Adaptation to the Interferon-Induced Antiviral State by Human and Simian Immunodeficiency Viruses

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The production of type I interferon (IFN) is an early host response to different infectious agents leading to the induction of hundreds of IFN-stimulated genes (ISGs). The roles of many ISGs in host defense are unknown, but their expression results in the induction of an “antiviral state” that inhibits the replication of many viruses. Here we show that prototype primate lentiviruses human immunodeficiency virus type 1 (HIV-1) and simian immunodeficiency virus of macaques (SIVMAC and SIVMNE) can replicate in lymphocytes from their usual hosts (humans and macaques, respectively), even when an antiviral state is induced by IFN-α treatment. In contrast, HIV-1 and SIVMAC/SIVMNE replication was hypersensitive to IFN-α in lymphocytes from unnatural hosts, indicating that the antiviral state can effectively curtail the replication of primate lentiviruses in hosts to which they are not adapted. Most of the members of a panel of naturally occurring HIV-1 and HIV-2 strains behaved like prototype strains and were comparatively insensitive to IFN-α in human lymphocytes. Using chimeric viruses engineered to overcome restriction factors whose antiretroviral specificities vary in a species-dependent manner, we demonstrate that differential HIV-1 and SIVMAC sensitivities to IFN-α in lymphocytes from humans and macaques could not be ascribed to TRIM5, APOBEC3, tetherin, or SAMHD1. Single-cycle infection experiments indicated that at least part of this species-specific, IFN-α-induced restriction of primate lentivirus replication occurs early in the retroviral life cycle. Overall, these studies indicate the existence of undiscovered, IFN-α-inducible antiretroviral factors whose spectrum of activity varies in a species-dependent manner and to which at least some HIV/SIV strains have become adapted in their usual hosts.

Recognition of viruses or other microbes through pathogen-associated molecular patterns leads to the expression of interferons (IFNs) (1) and constitutes one of the earliest host responses to an infection. Thereafter, the binding of type I IFN (IFN-α/β) to a receptor (IFNAR) that is present on many cell types activates a JAK-STAT signaling cascade, leading to the expression of hundreds of IFN-stimulated genes (ISGs) and the induction of an antiviral state (2). For both humans and macaques infected with human immunodeficiency virus type 1 (HIV-1) or simian immunodeficiency virus of macaques (SIVMAC), respectively, high levels of IFN-α (up to >1,000 U/ml) have been reported during acute infection (3–6). Moreover, in pathogenic primate lentivirus infections, a chronic activation of the immune system, including induction of ISGs, continues throughout the course of the disease (4, 7). Despite the induction of IFN-α expression, HIV-1 and SIVMAC replication persists.

Among the many genes induced by IFN-α are those that encode so-called “restriction factors.” These proteins have been shown to directly inhibit viral replication through various mechanisms (8, 9). To enable replication in the presence of restriction factors, primate lentiviruses have evolved ways to evade or counteract them, sometimes employing accessory genes as restriction factor antagonists. The activity of restriction factors and their antagonism by viral proteins vary in a highly species-dependent manner. Often, primate viruses have evolved to evade or antagonize restriction factors in their usual host species but remain sensitive to variants of the same factors in other hosts (8, 9). For example, HIV-1 and HIV-2 have evolved strategies to counteract human tetherin (10–12), while SIVMAC is sensitive to inhibition by human tetherin (13). In contrast, SIVMAC can efficiently antagonize macaque tetherin but HIV-1 and HIV-2 cannot (13, 14). Conceptually similar species-dependent evasion or antagonism phenomena are evident for each of the restriction factors that are known to target primate lentiviruses, namely, APOBEC3, TRIM5, tetherin, and SAMHD1 (8). Importantly, these restriction factors provide barriers to the cross-species transmission of primate lentiviruses and complicate the development of suitable animal models for the study of HIV-1 and other primate lentiviruses (15). Indeed, macaques, which are often used as a model system for HIV-1 infection in humans, express several factors that inhibit HIV-1, but generally not SIVMAC infection. Especially potent blocks to HIV-1 replication in macaque cells are imposed by TRIM5α, which inhibits incoming HIV-1 particles by recognizing the HIV-1 capsid (CA) protein, (16) and APOBEC3 proteins that catalyze the deamination of deoxycytidines in viral DNA during reverse transcription (17–19). Whereas the SIVMAC CA and Vif proteins can evade and antagonize the macaque TRIM5 and APOBEC3 proteins, respectively, the HIV-1 CA and Vif proteins do not.

