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Thymic HIV-2 Infection Uncovers Posttranscriptional Control of Viral Replication in Human Thymocytes

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ABSTRACT

A unique HIV-host equilibrium exists in untreated HIV-2-infected individuals. This equilibrium is characterized by low to undetectable levels of viremia throughout the disease course, despite the establishment of disseminated HIV-2 reservoirs at levels comparable to those observed in untreated HIV-1 infection. Although the clinical spectrum is similar in the two infections, HIV-2 infection is associated with a much lower rate of CD4 T-cell decline and has a limited impact on the mortality of infected adults. Here we investigated HIV-2 infection of the human thymus, the primary organ for T-cell production. Human thymic tissue and suspensions of total or purified CD4 single-positive thymocytes were infected with HIV-2 or HIV-1 primary isolates using either CCR5 or CXCR4 coreceptors. We found that HIV-2 infected both thymic organ cultures and thymocyte suspensions, as attested to by the total HIV DNA and cell-associated viral mRNA levels. Nevertheless, thymocytes featured reduced levels of intracellular Gag viral protein, irrespective of HIV-2 coreceptor tropism and cell differentiation stage, in agreement with the low viral load in culture supernatants. Our data show that HIV-2 is able to infect the human thymus, but the HIV-2 replication cycle in thymocytes is impaired, providing a new model to identify therapeutic targets for viral replication control.

IMPORTANCE

HIV-1 infects the thymus, leading to a decrease in CD4 T-cell production that contributes to the characteristic CD4 T-cell loss. HIV-2 infection is associated with a very low rate of progression to AIDS and is therefore considered a unique naturally occurring model of attenuated HIV disease. HIV-2-infected individuals feature low to undetectable plasma viral loads, in spite of the numbers of circulating infected T cells being similar to those found in patients infected with HIV-1. We assessed, for the first time, the direct impact of HIV-2 infection on the human thymus. We show that HIV-2 is able to infect the thymus but that the HIV-2 replication cycle in thymocytes is impaired. We propose that this system will be important to devise immunotherapies that target viral production, aiding the design of future therapeutic strategies for HIV control.

The thymus is the primary organ for T-cell production, and despite the age-associated decline, thymic function is maintained until late in life (1, 2). Thymic activity is vital in clinical settings requiring *de novo* T-cell generation, such as HIV infection (1, 3, 4). Accordingly, impairment of thymic output impacts the rate of HIV-1 disease progression, while the degree of immunological reconstitution achieved after antiretroviral therapy has been shown to rely on thymus recovery (1, 3, 4). Moreover, a functional cure for HIV infection is thought to entail a diverse T-cell repertoire, which can be generated only by the thymus.

HIV-1 targets the thymus in both children and adults, resulting in severe disruption of the thymic microenvironment, as demonstrated by the morphological changes and thymocyte depletion reported in the thymuses of HIV-1-infected individuals (5, 6). Several studies based on HIV-1 infection of the human thymus either *in vitro* (7–9) or *in vivo* using the SCID/hu mouse model (10, 11) have indicated that both direct infection of thymic cells and indirect viral effects upon the microenvironment play a role in HIV-1-associated thymic pathology. Furthermore, viral entry, viral replication kinetics, and the cytopathicity of HIV-1 in human thymocytes have been shown to be highly dependent on viral tropism, due to the predominance of CXCR4 (X4) versus CCR5 (R5) expression in the human thymus (9, 12, 13). Thymic disruption has also been described in nonhuman primate models of simian immunodeficiency virus infection (14).

Here we addressed, for the first time, the direct impact of HIV-2 infection on the human thymus. This is particularly relevant because HIV-2-infected individuals feature low rates of CD4 T-cell decline and disease progression (15–17). Moreover, they typically have low to undetectable levels of viremia, with this being observed even in AIDS patients with <200 CD4 T cells/μl (18, 19). The low levels of circulating virus account for the reduced horizontal and vertical transmission observed in HIV-2 infection (20, 21), as well as for its geographical confinement to West Africa and connected countries, such as Portugal. Despite the high preva-

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TABLE 1 HIV isolates used in the study

Virus	Major coreceptor(s) used	Source
Primary isolates		
HIV-$1_{92US660}$	R5	NIH[a]
HIV-$1_{92HT599}$	X4	NIH[a,b]
HIV-2_{60415K}	R5	NIH[a,c]
HIV-$2_{20.04}$[d]	X4	Nuno Taveira
Lab-adapted strains		
HIV-1_{NL4-3}	X4	MRC[e]
HIV-2_{ROD10}	R5, X4	MRC[e]

[a] From the Multicenter AIDS Cohort Study, NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH.
[b] Provided by Neal Halsey.
[c] Provided by Feng Gao and Beatrice Hahn.
[d] Previously represented as PTHCC20/2004 (26) and 19/2004 (27).
[e] NIBSC, Centre for AIDS Reagents, Medical Research Council, United Kingdom.

lence of HIV-2 in several regions of West Africa, such as in Guinea Bissau (8% in adults and up to 20% in people over 40 years of age) (22), there is no significant impact on the mortality of infected adults. HIV-2 thus constitutes a unique naturally occurring model of attenuated HIV disease valuable for the study of HIV pathogenesis.

