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HIV Migration Between Blood Plasma and Cellular Subsets Before and After HIV Therapy

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Abstract

The cellular source of HIV RNA circulating in blood plasma remains unclear. Here, we investigated whether sequence analysis of HIV RNA populations circulating before combination antiretroviral therapy (cART) and HIV DNA populations in cellular subsets (CS) after cART could identify the cellular sources of circulating HIV RNA. Blood was collected from five subjects at cART initiation and again 6 months later. Naïve CD4⁺ T cells, resting central memory and effector memory CD4⁺ T cells, activated CD4⁺ T cells, monocytes, and natural killer cells were sorted using a fluorescence-activated cell sorter. HIV-1 *env* C2V3 sequences from HIV RNA in blood plasma and HIV DNA in CSs were generated using single genome sequencing. Sequences were evaluated for viral compartmentalization (Fst test) and migration events (MEs; Slatkin Maddison and cladistic measures) between blood plasma and each CS. Viral compartmentalization was observed in 88% of all cellular subset comparisons (range: 77–100% for each subject). Most observed MEs were directed from blood plasma to CSs (52 MEs, 85.2%). In particular, there was only viral movement from plasma to NK cells (15 MEs), monocytes (seven MEs), and naïve cells (five ME). We observed a total of nine MEs from activated CD4 cells (2/9 MEs), central memory T cells (3/9 MEs), and effector memory T cells (4/9 MEs) to blood plasma. Our results revealed that the HIV RNA population in blood plasma plays an important role in seeding various cellular reservoirs and that the cellular source of the HIV RNA population is activated central memory and effector memory T cells.

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The information in this study has not previously been presented in any scientific meetings.

SUPPORTING INFORMATION

Additional supporting information may be found in the online version of this article at the publisher's web-site.

Keywords

HIV; compartmentalization; reservoir; viremia; antiretroviral therapy; cladistics analysis

INTRODUCTION

The use of combination antiretroviral therapy (cART) has dramatically improved the lives of individuals infected with human immunodeficiency virus (HIV) [Palella et al., 1998]. In HIV-1-infected patients, who respond optimally to cART, HIV RNA levels in blood plasma fall and remain below the limit of detection of ultrasensitive viral load assays [Hammer et al., 1997; Perelson et al., 1997]. However, in the vast majority of patients, stopping cART is associated with a quick rebound of viremia [Imamichi et al., 2001; Doyle et al., 2012; Li et al., 2014]. The source of rebounding virus that is untouched by cART represents a major obstacle to curing HIV [Finzi et al., 1997].

Latently infected resting memory CD4⁺ T cells are the best characterized reservoir for replication-competent latent HIV-1 populations [Chun et al., 1997]. However, proviral populations also persist in naïve CD4⁺ T cells, tissue macrophages, peripheral blood monocytes, and natural killer (NK) cells during cART [Orenstein et al., 1997; Blaak et al., 2000; Valentin et al., 2002; Zhu et al., 2002], and the cellular source of HIV RNA circulating in blood plasma remains unclear. HIV-1 populations in various tissues can differ in cellular tropism, diversity, and drug resistance [Si-Mohamed et al., 2000; Zhang et al., 2002; Pillai et al., 2006], and previous reports have shown that viral compartmentalization also exists between cellular subsets (CSs) [Fulcher et al., 2004; Potter et al., 2004; Delobel et al., 2005]. To date, the compartmentalization of HIV-1 within each cellular compartment has not been well described, whereas the dynamics of HIV migration between anatomical compartments has been extensively studied [Pillai et al., 2005; Smith et al., 2009; Butler et al., 2010; Gianella et al., 2012; Chaillon et al., 2014]. However, little is known about compartmentalization and viral gene flow between cellular compartments. In this study, we investigated whether sequence analysis of HIV RNA populations circulating before cART and HIV DNA populations in various CSs at baseline and 6 months after cART could identify cellular sources of circulating HIV RNA and the dynamics of HIV populations between blood plasma and various CSs.

MATERIALS AND METHODS**Patients and Samples**

Five HIV-infected subjects undergoing cART were enrolled for this study. The demographic and clinical characteristics of each subject are shown in Table I. Samples (20 ml) of whole blood anticoagulated with EDTA were obtained from each patient on day 1 of cART initiation and again 6 months later.

