Measuring the contribution of γδ T cells to the persistent HIV reservoir

Katherine S. James[a], Ilana Trumble[b], Matthew L. Clohosey[a], Matthew Moeser[c], Nadia R. Roan[d], Adaora A. Adimora[a], Sarah B. Joseph[c], Nancie M. Archin[a], Michael Hudgens[b] and Natalia Soriano-Sarabia[a]

Objective: To study the contribution of γδ T cells to the persistent HIV reservoir.

Design: Fifteen HIV-seropositive individuals on suppressive ART were included. We performed parallel quantitative viral outgrowth assays (QVOA) of resting $CD4^+$ T (rCD4) cells in the presence or absence of γδ T cells.

Methods: Resting αβ+$CD4^+$ T cells were magnetically isolated from PBMCs using two different custom cocktails, only one kit contained antibodies to deplete γδ T cells, resulting in two populations: rCD4 cells and rCD4 cells depleted of γδ cells. Frequency of infection was analyzed by QVOA and DNA measurements.

Results: Recovery of replication-competent HIV from cultures of rCD4 cells was similar in 11 individuals despite the presence of γδ T cells. In four donors, HIV recovery was lower when γδ T cells were present. Expression of the cytotoxic marker $CD16^+$ on Vδ2 cells was the only variable associated with the lower HIV recovery. Our results highlight the potency of those responses since a mean of 10 000 γδ T cells were present within 2.5 million rCD4 cells. However, despite the low frequency of γδ T cells, the presence of cytotoxic Vδ2 cells correlated with lower HIV recovery from cultures of rCD4 cells.

Conclusion: Results of this study show that quantification of the contribution of γδ T cells to the reservoir is challenging because of their low numbers compared with conventional rCD4 cells and highlights the potent antiviral function of γδ T cells and the impact of their presence on the frequency of latent HIV infection.

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Introduction

The major obstacle in achieving a cure for HIV infection is the existence of a persistent reservoir within latently infected cells that can produce replication-competent virus if antiretroviral therapy (ART) is stopped [1,2]. The HIV reservoir concentrates mainly in resting $CD4^+$ T (rCD4) cells [3], including $CD4^+$ T-cell subsets, such as T memory stem cells (TSCM) and T follicular helper (Tfh) [4,5]. In addition, we previously reported that γδ T cells can harbor replication-competent HIV [6,7] suggesting that these cells can contribute to the recovery of HIV upon latency reversal.

γδ T cells represent between 2 and 10% of circulating $CD3^+$ T cells [8] and express T-cell receptors (TCR)

[a]Division of Infectious Diseases, Department of Medicine, [b]Department of Biostatistics and Center for AIDS Research, [c]Department of Microbiology and Immunology, University of North Carolina, Chapel Hill, North Carolina, and [d]Department of Urology, University of California San Francisco, San Francisco, and Gladstone Institutes, San Francisco, California, USA.
Correspondence to Natalia Soriano-Sarabia, Department of Microbiology, Immunology and Tropical Medicine, School of Medicine & Health Sciences, Ross Hall 626, George Washington University, 2300 I Street, NW, Washington, DC 20037, USA.
Tel: +1 202 994 1172; e-mail: nataliasorsar@email.gwu.edu
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composed of γ and δ chains. They are classified according to their TCRδ chain usage into Vδ1 and Vδ2 cells [9]. Vδ2 cells are the major circulating subpopulation of γδ T cells in healthy individuals and constitute around 0.5–5% of total $CD3^+$ T cells [8]. However, Vδ2 cells are depleted during HIV infection via direct, through transient upregulation of the $CD4^+$ receptor [8], and indirect mechanisms [6,10]. Concomitantly with this depletion, the number of circulating Vδ1 cells, which are mainly found in tissues increases and the Vδ2/Vδ1 ratio relative to that in HIV-seronegative individuals is inversed [11]. Vδ2 cells can be productively infected, harbor high levels of HIV DNA [6,12] and constitute a novel reservoir of HIV infection [6]. However, in our previous study because of the low γδ T-cell numbers, we could not measure the extent of the contribution of this cell population to the total HIV reservoir. To overcome this difficulty, in the present study, we performed QVOA on parallel cultures of rCD4 cells in the presence (rCD4+γδ) or absence (rCD4−$\gamma\delta_{dep}$) of γδ T cells to analyze the contribution of γδ T cells to the inducible replication-competent HIV reservoir. We could not detect differences between QVOA performed in the presence or absence of γδ T cells highlighting the difficulty of measuring the contribution of γδ T cells to the cellular reservoir. However, we found that despite their scarce number, γδ T cells exerted a potent anti-HIV function that translated into a lower recovery of HIV in 27% of the donors.

Methods

HIV-seropositive and HIV-seronegative donors

Characteristics of the ART-suppressed cohort of HIV participants included in this study have been previously reported [13,14]. Briefly, all HIV-seropositive participants were recruited through the University of North Carolina (UNC) Aids Clinical Trials Unit and the Women Interagency HIV Study (WIHS) (UNC and University of California, San Francisco sites). Participants were on stable ART with plasma HIV-1 RNA less than 50 copies/ml for at least 10 months before enrollment and no blips (i.e. >50 copies/ml) were detected for at least 2 years prior to enrollment, whenever applicable. We included HIV-seropositive donors treated in chronic and acute HIV infection. HIV-seropositive donors treated in the acute phase of HIV infection started therapy within 45 days of the estimated date of infection. In addition, HIV-seronegative donors were screened for high CCR5 expression and following leukapheresis, isolated cells were frozen. All HIV-seropositive and seronegative participants provided written informed consent. Studies were approved by the Institutional Review Board of the University of North Carolina and the University of San Francisco. Buffy coats from HIV-seronegative donors were obtained from the New York Blood Center (Long Island City, New York, USA).