In spite of the low to undetectable HIV-2 plasma loads, HIV-2- and HIV-1-infected patients at equivalent stages of CD4 T-cell depletion feature comparable levels of cell-associated viral burden (18, 23, 24), indicating the establishment of disseminated HIV-2 reservoirs. They also feature similar levels of T-cell activation (19), suggesting distinct control of viral replication in the presence of cell activation in HIV-2 and HIV-1 infections.

Our previous data support the preservation of thymic function in HIV-2 infection, as estimated by signal joint (sj)/β T cell receptor excision circle (TREC) quantification (3) in circulating T cells of HIV-2-infected patients (25). However, there are no studies on the direct impact of HIV-2 infection on the human thymus either *in vivo*, due to the difficulty of obtaining thymic tissue from HIV-2-infected patients, or *in vitro*. We show here that HIV-2 is able to infect the human thymus but that this is associated with limited viral replication, irrespective of viral coreceptor tropism and thymocyte differentiation stage. HIV-2 infection of the human thymus thus offers a novel approach to investigate the mechanisms underlying the establishment of HIV reservoirs and the control of viral replication.

MATERIALS AND METHODS

Ethical statement. Thymic tissue specimens (from individuals ranging from newborns to children 4 years old) were obtained during routine thymectomy performed during pediatric corrective cardiac surgery at the Hospital de Santa Cruz, Carnaxide, Portugal, after the parents provided written informed consent. Buffy coats from healthy donors were provided by the Instituto Português do Sangue e da Transplantação after they provided written informed consent. The study was approved by the Ethical Boards of the Faculty of Medicine of the University of Lisbon and of the Hospital de Santa Cruz, Carnaxide, Portugal.

HIV stocks. The viruses used are described in Table 1. Viral stocks were propagated in pools of isolated peripheral blood mononuclear cells (PBMCs) stimulated for 3 days with phytohemagglutinin (PHA; 5 μg/ml; Sigma) and maintained with human recombinant interleukin-2 (IL-2; 10 U/ml; from Maurice Gately, Hoffmann-La Roche Inc., through the NIH AIDS Reagent Program), as described previously (28). Virus in cell-free culture supernatants was quantified by measuring reverse transcriptase (RT) activity using a Lenti-RT activity kit (Cavidi).

HIV infection of thymocyte suspensions. Total thymocytes were recovered through tissue dispersion and separation on a Ficoll-Paque Plus (GE Healthcare) density gradient. The CD4 single-positive (CD4SP) population was sorted from total thymocytes as $CD3^{high}$ CD8-negative ($CD8^{neg}$) cells (purity, >98%), using a FACSAria high-speed cell sorter (BD Biosciences). Viral stocks were ultracentrifuged for 30 min at 50,000 × *g* and 4°C (Beckman L8 ultracentrifuge), resuspended in complete medium (RPMI 1640 with 10% fetal bovine serum [FBS], 2 mM L-glutamine, 50 U/ml penicillin-streptomycin, and 50 μg/ml gentamicin [all from Gibco/Invitrogen] plus 3 μg/ml Polybrene [Sigma]), and added to the cells at 0.3 ng RT/10^6 thymocytes. Total and CD4SP thymocytes were cultured for 3 to 4 h at 10^8 and 3.5×10^7 cells/ml, respectively, in the absence or presence of virus. After infection, the cells were washed and cultured at 10^7 cells/ml in complete medium supplemented with IL-2 (20 U/ml), IL-4 (20 ng/ml; R&D), and IL-7 (10 ng/ml; R&D) at 37°C. A quarter of the medium was replaced every 3 to 4 days. On day 10, the thymocyte number per well was determined using 10-μm latex beads (Coulter), and the cells were analyzed by flow cytometry or stored as pellets at −80°C. Viral production in supernatants was quantified at day 10 postinfection of $CD11c^{neg}$ $CD14^{neg}$ $CD123^{neg}$ T-cell receptor γδ-negative ($TCR\gamma\delta^{neg}$) thymocytes by measuring RT activity using SYBR green product-enhanced RT (SG-PERT), as described previously (29, 30).

HIV infection of TOCs. Thymic tissue blocks (diameter, 1 to 2 mm) were placed on Millicell organotypic inserts (Millipore) in a 6-well plate containing 1 ml of thymic organ culture (TOC) medium (complete medium with 15% FBS, 10 mM HEPES, 1 mM sodium pyruvate, and 1% minimal essential medium nonessential amino acids [all from Gibco/Invitrogen]). TOCs were placed at 37°C in 5% CO_2 overnight, and half of the medium was replaced prior to infection. HIV infection was performed by placing a 5-μl drop containing 3 ng RT of virus on top of each TOC. A third of the medium was replaced every 2 to 3 days. On day 10 or 11 postinfection, TOCs were placed in 4% formaldehyde or mashed. Thymocytes were analyzed by flow cytometry or stored as cell pellets at −80°C. The role of Env proteins was assessed by culturing the TOCs for 7 days on Isopore membranes (Millipore) placed in TOC medium containing 1 μg/ml of purified recombinant glycoproteins: $gp105_{ROD}$ (MRC EVA621), $gp120_{Ba\text{-}L}$ (catalog no. 4961; NIH), and $gp120_{IIIB}$ (MRC EVA607) or anti-CD4 monoclonal antibody (MAb; BD Biosciences) as a control.