Cell Sorting

Blood was collected by phlebotomy, and plasma and peripheral blood mononuclear cells (PBMC) were separated by Ficoll-Hypaque density gradient centrifugation. Naïve CD4⁺ T

cells, central memory and effector memory CD4⁺ T cells, activated CD4⁺ T cells, CD14⁺ monocyte, and CD56⁺ NK cells were sorted using a fluorescence-activated cell sorter (FACS) (BD Biosciences, San Jose, CA). FITC-conjugated anti-CD3 mAbs (BioLegend, San Diego, CA) were added to label CD3 cells in the PBMC. Positively selected CD3 cells were then stained with PE-conjugated anti-CD4, APC-conjugated anti-CD45RA, perCP/Cy5.5-conjugated anti-CCR7, and APC/Cy7-conjugated anti HLA-DR monoclonal antibodies (mAbs). Negatively selected CD3⁻ cells were stained with perCP/Cy5.5-conjugated anti-CD14 and APC/Cy7-conjugated anti-CD56 mAbs. Cells were sorted by flow cytometry on a FACS Aria (BD Bioscience). Cell purity was determined by flow cytometry analysis of the sorted cells on the same instrument with the same instrument settings. The sorted cells populations were routinely >90% pure.

CD3⁺/CD4⁺/HLA DR⁻/CD45RA⁺/CCR7⁺ cells were considered resting naïve CD4 T cells. CD3⁺/CD4⁺/HLA DR⁻/CD45RA⁻/CCR7⁺ cells were considered resting central memory CD4 T cells. CD3⁺/CD4⁺/HLA DR⁻/CD45RA⁻/CCR7⁻ cells were considered resting effector memory CD4 T cells. CD3⁺/CD4⁺/HLA DR⁺ cells were considered activated CD4 T cells. CD3⁻/CD14⁺ cells were considered CD14⁺ monocytes. CD3⁻/CD56⁺ cells were considered NK cells.

Single Genome Amplification and Sequencing of C2V3

HIV RNA was extracted from blood plasma using the QIAmp Viral RNA Mini Kit (Qiagen, Hilden, Germany) and reverse-transcribed with the RETRO-script kit (Applied Biosystems, Foster City, CA) into complementary DNA (cDNA). Genomic DNA from each cellular compartment was isolated using the QIAmp DNA Mini Kit (Qiagen), according to the manufacturer's instructions. Nested PCR of env C2V3 was performed for single genome amplification and sequencing as previously described [Koelsch et al., 2003; Butler et al., 2009]. Nested PCR was performed using 10 µl of diluted cDNA or cellular DNA template added to 40 µl of reaction mixture for the first round. The reaction mixture consisted of 5.0 µl of 10× PCR buffer containing magnesium chloride and 1.0 µl of 10nM dNTP Mix (GeneAmp, Applied Biosystems), 0.25 µL of Taq DNA polymerase (Roche Diagnostics, Indianapolis, IN), 31.75 µl of molecular grade water, and 1 µl of each of two 20 µM primers, V3-Fout (5'-CAAAGGTATCCTTTGAGCCAAT-3') and V3-Bout (5'-ATTACAGTAGAAAAATCCCCCT-3'). The 50-µl samples were heated to 95 °C for 2 min and then subjected to 35 cycles of 30 s at 95 °C followed by 30 s at 50 °C followed by 60 s at 72 °C. After this, the samples were heated to 72 °C for 10 min and then held at 4 °C until use. Second round PCR used 5 µl of the first round product as the template added to 45 µl of reaction mixture for a total volume of 50 µl. This reaction mixture consisted of the same reagents, but the volume of molecular grade water was increased to 36.75 µl. For this round, the primers used were V3-Fin (5'-GAACAGGACCAGGATCCAATGTCAGCACAGTACAAT-3') and V3-Bin (5'-GCGTTAAAGCTTCTGGGTC CCCTCCTGAG-3'), and the thermal cycling parameters were the same as for the first round. Twenty PCR products from each sample were sequenced using Prism Dye terminator kits (ABI) on an ABI 3100 Genetic Analyzer.

