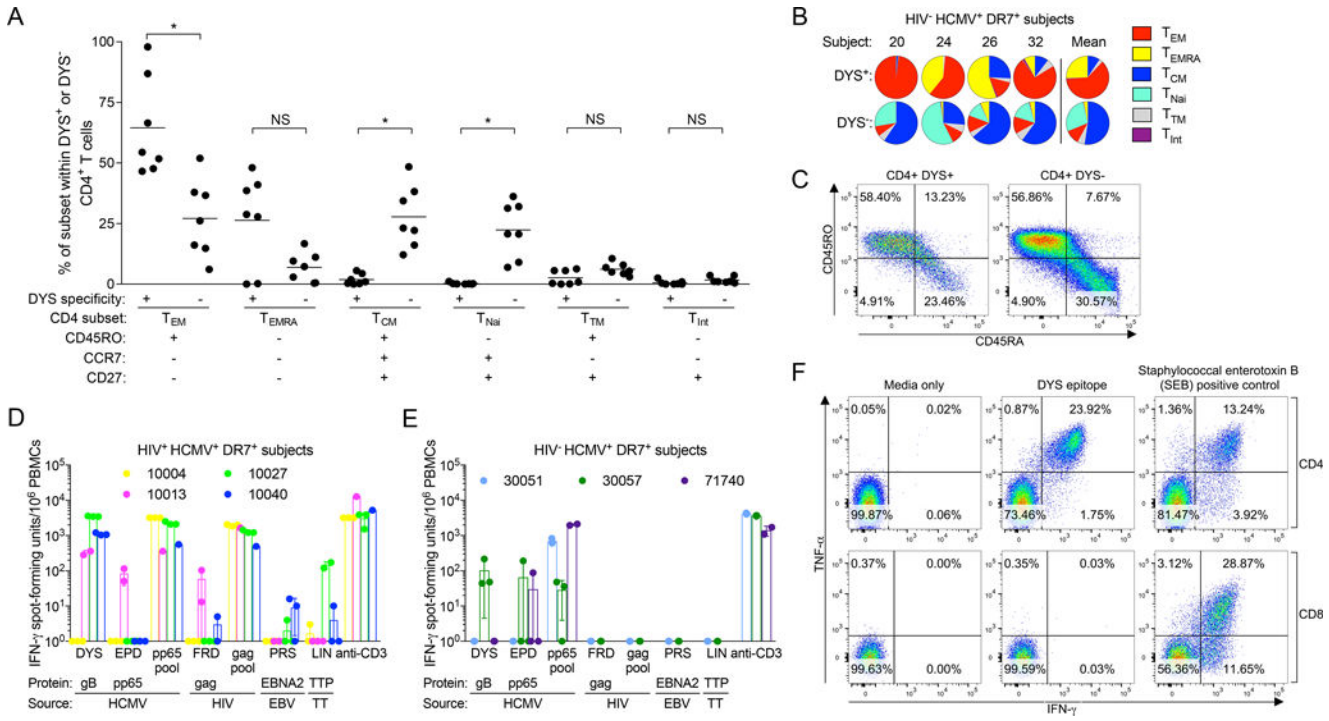

FIGURE 2.

$CD4^+$ T cells specific for DYS epitope consist of T_{EM} and T_{EMRA} subsets, and secrete cytokines upon DYS stimulation. (**A**) Response magnitude comparisons of DYS^+ and DYS^- $CD4^+$ T cell subsets from HIV^+ $HCMV^+$ $DR7^+$ subjects (n=7). PBMCs were stained with the tetramer and memory markers to identify the subsets. Plots show grand means and represent at least two biological replicates. (**B**) Normalized magnitudes of CD4 subsets within DYS^+ or DYS^- $CD4^+$ T cells from HIV^- $HCMV^+$ $DR7^+$ subjects with sufficient $tetramer^+$ response for analyses. (**C**) CD45RO and CD45RA staining of Subject 10027's PBMCs. **D**–**E**: Background-corrected IFN-γ ELISpot responses of PBMCs from (**D**) HIV^+ $HCMV^+$ $DR7^+$ (n=4; tp1) and (**E**) HIV^- $HCMV^+$ $DR7^+$ (n=3) subjects upon stimulation with 0.001 μg/ml of DYS, EPD, FRD, PRS, LIN epitopes, and of the following controls: HCMV pp65 overlapping 15-mer peptide pool, HIV's gag overlapping 15-mer peptide pool and anti-human CD3. Data represent technical triplicates except in conditions without mean ±SD. (**F**) Intracellular cytokine staining of Subject 10027's PBMCs after DYS or SEB stimulation.