Isolation of resting $CD4^+$ cells

Leukapheresis was performed and peripheral blood mononuclear cells (PBMCs) were isolated via Ficoll gradient. Total resting αβ+$CD4^+$ T cells (rCD4) were then magnetically isolated by negative selection from ART-suppressed HIV-seropositive participants using two different custom antibody cocktails from Stemcell Technologies in parallel. One cocktail contained antiγδTCR antibodies (pan-γδTCR) to deplete γδ T cells and therefore, rCD4 cells that did not contain γδ T cells (rCD4−$\gamma\delta_{dep}$) were recovered, whereas the other kit did not have antiγδTCR antibodies and rCD4 containing γδ T cells (rCD4+γδ) were isolated. Both cocktails contained the following antibodies: CD8, CD14, CD16, CD19, CD20, CD36, CD56, CD123, Glycophorin A, CD66b, CD25, HLADR and CD69. Isolations were performed in parallel following the manufacturer's instructions. An aliquot of the isolated fractions was analyzed by flow cytometry for purity, as described below.

Quantitative viral outgrowth assays

After rCD4 cell isolation, QVOA was performed as previously reported [15–19]. Briefly, an average of 42.6×10^6 rCD4 cells from each isolation method (rCD4−$\gamma\delta_{dep}$ and rCD4+γδ) were cultured in parallel in limiting dilution. The cells were activated with 2 μg/ml purified PHA (Thermofisher, Waltham, Massachusetts, USA), and 60U/ml IL-2, in the presence of allogeneic irradiated PBMCs from an HIV-seronegative donor. After 24 h of activation, the cells were washed to remove the PHA and feeder cells (PHA-activated PBMCs depleted of $CD8^+$ T cells from an HIV-negative donor displaying high CCR5 expression) were added to amplify outgrowth of the virus. Each participant is matched to a specific HIV-negative donor and feeder cells from the same donor are used throughout the experiment. Media was refreshed every 3–4 days and supernatants were harvested at day 15 to perform HIV p24 ELISA quantification (ABL, Rockville, Massachusetts, USA). Positivity of the HIV p24 ELISA was confirmed at day 19 of culture. Infectious units per million (IUPM) cells was calculated for each QVOA using the HIV p24 ELISA data, as described in the statistical methods.

Quantification of total HIV DNA levels

On the day of the HIV-seropositive participant's leukapheresis, if enough cells were available, aliquots of 5 million purified rCD4+γδ cells, and 5 million purified rCD4−$\gamma\delta_{dep}$ cells were pelleted, snap frozen, and stored at −80 °C. DNA was extracted from the two isolated cell populations using the DNeasy Blood & Tissue Kit (Qiagen, Germantown, Maryland, USA) following the manufacturer's instructions. The extracted DNA concentration and purity were determined using the Nanodrop 2000 spectrophotometer (Thermo Scientific, Waltham, Massachusetts, USA). Total HIV-1 DNA copies were assessed by quantitative PCR (qPCR) using

primers and probes to amplify HIV-1 *gag*, as previously described. Primers and probes used targeted conserved regions of HIV-1 *gag* [20]. Briefly, 500 ng of total cellular DNA were run per PCR reaction in triplicate and for each qPCR run, an HIV-1 *gag* standard curve was generated as described elsewhere [21]. Results were expressed as HIV-1 *gag* copies per million cells.

3′ Half genome sequencing

We used phylogenetic analysis to explore whether there is evidence that γδ T cells reduce virus production in QVOA by eliminating a genetically distinct subset of inducible, replication-competent viruses. The following protocol was used to sequence and perform phylogenetic analyses of outgrowth viruses from three HIV-seropositive participants (participants 354, 357 and 363). First, viral RNA was isolated from p24-positive QVOA wells and converted to cDNA using Superscript III Reverse Transcriptase and an oligo(dT) primer. 3′ half genomes (4924 to 9604 on HXB2) were amplified by PCR using barcoded primers, and the PCR products were gel-purified. The SMARTbell Template Prep Kit (PacBio, Menlo Park, California, USA) was used to add adaptors to amplicons and amplicons were sequenced using the PacBio Sequel platform (movie time of 10 h). Sequences were grouped by barcode and high-quality sequences were analyzed using the PacBio Long Amplicon Analysis (LAA) package. Sequences were visually screened to confirm that reading frames were intact. Sequences from each participant were aligned using MUSCLE (v3.8.1) and a neighbor-joining phylogenetic tree (XXCITE Capoferri) was constructed for each individual.

Flow cytometry

To estimate the frequency of γδ T cells in each population (rCD4+γδ and rCD4−$\gamma\delta_{dep}$), an aliquot from both cell populations was stained with monoclonal antibodies (mAbs) against CD3 (clone SK7), $CD4^+$ (clone OKT4), Vδ1 (clone TS8.2) and Vδ2 (clone M-T271) (all antibodies from Biolegend, San Diego, California, USA, except Vδ1 from Thermofisher). To analyze the expression of markers associated with cytotoxic functions in γδ T cells, PBMCs from the participants were stained with mAbs against $CD16^+$ (clone 3G8) and $CD56^+$ (clone HCD56). Cells were incubated in staining buffer (i.e. 10% FBS in PBS) for 20vmin on ice in the dark, washed and then fixed in 2% paraformaldehyde solution. Standard controls including compensation and fluorescence minus one controls (FMO), were used and data were acquired on an Attune Nxt instrument. Analysis was performed using Flow Jo version 10.1 (TreeStar, Ashland, Oregon, USA).

