A CCR5+ memory subset within HIV-1-infected primary resting CD4+ T cells is permissive for replication-competent, latently infected viruses in vitro

Kazutaka Terahara¹, Ryutarō Iwabuchi¹,²,³, Masahito Hosokawa⁴,⁵, Yohei Nishikawa²,³, Haruko Takeyama²,³,⁴,⁵, Yoshimasa Takahashi¹ and Yasuko Tsunetsugu-Yokota¹,⁶

Abstract

Objective: Resting CD4+ T cells are major reservoirs of latent HIV-1 infection, and may be formed during the early phase of the infection. Although CCR5-tropic (R5) HIV-1 is highly transmissible during the early phase, newly infected individuals have usually been exposed to a mixture of R5 and CXCR4-tropic (X4) viruses, and X4 viral DNA is also detectable in the host. Our aim was to identify which subsets of resting CD4+ T cells contribute to forming the latent reservoir in the presence of both X4 and R5 viruses.

Results: Primary resting CD4+ naïve T (TN) cells, CCR5− memory T (TM) cells, and CCR5+ TM cells isolated by flow cytometry were infected simultaneously with X4 and R5 HIV-1, which harbored different reporter genes, and were cultured in the resting condition. Flow cytometry at 3 days post-infection demonstrated that X4 HIV-1 cells were present in all three subsets of cells, whereas R5 HIV-1 cells were present preferentially in CCR5+ TM cells, but not in TN cells. Following CD3/CD28-mediated activation at 3 days post-infection, numbers of R5 HIV-1 cells and X4 HIV-1 cells increased significantly only in the CCR5+ TM subset, suggesting that it provides a major reservoir of replication-competent, latently infected viruses.

Keywords: HIV, Latent reservoir, Resting CD4+ T cells

Introduction

Current combination antiretroviral therapy has been successful in suppressing HIV-1 replication to undetectable levels. However, a barrier to the complete eradication of HIV-1 infection by combination antiretroviral therapy is the existence of a small reservoir of latently infected cells [1–4]. A prime candidate for this reservoir is resting CD4+ T cells since they are long-lived and can harbor replication-competent proviruses that remain transcriptionally silent in the absence of an activating stimulus [5–8]. Resting CD4+ T cells are heterogeneous populations that include naïve (TN) and memory (TM) cells. TM cells are further divided into central memory (TCM), transitional memory (TTM), and effector memory (TEM) cells. Resting CD4+ TM cells have been proposed to be major reservoirs of latent HIV-1 infection, on the evidence of high levels of HIV-1 DNA content [5, 9, 10]. However, it has also been suggested that resting CD4+ TN cells are an important reservoir of latent HIV-1 infection [11, 12].

A latent reservoir could be established during the early phase of HIV-1 infection [1, 6], during which CCR5-tropic (R5) HIV-1 is highly transmissible [13–15]. Notably, results from next-generation sequencing suggest that CXCR4-tropic (X4) HIV-1 may be more prevalent during the early phase of HIV-1 infection than previously reported [16], so that newly infected individuals have usually been exposed to a mixture of X4 and R5 viruses [17–20]. CD4+ T cells undergoing effector-to-memory
transient are permissive for HIV-1 latent infection [21]. Latency has also been shown to occur following direct infection of resting CD4+ T cells [22], but it is not yet known which subsets of resting CD4+ T cells are involved in the latent infection by X4 and R5 HIV-1.

We previously constructed a recombinant X4 HIV-1 (HIV-1NL-E) harboring EGFP reporter gene for expression of a green fluorescent protein, along with an isogenic R5 HIV-1 (HIV-1NLAD8-D) harboring DsRed gene, for expression of a red fluorescent protein, enabling us to distinguish between these viruses in productively infected cells [23]. Here, we investigated the infectivity of these viruses in isolated, primary human resting CD4+ T cell subsets (TN, CCR5+ TM, and CCR5+ TM) in a dual-infection model.

Main text

Methods

Virus preparation

To generate HIV-1NL-E and HIV-1NLAD8-D stocks, HEK293T cells were transfected with the corresponding proviral DNA plasmid using the calcium phosphate precipitation method as described previously [23]. The amount of p24 Gag in the culture supernatant was measured with an in-house enzyme-linked immunosorbent assay [24]. The supernatant was then filtered, aliquoted, and stored at −80 °C.