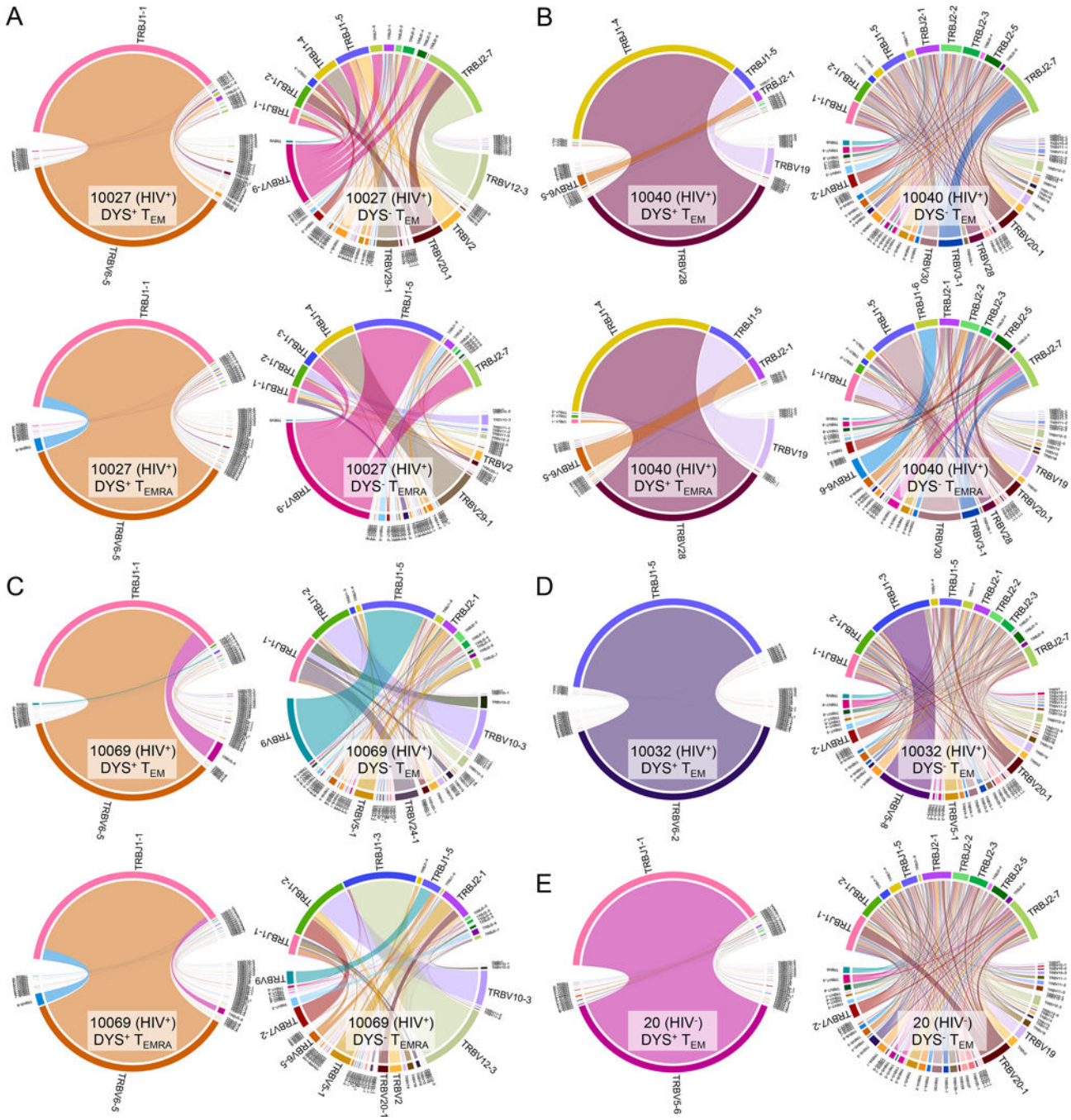

FIGURE 3.