Overcoming TRIM5α- and APOBEC3-based restrictions by engineering HIV-1 strains that encode the SIVMAC CA and Vif proteins has made it possible to develop a simian-tropic HIV-1 strain (stHIV) that is able to replicate in rhesus macaque lymphocytes (20). Moreover, the discovery that pigtailed macaques express a TRIM5-cyclophilin A fusion protein (TRIMCyp), that is...
not active against HIV-1 (21–23) enabled the construction of an stHIV strain in which the only alteration was the inclusion of SIVMAC Vif (24). This stHIV strain replicates efficiently in pigtailed macaque lymphocytes in vitro. However, although viremia is robust during the acute infection phase in vivo, it progressively declines over time and no disease is evident (24), suggesting the possibility that additional species-dependent restrictions to primate lentiviruses exist that limit stHIV replication in vivo.

Here we have examined whether the IFN-α-induced host response imposes a host species-dependent block to primate lentivirus replication. We found that this is indeed the case and that stHIV and two prototype SIVs are hypersensitive to inhibition by IFN-α when asked to replicate in primary lymphocytes from unnatural hosts. We demonstrated that these blocks are not attributable to TRIM5, APOBEC3, tetherin, or SAMHD1 and are manifested, at least in part, during the early stages of the virus life cycle. Moreover, naturally occurring HIV-1 and HIV-2 strains are generally less sensitive to IFN-α than the prototype SIVs in human lymphocytes. Overall, these studies indicate the existence of novel IFN-α-inducible factors whose antiretroviral activity varies in a manner that is dependent on the particular primate lentivirus and the host cell species.

MATERIALS AND METHODS

Plasmid construction. The proviral plasmids stHIV and stHIV(SCA), which are based on the HIV-1 clone NL4-3, have been described previously (20, 24). In stHIV, the Vif coding sequences have been replaced with those of SIVMAC239 and the Env coding sequences have been replaced with those of the macaque-adapted HIV-1 envelope from SHIV/K9B9. In stHIV(SCA), the HIV-1 CA-encoding sequences have also been replaced with those of SIVMAC239.

The proviral plasmid stHIVΔVpu was generated by using overlap extension PCR to replace the first two codons of the stHIV Vpu codon with six nucleotides encoding a BamHI site (GGATCC). For the construction of stHIVΔVpuΔenv, overlap extension PCR was used to replace the stHIV Vpu coding sequences with those from SIVΔVpuΔenv and SIVΔenvVpu. Because the Vpu and Env sequences overlap, this manipulation resulted in the replacement of the K9B Env amino terminus with that of SIVΔenv Env or SIVΔenv Env. However, the Vpu-Env overlap is confined to the Env signal peptide, so the amino acid sequence of mature, functional KB9 Env was unaltered (see Results).

The proviral plasmid stHIVΔenvGFP has been described previously (20). Derivatives thereof (stHIVΔvpuΔenvGFP, stHIVΔenvΔvpuΔenvGFP, and stHIVΔvpuΔenvGFP) were generated by combining fragments of stHIVΔenvGFP with stHIVΔvpu stHIVΔenvVpu or stHIVΔenv.

To generate stHIVVpx, overlap extension PCR was used to insert SIVMAC239 Vif and Vpx sequences, rather than Vif alone, into the 3′ end of HIV-1 pol and the 5′ end of HIV-1 vpr, as was described for the original generation of stHIV (20, 24) (see Results). A proviral plasmid (stHIVSp6) encoding the Vpx packaging signal from SIVMAC239 p6 was generated essentially as previously described (25). The stHIVSp6Vpx proviral plasmid (see Fig. 5A) was generated by combining fragments of stHIVSp6 and stHIVVpx.

A plasmid containing the full-length SIVMAC239 proviral DNA was generated on the basis of a px33-derived low-copy-number vector termed V1 and using p239Spsp5 and p239SpEL3′ obtained from the NIH AIDS Research and Reference Reagent Program and contributed by R. C. Desrosiers, J. S. Gibbs, and D. Regier (26). A SIVMAC239ΔNef proviral plasmid was similarly derived. For the generation of SIVMAC239ΔVpx, the Vpx initiation codon was mutated without changing the amino acid sequence in the overlapping Vif protein. The SIVMAC239ΔNef proviral plasmid has been described elsewhere (27).