Flow cytometry. Surface staining was performed for 20 min at room temperature and always included fixable viability dye (eBioscience) for dead cell exclusion. Thymocytes were fixed, permeabilized, and stained using an intracellular staining kit (eBioscience), as described previously (31). The anti-human MAbs used were (clone designations are given in parentheses) CD3 (UCHT1), CD4 (RPA-T4), CD8α (RPA-T8), CD11c (3.9), CD14 (61D3), CD16 (eBioCB16), CD19 (HIB19), CD123 (6H6), and TCRγδ (B1.1) from eBioscience. The following anti-Gag MAbs were used: KC57 (Beckman Coulter), anti-p24 (MAb Kal-1; Dako), anti-p24 (MAb AG3.0 or 183-H12-5C; NIH), and anti-p27 (MAb ARP396/397; P. Szawlowski, NIBSC, Centre for AIDS Reagents, Medical Research Council [MRC]). Alexa Fluor 488 goat anti-mouse IgG (H+L) antibody (Molecular Probes) was used for secondary detection. Cells were acquired using a LSRFortessa cell analyzer (BD Biosciences), and data were analyzed with FlowJo software (TreeStar).

Immunohistochemistry. Fixed TOCs were embedded in paraffin and cut into 3-μm sections (Minot microtome Leica RM2145). Epitope retrieval was performed at pH 9 (Leica Biosystems buffer) for 15 min using a microwave (800 W). Samples were stained with the appropriate anti-Gag primary antibodies, incubated with a peroxidase-diaminobenzidine detection system (EnVision; Dako), and counterstained with Harris's hematoxylin (BioOptica). Images were acquired using a Leica DM2500 microscope.

Quantification of total HIV DNA and *gag* mRNA by real-time PCR. Total HIV DNA was quantified in cell lysates prepared by treating cell

pellets with 100 μg/ml proteinase K in 10 mM Tris-HCl for 1 h at 56°C, followed by 10 min of enzyme inactivation at 95°C. For *gag* mRNA quantification, 200 ng of total RNA, purified using a mirVana microRNA isolation kit (Ambion), was used to synthesize cDNA using oligo(dT)$_{20}$ and SuperScript III RT (Invitrogen) according to the manufacturer's instructions. Real-time PCR was performed using Platinum *Taq* plus carboxy-X-rhodamine (ROX) or TaqMan gene expression master mix (Applied Biosystems) with the primers and probes described in Table S1 in the supplemental material. Standard curves were generated from serial dilutions of cDNA prepared from the mRNA of 3 different thymuses (for GAPDH [glyceraldehyde-3-phosphate dehydrogenase] quantification) or of plasmids containing the amplicons of HIV-1 *gag* and CD3γ (a kind gift from Rémi Cheynier) (32) or of HIV-2 *gag*. A plasmid carrying HIV-2 *gag* was generated by inserting a sequence including the sequences for the long terminal repeat/*gag* from HIV-2$_{ROD10}$ into the pGEM-T Easy vector (Promega). Quantification was performed using an Applied Biosystems 7500 Fast real-time PCR system.

Statistical analysis. Statistical analysis was performed using GraphPad Prism (v5.01) software (GraphPad Software Inc.). Data from two samples were compared using the Wilcoxon matched-pairs test. Data from more than two samples were compared using the Friedman test or the Kruskal-Wallis test with Dunn's multiple-comparison posttest. *P* values of <0.05 were considered significant.

RESULTS

HIV-2 infects the human thymus. We investigated HIV-2 infection of the human thymus *in vitro* using both TOCs, which preserve the thymic microenvironment and have been shown to be permissive to HIV-1 infection (7, 8), and thymocyte suspensions. TOCs and thymocyte suspensions were infected with HIV-2 or HIV-1 primary isolates with selective R5 or X4 coreceptor specificity and cultured for 10 days. RT activity was used to normalize the viral input, as HIV-1 and HIV-2 RT enzymes were shown to possess similar specific activities (33).

We found that HIV-2 was able to infect both thymic tissue and total thymocyte suspensions, as indicated by the levels of total HIV DNA, which includes unintegrated and integrated proviral DNA, in HIV-2-infected samples (Fig. 1A). Furthermore, we observed that HIV-2 infection of both thymic tissue and total thymocyte suspensions was less coreceptor dependent than HIV-1 infection.