Productive TCRβ V and J gene pairs of bulk-sorted, inflated DYS^+ $CD4^+$ T cells are highly restricted. TCRβV and TCRβJ gene family pairings of T_{EM} and/or T_{EMRA} subsets of productive DYS^+ and DYS^- CD4 TCRs from Subjects (**A**) 10027, (**B**) 10040, (**C**) 10069, (**D**) 10032, and (**E**) 20. Data shown represent single experiments. V and J gene pairs are connected by stems between their arcs. Arc lengths reflect gene family proportions within the sample's repertoire. High magnitude V and J gene families are emphasized.

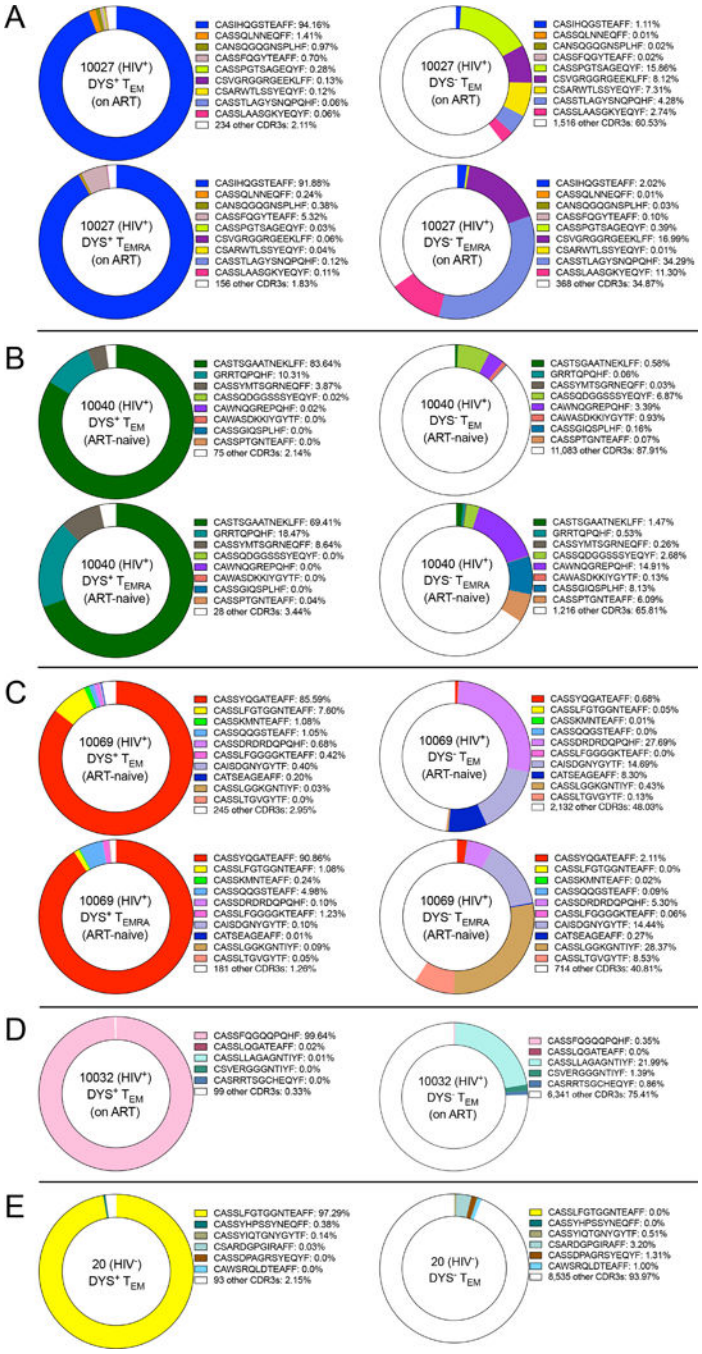

FIGURE 4.

Inflated DYS$^+$ CD4$^+$ T cells have nearly monoclonal productive CDR3s. Productive and unique TCRβ CDR3 clones of DYS$^+$ and DYS$^-$ T_{EM} and/or T_{EMRA} subsets from Subjects (**A**) 10027, (**B**) 10040, (**C**) 10069, (**D**) 10032 and (**E**) 20. Data represent single experiments.