Sequence Analysis

Sequences were initially edited and aligned by Clustal W [Thompson et al., 1994; Larkin et al., 2007]. The alignment was manually edited in Bioedit, version 7.05 to preserve frame insertions and deletions if present. Because recombination may confound the results of phylogeographic inference [Schierup and Hein, 2000], all data sets for phylogeographic analyses were verified and tested negative for recombination using RIP 3.0 [Siepel et al., 1995] and GARD [Pond et al., 2006]. The sequences of each subject have been submitted to GenBank (GenBank accession number: KP796426– KP797835).

Analysis of HIV-1 diversity and divergence—Viral intra-subject diversity (number of base substitutions per site) and ratio of diversity within each compartment from an average of overall individual diversity were conducted using the Tamura 3-parameter model in MEGA5 [Tamura, 1992; Tamura et al., 2011]. The mean divergence was calculated between blood plasma and all other CSs for each subject. HIV population diversity in CSs was estimated as the average pair-wise genetic distances and was calculated using PAUP* version 4.0b10 with the corresponding evolutionary model. The mean diversity and divergence were calculated for each subject's CSs.

Compartmentalization analysis—Viral compartmentalization and population subdivision among each CS were assessed by computing both tree-based [Slatkin and Maddison, 1989] and distance-based methods (F_{ST} and Snn tests) [Hudson et al., 1992; Hudson, 2000; Zarate et al., 2007] for each individual data set using the HyPhy software package [Pond et al., 2005]. Statistical significance was derived via a 1,000 population-structure randomization/ permutation test, as previously described [Bull et al., 2009; Boeras et al., 2011; Sturdevant et al., 2012]. Distance matrices were calculated using the Tamura-Nei 93 genetic distance model [Tamura, 1992].

Cladistic measure of gene flow—The Slatkin–Maddison tree-based method [Slatkin and Maddison, 1989] was used to evaluate compartmentalization and the number of migration events (MEs) among each cellular subset for each individual at both time points. This cladistic measure of MEs determined the minimum number of MEs between compartments that result in the phylogenetic tree under evaluation. We estimated the number of MEs between populations and determined whether the estimated number of MEs was less than expected without compartmentalization. Statistical support was determined by comparing this number of MEs to the number of MEs expected in a randomly structured population [Slatkin and Maddison, 1989; Zarate et al., 2007]. These results were compared with the number of MEs for 1,000 randomizations of compartment labels on the same tree. Evidence of restricted gene flow (i.e., compartmentalization) was documented (at a P value of 0.05) when <5% of the replicates required as many or fewer MEs as in the observed tree.

Phylogenetic analyses—A Bayesian Markov chain Monte Carlo (BMCMC) approach was used to reconstruct phylogenies from sequences for each individual. Sampling dates were used to calibrate the time scale of the trees in days. To incorporate the different CS, we used the discrete Bayesian asymmetric diffusion approach implemented in the BEAST software package [Drummond et al., 2012]. Briefly, BMCMC chains of 100 million

generations were performed for each individual's sequences with a GTR+ Γ 4 substitution model and a Bayesian Sky-grid tree prior [Gill et al., 2013]. Analyses were performed using an uncorrelated lognormal relaxed molecular clock [Drummond et al., 2012]. Tracer, version 1.6 (available at: <http://beast.bio.ed.ac.uk/Tracer>) was used to check for convergence. Trees were visualized in FigTree, version 1.4.2, after selecting the location state set for the nodes and their posterior probability.

Ethics Statement

This study was approved by Severance Hospital IRB (Approval No 4-2011-0295). All subjects provided written informed consent. Human experimentation guidelines of the United States Department of Health and Human Services and those of Severance Hospital were followed.

RESULTS

Subjects and Sample Characteristics

In this study, HIV RNA and HIV DNA populations were sequenced from blood plasma and CSs, respectively, from five subjects at cART initiation and 6 months later. Demographic and clinical characteristics of the enrolled subjects are shown in Table I. At 6 months, viral loads were <20 copies/ml in three patients and <200 copies/ml in two patients. A mean of 282 C2V3 *env* sequences (range: 277–292) per subject and 20 sequences per plasma and CS (range: 17–24) was identified. Within subjects, the mean viral population diversity varied from 0.008 to 0.07 substitute/ site (mean 0.02 substitute/site). Close inspection of viral diversity within each compartment compared to overall intra-individual diversity revealed a higher diversity in monocytes (mean diversity ratio: 2.34, range: 0.22–4.99) and NK cells (1.45 [0–3.18]) and a lower viral diversity in effector memory T cells (0.20 [0–0.27]), and naïve T cells (0.35 [0–1.30]).