A proviral plasmid, SIV-GFP, encoding green fluorescent protein (GFP) in place of SIVMAC239Nef has been described previously (28). To obtain stHIV-GFP and stHIV(SCA)-GFP, an stHIV fragment containing SIVMAC Vif was introduced into HIV/KB9-GFP and HIV(SCA)KB9-GFP, respectively, which have been described previously (20).

The plasmid coding for hemagglutinin (HA)-tagged human tetherin has been described previously (29). A similar plasmid encoding pigtailed HA-tagged macaque tetherin (13) was constructed by using similar approaches. PCR primer sequences used for the generation of the above constructs can be obtained upon request. The plasmid encoding rHAPOBEC3G has been previously described (19).

Proviral plasmids encoding transmitted founder HIV-1 strains were obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH (Panel of Infectious Molecular Clones; catalog number 11919), from John Kappes (30–34).

Cell culture. The adherent cell lines 293T and TZM were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum (FCS) and gentamicin. CEMx174 suspension cells were grown in RPMI medium with 10% FCS and gentamicin. Human peripheral blood mononuclear cells (huPBMCs) were isolated from blood by Ficoll-Paque gradient centrifugation. Isolated cells were activated with phytohemagglutinin (PHA-P; Sigma) and 5% interleukin-2 (IL-2; Hemagen) for 48 h and then grown in the presence of 5% IL-2 in RPMI medium supplemented with 10% FCS. Frozen stocks of pigtailed macaque PBMCs (pPBMCs) were kindly provided by Jeff Lifson. After thawing, cells were activated with 3 μg/ml staphylococcal enterotoxin B and 5% IL-2 for 48 h and then maintained in RPMI medium with 10% FCS and 5% IL-2.

Transfection and virus production. 293T cells were seeded at 2.5 × 10^5/well (24-well plate) or 6 × 10^5/10-cm dish and transfected the following day using polyethylenimine (Polysciences). For the generation of virus stocks, 293T cells in a 10-cm plate were transfected with 15 μg of proviral plasmids and placed in fresh medium after 24 h. At 40 h posttransfection, virus-containing cell supernatant was harvested, clarified by low-speed centrifugation, filtered (0.22 μm), and stored at −80°C. To generate vesicular stomatitis virus glycoprotein G (VSV-G)-pseudotyped virus stocks, 1 μg of a VSV-G expression plasmid was added to the transfection mixture. To compare the activities of Vif proteins against rHAPOBEC3G, a myc-tagged APOBEC3G plasmid (19) was cotransfected with full-length viruses and at 48 h posttransfection, cell lysates were harvested and analyzed by immunoblotting.

To determine infectious virus titers, serial dilutions of the virus stock were used to infect TZM reporter cells seeded into 96-well plates at a density of 3 × 10^4/well. At 48 h postinfection, the cells were fixed with 0.5% glutaraldehyde and stained with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) to determine the number of infected foci. Titers of GFP reporter viruses were determined by infecting CEMx174 cells with serial dilutions of the stock. At 48 h postinfection, the number of infected cells was determined by fluorescence-activated cell sorting (FACS).

Cell-free HIV-2 stocks were obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH (HIV-2 7294A, HIV-2 7312A, and HIV-2 60415K from Feng Gao and Beatrice Hahn and HIV-2 CDC310072, HIV-2 CDC310319, and HIV-2 CDC77618 from Stefan Wiktor and Mark Rayfield) (35,36). HIV-2 stocks were generated by short-term culture (to avoid any potential adaptation) in huPBMCs.

rHAPOBEC3G antagonism. To determine activity against rHAPOBEC3G, 350 ng of HIV-1, stHIV, or SIVMAC proviral plasmid expressing GFP in place of Nef was cotransfected with 350, 175, 87.5, or 43.75 ng of an rHAPOBEC3G expression plasmid or a control empty vector. The total amount of DNA was held constant by supplementing the transfection with empty expression vector. At 48 h posttransfection, supernatants were harvested, clarified by low-speed centrifugation, and filtered (0.22 μm) and titers were determined by infecting CEMx174 cells.