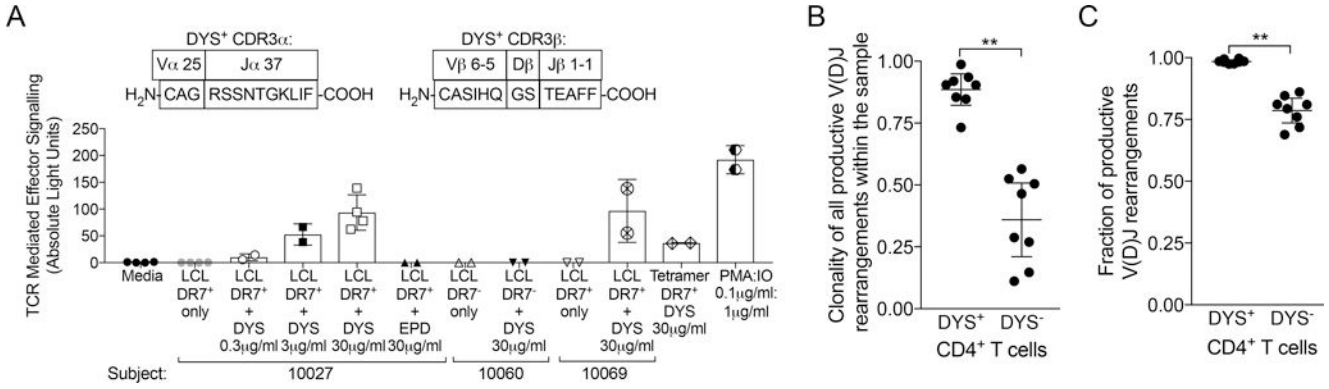

FIGURE 5.

Expressed clonal TCR of inflated DYS$^+$ CD4$^+$ T cells recognize DR7-restricted DYS epitope. (**A**) Subject 10027's DYS$^+$ single cell α:β TCR CDR3 sequences, and NFAT-mediated luciferase luminescence response of DR7$^+$ LCL-presented, serially diluted DYS epitope stimulation of DYS$^+$ TCR. Subject 10027's DYS$^+$ α:β TCR gene sequences were determined from single cell sorting and expressed using plasmids in an NFAT-luciferase reporter Jurkat cell line for stimulation. Graph shows mean±SD: conditions with two data-points represent technical replicates, while those with four represent two biological replicates of two technical replicates. (**B**) Clonality comparison of productive CDR3s of DYS$^+$ to DYS$^-$ CD4$^+$ T_{EM} and T_{EMRA} subsets from Subjects 10027, 10040, 10069, 10032 and 20. Clonality fractions were bioinformatically determined after productive entropy normalization. Values near 1: more clonal. (**C**) Fractional comparison of productive V(D)J rearrangements of DYS$^+$ to DYS$^-$ CD4$^+$ T_{EM} and T_{EMRA} subsets from all five subjects. Values near 1: fewer out-of-frame sequences or stop codons. **B**–**C**: Data represent single experiments for each subject with mean±95% CI.

