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BRIEF REPORT

Follicular Helper T Cells Are Major Human Immunodeficiency Virus-2 Reservoirs and Support Productive Infection

Ana Godinho-Santos, Russell B. Foxall,[a] Ana V. Antão,[b] Bárbara Tavares, Tiago Ferreira,[c] Ana Serra-Caetano, Paula Matoso,[d] and Ana E. Sousa

Instituto de Medicina Molecular João Lobo Antunes, Faculdade de Medicina, Universidade de Lisboa, Portugal

Follicular helper T cells (Tfh), CD4 lymphocytes critical for efficient antibody responses, have been shown to be key human immunodeficiency virus (HIV)-1 reservoirs. Human immunodeficiency virus-2 infection represents a unique naturally occurring model for investigating Tfh role in HIV/acquired immune deficiency syndrome, given its slow rate of CD4 decline, low to undetectable viremia, and high neutralizing antibody titers throughout the disease course. In this study, we investigated, for the first time, Tfh susceptibility to HIV-2 infection by combining in vitro infection of tonsillar Tfh with the ex vivo study of circulating Tfh from HIV-2-infected patients. We reveal that Tfh support productive HIV-2 infection and are preferential viral targets in HIV-2-infected individuals.

Keywords. follicular helper T cells; HIV-2; HIV/AIDS; Tfh; viral reservoirs.

Life-long antiretroviral therapy (ART) is currently required to control viral replication in the 36 million people with human immunodeficiency virus (HIV). A better understanding of the establishment and maintenance of the persistent HIV reservoirs is critical to identify strategies to prevent viral rebound soon after ART interruption.

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[a]Present Affiliation: Centre for Cancer Immunology and Faculty of Medicine, University Hospital Southampton, Southampton, United Kingdom.

[b]Present Affiliation: Cytokines and Viral Infections Unit, Department of Infection, Immunity and Inflammation, Institut Cochin, INSERM, Paris, France.

[c]Present Affiliation: Laboratory of Oncolytic Virus Immuno-Therapeutics, Deutsches Krebsforschungszentrum, Heidelberg, Germany.

[d]Present Affiliation: Lymphocyte Physiology Unit, Instituto Gulbenkian de Ciência, Oeiras, Portugal.

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Correspondence: Ana E. Sousa, MD, PhD, Instituto de Medicina Molecular João Lobo Antunes, Faculdade de Medicina da Universidade de Lisboa, Avenida Professor Egas Moniz, 1649-028 Lisboa, Portugal (asousa@medicina.ulisboa.pt).

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Follicular helper T cells (Tfh), CD4 T cells specialized in B-cell help and thus essential for durable antibody (Ab) responses, have been shown to be key HIV-1 reservoirs [1]. Follicular helper T cell biology and activation state may facilitate HIV infection within the germinal centers, which can be viewed as HIV sanctuaries even in individuals receiving ART [1, 2]. Although various studies have addressed the role of Tfh in HIV/acquired immune deficiency syndrome (AIDS), there are important gaps concerning the relationship between the dynamics of Tfh viral reservoirs and its impact on development of neutralizing Abs (nAbs). Human immunodeficiency virus-2, a naturally occurring attenuated form of HIV/AIDS, provides a unique model to investigate the interplay between Tfh and HIV. Human immunodeficiency virus-2 infection is associated with the establishment of disseminated reservoirs similarly to HIV-1 [3]. Notwithstanding this, HIV-2-infected patients feature the following: (1) low to undetectable plasma viral load in the absence of ART [3, 4]; (2) slow rate of CD4 decline with reduced impact on the survival of infected adults [4]; (3) progressive immune activation directly associated with the degree of CD4 decline [4]; and (4) high titers of nAbs throughout the disease course [5]. There are limited data on the impact of HIV-2 on Tfh. In this study, we investigated, for the first time, the ability of Tfh to support HIV-2 infection in vitro and ex vivo through the study of purified circulating Tfh (cTfh) from HIV-2-infected individuals.