Western blot analyses. Cell lysates and virions pelleted through 20% sucrose (20,000 × g for 2 h at 4°C) were separated on NuPage Novex 4 to
12% Bis-Tris Mini Gels (Invitrogen). Proteins were blotted onto nitrocellulose membranes. Thereafter, blots were blocked in blocking solution (Rockland) and probed with the primary antibody and a corresponding IRDye 800CW- or IRDye 680-conjugated secondary antibody. Fluorescent signals were detected and quantified with an Odyssey scanner (LI-COR Biosciences). HIV-1 CA was detected with the mouse monoclonal antibody 37 (37) from Bruce Chesebro and Hardy Chen. SIVMAC239 Vpx was detected with a Vpx monoclonal antibody (6D2.6) (38) from John C. Kappes. HIV-1 gp41 was detected with monoclonal antibody F240 (39) from Marshall Posner and Lisa Cavacini, and tetherin was detected with a polyclonal rabbit serum (40) from Klaus Strebel and Amy Andrews. Each of the aforementioned antibodies was acquired through the NIH AIDS Research and Reference Reagent Program.

Virus replication assay. Freshly activated PBMCs were infected with HIV-1, stHIV, HIV-2, or SIV strains at a multiplicity of infection (MOI) of 0.001 (infectious titers were determined on TZM cells). Alternatively, viral stocks were normalized for reverse transcriptase (RT) activity prior to infection. At 16 h postinfection, cells were washed and divided into two or three wells containing 0, 100, or 1,000 U/ml IFN-α (Sigma). Supernatants were collected every 48 h for the following 15 days, and RT activity was measured with RT activity kit (Lenti RT Activity kit; CaviDi).

Virus release assays. Provirial plasmids (500 μg) were cotransfected into 293T cells with increasing amounts of plasmids expressing human or pigtailed macaque tetherin (0 ng, 11 ng, 33 ng, and 100 μg). At 48 h posttransfection, virion-containing supernatants were harvested, clarified by low-speed centrifugation, and filtered. Infectious virus release was determined by inoculating TZM indicator cells, which had been plated the previous day in 96-well plates at 8 × 10⁴ cells/well, with 50 μl of serially diluted supernatants. At 48 h after infection, β-galactosidase activity was determined with GalactoStar reagent (PerkinElmer). The remainder of the virus-containing supernatant (750 μl) was layered onto 400 μl of 20% sucrose in phosphate-buffered saline and centrifuged at 20,000 × g for 2 h at 4°C. Virion pellets and corresponding virion-producing cells were dissolved in SDS-PAGE loading buffer, and Western blot analysis was performed as described above.

Single-cycle PBMC infection assays. Freshly activated huPBMCs or pgtPBMCs seeded at a density of 5 × 10⁴/well of a 96-well plate were treated with 100 or 1,000 U/ml IFN-α or left untreated. After 24 h, cells were infected with GFP-expressing reporter viruses. After a further 24 h, the medium was changed and 100 ng/ml lamivudine (3TC) was added to prevent further virus spreading. At 48 h postinfection, the cells were fixed and the percentage of GFP-positive cells was determined by FACS.

CD4 downregulation assay. CEMx174 cells were infected with VSV-G-pseudotyped stHIV Δenv GFP reporter viruses expressing the various Vpu proteins. The cells were stained at 48 h postinfection with a mouse anti-human CD4 antibody (RPA-T4) conjugated to Alexa 700 (BD PharMingen) and fixed in paraformaldehyde. Cell-associated fluorescence in the 700-nm and GFP channels was measured with an LSR II flow cytometer (BD), and data were analyzed with FlowJo software.