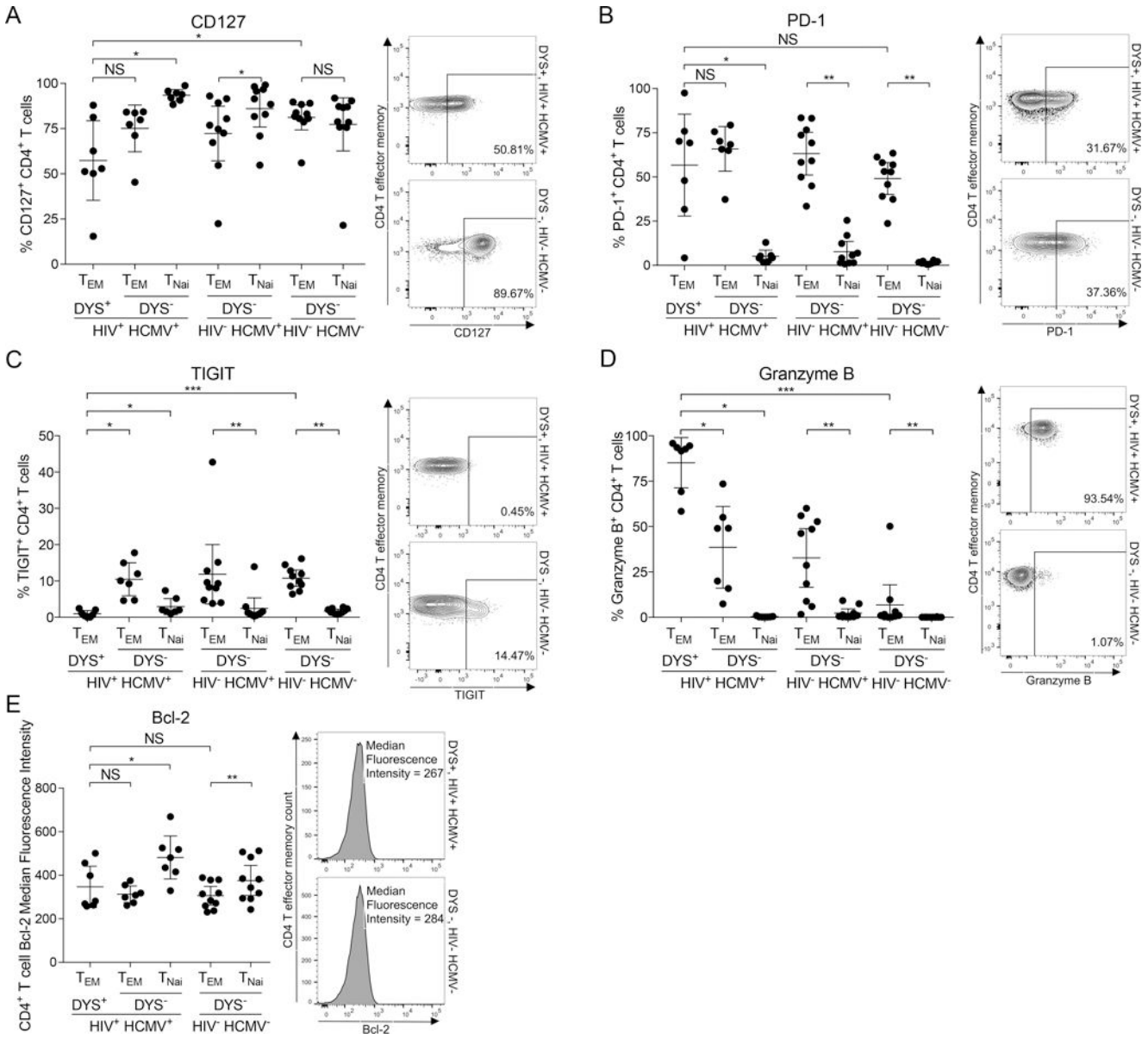

FIGURE 6.

Inflated DYS$^+$ CD4$^+$ T cells have a CD127$^-$ TIGIT$^-$ Granzyme B$^+$ phenotype. *Ex vivo* comparisons of (**A**) CD127, (**B**) PD-1, (**C**) TIGIT, (**D**) Granzyme B expressions or (**E**) Bcl-2 median fluorescence intensity (MFI) of DYS$^+$ CD4$^+$ T_{EM} of HIV$^+$ HCMV$^+$ subjects to DYS$^-$ CD4$^+$ T_{EM} of HIV$^-$ HCMV$^-$ controls. PBMCs were stained with tetramer and mAbs for either surface PD-1, CD127 and TIGIT or intracellular granzyme B and Bcl-2 proteins. HIV$^+$ HCMV$^+$: n=7, HIV$^-$ HCMV$^+$: n=10 and HIV$^-$ HCMV$^-$: n=10. Graphs represent single experiments for each subject, with mean±95% CI for all subjects.