HIV inhibition assays

Viral inhibition assays were performed as previously described [22]. Briefly, HIV-infected $CD4^+$ T cells were cultured alone as a control of HIV production, or co-cultured with Vδ2 cells in triplicate at a 1 : 10 effector : target ratio. Supernatants were harvested at day 7 and stored at −20 °C until HIVp24 ELISA quantification (ABL_{inc}, Rockville, Masssachusetts, USA) was performed. Results of 1 : 10 effector : target cell ratio cocultures are shown. Results are expressed as percentage of viral inhibition normalized to HIV p24 production when target $CD4^+$ T cells were cultured alone.

Statistical analysis

Infectious units per million (IUPM) for rCD4+γδ and rCD4−$\gamma\delta_{dep}$ samples were estimated using the SLDAssay R software package [23]. The bias-corrected maximum likelihood estimate, corresponding exact confidence intervals, and goodness-of-fit *P* values (PGOF) were computed. For each individual, the log of the ratio of the IUPM estimates between rCD4+γδ and rCD4−$\gamma\delta_{dep}$ cells was calculated. In addition, the IUPM difference between rCD4+γδ and rCD4−$\gamma\delta_{dep}$ and a corresponding exact 95% confidence interval (CI) was calculated for each individual by inverting a likelihood ratio test. In order to assess associations between covariates of interest and the change in IUPM between rCD4+γδ and rCD4−$\gamma\delta_{dep}$ cells within an individual, the log of the ratio of IUPM estimates was compared with covariates. To assess the association between the log of the ratio of IUPM estimates and categorical covariates with two levels (sex and acute classification), the Wilcoxon rank sum test was used. For categorical covariates with more than two levels (race), the Kruskal–Wallis test was used. For continuous covariates, the Spearman rank correlation *P* value with permutation and the mid-ranks tie handling method was used. All statistical analyses were conducted using R version 3.4.3 (R Foundation, Vienna, Austria). Following recommendations from the American Statistical Association [24], instead of presenting results according to the dichotomy of $P < 0.05$ ('statistically significant') or *P* at least 0.05 ('no statistical difference'), *P* values are presented as continuous statistics as well as other numerical results, such as confidence intervals, which provide additional context.

Results

Participants' characteristics

The 15 HIV-seropositive participants had been suppressed for a median period of 3.6 years (range 0.87–9.35 years). The median time on ART was 4.8 years (range 0.9–19.9 years). Eleven (73.3%) participants were male and four (26.6%) were female. Six (40%) were Caucasian, six African American (40%), two (13.3%) belonged to other races, and one was of unknown race (6.7%). Four (26.7%) participants were treated in the acute phase of infection, and 11 (73.3%) were treated in the chronic phase of infection.

Efficacy of γδ T-cell depletion

rCD4 cells isolated from PBMCs using two different cocktails in parallel resulted in two different cell

Table 1. Difference in rCD4+γδ and rCD4−$\gamma\delta_{dep}$ bias-corrected infectious units per million estimates per participant, with corresponding exact 95% confidence interval.

	Participant ID	Difference in IUPM	Exact CI		Efficacy of γδ depletion Vδ2	Vδ1
1	321	−0.22	−3.26	2.56	100	70
2	327	−0.07	−0.33	0.17	100	55
3	305	−0.06	−0.47	0.23	100	73
4	376	−0.05	−0.82	0.67	43	86
5	355	−0.03	−5.45	3.96	100	100
6	347	0.02	−0.01	0.16	70	74
7	324	0.03	−0.10	0.18	100	87
8	281	0.05	−0.33	0.51	98	82
9	351	0.14	−0.24	0.59	100	100
10	263	0.29	−0.11	0.92	63	100
11	348	1.43	−0.52	6.32	93	51
12	**357**	**0.49**	**−0.00**	**1.21**	**100**	**94**
13	**313**	**0.80**	**0.05**	**2.44**	**50**	**92**
14	**363**	**0.92**	**0.16**	**2.87**	**100**	**100**
15	**354**	**3.15**	**0.49**	**11.43**	**100**	**95**

Bolded text in rows indicate exact CIs with lower endpoint ≥ 0.0, which indicates higher recovery of replication-competent HIV when γδ T cells were depleted from cultures of rCD4 cells BC, bias-corrected; CI, confidence interval; IUPM, infectious units per million.

populations: rCD4 cells with γδ T cells (rCD4+γδ cells), and rCD4 cells depleted of γδ T cells (rCD4−$\gamma\delta_{dep}$). Frequency of Vδ1 and Vδ2 cells after rCD4 cell isolation was compared by flow cytometry (Supplemental Fig. 1, http://links.lww.com/QAD/B577), and the efficacy of the depletion was calculated as the difference between the frequency of γδ T cells present within rCD4+γδ cells minus the frequency of γδ T cells within rCD4−$\gamma\delta_{dep}$ cells (Table 1). Within rCD4+γδ cells, mean frequency of CD3+ Vδ2+ cells was 0.2% (range 0.03–0.70), and mean frequency of CD3+ Vδ1+ cells was 1.06% (range 0.6–1.9). However, in cultures of rCD4−$\gamma\delta_{dep}$ cells, the mean frequency of Vδ2 cells decreased to 0.02% (range 0–0.1) and the mean frequency of Vδ1 cells decreased to 0.2% (range 0–0.75).