METHODS

Human Samples

Tonsil specimens were obtained through routine pediatric tonsillectomy at Centro Hospitalar Universitário Lisboa Norte ([CHULN] Lisbon, Portugal). Human immunodeficiency virus-2-infected patients were under follow-up at Clinica Universitária de Doenças Infecciosas of CHULN. The study was conducted under the approval of the Ethical Boards of the CHULN/Faculdade de Medicina da Universidade de Lisboa. Samples were collected after written informed consent from each participant or their legal representatives.

Cell Isolation

Tonsillar mononuclear cells were recovered through tissue dispersion, isolated via Ficoll-Paque Plus (GE Healthcare) density gradient separation, and subsequently magnetically enriched (untouched Human CD4 T Cell Isolation Kit, MojoSort; BioLegend). Follicular helper T cells were then isolated from these cells as illustrated in Figure 1A (purity >98%; FACSAria high-speed cell sorter; BD Biosciences). Peripheral blood mononuclear cells, isolated by Ficoll-Paque Plus, were magnetically enriched for CD4 T cells (untouched Human CD4 T cell Enrichment kit; Stem Cell Technologies) and subsequently sort-purified (purity >98%; FACSAria) as illustrated in Figure 2A.

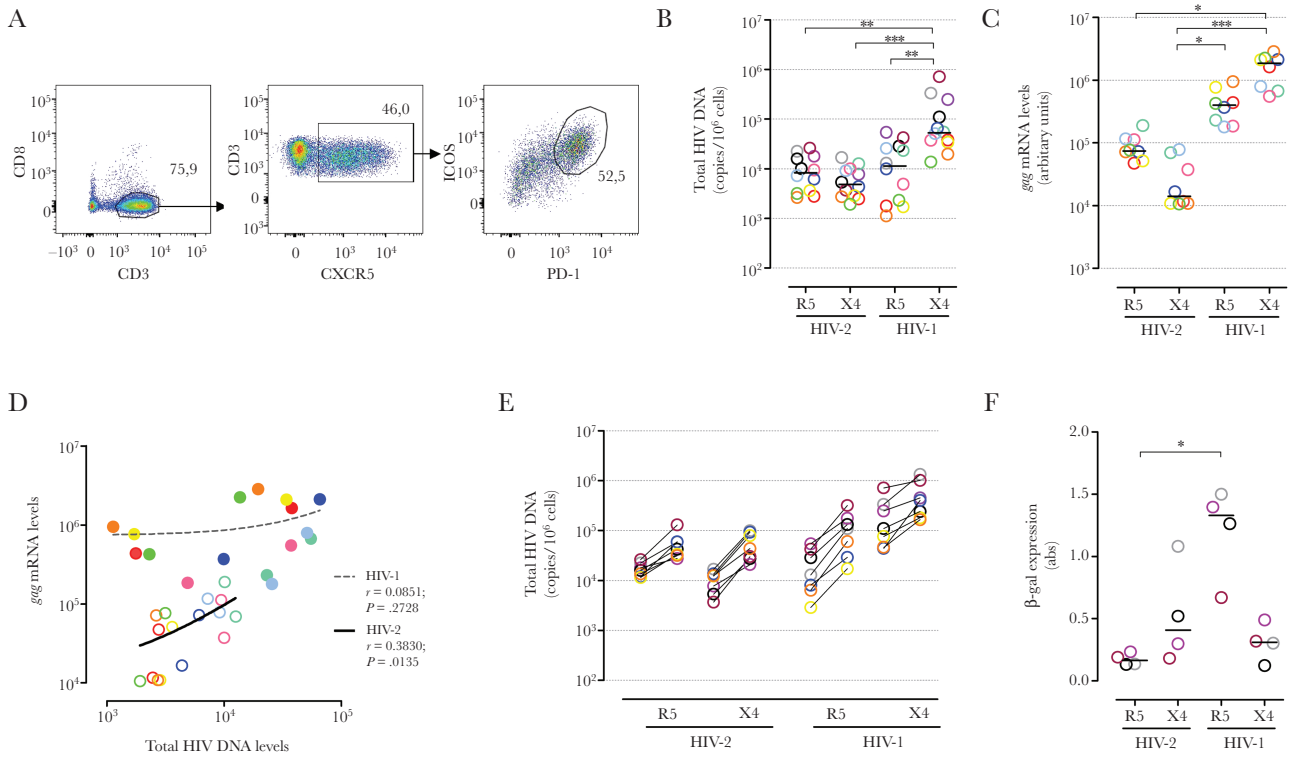

Figure 1. In vitro infection of tonsillar follicular helper T cells (Tfh) by human immunodeficiency virus (HIV)-2. (A) Illustrative dot plots of the sorting strategy, based on CXCR5, PD-1, and ICOS expression, used to isolate Tfh from human tonsillar mononuclear cells enriched for CD4 T cells by magnetic isolation. (B) Total HIV