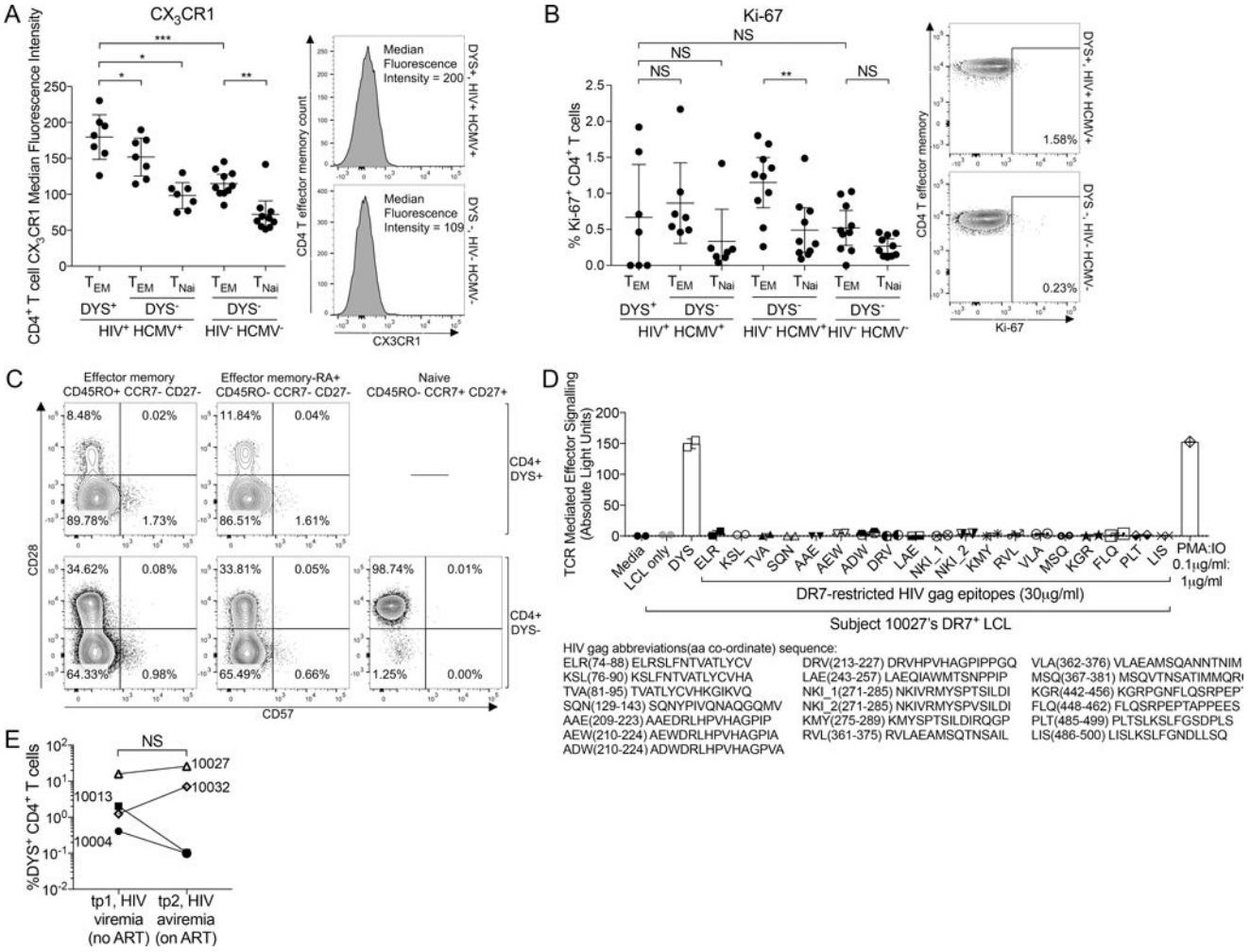

FIGURE 7.

Inflated DYS$^+$ CD4 T cells are CX$_3$CR1^{high}, not replicatively senescent and do not cross-react with HIV gag proteins. **A**–**B**: *Ex vivo* comparisons of (**A**) CX$_3$CR1 MFI and (**B**) Ki-67 response magnitude of DYS$^+$ CD4$^+$ T$_{EM}$ of HIV$^+$ HCMV$^+$ subjects to DYS$^-$ CD4$^+$ T$_{EM}$ of HIV$^-$ HCMV$^-$ controls. HIV$^+$ HCMV$^+$: n=7, HIV$^-$ HCMV$^+$: n=10 and HIV$^-$ HCMV$^-$: n=10. (**C**) CD57 and CD28 expressions of Subject 10027 DYS$^+$ and DYS$^-$ CD4$^+$ T cells. (**D**) Luciferase luminescence response of 19 DR7-presented HIV gag epitopes stimulation of Subject 10027's DYS$^+$ TCR expressed in the Jurkat cell line. (**E**) DYS$^+$ CD4 magnitude change from tp1 (HIV viremia) to tp2 (HIV aviremia). **A**–**B**, **E**: graphs show mean±95% CI for all subjects and represent single experiments with no replicates except (**E**) showing technical replicates. **D**: data shows mean±SD of technical duplicates.