Impact of the presence of γδ T cells on the recovery of replication-competent HIV

A mean of 42.6 million rCD4 cells were assayed in parallel cultures and bias corrected (BC)-IUPM values were calculated (Table 2). The mean IUPM was 0.583 (95% CI 0.312–1.311) for rCD4+γδ cells compared with 1.044 (95% CI 0.557–2.484) for rCD4−$\gamma\delta_{dep}$ cells ($P = 0.07$, Fig. 1a). Interestingly, the pattern of HIV recovery was heterogeneous and some donors had a higher recovery in cultures when γδ T cells were present and vice versa. IUPM estimates and corresponding CIs for rCD4+γδ and rCD4−$\gamma\delta_{dep}$ cells for each individual are displayed in Fig. 1b. For most participants, IUPM was similar in wells with or without γδ cells with observed differences likely to be due solely to assay variability. However, in 27% of the participants (313, 363, 354 and 357), the difference in IUPM between wells with and without γδ T cells was greater than that expected from only random assay variation; this is reflected by the 95% confidence intervals in Table 2 having lower bounds greater than or equal to 0. In these four participants, the recovery of replication-competent HIV was lower when γδ T cells were present in cultures of rCD4 cells. Finally, in the seven participants with available cells, total *gag* HIV DNA levels were similar between rCD4+γδ and rCD4−$\gamma\delta_{dep}$ cells ($P = 0.15$, Fig. 1c). As the depletion of γδ T cells was not 100% effective (Table 1), we performed correlations between the differences in IUPM in rCD4+γδ and rCD4−$\gamma\delta_{dep}$ cells and the efficacy of the depletion showing no correlation (Table 3).

Expression of CD16$^+$ on Vδ2 cells is associated with a lower recovery of replication-competent HIV

We hypothesized that the lower recovery of HIV from cultures of rCD4+γδ cells in some donors, was because

Table 2. Culture conditions and estimated infectious units per million rCD4+γδ and rCD4−$\gamma\delta_{dep}$ cells.

			rCD4+γδ				rCD4−$\gamma\delta_{dep}$			
Donor	Cells (×10^6)	Total wells	Positive wells	BC-IUPM	Lower CI	Upper CI	Positive wells	BC-IUPM	Lower CI	Upper CI
263	33.6	24	5	0.178	0.063	0.424	10	0.476	0.247	0.959
281	33.75	30	6	0.264	0.117	0.532	8	0.323	0.148	0.664
305	33.6	24	6	0.217	0.076	0.477	5	0.157	0.058	0.355
313	33.75	30	10	0.438	0.214	0.843	16	1.239	0.736	2.610
321	33.75	30	17	1.656	0.919	4.043	16	1.440	0.788	3.498
324	63.6	36	4	0.067	0.024	0.167	6	0.103	0.037	0.215
327	63.75	42	14	0.268	0.156	0.451	10	0.194	0.100	0.359
347	48.6	30	1	0.021	0.001	0.107	2	0.042	0.008	0.148
348	33.6	24	17	1.042	0.648	2.011	19	2.468	1.381	5.946
351	48.6	30	10	0.270	0.137	0.507	13	0.406	0.221	0.720
354	33.6	24	13	0.813	0.446	1.682	16	3.966	1.793	11.617
355	33.6	24	17	2.087	1.052	5.870	18	2.057	1.136	4.862
357	48.6	30	12	0.338	0.180	0.612	20	0.827	0.519	1.464
363	48.6	30	12	0.370	0.205	0.679	17	1.292	0.780	2.654
376	48.6	30	18	0.716	0.439	1.261	17	0.667	0.394	1.185

BC, bias-corrected; CI, confidence interval; IUPM, infectious units per million.