RESULTS

Host cell species-dependent effects of IFN-α on HIV/SIV replication. To test the hypothesis that unidentified ISGs might inhibit primate lentivirus replication in unnatural-host cells, we compared the replication of SIVMAC239, SIVMNE027, and stHIV in huPBMCs and pgtPBMCs in the presence or absence of IFN-α. The stHIV strain used was based on NL4-3 and the Env protein is from SIVMAC239, and the Env protein is from SHIV/KB9. All other sequences are from NL4-3. (B) Equivalent amounts of HIV-1, stHIV, and SIVMAC proviruses expressing GFP were cotransfected with various amounts of rhAPOBEC3G. Supernatants were collected at 48 h posttransfection and assayed for infectivity on CEM cells. (C and D) Activated huPBMCs (B) or pgtPBMCs (C) were infected with stHIV or SIVMAC239/SIVMNE027 at an MOI of 0.001. At 24 h postinfection, the cells were washed and samples were split into three wells, two of which were treated with 100 or 1,000 U/ml IFN-α. Supernatants were collected every 48 h for the following 15 days, and RT activity was measured with an enzyme-linked immunosorbent assay (CaviDi).
Figure 2. Functional replacement of the stHIV Vpu protein with SIVden Vpu or SIVgsn Vpu. (A) huPBMCs left untreated or treated with 100 or 1,000 U/ml IFN-α for 24 h were lysed and subjected to Western blot analysis with antitetherin and antitubulin antibodies. Note that tetherin is highly and heterogeneously glycosylated and migrates as a smear at 50 to 64 kDa. (B) Schematic representation of the stHIVdenU and stHIVgsnU genomes. The Vpu proteins and Env signal peptides are from SIVden or SIVgsn. All other sequences are from the same stHIV construct described in Fig. 1A. (C) and (D) 293T cells were transfected with stHIV, stHIVΔvpu, stHIVdenU, or stHIVgsnU. At 48 h posttransfection, the virions were harvested, cells were lysed, and both were subjected to Western blot analysis with anti-CA (B) or anti-gp41 (C) antibodies. (E) Virion-containing supernatants were titrated on TZM reporter cells, and infected foci were revealed by X-Gal staining and counted. (F) CEMx174 cells were infected with stHIVΔenvGFP reporter viruses pseudotyped with VSV-G containing the gene for GFP in the place of the gene for Nef. At 48 h postinfection, the cells were fixed and stained for CD4 and analyzed by flow cytometry. LTR, long terminal repeat.
IFN-α (100 or 1,000 U/ml) used in Fig. 1 are within the range of those induced by HIV-1 or SIV<sub>MAC239</sub> replication in the respective hosts (3–6).

These data suggest that one or more IFN-α-induced antiretroviral activities are expressed in the normal target cells of primate lentiviruses and specifically inhibit viruses that are not adapted to replicate in that particular species. Notably, such antiretroviral activities are unlikely to reflect the activity of TRIM5 proteins since neither HIV-1 nor SIV<sub>MAC239</sub> is sensitive to inhibition by the human or pigtailed macaque forms of these factors (41). Moreover, since stHIV and SIV<sub>MAC239</sub> encode identical Vif proteins, these species-specific effects of IFN-α cannot be ascribed to the APOBEC3 proteins that are targeted by Vif and are known to be active against primate lentiviruses (Fig. 1B).

**Tetherin does not account for species-dependent effects of IFN-α on HIV/SIV replication.** Another antiviral protein that could, in principle, be responsible for the virus- and host-specific inhibition of primate lentiviruses by IFN-α is tetherin. stHIV encodes an HIV-1 Vpu protein that is unable to antagonize tetherin variants in most nonhuman primates, including macaques (14, 29). Because tetherin is induced severalfold in PBMCs following IFN-α treatment (11) (Fig. 2A), it might have been responsible for the IFN-α-induced inhibition of stHIV replication in pgtPBMCs.

We recently identified Vpu proteins from SIV strains that are effective antagonists of macaque tetherin proteins (42), and so we used these to replace the HIV-1 Vpu protein in stHIV. Specifically, we generated two stHIV variants, namely, stHIV<sub>denU</sub> and stHIV<sub>gsnU</sub>, that express the Vpu proteins from SIV<sub>den</sub> (43) and SIV<sub>gsn</sub>71 (44), respectively (Fig. 2B). We also generated an stHIV variant that does not express a Vpu protein (stHIV<sub>H9004</sub>). When 293T cells were transfected with stHIV, stHIV<sub>H9004</sub>, stHIV<sub>denU</sub>, or stHIV<sub>gsnU</sub> proviral plasmids, they expressed viral Gag proteins and generated extracellular virions at levels comparable to those obtained with stHIV (Fig. 2C). Because the 3′ portion of the Vpu sequence overlaps the Env signal peptide sequence, both stHIV<sub>denU</sub> and stHIV<sub>gsnU</sub> contain signal peptides that are not native to HIV-1 (Fig. 2B). However, these are cleaved upon Env maturation and were not predicted to affect the expression or function of stHIV Env. Indeed, Env expression and incorporation into particles were similar for stHIV, stHIV<sub>ΔU</sub>, stHIV<sub>H9004</sub>, and...
Additionally, each construct yielded particles similar in infectiousness (Fig. 2E).