Viral Compartmentalization Between the Various CSs

At baseline, viral compartmentalization, defined as a restriction of viral gene flow [Nickle et al., 2003] between blood plasma and CSs, was detected by one or more statistical tests in all CSs and by all four tests in 76.7% of the CSs (Table II). Longitudinal analyses between viral populations in blood plasma sampled at baseline and CSs sampled after 6 months of cART revealed that viral compartmentalization persisted (Table II), and there were no significant differences in compartmentalization between blood plasma and CSs or between the various CSs before and after the start of cART. Tree topologies were consistent with the compartmentalization of independent well-segregated stable HIV subpopulations within the different CSs (Fig. 1).

Spatial Dynamics of HIV-1 Among CSs

HIV RNA in blood plasma as the source of HIV in CSs—We estimated the spread of HIV-1 variants between blood plasma to CSs using tree-based SM statistical analyses [Slatkin and Maddison, 1989]. We quantified viral genetic exchanges between HIV populations circulating in blood plasma at baseline and HIV populations in CSs at baseline and between HIV RNA in blood plasma at baseline and in CSs after 6 months on cART

(Table III and Fig. 2). As the number of MEs depends on the number of sequences analyzed from each cellular subset, a randomization test was performed to estimate *P* values to assess the significance of compartmentalization (Table III). Overall, HIV migration was mostly directed from blood plasma to all CSs with 85% (52 of 61 ME) of all viral lineage movements originating from blood plasma to CSs at baseline (Fig. 2A). The CS groups with the highest number of MEs from blood plasma were the central memory T cells and NK cells, with 13 (25%) and 15 (28.8%) of all MEs from originating from plasma, respectively.

We applied a discrete Bayesian diffusion approach to estimate the spatiotemporal dynamics of viral gene flow between plasma and CSs. Convergence of analyses was reached for all but one subject (subject B) because of a limited phylogenetic signal. Analyses of the four remaining individuals also allowed us to infer the ancestral spatial location of HIV variants during the course of infection and to infer the direction of viral gene flow to populate the different CSs (Fig. 1). For example, in subject C, the vast majority of HIV variants sampled after 180 days in NK cells originated from plasma samples (Fig. 1C), whereas phylogenetic analysis of sequences derived from Subject E found a broader range of viral genetic flow from plasma and CSs (Fig. 1E).

HIV population dynamics among CSs during cART—The study of viral gene flow after 6 months of cART revealed similar patterns of viral migration (Table III and Fig. 2B) with a persistence of viral lineage export from HIV RNA populations sampled in blood plasma at baseline to HIV DNA populations sampled in CSs 6 months after cART. The number of observed MEs differed across individuals and between CSs. Of all 61 MEs observed, 29 (47.5%) were found in subject E (26 MEs from blood plasma to CSs and four MEs from CSs to blood plasma), whereas the fewest MEs during cART were observed in subjects B and D with a total of five MEs for both subjects (Table III). Interestingly, HIV population mobility was observed to be only unidirectional from blood plasma to CSs in NK cells (15 vs. 0 MEs), monocyte (7 vs. 0), and naïve CD4⁺ T-cells (5 vs. 0) across all subjects (Fig. 2). We observed some MEs (a total of nine) at baseline in HIV DNA populations from CSs to HIV RNA populations in blood plasma from activated CD4 cells (two MEs), central memory T cells (three MEs), and effector memory T cells (four MEs).

DISCUSSION

Cellular reservoirs of HIV-1 remain a major obstacle to virus eradication, so it is important to clearly understand the origin and establishment of viral populations within various CSs, especially how these cellular reservoirs persist during cART. In this study, we addressed these issues by sampling and sequencing HIV populations in blood plasma and six different CSs before and after 6 months of cART and analyzed these sequences using cladistics techniques to gain new insights into the establishment and persistence of HIV DNA populations in CSs. In this study, we observed a sustained cell-specific compartmentalization of HIV DNA subpopulations across the six different CSs, and compartmentalization was also observed with HIV RNA populations circulating in blood plasma and HIV DNA populations in CSs both before and during cART. The finding of persistent compartmentalization between CSs is consistent with previous studies that observed distinct HIV DNA populations in blood monocytes and naïve and memory CD4 T