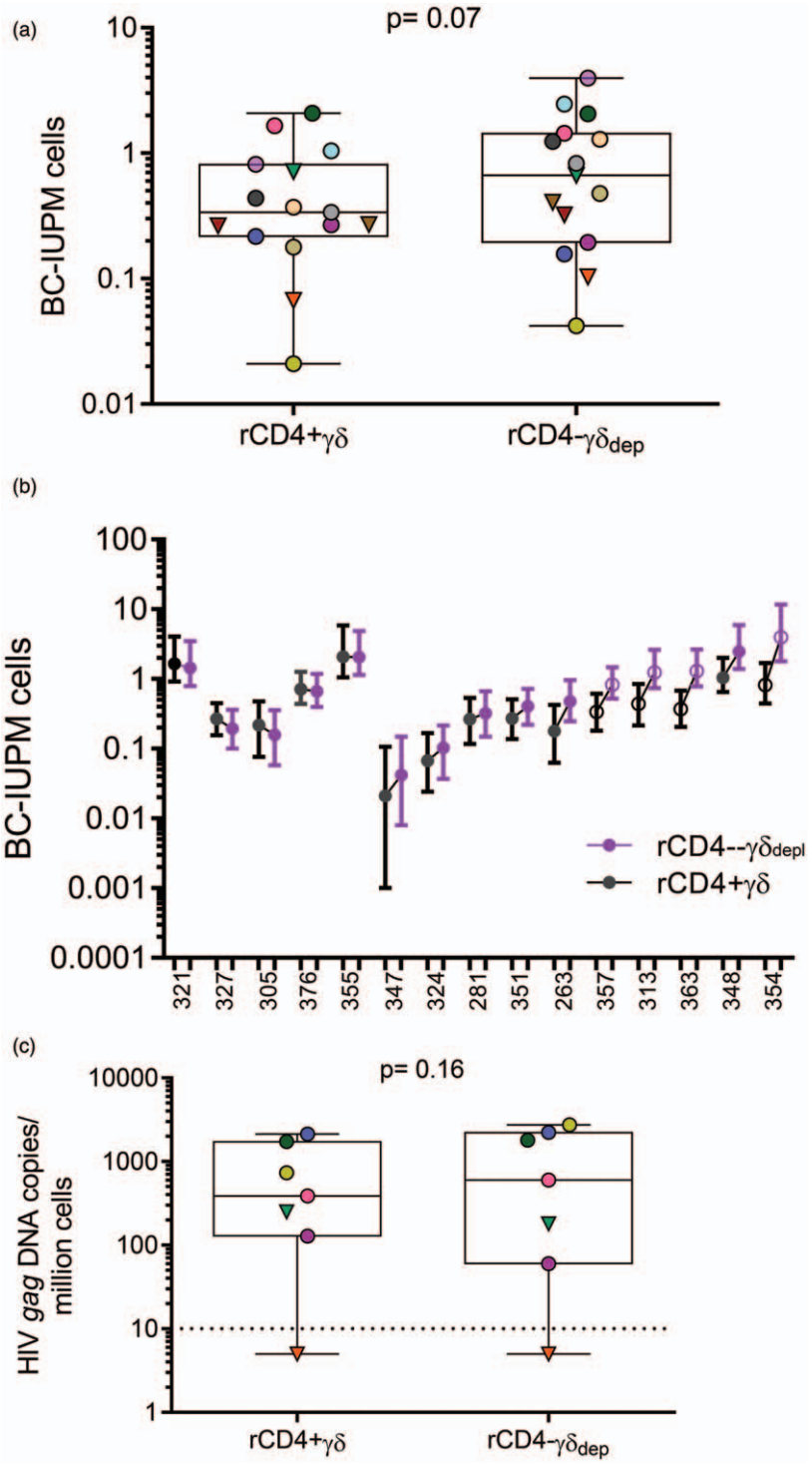

Fig. 1. Frequency of persistent HIV infection. (a) Recovery of replication-competent HIV by QVOA. BC-IUPM from 15 HIV-seropositive participants was calculated in parallel cultures of rCD4 cells in the presence (rCD4+γδ cells) or absence of γδ T cells (rCD4−$\gamma\delta_{dep}$). Cultures depleted of γδ T cells tended to have a higher BC-IUPM ($P = 0.07$, Wilcoxon rank sum test). (b) Infectious units per million (IUPM) rCD4+γδ cells and IUPM rCD4−$\gamma\delta_{dep}$. Bias corrected (BC)-IUPM estimates and corresponding exact 95% CI are represented for the 15 HIV-seropositive participants included in the study. Open symbols represent donors where the recovery of replication competent HIV was greater in cultures of rCD4−$\gamma\delta_{dep}$ cells than in cultures of rCD4+γδ cells. (C) Levels of total *gag* HIV DNA. HIV DNA levels were analyzed in 7 of the 15 participants' similar levels between cultures of rCD4+γδ cells and rCD4−$\gamma\delta_{dep}$ ($P = 0.16$, Wilcoxon rank sum test). In (a) and (b), circles represent HIV-seropositive participants treated in the chronic phase of the infection and triangles represent participants treated in the acute phase of the infection. BC, bias-corrected; CI, confidence interval; IUPM, infectious units per million; QVOA, quantitative viral outgrowth assays.

Table 3. Tests for association between covariates of interest and log(IUPM rCD4+γδ/IUPM rCD4−$γδ_{dep}$).

Covariate	*P* value	Spearman rank correlation
Age	0.77	0.08
Sex	0.85[a]	N/A
Race	0.79[b]	N/A
Nadir $CD4^+$	0.54	−0.16
$CD4^+$	0.93	0.03
$CD8^+$	0.65	0.13
Number of cells cultured	0.58	−0.15
Number of wells plated	0.44	−0.21
Presence of Vδ2 in rCD4+γδ	0.10	0.45
Presence of Vδ2 in rCD4−$γδ_{dep}$	0.41	0.22
Presence of Vδ1 in rCD4+γδ	0.48	0.19
Presence of Vδ1 in rCD4−$γδ_{dep}$	0.83	−0.06
Expression of CD16 in Vδ2 cells	0.03	0.60
Expression of CD56 in Vδ2 cells	0.41	−0.27
Time on ART	0.27	0.30
Treated in acute phase of infection	0.41[a]	N/A
Years of suppression	0.46	0.21
HIV DNA levels in rCD4+γδ	0.49	−0.26
HIV DNA levels in rCD4−$γδ_{dep}$	0.80	−0.10
Ratio of HIV DNA levels in rCD4+γδ to rCD4−$γδ_{dep}$	0.38	−0.36

The Spearman rank correlation *P* value was used for continuous covariates. The Spearman rank correlation estimate is also provided whenever applicable. IUPM, infectious units per million.
[a]The Wilcoxon rank sum test was used for categorical covariates with two levels.
[b]The Kruskal–Wallis was used for categorical covariates with more than two levels.

of a more potent antiviral function from γδ T cells. In order to test this hypothesis, we analyzed the expression of the cytotoxic markers $CD16^+$ on Vδ2 cells, and $CD56^+$ on both Vδ1 and Vδ2 T cell populations (Fig. 2a) in the HIV-seropositive donors. As the original flow cytometry panel did not include $CD16^+$ to be measured on Vδ1 cells, only the frequency of Vδ2 cells expressing $CD16^+$ was accounted for in the analysis.