Cell preparation

Human peripheral blood was donated by healthy Japanese adult volunteers. Peripheral blood mononuclear cells (PBMCs) were separated by the Lymphocyte Separation Medium 1077 (PromoCell, Heidelberg, Germany). CD4+ T cells were first negatively enriched from PBMCs using the EasySep Human CD4+ T cell Enrichment Kit (StemCell Technologies, Vancouver, BC, Canada). Enriched CD4+ T cells were stained with the following antibodies: CD69-FITC (FN50; ThermoFisher Scientific, Waltham, MA, USA), HLA-DR-Alexa Fluor 488 (L243; BioLegend, San Diego, CA, USA), CD8-PerCP (RPA-T8, BioLegend), CD19-PerCP (HIB19; BioLegend), CD27-Alexa Fluor 700 (O323; BioLegend), CD45RA-PE-Cy7 (H1100; BioLegend), and CCR5-Alexa Fluor 647 (T312; [25]). Notably, the anti-CCR5 monoclonal antibody T312 does not interfere with the binding of R5 HIV-1 to CCR5 [25]. Three subsets of resting (CD69−HLA-DR−) CD4+ T cells [TN (CD45RA+CD27−CCR5−), CCR5+ TM (CD45RA−CCR5+), and CCR5+ TM (CD45RA−CCR5+)] were isolated by flow-cytometric sorting using a FACSAria III (BD Biosciences, San Diego, CA, USA). Dead cells stained with the Live/Dead Fixable Violet Dead Cell Stain (ThermoFisher Scientific) were not isolated.

Infection and culture

Resting CD4+ T-cell subsets isolated by flow cytometry were infected with a 1:1 mixture of X4 and R5 viruses (determined by the amount of p24) at a range of 10–20 ng total p24 per 10^5 cells by the spinoculation method as previously described [26]. After spinoculation, cells were washed and then incubated in R-10 medium [RPMI-1640 with 10% fetal bovine serum, 100 µg/ml penicillin, 100 µg/ml streptomycin, and 1% GlutaMAX solution (ThermoFisher Scientific)] for 2 h at 37 °C to induce viral fusion. Cells were then washed and cultured in R-10 medium supplemented with 1.25 µM saquinavir, to prevent viral spread, for up to 5 days. At 3 days post-infection, half of the culture medium was replaced with fresh medium, and the cultures were either kept in the resting condition or subjected to activation via the T-cell receptor using the Dynabeads Human T-Activator CD3/ CD28 (ThermoFisher Scientific) in the presence of 5% heat-inactivated human AB serum and 50 U/ml IL-2 for 2 days. Flow cytometry was performed to detect productively infected cells at 3 days and 5 days post-infection using a FACSCanto II (BD Biosciences). All experiments with HIV-1 were conducted in a biosafety level 3 containment facility at National Institute of Infectious Diseases (NIID; Tokyo, Japan).

Analysis for flow cytometry

Data obtained from flow cytometry were saved as FCS files and analyzed using FlowJo v10.5.0 (BD Biosciences).

Statistical analysis

The significance of data comparisons was evaluated by repeated-measures one-way ANOVA and Tukey’s multiple comparison test using GraphPad Prism version 8 (Graph Pad Software, San Diego, CA, USA). A P-value of <0.05 was considered statistically significant.

Results

Profiles of resting TN, CCR5− TM−, and CCR5+ TM−cell populations prior to sorting

For HIV-1 infection and culture experiments, negatively enriched CD4+ T cells were prepared from 200 ml each of peripheral blood from three donors. The enriched CD4+ T cells were stained with fluorochrome-conjugated antibodies and subjected to flow-cytometric sorting. Resting CD4+ T cells were defined as CD69 and HLA-DR double-negative, as described elsewhere [27], and represented 83.1–94.3% of the enriched CD4+ T cells (Fig. 1a). The resting CD4+ T cells consisted of CD45RA−CD27−CCR5− TN, CD45RA−CCR5− TM, and CD45RA−CCR5+ TM cells, with CCR5 expressed exclusively on CD45RA− TM cells. The resting CCR5+
TM cells were the minor population, with yields of $0.9 \times 10^6$ to $2.2 \times 10^6$ cells from the three donors. All of the sorted cells were used for HIV-1 infection and culture experiments. In a pilot experiment, the cell-sorting protocol was applied to small-scale samples from two more donors. Resting CCR5$^+$ TM cells were isolated at 86.9–93.0% purity, whereas resting $T_N$ cells and CCR5$^-$ TM cells were isolated at > 97% purity (Fig. 1b).