deoxyribonucleic acid (DNA) and (C) *gag* messenger ribonucleic acid (mRNA) quantified after 24-hour infection with R5- or X4-tropic HIV-2 or HIV-1 primary isolates. (D) Correlation of total HIV DNA and viral *gag* mRNA levels. (E) Comparison of total viral DNA at 24 hours postinfection and after 48-hour T-cell receptor (TCR)-mediated stimulation with α-CD3i/α-CD28s. (F) Infectivity of culture supernatants harvested after 48-hour TCR stimulation of HIV-infected Tfh assessed using a TZM-bl reporter cell line and chlorophenolred-β-D-galactopyranoside colorimetric assay (CPRG). Each dot represents 1 independent experiment, and each color refers to a different tonsil donor. $^{*}P < .05$, $^{**}P < .01$, and $^{***}P < .001$ are shown (Friedman test with Dunn's multiple-comparison posttest).

In Vitro Infections

The following primary isolates were used as previously described [6]: CCR5- (R5-) tropic HIV-2, 60415K, subtype A; CXCR4- (X4-) tropic HIV-2, 20.04, subtype A; and HIV-1 R5-tropic, 92US660, subtype B; HIV-1 X4-tropic, 92HT599, subtype B. All obtained from NIH AIDS Reagent Program, except 20.04 (provided by Nuno Taveira, Research Institute for Medicines, Faculdade de Farmácia, Universidade de Lisboa, Portugal). Sorted Tfh (2.5×10^5) were infected with primary isolates (1.6 ng reverse transcriptase [RT]/10^6 cells), washed after 3 hours with phosphate-buffered saline, and cultured (2.5×10^6 cells/mL) at 37°C/5% CO_2 in Roswell Park Memorial Institute 1640 medium with 10% fetal bovine serum, 2 mM L-glutamine, 50 U/mL penicillin-streptomycin, 50 µg/mL gentamicin