We calculated the log ratio of the two IUPMs defined as the log(IUPMγδ+/IUPMγδ-) to evaluate what covariates were involved in the difference in IUPMs of rCD4+γδ and rCD4−$γδ_{dep}$ (Table 3). Covariates included: demographic characteristics (age, sex and race); immune status (nadir $CD4^+$, and $CD4^+$ and $CD8^+$ counts at the time of leukapheresis); culture-related data (number of cells and total wells cultured); frequency of Vδ1 and Vδ2 cells in the cultures; expression of cytotoxic markers ($CD16^+$ and $CD56^+$) on Vδ1 and Vδ2 cell T cells; and ART-related data (including time on ART, treatment in acute/chronic infection and time participants had been suppressed). Interestingly, expression of the cytotoxic marker $CD16^+$ on Vδ2 cells was associated with a lower recovery of replication-competent HIV ($R = 0.598$, $P = 0.03$, Table 1).

Strengthening our hypothesis, seven of the donors analyzed in this study, had been included in a previous study where the antiviral capacity of their Vδ2 cells had been directly measured in HIV inhibition assays [22]. In that study, isolated Vδ2 cells showed a mean inhibition capacity of 84% when cocultured with isolated autologous $CD4^+$ T cells. Interestingly, in the present study, donors with a more potent antiviral capacity (313, 363, 354 and 357) showed a lower HIV recovery from cultures of rCD4 cells when γδ T cells were present (Fig. 2b).

Similar viral sequences between cultures of rCD4 cells containing or lacking γδ T cells

In order to explore whether viral sequences generated from cultures with or without γδ T cells may be

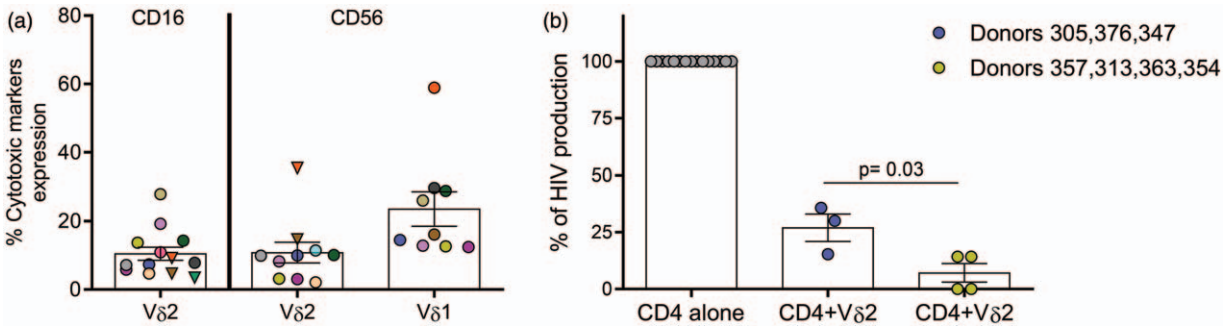

Fig. 2. Expression of cytotoxic markers $CD16^+$ and $CD56^+$ in γδ T-cell populations and association with HIV recovery from quantitative viral outgrowth assays. (a) Expression of $CD16^+$ in Vδ2 cells and $CD56^+$ in Vδ2 and Vδ1 cells. Mean $CD16^+$ expression on Vδ2 cells was 10.3%, whereas $CD56^+$ was expressed by a mean of 10.8% in Vδ2 cells compared with a mean of 23.3% in Vδ1 cells. Circles represent HIV-seropositive participants treated in the chronic phase of the infection and triangles represent participants treated in the acute phase of the infection. (B) Enhanced viral inhibition capacity by Vδ2 cells from donor 357, 313, 363 and 354. HIV inhibition assays from isolated Vδ2 cells cocultured with autologous isolated $CD4^+$ cells from ART-suppressed HIV-infected donors. HIV p24 production (measured by ELISA) normalized to the condition where only isolated $CD4^+$ cells were cultured is represented. Donors 305, 376 and 327, represented in blue, did not show differences in HIV recovery from cultures of rCD4+γδ and rCD4−$γδ_{dep}$. In donors 357, 313, 363 and 354, HIV recovery was higher when γδ T cells had been depleted. The capacity to inhibit viral replication from autologous isolated $CD4^+$ cells, was higher in donors 357, 313, 363 and 354 compared with 305, 376 and 327 ($P = 0.03$). ART, antiretroviral therapy.

identifiable as a genetically distinct portion of the inducible HIV-1 reservoir, we sequenced and phylogenetically compared outgrowth viruses from three donors (354, 357 and 363) that had lower IUPM estimates in rCD4+γδ compared with rCD4−$\gamma\delta_{dep}$. We did not observe evidence of sequence differences between rCD4+γδ and rCD4−$\gamma\delta_{dep}$ cells (Supplemental Fig. 2, http://links.lww.com/QAD/B578). Similar numbers of sequences from the rCD4 cells with γδ T cells (participant 354 = 29; participant 357 = 52, and participant 363 = 21) and those depleted of γδ T cells (participant 354 = 28; participant 357 = 22, and participant 363 = 21) were generated. Sequences from both populations were interspersed in the phylogenetic tree and therefore, there was no evidence of a genetically distinct viral lineages found in cultures of rCD4−$\gamma\delta_{dep}$ and rCD4+γδ cells (Supplemental Fig. 2, http://links.lww.com/QAD/B578). Although we did not detect evidence that specific viral lineages are different from rCD4 cell cultures containing versus lacking γδ T cells, we did not sequence a sufficient number of viruses to detect changes in rare cell populations.