**Productive HIV-1 infection in resting cells**

The protocol for the study of HIV-1 infection and culture is summarized in Fig. 2a. The results of
flow cytometry showed that X4 HIV-1 productively infected all resting subsets, with EGFP expression in 0.50% ± 0.25% (mean ± SD) of TN cells, 1.83% ± 1.99% of CCR5− TM cells, and 1.76% ± 1.43% of CCR5+ TM cells at 3 days post-infection (Fig. 2b, c and Additional file 1). The percentage of resting cells with R5 HIV-1 infection was highest in CCR5+ TM cells (4.35% ± 0.42%), considerably less in CCR5− TM cells (0.47% ± 0.25%), and very
In TN cells, extending the culture from 3 to 5 days in the infection condition substantially enhanced both the intensity and frequency of expression of the EGFP reporter, suggesting that productive infection in resting TN cells proceeded more slowly than in resting TM cells.

**Activation increases the frequency of HIV-1 infection in CCR5+ TM cells**

Proportions of HIV-1+ cells 5 days post-infection were compared in resting cells and cells that were activated on day 3 post-infection. In resting condition substantially enhanced both the intensity and frequency of expression of the EGFP reporter, suggesting that productive infection in resting TN cells proceeded more slowly than in resting TM cells.

We found that the intensity of the viral reporter fluorescence in resting CD4+ TN cells was lower than in resting CD4+ TM cells at 3 days post-infection. Previous studies indicated that the low intensity of the viral reporter fluorescence may suggest the presence of unintegrated viral DNA, which is capable of generating infectious virions [28, 29]. Although we did not determine the levels of integrated proviral DNA and unintegrated viral DNA, our results highlight a replicative advantage of CCR5+ memory subsets for both X4 and R5 HIV-1, even in the resting condition.

**Discussion**

In this study, we demonstrated by flow cytometry that resting CD4+ T cells are subject to productive infection. This result is consistent with findings from other studies, where primary resting CD4+ T cells were infected with HIV-1 expressing fluorescent reporter proteins [22, 28, 29]. The profile of productive infection that we observed was associated with the expression pattern of the coreceptors. In particular, CCR5 is expressed on TM cells, especially the TEM subset, and is rarely detectable on TN cells, whereas CXCR4 is detectable in all CD4+ T-cell subsets [30, 31]. The R5 HIV-1+ cells that we detected in the CCR5− TM subset might result from the presence of R5 HIV-1-permissive cells that were not detected for CCR5 expression by flow cytometry, as low levels of CCR5 are sufficient for R5 HIV-1 infection if they are associated with sufficient cell-surface expression of CD4 [32].

First, we did not investigate whether, after exposure to virus, reporter-negative resting cells possessed proviruses that were induced for viral replication following activation. Second, because we identified preferential infection of R5 HIV-1 over X4 HIV-1 in resting CCR5+ TM cells, it was assumed that R5 HIV-1 is more permissive for latent infection in resting CCR5+ TM cells than X4 HIV-1.
However, it has been reported that X4 laboratory strains such as NL4-3, from which our X4 HIV-1 (HIV-1NL-E) was derived, are less effective at infecting cells expressing low levels of CXCR4 than primary isolates [34]. Indeed, CXCR4 expression levels in CCR5+ T_M cells are lower than in T_N cells (Additional file 2) [31]. Our separate experiments demonstrated that the predominance of R5 HIV-1 over X4 HIV-1 had already begun at the binding stage of the infection of CCR5+ T_M cells (Additional file 3). Therefore, X4 primary isolates should be tested to evaluate the predominance of latent infection between X4 and R5 viruses.

Additional files

Additional file 1: Fig. S1. Flow-cytometry profiles of Donor #2 and Donor #3 at day 3 and day 5 post-infection.

Additional file 2: Fig. S2. Comparison of CXCR4 expression between T_M and CCR5+ T_M cells within resting CD4+ T cells.

Additional file 3: Fig. S3. Data from separate experiments for the evaluation of HIV-1 binding (S3-1), entry (S3-2), and reverse transcription (S3-3) in CCR5+ T_M cells.

Abbreviations

NIID: National Institute of Infectious Diseases; PBMC: peripheral blood mononuclear cell; R5: CCR5-tropic; T_M cell: central memory T cell; TEM cell: effector memory T cell; TM cell: memory T cell; TN cell: naïve T cell; TTM cell: transitional memory T cell; X4: CXCR4-tropic.

Authors’ contributions

KT conceived the study design, obtained funding and ethical approvals, performed experiments, interpreted the data, wrote the manuscript, and supervised the study. RI performed experiments, searched related studies, and discussed the data interpretation. MH, YN, and HT performed experiments. YT advised on the study design and data interpretation. YT-Y advised on the data interpretation. MH, YN, and HT performed experiments. RI performed experiments, searched related studies, and discussed the data interpretation. MH, YN, and HT performed experiments. YT advised on the study design and data interpretation. YT-Y advised on the data interpretation. MH, YN, and HT performed experiments. RI performed experiments, interpreted the data, wrote the manuscript, and approved the final manuscript.