cells during cART [Fulcher et al., 2004; Delobel et al., 2005] and studies that identified central memory and transitional memory CD4⁺ T cells as the major cellular reservoirs for HIV [Chomont et al., 2009]. Our study further clarified the picture of viral dynamics among cellular compartments and the role of HIV RNA populations circulating in blood plasma as the source of the provirus in CSs. Before cART, we showed that viral subpopulations in blood plasma contributed to the establishment of HIV DNA populations in all CSs except for monocytes. The identified viral migration suggests that activated T cells, resting central memory T cells, and resting effector memory T cells are the main source of circulating HIV RNA populations in blood plasma, whereas the observation of only MEs from blood plasma to NK cells, monocytes, and naïve CD4⁺ T-cells suggests that these CSs might be considered viral “sinks” and likely not major sources of circulating HIV RNA populations.

The very limited viral evolution within each CS (mean nucleotide divergence = 0.0061subst./site, range: 0.0014–0.0373) over 6 months on cART (Supplementary Table SI) supports the hypothesis that residual HIV replication does not greatly contribute to the maintenance of HIV DNA populations in CSs during cART. Previous studies determined that the HIV DNA reservoir in CD4⁺ T cells has a slow decay with a half-life up to 44 months [Siliciano et al., 2003]. Although non-mutually exclusive explanations of these long lifespan HIV reservoirs and numerous mechanisms of viral persistence are still debated [Pierson et al., 2000], our observations suggest that cellular HIV DNA populations are stable and are likely not replenished through residual replication. Despite the short follow-up period and the narrow evolutionary resolution, the Bayesian phylogeographic inference framework used in this study allowed us to estimate how circulating HIV RNA in plasma populates various CSs in four of our five subjects. Given the limited viral evolution over the 180-day period of sampling and variability of MEs observed in each subject, our results need to be interpreted cautiously. Future investigations with more subjects and multiple time points should be performed to define reservoirs and sinks of HIV populations and more precisely investigate the temporal dynamics of viral dispersal within multiple CSs during the course of infection.

There were a few other limitations to our study. First, because we did not evaluate the replication competence of cellular DNA, we cannot precisely comment as to whether the HIV DNA populations were replication competent even if the CD4⁺ T cells are the major source of the latent HIV [Bukrinsky et al., 1991; Siliciano et al., 2003]. Second, we did not evaluate clinical or other immunologic factors that could affect the viral evolution and migration within blood plasma and CS compartments. Future studies should characterize and analyze such factors to better clarify which elements could be associated with CS infection, migration, and persistence. Third, the small size of the evaluated sequences and the potential contamination of analyzed cell types during the sorting process may have affected the directionality of viral migration events and compartmentalization.

In conclusion, our results revealed that the HIV RNA population circulating in blood plasma seemed to mostly originate from activated, central memory and effector memory CD4⁺ T cells and not from NK cells, monocytes, or naïve CD4⁺ T cells; the blood plasma HIV plays an important role in seeding various cellular reservoirs when viral replication is unrestrained by cART. We suggest that the cellular HIV DNA populations established before the start of

cART remain stable during cART. Understanding the establishment and persistence of HIV populations within multiple CSs and what mechanisms are involved in the temporal establishment and maintenance of these HIV populations is crucial for planning HIV eradication studies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. Time-scaled Bayesian phylogenetic trees. Plasma branches are colored in red according to the most probable location state of their descendant nodes. Colored triangles represent variants from blood plasma (red), activated CD4+ T cells (yellow), NK cells (brown), central memory T cells (TCM) (blue), effector memory T cells (TEM) (cyan), monocytes (green), and naïve CD4+ T cells (purple).

Fig. 2.

Overall cladistics measure of HIV migration events between blood plasma and viral cellular subsets. The Slatkin–Maddison (SM) test [Slatkin and Maddison, 1989] uses an estimate of the number of migration events (MEs) between subpopulations. Statistical significance ($P < 0.05$) of the SM measure was assessed after 1,000 resampling replicates. The total number of MEs from (red) and to (blue) blood plasma for all five subjects are indicated before cART (A) and after 6 months of cART (B).