Next we sorted purified mature CD4SP thymocytes, which constitute a target of HIV-1 (11), and cultured them for 10 days after infection with the above-mentioned viruses. In contrast to HIV-2 infection of TOCs and thymocyte suspensions, HIV-2 infection of CD4SP thymocytes resulted in coreceptor-dependent levels of total HIV DNA (Fig. 1A). The limited ability of R5-tropic HIV-2 to infect the CD4SP subset was in contrast to our observations in TOCs and total thymocyte suspensions (Fig. 1A), suggesting that R5-tropic HIV-2 targeted other human thymocyte populations.

We next assessed the levels of ongoing viral transcription in HIV-2-infected TOCs and thymocytes by measuring viral *gag* mRNA. Both R5- and X4-tropic HIV-2 isolates generated *gag* mRNA levels comparable to those found upon infection with the X4-tropic HIV-1 isolate (Fig. 1B), indicating efficient integration and transcription of HIV-2 in TOCs and thymocyte cultures. Of note, HIV-2 *gag* mRNA levels showed coreceptor dependency in the case of purified CD4SP thymocytes but not in TOCs or thymocyte suspensions (Fig. 1B), in line with the total HIV DNA levels that were observed in the same samples.

In summary, our results indicated that HIV-2 is able to successfully enter, reverse transcribe, and integrate in human thymocytes in TOCs and in suspension and that viral transcription is ongoing in these systems.

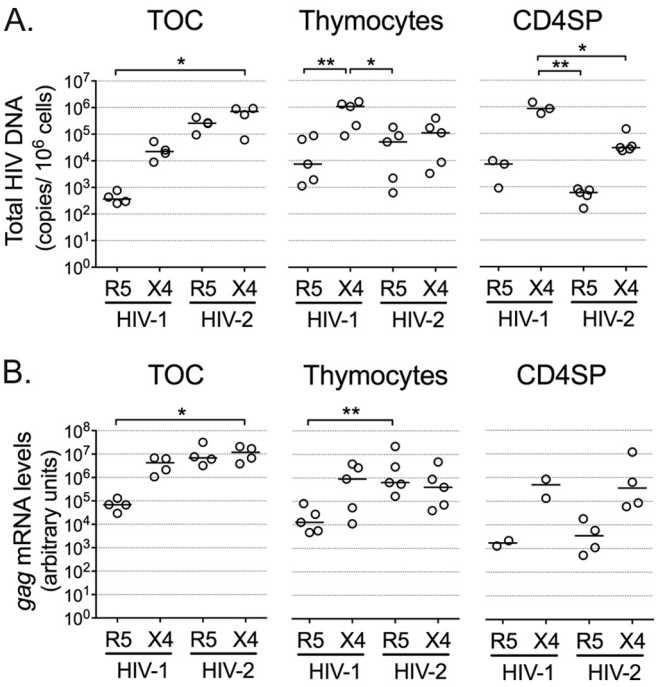

FIG 1 HIV-2 infects human thymic tissue and isolated thymocytes. TOCs were infected with R5- or X4-tropic HIV-2 or HIV-1 primary isolates and cultured for 10 or 11 days on Millipore inserts. Single-cell suspensions of total thymocytes or CD4SP thymocytes, sorted as CD3^{high} CD8^{neg} cells, were also infected with the above-mentioned viruses and cultured for 10 days. Graphs show total HIV DNA (A), as assessed by quantitative real-time PCR, or viral *gag* mRNA (B), as assessed by real-time RT-PCR (the level of mRNA expression was normalized to the level of GAPDH expression). Each dot represents a single thymus. Lines indicate median values. *, $P < 0.05$; **, $P < 0.01$.

Limited viral production in HIV-2 infection of human thymocytes. Next we assessed viral production in HIV-2-infected thymic tissue using immunohistochemistry. The Gag protein was detected in R5- and X4-tropic HIV-2-infected TOCs after culture (Fig. 2A), using validated antibodies (see Fig. S1 in the supplemental material) (34). Gag protein expression was both cell associated and extracellular (Fig. 2B), with the latter likely corresponding to virus produced by HIV-infected cells during TOC, as no Gag staining was observed on day 1 (Fig. 2A). Of note, Gag protein levels were often indistinguishable in X4- and R5-tropic HIV-1 TOC infections (Fig. 2A), despite the differences in the levels of the cell-associated viral burden (Fig. 1), indicating a lack of correlation between the amount of virus detected by immunohistochemistry and the levels of thymocyte viral production. Therefore, although our immunohistochemistry data confirmed the ability of HIV-2 to productively infect the human thymus, it was not possible to infer the specific contribution of thymocytes versus that of the thymic stroma to the viral protein detected in the histological analysis.