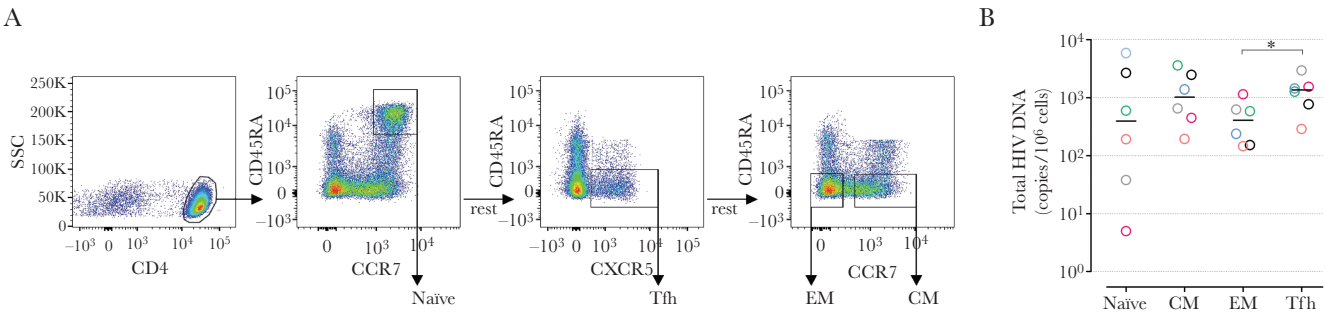

Figure 2. Total human immunodeficiency virus (HIV) deoxyribonucleic acid (DNA) in circulating follicular helper T cells (Tfh) from HIV-2-infected patients. (A) Illustrative dot-plots of the sorting strategy used to obtain peripheral CD4 T-cell subsets based on CD45RA, CCR7, and CXCR5 with a FACSAria, after magnetic enrichment of CD4 T cells from peripheral blood mononuclear cells. Purity of the sorted subsets was always higher than 98%. (B) Total viral DNA levels in naive, Tfh, central memory (CM), and effector memory (EM) subsets. Each color dot represents 1 individual. *, $P < .05$ (Wilcoxon matched-pairs signed-rank test).

(Gibco/Invitrogen) plus 3 μg/mL Polybrene (Sigma) in 96-well plates (Costar). After 24 hours, 5×10^4 cells were collected for deoxyribonucleic acid (DNA)/ribonucleic acid (RNA) extraction, and the remaining cells were stimulated with immobilized anti-CD3 (α-CD3i, 1 μg/mL; eBioscience) and soluble anti-CD28 (α-CD28s, 1 μg/mL; eBioscience) in 96-well plates. Both cells and supernatants were collected after 48 hours and stored at −80°C.

Quantification of Cell-Associated Human Immunodeficiency Virus Deoxyribonucleic Acid and *gag* Messenger Ribonucleic Acid by Real-Time Polymerase Chain Reaction

Total DNA and RNA were extracted from cell pellets of in vitro-infected Tfh and sort-purified blood CD4 T-cell subsets, using AllPrep DNA/RNA Micro kit (QIAGEN) and ZR-Duet DNA/RNA MiniPrep kit (Zymo Research), respectively. For *gag* messenger RNA (mRNA) quantification, 80 ng of total RNA was used to synthesize complementary DNA using oligo(dT)20 and SuperScript III RT (Invitrogen). Real-time polymerase chain reaction (PCR) was performed with ABI 7500 Fast Real-Time PCR System (Applied Biosystems) using TaqMan gene expression master mix (Applied Biosystems); primers and probes used are described in Supplementary Table 1. Positive controls were generated as previously described [6].

Viral Infectivity Assay

TZM-bl cells, carrying β-galactosidase gene under HIV-1 long terminal repeat control, were infected with supernatants harvested from in vitro-infected Tfh, in duplicates. Cells were lysed after 40 hours to evaluate Tat-inducible β-galactosidase expression by chlorophenolred-β-D-galactopyranoside colorimetric assay (CPRG; Roche). The background absorbance of lysed uninfected cells was subtracted from results.

Statistical Analysis

Wilcoxon matched-pairs signed rank test or the Friedman test with Dunn's multiple-comparison posttest were performed using GraphPad Prism (v6.01) software. $P < .05$ were considered significant.

RESULTS

To assess their susceptibility to HIV-2 infection, we sort-purified Tfh from human tonsillar tissue based on CXCR5, PD-1, and ICOS expression (Figure 1A) and challenged them with HIV-2 and HIV-1 primary isolates with selective CCR5 or CXCR4 coreceptor usage, using RT activity to normalize the viral input. We found that HIV-2 were able to infect Tfh, irrespectively of coreceptor usage as shown by total HIV DNA levels measured after 24 hours of infection (Figure 1B). As anticipated, Tfh were susceptible to HIV-1 in our in vitro system, independently of their tropism, which is in agreement with previous reports [1, 7, 8]. Moreover, as expected given the higher expression of CXCR4 compared with CCR5 on Tfh, HIV-1 X4-tropic virus yielded increased copies of *gag* than its R5-tropic counterpart (Figure 1B). It is interesting to note that, in contrast with the pattern observed for HIV-1, CXCR4 usage was not associated with higher total HIV-2 DNA in the case of HIV-2 primary isolates (Figure 1B). The levels of HIV-1 X4-tropic virus were also significantly higher than those obtained with either R5- or X4-tropic HIV-2 (Figure 1B).