To verify that the various Vpu proteins were expressed in a functional form, we generated Env-defective derivatives of stHIV, stHIVdenU, stHIVgsnU, and stHIV/H9004U that expressed GFP in place of Nef. In these constructs, the only viral protein that is capable of inducing CD4 downregulation is Vpu. Infection of CEMx174 cells with VSV-G-pseudotyped virions generated with each of the constructs (with the exception of stHIV/H9004U) induced efficient CD4 downregulation (Fig. 2F). Finally, to confirm that each Vpu protein possessed tetherin antagonist activity when expressed in the context of stHIV, we cotransfected the panel of stHIV constructs with increasing amounts of plasmids expressing either human tetherin or pigtailed macaque tetherin and measured particle release by Western blot (Fig. 3A) and infectivity (Fig. 3B) assays. As expected, stHIV, which expresses HIV-1 Vpu, was largely resistant to human tetherin but sensitive to pigtailed macaque tetherin, while stHIV/H9004U was sensitive to both tetherin proteins (Fig. 3A and B). In contrast, stHIVdenU and stHIVgsnU were largely resistant to pigtailed macaque tetherin but displayed intermediate sensitivity to human tetherin (Fig. 3A and B). Importantly, because stHIVdenU and stHIVgsnU were significantly less sensitive than stHIV to pigtailed macaque tetherin, these experiments established reagents that could test whether the host...
cell species-dependent effects of IFN-α on stHIV replication could be attributed to tetherin. Specifically, if tetherin is the major effector of the IFN-α/H9251-induced block to stHIV replication, then stHIVdenU and stHIVgsnU should be less sensitive than stHIV and stHIV/H9004U to IFN-α/H9251 in pgtPBMCs and more sensitive than stHIV to IFN-α/H9251 in huPBMCs. As a complementary approach, we also used an SIVMAC239/H9004Nef construct. Nef is the only SIVMAC239 protein that can antagonize tetherin (13, 14). Therefore, SIVMAC239/H9004Nef should be more sensitive to IFN-α/H9251 in pgtPBMCs if tetherin is the sole (or major) effector of IFN-α-induced antiretroviral activity therein.

In the absence of IFN-α, all of the aforementioned viruses replicated to comparable levels in huPBMCs and pgtPBMCs (Fig. 4A and B). In the presence of 100 U/ml or 1,000 U/ml IFN-α, the stHIVΔpu, stHIVdenU, and stHIVgsnU variants replicated well in huPBMCs, but a modest delay in replication was evident compared to stHIV (compare day 3 and day 5 RT values in Fig. 4A; data not shown). This delay was most likely due to the inability of these variants to efficiently counteract human tetherin, which, as previously reported, is upregulated in huPBMCs in the presence of IFN-α (11) (Fig. 2A). Importantly, stHIV, stHIVΔpu, stHIVdenU, and stHIVgsnU were equally sensitive to IFN-α in pgtPBMCs, which abolised replication therein (Fig. 4B). These findings indicate that IFN-α-induced inhibitors other than tetherin are the major factors limiting stHIV replication in IFN-α-treated pgtPBMCs. In support of this conclusion, SIVMAC239ΔNef did not exhibit increased sensitivity to IFN-α in pgtPBMCs, while both wild-type SIVMAC239 and SIVMAC239ΔNef were very sensitive to IFN-α in huPBMCs (Fig. 4C and D).

**SAMHD1 does not account for species-dependent effects of IFN-α on HIV/SIV replication.** One major difference between the HIV-1 and SIVMAC lineages is that the latter encodes a Vpx protein that is known to counteract SAMHD1 (45, 46). SAMHD1 expression is modestly induced by IFN-α in some cell types (P.D.B., unpublished observations) and is a deoxynucleoside triphosphate triphosphohydrolase (47) that reduces the levels of intracellular deoxynucleoside triphosphates below the concentration needed for efficient reverse transcription (48). Vpx proteins, delivered to target cells as a component of virions, can interact with SAMHD1 and target it for proteasomal degradation, thereby alleviating restriction (45, 46). Although SIVMAC Vpx is able to counteract both human and macaque SAMHD1 (49, 50), it was possible that quantitative differences in the efficiency of Vpx-mediated antagonism of human versus macaque SAMHD1 might affect the differential sensitivity of stHIV and SIVMAC239 to IFN-α in pgtPBMCs. Alternatively, it was possible that Vpx might have some other function that enabled replication in IFN-α-treated pgtPBMCs but was unnecessary in huPBMCs.