We thus evaluated viral production at the single-cell level by flow cytometry. Notably, we found very low levels of intracellular Gag protein in cells isolated from HIV-2-infected TOCs or in thymocyte suspensions, irrespective of the virus used (Fig. 3) and the time point analyzed (see Fig. S2 in the supplemental material). In contrast, Gag protein expression was clearly detected in cells

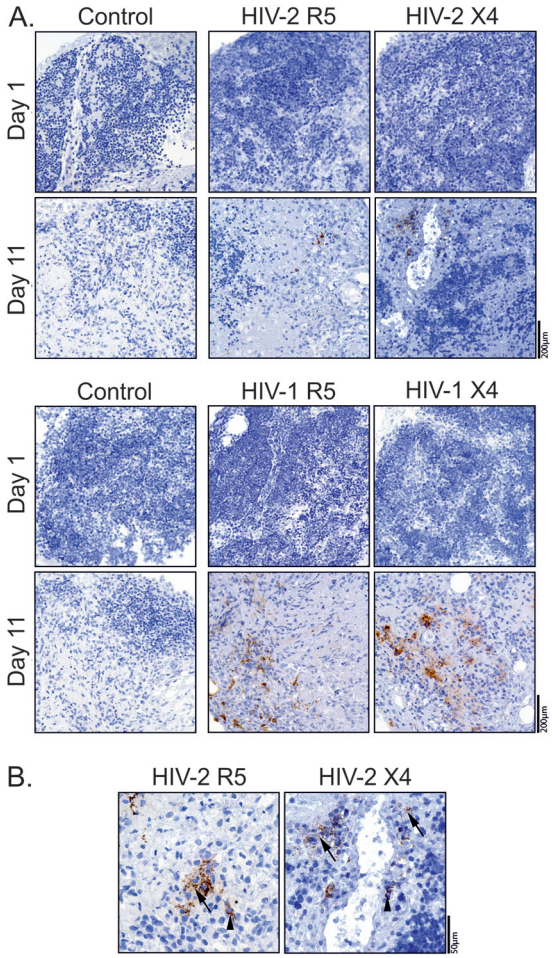

FIG 2 Viral production in HIV-2-infected TOCs. TOCs infected with R5- or X4-tropic HIV-2 or HIV-1 primary isolates were analyzed for viral production by immunohistochemistry using anti-Gag antibodies (brown) and hematoxylin counterstain (blue). (A) Viral production at days 1 and 11 after infection with HIV-2 (top) or HIV-1 (bottom), as assessed using an anti-p27 MAb (ARP396/397 from MRC) or an anti-p24 MAb (Kal-1 from Dako), respectively. (B) Extracellular (arrow) and cytoplasmic (arrowhead) Gag expression in R5- or X4-tropic HIV-2-infected TOCs at day 11 (determined with the ARP396/397 anti-p27 antibody).

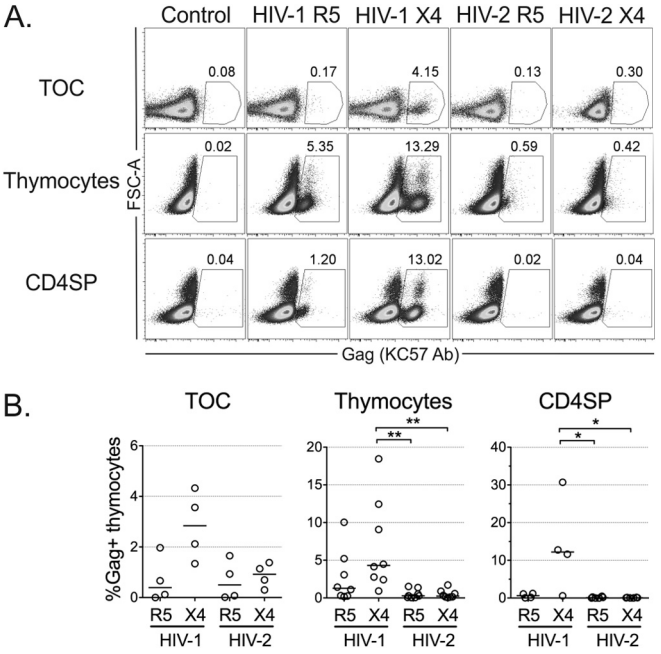

FIG 3 Limited replication of HIV-2 in human thymocytes. Viral production at the single-cell level was determined by flow cytometry using the anti-Gag antibody (Ab) KC57 at 10 days postinfection of TOCs, total thymocytes, or CD4SP thymocytes with R5- or X4-tropic HIV-1 and HIV-2 primary isolates. Representative dot plots of intracellular Gag expression (numbers show the proportion of cells inside the gate) (A) and frequency of productively infected KC57-positive thymocytes (B) under each condition are shown. Flow cytometric analysis was performed after exclusion of dead cells and aggregates. Each dot represents a single thymus. Lines indicate median values. *, $P < 0.05$; **, $P < 0.01$. FSC, forward scatter.

infected with HIV-1, particularly with the X4-tropic isolate (Fig. 3), excluding the possibility that the low level of Gag detection in HIV-2 infections was due to sample processing. Of note, intracellular Gag protein was also barely detected upon thymocyte infection with the lab-adapted strain HIV-2_{ROD} (see Fig. S2 in the supplemental material), despite the high levels of total HIV DNA detected (181,499 copies/10^6 cells in the example presented). Low Gag levels upon HIV-2 infection were particularly evident in CD4SP thymocytes, which featured extremely low levels of intracellular Gag after infection with HIV-2 primary isolates or HIV-2_{ROD} (Fig. 3; see also Fig. S2 in the supplemental material).