Author details

1 Department of Immunology, National Institute of Infectious Diseases, 1-23-1 Toyama, Shinjuku-ku, Tokyo 162-8640, Japan. 2 Department of Life Science and Medical Bioscience, Waseda University, 2-2 Wakamatsu-cho, Shinjuku-ku, Tokyo 162-8480, Japan. 3 Computational Bio Big-Data Open Innovation Laboratory, National Institute of Advanced Industrial Science and Technology, 3-4-1 Okubo, Shinjuku-ku, Tokyo 169-8555, Japan. 4 Research Organization for Nano & Life Innovation, Waseda University, S13 Wasedatsumurakicho, Shinjuku-ku, Tokyo 162-0041, Japan. 5 Institute for Advanced Research of Biosystem Dynamics, Waseda Research Institute for Science and Engineering, Waseda University, 2-2 Wakamatsu-cho, Shinjuku-ku, Tokyo 162-8480, Japan. 6 Department of Medical Technology, School of Human Sciences, Tokyo University of Technology, 5-23-22 Nishikamata, Ota-ku, Tokyo 144-8535, Japan.

Acknowledgements

We thank Dr. Y. Tanaka (University of the Ryukyus, Okinawa, Japan) for kindly providing anti-CCRF monoclonal antibody (T312). We also thank R. Iwaki (NIID) for technical support.

Competing interests

The authors declare that they have no competing interests.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Consent for publication

Not applicable.

Ethics approval and consent to participate

Human peripheral blood was obtained from healthy Japanese adult volunteers after receiving written informed consent. The use of human peripheral blood was approved by the Institutional Ethical Committee of the National Institute of Infectious Diseases (Reference Number 887).

Funding

This work was supported by JSPS KAKENHI under Grant Number JP17K08800 and AMED under Grant Number JP18K101003. The funding bodies had no role in the design of the study; the collection, analysis, or interpretation of data or the writing of the manuscript.

Publisher’s Note

Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Received: 1 April 2019 Accepted: 25 April 2019 Published online: 29 April 2019

References

1. Castro-Gonzalez S, Colomer-Lluch M, Serra-Morenzo R. Barriers for HIV cure: the latent reservoir. AIDS Res Hum Retroviruses. 2018;34:739–59.
2. Finzi D, Blankson J, Siliciano JD, Margolick JB, Chadwick K, Pierson T, Smith K, Lisziewicz J, Lori F, Flexner C, et al. Latent infection of CD4+ T cells provides a mechanism for lifelong persistence of HIV-1, even in patients on effective combination therapy. Nat Med. 1999;5:S12–7.
3. Finzi D, Hermankova M, Pierson T, Carmuth LM, Buck C, Chaisson RE, Quinn TC, Chadwick K, Margolick J, Brookmeyer R, et al. Identification of a reservoir for HIV-1 in patients on highly active antiretroviral therapy. Science. 1997;278:1295–300.
4. Ho YC, Shan L, Hosmane NN, Wang J, Laskey SB, Rosenbloom DI, Lai J, Blankson JN, Siliciano JD, Siliciano RF. Replication-competent noninduced proviruses in the latent reservoir increase barrier to HIV-1 cure. Cell. 2013;155:S40–S1.
5. Chomont N, El-Far M, Ancuta P, Trautmann L, Procopio FA, Yassine-Diab B, Boucher G, Boulasell MR, Ghattas G, Brenchley JM, et al. HIV reservoir size and persistence are driven by T cell survival and homeostatic proliferation. Nat Med. 2009;15:893–900.
6. Chun TW, Engel D, Berrey MM, Shea T, Corey L, Fauci AS. Early establishment of a pool of latently infected, resting CD4+ T cells during primary HIV-1 infection. Proc Natl Acad Sci USA. 1998;95:8869–73.
7. Pallikkuth S, Sharkey M, Babic DJ, Gupta S, Stone GW, Fischl MA, Stevenson M, Pahwa S. Peripheral T follicular helper cells are the major HIV reservoir within central memory CD4 T cells in peripheral blood from chronically HIV-infected individuals on combination antiretroviral therapy. J Virol. 2015;90:2718–28.
8. Sengupta S, Siliciano RF. Targeting the latent reservoir for HIV-1. Immunity. 2018;48:872–95.
9. Bacchus C, Chret A, Avettand-Fenoel V, Nembot G, Melad A, Blanc C, Lacouix-Combe C, Slama L, Allegre T, Allavena C, et al. A single HIV-1 cluster and a skewed immune homeostasis drive the early spread of HIV among resting CD4+ cell subsets within one month post-infection. PLoS ONE. 2013;8:e64219.
10. Saez-Cirion A, Bacchus C, Hocquedeaux L, Avettand-Fenoel V, Girault L, Lecourex C, Potard V, Versmisse P, Melad A, Pruzuck T, et al. Post-treatment HIV-1 controllers with a long-term virological remission after the interruption of early initiated antiretroviral therapy. ANRS VISCONTI Study. PLoS Pathog. 2013;9:e1003211.
23. Yamamoto T, Tsunetsugu-Yokota Y, Mitsuki YY, Mizukoshi F, Tsuchiya T, Terahara T, Inagaki Y, Yamamoto N, Kobayashi K, Inoue J. Selective transmission of RS HIV-1 over X4 HIV-1 at the dendritic cell-T cell infectious synapse is determined by the T cell activation state. PLoS Pathog. 2009;5:e1000279.