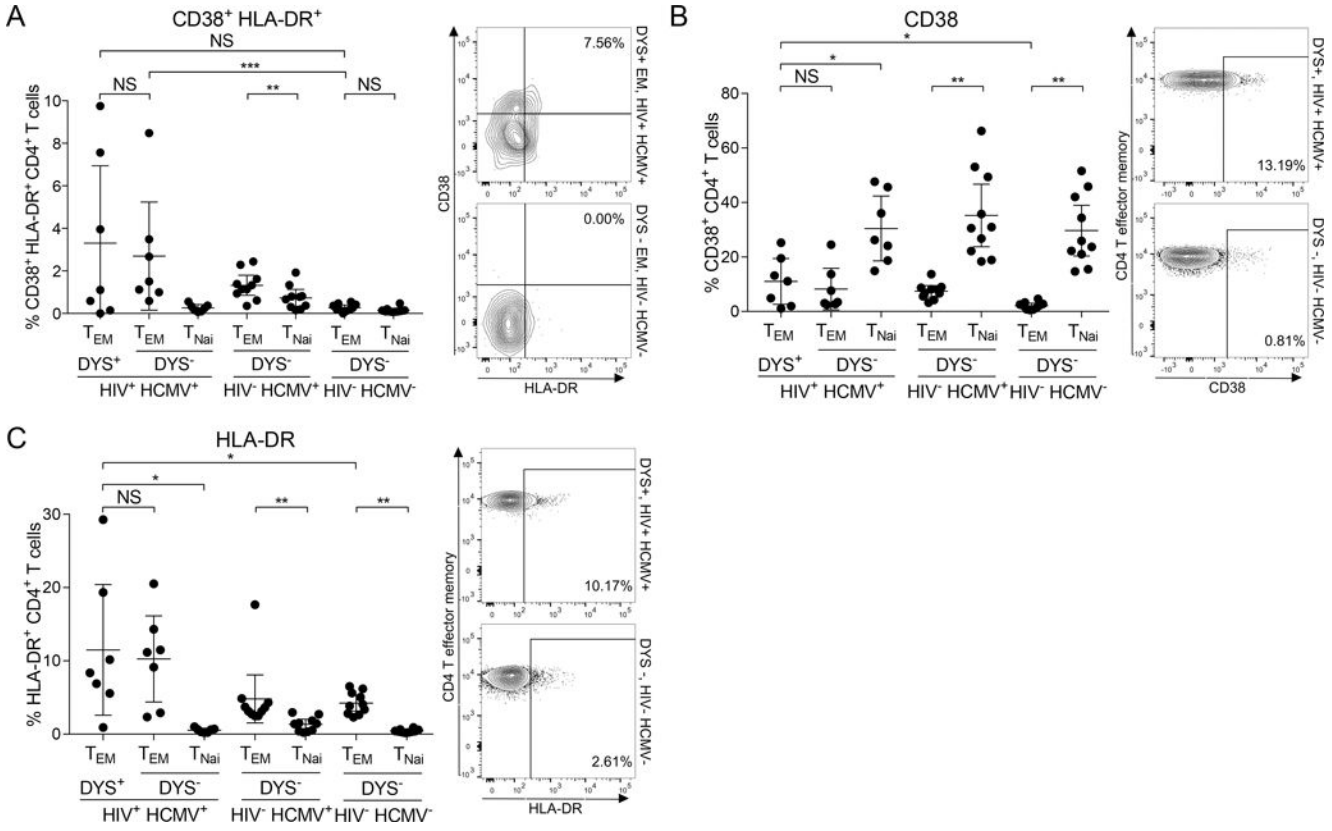

FIGURE 8.

Inflated DYS$^+$ CD4$^+$ T cells have a wider but not significantly higher response magnitude of CD38$^+$HLA-DR$^+$ co-expression. *Ex vivo* comparisons of (**A**) CD38$^+$HLA-DR$^+$ (**B**) CD38 and (**C**) HLA-DR magnitudes on DYS$^+$ CD4$^+$ T_{EM} of HIV$^+$ HCMV$^+$ subjects to DYS$^-$ CD4$^+$ T_{EM} of HIV$^-$ HCMV$^-$ controls. HIV$^+$ HCMV$^+$: n=7, HIV$^-$ HCMV$^+$: n=10 and HIV$^-$ HCMV$^-$: n=10. Graphs represent single experiments for each subject and mean±95% CI for all subjects.

Table I

Characteristics of HIV^+ $HCMV^+$ $DR7^+$ subjects.