The antibody used (KC57) has previously been shown to be specific for both HIV-2 Gag and HIV-1 Gag (35). We further confirmed the ability of the antibody to efficiently detect HIV-2 Gag by flow cytometry in activated PBMCs infected with the HIV-2 strains used in the current study (see Fig. S3 in the supplemental material). Moreover, the level of KC57 staining was comparable to that obtained with other available anti-Gag antibodies in HIV-2-infected PBMCs or thymocytes (see Fig. S2 and S3 in the supplemental material), confirming the low level of viral production in HIV-2-infected thymocytes.

The low levels of intracellular Gag protein translated into reduced viral production in HIV-2 infections, as indicated by the lower levels of virus in the supernatant of HIV-2-infected thymocyte cultures than in the supernatant of their HIV-1-infected counterparts (RT activities, 5.97 ± 3.23 pg/ml for R5-tropic HIV-1, 1,318 ± 583.5 pg/ml for X4-tropic HIV-1, 0.31 ± 0.15 pg/ml for R5-tropic HIV-2, and 8.54 ± 3.40 pg/ml for X4-tropic HIV-2; $n = 3$).

Overall, we found that HIV-2 infection of human thymocytes occurred without significant viral production per cell, despite evidence of cell-associated viral burden and active ongoing viral transcription.

Impact of HIV-2 infection on thymocyte populations. Finally, we assessed the impact of HIV-2 on the distribution of thymocyte populations in infected TOCs and thymocyte suspensions. TOCs infected with X4-tropic viruses, whether they were HIV-2 or HIV-1, featured a dramatic decrease in $CD4^+$ thymocytes compared to the amounts in TOCs infected with R5-tropic viruses (Fig. 4A and B). This was also observed in thymocyte suspensions infected with X4-tropic HIV-1 but not with X4-tropic HIV-2, infection with which did not result in significant alterations of the thymocyte phenotype in culture (Fig. 4C and D). One possible explanation for these results is that downregulation of the