We next investigated the levels of viral transcription in HIV-2-infected Tfh by quantifying viral *gag* mRNA upon 24 hours of infection. As shown in Figure 1C, both R5- and X4-tropic HIV-2 viruses generated *gag* mRNA transcripts, demonstrating ongoing viral transcription in HIV-2-infected Tfh. It is interesting to note that, in the case of HIV-2-infected Tfh, there was a significant direct correlation between the levels of total HIV DNA and *gag* mRNA, which was not observed in the case of HIV-1 infection (Figure 1D).

To evaluate whether Tfh can support productive HIV-2 infection, we quantified the amount of viral production upon T-cell receptor (TCR) activation with α-CD3i/α-CD28s after 24 hours of infection. We observed an increase of cell-associated total viral DNA levels in both R5- and X4-tropic HIV-2 isolates after 48 hours of stimulation (Figure 1E), which suggests that Tfh support productive HIV-2 infection irrespectively of coreceptor usage. As expected, we also observed a comparable increase for both HIV-1 primary isolates. To provide further evidence of productive infection, supernatants from TCR-stimulated HIV-infected Tfh were collected and evaluated for their infectivity using the TZM-bl reporter cell line. The results indicated that the newly produced virions released by HIV-2-infected Tfh are infectious (Figure 1F). Hence, Tfh are indeed able to support productive HIV-2 infection.

To confirm that in vitro Tfh susceptibility is recapitulated in vivo, we assessed whether Tfh isolated from HIV-2-infected individuals harbored HIV-2. CD4 T-cell subsets were purified from the peripheral blood of 6 ART-naive HIV-2-infected individuals, all females, median age 60, 3 "Caucasians," with undetectable viremia (less than 40 copies viral RNA/mL), preserved CD4 T-cell counts (median 882 cells/μL) and no history of opportunistic infections. Total HIV DNA was quantified in cTfh, defined as CD45RAnegCXCR5$^+$ cells (Figure 2A), and compared with the levels measured in sorted naive (CCR7$^+$CD45RA$^+$), central memory (CCR7$^+$CD45RAnegCXCR5neg), and effector memory ([EM] CCR7negCD45RAnegCXCR5neg) CD4 T-cell subsets (Figure 2B). We found that HIV-2 can infect Tfh in vivo, and, in addition, that blood Tfh showed the highest median levels of total HIV DNA, which were significantly different from the EM subset (Figure 2B). Of note, this reservoir was effectively controlled, because these patients featured no detectable viremia in the absence of ART. Therefore, our data support that Tfh are a major HIV-2 reservoir in vivo.

DISCUSSION

This first study addressing the ability of HIV-2 to infect Tfh revealed that HIV-2 infects purified tonsillar Tfh in vitro and