24. Tsunetsugu-Yokota Y, Akagawa K, Kimoto H, Sasaki Y, Iwasaki M, Yasuda S, Hauser G, Hultgren C, Meyerhans A, Takekomi T. Monocyte-derived cultured dendritic cells are susceptible to human immunodeficiency virus infection and transmit virus to resting T cells in the process of nominal antigen presentation. J Virol. 1995;69:4544–7.

25. Tanaka R, Yoshida A, Murakami T, Baba E, Lichtenfeld J, Omori T, Kimura T, Tsunetani N, Fuji N, Wang ZX, et al. Unique monoclonal antibody recognizing the third extracellular loop of CXCR2S induces lymphocyte agglutination and enhances human immunodeficiency virus type 1-mediated syncytium formation and productive infection. J Virol. 2001;75:11534–43.

26. O’Doherty U, Swiggard WJ, Malim MH. Human immunodeficiency virus type 1 spinoculation enhances infection through virus binding. J Virol. 2000;74:10074–80.

27. Swiggard WJ, Baytop C, Yu JJ, Dai J, Li C, Schretzenmair R, Theodospoulos T, O’Doherty U. Human immunodeficiency virus type 1 can establish latent infection in resting CD4+ T cells in the absence of activating stimuli. J Virol. 2005;79:14179–88.

28. Chan CN, Trinite B, Lee CS, Mahajan S, Anand A, Wodarz D, Sabbaj S, Bansal A, Goepfert PA, Levy DN. HIV-1 latency and virus production from unintegrated genomes following direct infection of resting CD4+ T cells. Retrovirology. 2016;13:1.

29. Trinite B, Ohlson EC, Voznesensky I, Rana SP, Chan CN, Mahajan S, Alster J, Burke SA, Wodarz D, Levy DN. An HIV-1 replication pathway utilizing reverse transcription products that fail to integrate. J Virol. 2013;87:12701–20.

30. Groot F, van Capel TM, Schuitemaker J, Dekker J, van der Hoek L, Sol C, Coutinho R, et al. Syncytium-inducing (SI) phenotype suppression at seroconversion after intramuscular inoculation of a non-syncytium-inducing/SI phenotypically mixed human immunodeficiency virus population. J Virol. 1995;69:1810–8.

31. Mariani SA, Vicenzi E, Poli G. Asymmetric HIV-1 coreceptor use and homeostatically maintained resting naive CD4+ T lymphocytes. J Transl Med. 2010;9(Suppl 1):S8.

32. Platt EJ, Wehrly K, Kuhmann SE, Chesebro B, Kabat D. Effects of CCR32 and CCR4 on mucosal HIV tropism and disease progression in rhesus macaques. J Gen Virol. 2000;81:2285–96.

33. Tsunetsugu-Yokota Y, Kobayashi-Ishihara M, Wada Y, Terahara K, Takeyama H, Kawana-Tachikawa A, Tokunaga K, Yamagishi M, Martinez JP, Meyerhans A. Homeostatically maintained resting naive CD4+ T cells resist latent HIV reactivation. Front Microbiol. 2016;7:1944.

34. Tokunaga K, Greenberg ML, Morse MA, Cumming RI, Lyerly HK, Cullen BR. Molecular basis for cell tropism of CXCRR4-dependent human immunodeficiency virus type 1 isolates. J Virol. 2001;75:6767–85.