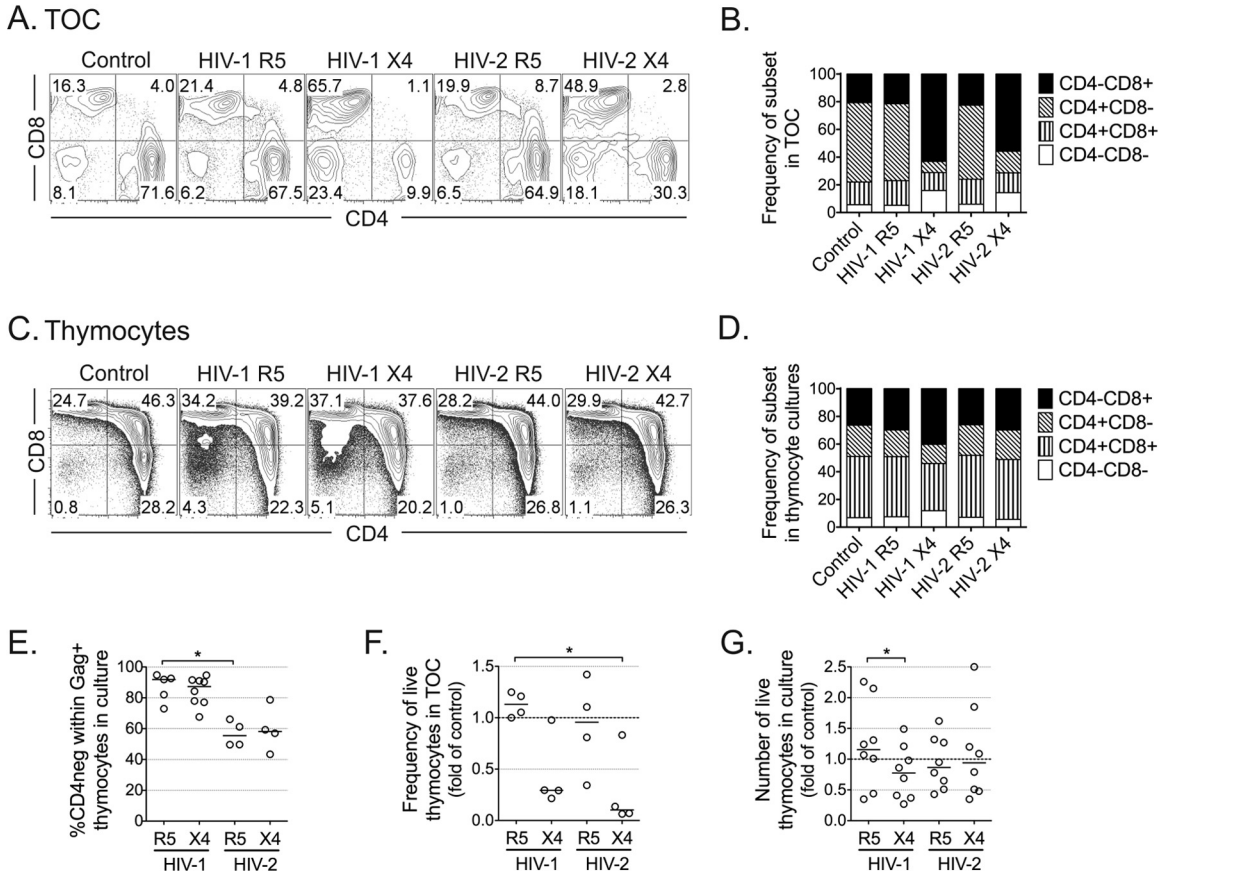

FIG 4 Distinct cytopathic impact of HIV-2 infection on TOCs and thymocytes. HIV-infected TOCs (A and B) or thymocyte suspensions (C and D) were cultured for 10 days. Representative dot plots of CD4 and CD8 expression in cells in TOCs (A) and in total thymocyte suspensions (C), with the graphs showing the mean frequency of thymocyte subsets in all thymuses analyzed ($n = 4$ for panel B and $n = 8$ for panel D). TOC analysis was performed in $CD14^{neg}$ $CD16^{neg}$ $CD19^{neg}$ $CD123^{neg}$ cells. Dead cells and aggregates were excluded from the flow cytometric analysis. Numbers in dot plots represent the proportion of cells inside quadrants. (E) Frequency of $CD4^{neg}$ cells within Gag-positive (KC57 antibody-positive) thymocytes in HIV-infected thymocyte suspensions. (F) Fold change in the frequency of live cells in TOCs relative to that in the uninfected controls, as assessed by flow cytometry. (G) Fold change in the number of live thymocytes in culture relative to that in the uninfected control. Lines indicate median values. *, $P < 0.05$.

CD4 coreceptor is distinctly induced by HIV-2 and HIV-1. Accordingly, our analysis of productively infected cells indicated that HIV-2 was less efficient than HIV-1 at downregulating CD4 (Fig. 4E).

The effects on the subset distribution were mirrored by the cytopathic effect of the respective virus: both X4-tropic HIV-2 and X4-tropic HIV-1 but not R5-tropic viruses were cytopathic in TOCs (Fig. 4F), while X4-tropic HIV-1 had a small effect on cell viability in thymocyte suspensions (Fig. 4G). The low level of cell recovery in X4-tropic HIV-2-infected TOCs, together with the imbalance in cell subsets and the apparent low impact of HIV-2 infection on CD4 downregulation, suggested that X4-tropic HIV-2 infection of TOCs directly or indirectly led to the death of $CD4^+$ thymocytes. This occurred despite the lack of impact of X4-tropic HIV-2 infection on total thymic cell suspensions, supporting the contribution of other cell populations present in the whole tissue.

Given our previous data supporting the immunosuppressive properties of the HIV-2 envelope proteins in peripheral blood lymphocytes mediated, at least in part, by monocytes (36–38), we also evaluated whether the alterations observed in X4-tropic HIV-2-infected TOCs were induced by the direct action of viral envelope proteins. We cultured TOCs in the presence of recombinant HIV-2 and HIV-1 Env proteins binding to either X4 (HIV-2 $gp105_{ROD}$ and HIV-1 $gp120_{IIIB}$) or R5 (HIV-1 $gp120_{Ba-L}$). In our system, Env proteins did not induce $CD4^+$ thymocyte depletion or subset imbalances (Fig. 5), indicating that the effects observed may require viral entry and/or postentry steps.

Altogether, our data support a cytopathic effect of X4-tropic HIV-2 on the human thymus that was not observed in thymocyte suspensions, suggesting a dependency on the stromal compartment. Importantly, R5-tropic HIV-2 had no significant cytopathic effect on either TOCs or thymocyte suspensions.

DISCUSSION

We addressed here, for the first time, the ability of HIV-2 to infect the human thymus. We showed that HIV-2 is able to infect thymic tissue and thymocyte suspensions in the absence of exogenous stimulation, although with a very low level of viral production per thymocyte, supporting the existence of posttranscriptional control in viral replication.

HIV-2 infection of the human thymus was confirmed by the levels of cell-associated HIV DNA and *gag* mRNA measured following HIV-2 infection of either TOCs or thymocyte suspensions.

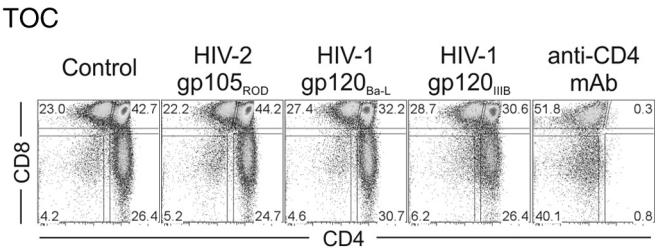

FIG 5 The X4-tropic HIV-2 envelope per se does not impact the thymocyte distribution in TOCs. TOCs were cultured for 7 days with medium only (control) or in the presence of the recombinant envelope protein HIV-2 $gp105_{ROD}$, HIV-1 $gp120_{Ba\text{-}L}$, or HIV-1 $gp120_{IIIB}$ (all at 1 μg/ml) or anti-CD4 MAb. The dot plots show a representative example (from one of three independent experiments with different thymuses) of the frequency of subsets in TOCs, as determined by CD4 and CD8 expression. Numbers correspond to the proportion of cells inside gates.