identified peripheral blood Tfh as a key viral reservoir in HIV-2-infected patients. Human immunodeficiency virus-2 primary isolates infected tonsillar Tfh irrespective of their tropism as attested by cell-associated HIV DNA and *gag* mRNA levels. Follicular helper T cells are thought to be mainly infected at the stage of Tfh precursors, which express high levels of CCR5 [8]. Nonetheless, we showed that differentiated Tfh are susceptible to R5-tropic viruses, confirming that low levels of CCR5 are enough for efficient HIV-1 and HIV-2 infection. Of note, these results were obtained in the absence of extrinsic cell activation. Although polybrene was used to increase infection efficiency [9], this method does not affect cell activation or viability, and viral entry mechanisms were not the focus of this work. Our data on HIV-1 infection are in agreement with previous studies revealing high permissiveness of Tfh to HIV-1 [1, 10, 11]. X4-tropic HIV-1 generated higher amounts of total HIV DNA than R5-tropic HIV-1, a typical pattern of coreceptor dependency that was not observed in HIV-2 primary isolates. It has been previously reported that HIV-2 cytopathicity in human lymphoid tissue is coreceptor dependent and comparable to that of HIV-1 [6]. Further studies using smaller viral inputs and a range of HIV-2 primary isolates are required to evaluate the underlying mechanisms.

Our results indicate that HIV-2 cannot only complete the early phase of the replication cycle until integration, but it can also undergo viral transcription in Tfh. It is interesting to note that a stronger correlation was observed between the levels of total HIV DNA and *gag* mRNA in HIV-2-infected Tfh than in those infected by HIV-1, which might suggest a distinct regulation of viral transcription. It is notable that the structure of HIV-2 transactivation region (TAR) differs from that of the HIV-1 TAR stem loop, being much longer and folded into a fork motif [12], providing a possible explanation for the changes in the transcription of partially spliced and unspliced viral transcripts upon Tat-TAR interaction. Because tonsillar Tfh, unlike peripheral CD4 T cells, support infection without exogenous stimulation, these cells represent a very relevant model to study latency and viral transcription.

Follicular helper T cells supported productive HIV-2 infection, as demonstrated by the increase in cell-associated viral DNA levels after TCR stimulation and the release of infectious virions into culture, as evaluated by the TZM-bl reporter cell line. Of note, this reporter system was not optimized to allow a quantitative evaluation of viral production, precluding the investigation of any possible posttranscriptional control, a phenomenon we previously observed in in vitro-infected thymocytes [6].

It could be argued that Tfh derived from inflamed tonsillar tissue may favor HIV infection. Nevertheless, our data obtained in peripheral blood Tfh from HIV-2-infected patients support Tfh susceptibility to HIV-2, and, importantly, showed that Tfh are a major HIV-2 reservoir in vivo. Follicular helper T cells have been identified as prime targets for HIV-1 infection [13]. Accordingly, we observed higher levels of cell-associated viral HIV-2 DNA in Tfh when compared with CD4 effector memory cells. It is notable that these patients featured no detectable viremia in the absence of ART and had preserved blood CD4 T-cell counts, indicating that HIV-2 infection is being naturally controlled regardless of disseminated viral reservoirs. The control of ongoing HIV-2 replication in these patients is likely due to particular host-pathogen interactions, because it has been shown that HIV-2 can efficiently replicate in vitro [6]. In support of a role for Tfh in this interplay, HIV-2 infection is associated with sustained titers of nAbs [5], irrespective of disease stage, which we have also shown to be accompanied by progressive memory B-cell depletion [14]. It is remarkable that a relatively preserved cTfh ability to provide adequate B-cell help has been documented in HIV-1 elite controllers in comparison to chronic HIV-1-infected individuals [15]. Future studies are required to better understand how Tfh infection impacts B-cell help in HIV-2-infected patients, which will ultimately provide insights into nAb responses.

CONCLUSIONS

Our results, taken together, show that Tfh support productive HIV-2 infection and represent a key HIV-2 reservoir. Thus, HIV-2 infection of Tfh provides a unique model to identify host factors playing a role in the natural control of HIV-2 replication and to further investigate the generation of HIV nAbs.

Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

Supplementary Table 1. Primers and probes used for quantification of total human immunodeficiency virus (HIV) deoxyribonucleic acid (DNA) and *gag* messenger ribonucleic acid (mRNA) by quantitative real-time polymerase chain reaction (PCR).

Notes

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Potential conflicts of interest. All authors: No reported conflicts of interest. All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest.

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