Subject ID	HLA-DRB1 alleles	Time-point	Age (years)	Years of HIV infection	CD4 T cell count/μl blood	HIV load (RNA copies/ml plasma)	Time on ART (years)
10004	07:01, 03:01	1	58	22	203	<50	–
		2	62	26	181	<50	2.17
10013	07:01, 04:08	1	47	22	420	5,354	–
		2	60	35	380	<50	5.0
10027	07:01, 03:01	1	66	14	378	7,340	–
		2	75	23	977	<50	7.17
10032	07:01, 03:01	1	46	5	543	2,837	–
		2	56	15	886	<50	4.42
10040	07:01, 13:01	1	47	14	1,161	<50	–
10030	07:01, 09:01	1	60	13	856	<50	–
10066	07:01, 07:01	1	52	14	1,063	<50	–
10069	07:01, 08:04	1	46	6	903	2,470	–

Table II

Dominant CDR3βs of inflated DYS$^+$ CD4$^+$ T cells share conserved polar, neutral amino acids.

Subject ID	CDR3 freq.	TCRβV family	V segment (start of CDR3)	N1	D segment	N2	J segment	TCRβJ family
10027	94%	6-5	C A S	I H Q	G S		T E A F F	1-1
			tgt gcc agc	atc cat caa	ggg agc		act gaa gct ttc ttt	
10040	84%	28	C A S	T	S G	A A T N E K L F F		1-4
			tgt gcc agc	act	tca ggg	gcg gca act aat gaa aaa ctg ttt ttt		
10069	91%	6-5	C A S S Y		Q G	A T E A F F		1-1
			tgt gcc agc agt tat		cag ggc	gcc act gaa gct ttc ttt		
10032	99%	6-2	C A S S	F	Q G	Q Q P Q H F		1-5
			tgt gcc agc agt	ttc	cag ggg	caa cag ccc cag cat ttt		
20	96%	5-6	C A S S L	F G	T G G	N T E A F F		1-1
			tgt gcc agc agc ttg	ttc ggg	aca ggg ggg	aac act gaa gct ttc ttt		

Conserved glutamine (Q), serine (S) and threonine (T) within the D-segment are underlined. The dominant CDR3s were identical for DYS$^+$ CD4$^+$ T_{EM} and T_{EMRA} in all subjects.

Table III

Dominant CDR3βs of DYS^{-} $CD4^{+}$ T cells do not share conserved polar, neutral amino acids.

Subject	CD4 subset	CDR3 Freq.	TCRβV family	V gene start of CDR3						N1			D gene			N2					J gene				TCRβJ family
10027	T_{EM}	16%	12-3	C	A	S	S				P		G	T	S	A	G				E	Q	Y	F	2-7
				tgt	gcc	agc	agt				ccg		ggg	act	agc	gcc	ggc				gag	cag	tac	ttc	
	T_{EMRA}	34%	7-9	C	A	S	S			T	L	A	G			Y		S	N	Q	P	Q	H	F	1-5
				tgt	gcc	agc	agc			acg	ttg	gcg	gga			tat		agc	aat	cag	ccc	cag	cat	ttt	
10040	T_{EM}	7%	3-1	C	A	S	S	Q	D				G	G	S		S		S	Y	E	Q	Y	F	2-7
				tgt	gcc	agc	agc	caa	gac				ggc	ggg	agt		agc		tcc	tac	gag	cag	tac	ttc	
	T_{EMRA}	15%	30	C	A	W				N		Q	G				R			E	P	Q	H	F	1-5
				tgt	gcc	tgg				aat		cag	ggg				cgc			gag	ccc	cag	cat	ttt	
10069	T_{EM}	28%	9	C	A	S	S			D		R	D	R					D	Q	P	Q	H	F	1-5
				tgt	gcc	agc	agc			gac		cgg	gac	agg					gat	cag	ccc	cag	cat	ttt	
	T_{EMRA}	28%	12-3	C	A	S	S				L		G	G		K			G	N	T	I	Y	F	1-3
				tgt	gcc	agc	agc				ctg		ggg	ggg		aaa			gga	aac	acc	ata	tat	ttt	
10032	T_{EM}	22%	5-8	C	A	S	S	L			L	A	G					A	G	N	T	I	Y	F	1-3
				tgt	gcc	agc	agc	tta			cta	gca	ggg					gct	gga	aac	acc	ata	tat	ttt	
120	T_{EM}	3%	20-1	C	S	A	R		D		G	P	G			I	R					A	F	F	1-1
				tgc	agt	gct	aga		gat		ggt	cca	ggg			att	cga					gct	ttc	ttt	

Glutamine (Q) within the D-segment is underlined.

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