Discussion

In this study, we aimed to assess the extent of γδ T-cell contribution to the persistent reservoir. We previously demonstrated the capacity of these cells to harbor replication-competent HIV in long-term ART-suppressed individuals [6]. We show that since γδ T-cell frequency in peripheral blood is very low and conventional $CD4^+$ cells outnumber γδ T cells, measuring their contribution to the total latent reservoir is challenging. However, despite the low frequency of γδ T cells, they exert a potent antiviral function that can directly have an impact on the recovery of replication-competent HIV from cultures of rCD4 cells, as supported by the results of this study.

We performed QVOA on cultures of isolated rCD4 cells that had been depleted of γδ T-cell populations and compared them with cultures of isolated rCD4 cells with γδ T cells. When possible, experiments were performed in parallel using the same numbers of cells and limiting dilution conditions to avoid assay fluctuations. Unfortunately, we could not measure the extent of the contribution of γδ T cells to the replication-competent virus most likely because of the scarce number of γδ T cells within cultures of rCD4 cells, highlighting the difficulty of working with rare populations. However, for some of the participants included herein, the frequency of infection within isolated γδ T cells was quantified in a previous work [6] and in ongoing studies. In participants 263, 321, 354 and 357 HIV was previously recovered from isolated Vδ2 cells [6]. However, when cultured within rCD4 cells, the specific contribution to the viral reservoir from γδ T cells was not quantifiable, possibly because classical rCD4 cells vastly outnumber γδ T cells. Analysis of half 3' half-genome sequences from HIV p24 positive wells from QVOA showed no evidence of genetic differences between the cultures with versus without γδ T cells, suggesting that virus contained within classical $CD4^+$ T cells and γδ T cells are not different or if different, we were not able to detect them possibly because of the low frequency. Ongoing work will help elucidate the nature of viral isolates from γδ T cells.

Interestingly, 27% of the donors showed increased frequency of infection when γδ T cells were depleted from cultures of rCD4 cell demonstrating the potent antiviral capacity of these cells given their low frequency (less than 10 000 γδ T cells in 2.5×10^6 rCD4 cells). This finding is consistent with our prior work showing that antiviral function of γδ T cells reduced the recovery of replication-competent virus from isolated Vδ2 cells [6]. Although γδ T cells are recognized for their antiviral functions, their potency to reduce the HIV production in cultures of rCD4 cells has not been previously shown. We hypothesized that the potent antiviral function from γδ T cells was responsible for the lower recovery of virus upon reactivation. To test this hypothesis, we interrogated the implication of different covariates, including markers of cytotoxicity in γδ T cells [25,26], in the outcome of HIV recovery. Similar to natural killer (NK) cells, CD16 expression on γδ T cells is associated with cytotoxic function. $CD16^+$ expression on Vδ2 cells identifies two different subsets with different responses. Specifically, Vδ2 cells expression $CD16^+$ are more phenotypically similar to NK cells, express NK receptors, and high levels of perforin [27]. In addition, a functional linkage has been reported between CD16 and CD3 expression that points to a mechanism to be explored [26]. Our correlation analyses showed that the frequency of Vδ2+ CD16+ cells was associated with a lower recovery of HIV in cultures of rCD4 cells, although we cannot exclude the involvement of other factors, from either γδ T-cell population. Strengthening our hypothesis, seven of the donors analyzed in this study, had been included in a previous study where their antiviral capacity had been directly measured in HIV inhibition assays [22]. In the four donors, where HIV recovery was lower when γδ T cells were present, we saw a more potent γδ antiviral activity when compared with the other participants in that study. Other covariates, including demographics, immune status at the time of leukapheresis, time on ART or ART initiation in the acute or chronic phase of the infection, frequency of remaining γδ T cells in the cultures after rCD4 cell isolation, were analyzed and did not seem to have a significant role on the recovery of replication-competent HIV.

In conclusion, results of this study show that quantification of the contribution of γδ T cells to the reservoir is challenging because of their low frequency compared

with rCD4 cells. However, despite their low frequency, our results show that γδ T cells from some participants, have the ability to reduce the production of replication-competent HIV recovered in cultures of rCD4 cells and highlights the importance of depleting γδ T cells from cultures of rCD4 cells for accurate QVOA measurements and when testing the efficacy of LRAs.

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Conflicts of interest

There are no conflicts of interest.