The high levels of total HIV DNA observed were consistent with those from previous studies where unintegrated plus integrated proviral DNA were quantified (39, 40). HIV-1 infection of human thymocytes was shown to be coreceptor dependent (9, 12), and our data recapitulated these results. Conversely, we showed that HIV-2 infection of TOCs and thymocyte suspensions was much less dependent on coreceptor tropism than HIV-1 infection in terms of both cell-associated HIV DNA and viral RNA, which could be related to the broader coreceptor usage reported for HIV-2 than for HIV-1 (41).

On the other hand, HIV-2 cytopathicity in human thymic tissue was coreceptor dependent, with X4-tropic virus inducing significant T-cell death in TOCs, which was not observed for R5-tropic HIV-2. This was in line with the findings of a previous study of HIV-2 infection of lymphoid tissue, where lymphocyte depletion also occurred with X4- but not R5-tropic isolates (42). Importantly, we showed that X4-tropic HIV-2 cytopathicity in TOCs was not due to the direct action of HIV-2 Env, indicating a requirement for viral entry and/or postentry steps for $CD4^+$ thymocyte depletion in the human thymus.

The observed cytopathicity caused by X4-tropic HIV-2 in tissue but not in thymocyte suspensions could be related to the infection of components of the thymic stroma that are present in TOCs. Thymic stromal cells, including dendritic cells, macrophages, and even thymic epithelial cells, have been reported to be permissive to HIV-1 infection (43–45). It is not known whether HIV-2 infects the thymic stroma, and due to alterations in tissue morphology that occurred during the culture process, we were not able to directly infer the type of infected cells from our immunohistochemistry data. However, in contrast to the HIV-1 genome, the HIV-2 genome encodes the lentiviral accessory protein Vpx, which targets for degradation the restriction factor sterile alpha motif and histidine/aspartic acid domain-containing protein 1 (SAMHD1), a factor that has been shown to limit the productive infection of HIV-1 in myeloid cells (46). Moreover, additional cell targets may be considered, given the broader coreceptor usage reported for HIV-2 (41). We are currently addressing the possibility that infection of thymic stroma components might have distinct consequences in HIV-1 and HIV-2 infections.

We had previously reported that thymic function, estimated via sj/βTREC measurement, was better preserved in HIV-2-infected than HIV-1-infected patients (25). It is likely that most of these individuals were infected with R5-tropic HIV-2 (25). Importantly, we showed here that R5-tropic HIV-2 infection did not significantly impact TOCs or thymocyte suspensions in terms of cytopathicity or the subset distribution or induce CD4 downregulation in the latter. Nevertheless, the elevated levels of total HIV DNA and viral mRNA support the potential establishment of HIV-2 reservoirs in human thymocytes. It would thus be important to investigate whether, as reported for HIV-1 (32), $CD4^+$ recent thymic emigrant T cells from HIV-2-infected patients may constitute viral reservoirs.

Our data suggest that, in our system, distinct regulation of the replicative cycle of HIV-2 and HIV-1 occurs at the posttranscriptional/translational level. The discrepant levels of Gag protein in the two infections markedly contrasted with the similarly high levels of total HIV DNA and viral mRNA documented. HIV-2 and HIV-1 have been reported to differ significantly in their mechanisms of translation initiation (47). For instance, HIV-2 was described to have a lower translational efficiency than HIV-1 due to differences in the 5′ untranslated region (5′ UTR) of viral genomic RNA (48, 49). In agreement with our observations, in the presence of equivalent T-cell-associated viral *gag* mRNA levels, the levels of Gag protein production were reported to be lower in T-cell lines and macrophages infected with HIV-2 than those infected with HIV-1 (49). Importantly, and in relation to our data, this was not due to the lower stability of the Gag protein or to the higher rate of viral particle release (49). Interestingly, the authors raised the possibility that host factors may differentially regulate the initiation of Gag translation in HIV-2 and HIV-1 (49). HIV-2 infection of the human thymus provides, to our knowledge, the first model of HIV-2 posttranscriptional regulation in *ex vivo* T cells using primary isolates, providing a unique opportunity to study the molecular factors and mechanisms involved in the regulation of HIV translation.

Human thymocytes represent an ideal system for the study of HIV latency due to their ability to become infected in the absence of external activation (50). Models using peripheral $CD4^+$ T cells require cellular activation followed by quiescence induction (51), thus inducing molecular modifications that may impact the processes under study. The system described here, utilizing HIV-2 infection of the human thymus in the absence of exogenous stimulation, thus provides an important cellular model for the study of latency and reservoir generation in HIV pathogenesis.

Our results regarding HIV-2 infection also highlight the potential importance of posttranscriptional control of viral production, possibly through the regulation of translation, in viral pathogenesis. Further studies using this model will enable the discovery of potential molecular targets to be used as the basis for new immunotherapies aimed at achieving a functional cure for HIV infection.

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