TABLE I

Subjects and HIV-1 Sequencing Characteristics

ID	Sex	Age (years)	Plasma HIV RNA (copy/ml)		CD4 T cell count (cells/ml)		SGA sequences		
			Baseline	6 months	Baseline	6 months	Number	Length (nt)	Mean diversity (SE)
A	Male	26	61,800	<20	176	361	278	114	0.015 (0.001)
B	Male	25	3,150,000	141	84	183	282	114	0.008 (0.002)
C	Male	51	85,300	58	244	703	292	114	0.070 (0.02)
D	Male	26	14,300	<20	324	323	277	114	0.011 (0.001)
E	Male	31	12,100	<20	453	531	280	114	0.020 (0.003)

C2V3 HIV-1 *env* sequences were obtained within each compartment by single genome amplification (SGA) (see Methods section). Intra-individual diversity analyses and standard error (SE) estimate(s) were conducted using the Tamura 3-parameter model [Tamura, 1992] in MEGA5 [Tamura et al., 2011]. All positions containing gaps and missing data were eliminated.

Cross-Sectional Analyses of HIV Compartmentalization Between Blood Plasma and Other Cellular Subsets

TABLE II

Subject	Study month	Number of significant tests between plasma and other cellular subsets (CSs)						
		Activated CD4+ T cells	TEM	TCM	NK	Monocytes	Naïve CD4+ T cells	Total
A	0	4/4	1/4	4/4	3/4	4/4	4/4	20/24
	6	4/4	3/4	4/4	4/4	3/4	4/4	22/24
B	0	4/4	4/4	4/4	4/4	4/4	4/4	24/24
	6	4/4	4/4	4/4	3/4	4/4	4/4	23/24
C	0	1/4	4/4	4/4	4/4	1/4	4/4	18/24
	6	4/4	0/4	4/4	4/4	4/4	4/4	20/24
D	0	4/4	4/4	4/4	4/4	4/4	4/4	24/24
	6	4/4	4/4	4/4	4/4	4/4	4/4	24/24
E	0	4/4	1/4	4/4	4/4	2/4	1/4	16/24
	6	4/4	3/4	4/4	3/4	3/4	4/4	21/24
Total	0	17/20	14/20	20/20	19/20	15/20	17/20	102/120
	6	20/20	14/20	20/20	18/20	18/20	20/20	110/120

Viral compartmentalization and population subdivision among each cellular subset were assessed by four tests* for each individual before cART and after 6 months on suppressive cART using the HyPhy software package [Pond et al., 2005].

* Tests: Slatkin–Maddison (SM) [Slatkin and Maddison, 1989]; Hudson nearest neighbor (Snn) [Hudson et al., 1992]; branch count correlation coefficient (rb); branch length (r) correlation coefficient [Crichtlow et al., 2000].

EM, Effector Memory CD4 T cells; TCM, central memory CD4 T cells; NK, natural killer

TABLE III
Cladistics Measure of HIV Migration Events From/to Blood Plasma and Other Cellular Subsets

Subject	Study month	Activated CD4+ T cells	TEM	TCM	NK cells	Monocytes	Naïve CD4+ T cells	Total
A	0	0/1	1/0	0/0	3/0	0/0	0/0	4/1
	6	1/0	1/0	2/0	1/0	1/0	2/0	8/0
B	0	0/0	0/1	0/0	0/0	0/0	1/0	1/1
	6	1/1	0/0	0/0	1/0	0/0	0/0	2/1
C	0	1/0	0/0	0/0	2/0	0/0	0/0	3/0
	6	0/0	2/0	0/0	1/0	2/0	0/0	5/0
D	0	0/0	0/0	0/0	1/0	0/0	0/0	1/0
	6	0/0	0/2	0/0	0/0	2/0	0/0	2/2
E	0	1/0	0/1	5/1	4/0	0/0	1/0	11/2
	6	1/0	3/0	6/2	2/0	2/0	1/0	15/2
Total	0	2/1	1/2	5/1	10/0	0/0	2/0	20/4
	6	3/1	6/2	8/2	5/0	7/0	3/0	32/5

The Slatkin Madison (SM) test [Slatkin and Maddison, 1989] uses an estimate of the number of migrations events (MEs) between subpopulations. The statistical significance ($P < 0.05$) of the SM measure was assessed after 1,000 resampling replicates.

TEM, effector memory CD4 T cells; TCM, central memory CD4 T cells; NK: natural killer