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References

1. Wong JK, Hezareh M, Günthard HF, Havlir DV, Ignacio CC, Spina CA, *et al*. **Recovery of replication-competent HIV despite prolonged suppression of plasma viremia.** *Science* 1997; **278**:1291–1295.
2. Chun TW, Stuyver L, Mizell SB, Ehler LA, Mican JA, Baseler M, *et al*. **Presence of an inducible HIV-1 latent reservoir during highly active antiretroviral therapy.** *Proc Natl Acad Sci USA* 1997; **94**:13193–13197.
3. Chun T-W, Carruth L, Finzi D, Shen X, DiGiuseppe JA, Taylor H, *et al*. **Quantification of latent tissue reservoirs and total body viral load in HIV-1 infection.** *Nature* 1997; **387**:183–188.
4. Buzon MJ, Sun H, Li C, Shaw A, Seiss K, Ouyang Z, *et al*. **HIV-1 persistence in CD4+ T cells with stem cell–like properties.** *Nat Med* 2014; **20**:139–142.
5. Perreau M, Savoye A-L, De Crignis E, Corpataux J-M, Cubas R, Haddad EK, *et al*. **Follicular helper T cells serve as the major CD4 T cell compartment for HIV-1 infection, replication, and production.** *J Exp Med* 2013; **210**:143–156.
6. Soriano-Sarabia N, Archin NM, Bateson R, Dahl NP, Crooks AM, Kuruc JD, *et al*. **Peripheral Vγ9Vδ2 T cells are a novel reservoir of latent HIV infection.** *PLoS Pathog* 2015; **11**:e1005201.
7. Barton K, Winckelmann A, Palmer S. **HIV-1 reservoirs during suppressive therapy.** *Trends Microbiol* 2016; **24**:345–355.
8. Hayday AC. **γδ cells: a right time and a right place for a conserved third way of protection.** *Annu Rev Immunol* 2000; **18**:975–1026.
9. Bottino C, Tambussi G, Ferrini S, Ciccone E, Varese P, Mingari MC, *et al*. **Two subsets of human T lymphocytes expressing gamma/delta antigen receptor are identifiable by monoclonal antibodies directed to two distinct molecular forms of the receptor.** *J Exp Med* 1988; **168**:491–505.
10. Li H, Pauza CD. **HIV envelope-mediated, CCR5/alpha4beta7-dependent killing of CD4-negative gammadelta T cells which are lost during progression to AIDS.** *Blood* 2011; **118**:5824–5831.
11. Autran B, Triebel F, Katlama C, Rozenbaum W, Hercend T, Debre P. **T cell receptor gamma/delta+ lymphocyte subsets during HIV infection.** *Clin Exp Immunol* 1989; **75**:206–210.
12. Imlach S, Leen C, Bell JE, Simmonds P. **Phenotypic analysis of peripheral blood γδ t lymphocytes and their targeting by human immunodeficiency virus type 1 in vivo.** *Virology* 2003; **305**:415–427.
13. Archin NM, Vaidya NK, Kuruc JD, Liberty AL, Wiegand A, Kearney MF, *et al*. **Immediate antiviral therapy appears to restrict resting CD4+ cell HIV-1 infection without accelerating the decay of latent infection.** *Proc Natl Acad Sci USA* 2012; **109**:9523–9528.
14. Gay C, Dibben O, Anderson JA, Stacey A, Mayo AJ, Norris PJ, *et al*. **Cross-sectional detection of acute HIV infection: timing of transmission, inflammation and antiretroviral therapy.** *PloS One* 2011; **6**:e19617.
15. Archin NM, Eron JJ, Palmer S, Hartmann-Duff A, Martinson JA, Wiegand A, *et al*. **Valproic acid without intensified antiviral therapy has limited impact on persistent HIV infection of resting CD4+ T cells.** *AIDS* 2008; **22**:1131–1135.
16. Eriksson S, Graf EH, Dahl V, Strain MC, Yukl SA, Lysenko ES, *et al*. **Comparative analysis of measures of viral reservoirs in HIV-1 eradication studies.** *PLoS Pathog* 2013; **9**:e1003174.
17. Soriano-Sarabia N, Bateson RE, Dahl NP, Crooks AM, Kuruc JD, Margolis DM, *et al*. **Quantitation of replication-competent HIV-1 in populations of resting CD4+ T cells.** *J Virol* 2014; **88**:14070–14077.
18. Siliciano JD, Siliciano RF. **Enhanced culture assay for detection and quantitation of latently infected, resting CD4+ T-cells carrying replication-competent virus in HIV-1-infected individuals.** In: Zhu T. editor. *Human retrovirus protocols: virology and molecular biology* Totowa, NJ: Humana Press; 2005. pp. 3–15.
19. Crooks AM, Bateson R, Cope AB, Dahl NP, Griggs MK, Kuruc JD, *et al*. **Precise quantitation of the latent HIV-1 reservoir: implications for eradication strategies.** *J Infect Dis* 2015; **212**:1361–1365.
20. Israel-Ballard K, Ziermann R, Leutenegger C, Di Canzio J, Leung K, Strom L, *et al*. **TaqMan RT-PCR and VERSANT® HIV-1 RNA 3.0 (bDNA) assay: quantification of HIV-1 RNA viral load in breast milk.** *J Clin Virol* **2005; 34:253–256.**
21. Robinson LH, Gale CV, Kleim J-P. **Inclusion of full length human immunodeficiency virus type 1 (HIV-1) gag sequences in viral recombinants applied to drug susceptibility phenotyping.** *J Virol Methods* 2002; **104**:147–160.
22. Garrido C, Clohosey ML, Whitworth CP, Hudgens M, Margolis DM, Soriano-Sarabia N. **γδ T cells: an immunotherapeutic approach for HIV cure strategies.** *JCI Insight* 2018; **3**:pii: 120121.
23. Trumble IM, Allmon AG, Archin NM, Rigdon J, Francis O, Baldoni PL, *et al*. **SLDAssay: a software package and web tool for analyzing limiting dilution assays.** *J Immunol Methods* 2017; **450**:10–16.

24. Wasserstein RL, Schirm AL, Lazar NA. **Moving to a world beyond '*P* < 005'.** *Am Stat* 2019; **73 (Suppl 1)**: 1–19.
25. Ryan PL, Sumaria N, Holland CJ, Bradford CM, Izotova N, Grandjean CL, *et al*. **Heterogeneous yet stable Vδ2(+) T-cell profiles define distinct cytotoxic effector potentials in healthy human individuals.** *Proc Natl Acad Sci USA* 2016; **113**:14378–14383.
26. Braakman E, van de Winkel JGJ, van Krimpen BA, Jansze M, Bolhuis RLH. **CD16 on human γδ T lymphocytes: expression, function, and specificity for mouse IgG isotypes.** *Cell Immunol* 1992; **143**:97–107.
27. Angelini DF, Borsellino G, Poupot M, Diamantini A, Poupot R, Bernardi G, *et al*. **FcγRIII discriminates between 2 subsets of Vγ9Vδ2 effector cells with different responses and activation pathways.** *Blood* 2004; **104**:1801–1807.