The HIV-1 p6 domain of Gag is unable to recruit SIVMAC239 Vpx into particles (51, 52). However, introduction of a Vpx packaging signal from SIVMAC239 p6 into HIV-1 p6 allows the efficient incorporation of SIVMAC239 Vpx into HIV-1 particles (25). On the basis of this finding, we generated three stHIV-based proviral plasmids that included the minimal SIVMAC239 Vpx packaging signal within p6 (25) (stHIVSp6Vpx), the SIVMAC239 Vpx protein (stHIVVpx), or both (stHIVSp6Vpx) (Fig. 5A). For each construct, Gag expression and particle release upon the transfection of 293T cells were comparable to those of stHIV (Fig. 5B). Fur-
thermore, all of the constructs yielded similar titers of infectious virions using TZM indicator cells (Fig. 5C). As expected, although both stHIVVpx and stHIVSp6Vpx expressed Vpx, only stHIVSp6Vpx was able to package Vpx into particles (Fig. 5B).

Notably, stHIV, stHIVSp6, stHIVVpx, and stHIVSp6Vpx all replicated to similar levels in huPBMCs and exhibited a similar relative lack of sensitivity to IFN-α/H9251 (Fig. 6A). Moreover, each of these viruses replicated to similar levels in pgtPBMCs and, importantly, replication was completely suppressed in the presence of IFN-α/H9251 (Fig. 6B). These results indicate that the putative IFN-α-induced inhibitors of HIV-1 replication in pgtPBMCs are not antagonized by SIVMAC239 Vpx. To corroborate these findings, the replication of SIVMAC239 and SIVMAC239ΔVpx was analyzed in huPBMCs and pgtPBMCs and pgtpBMCM donors are shown. Note that the virus replication curves in the presence of 100 or 1,000 U/ml of IFN-α (gray and open circles) are superimposed in panels B and C (bottom). p.i., postinfection.

FIG 6 Insertion of Vpx into or its removal from the stHIV or SIVMAC239 genome does not confer resistance or sensitivity to IFN-α. (A to D) huPBMCs (A and C) or pgtPBMCs (B and D) were infected at an MOI of 0.001 with stHIV, stHIVSp6, stHIVVpx, or stHIVSp6Vpx (A and B) or with SIVMAC239 or SIVMAC239ΔVpx (C and D). The next day, cells were washed and divided among three wells that were treated with 0, 100, or 1,000 U/ml IFN-α. Supernatants were collected every 48 h for the following 15 days, and RT activity was measured with an enzyme-linked immunosorbent assay-based assay (Cavidi Tech). Results from two huPBMC and pgtPBMC donors are shown. Note that the virus replication curves in the presence of 100 or 1,000 U/ml of IFN-α (gray and open circles) are superimposed in panels B and C (bottom). p.i., postinfection.

IFN-α induces an early, species-dependent but TRIM5-independent block in HIV/SIV infection. The above data strongly suggested that one or more undiscovered IFN-α-induced factors are responsible for a species-specific restriction of lentiviral replication. Restriction factors have been shown to act at various stages
of the lentiviral life cycle, so to gain some insight into where such novel factors might act, we conducted single-cycle infection assays of huPBMCs or pgtPBMCs. Derivatives of stHIV or SIVMAC239 each harboring the GFP protein in the place of Nef were used to quantify infected cells, and to limit the assay to a single cycle of infection, an RT inhibitor (3TC) was added at 16 h postinoculation.

In this assay format, IFN-α treatment of huPBMCs target cells inhibited single-cycle SIVMAC239/GFP infectivity in a dose-dependent manner up to ~8- to 10-fold (Fig. 7A). The same treatment inhibited single-cycle stHIV/GFP infectivity by only ~2- to 3-fold. In contrast, IFN-α treatment of pgtPBMC targets inhibited single-cycle SIVMAC239/GFP infectivity by ~3-fold but inhibited single-cycle stHIV/GFP infectivity by ~8 to 10-fold (Fig. 7B). The greater potency with which IFN-α inhibited single-cycle SIVMAC239/GFP and stHIV/GFP infectivity in PBMCs from an unnatural host species was concordant with the effects in spreading replication assays and suggests that at least part of IFN-α’s antiretroviral effect is mediated by a factor(s) inhibiting an early event(s) in the lentiviral life cycle, namely, entry, uncoating, reverse transcription, integration, and/or gene expression.

While the TRIM5 proteins found in humans and pigtailed macaques do not restrict either HIV-1 or SIVMAC239 (41), the finding that at least part of the species-dependent effects of IFN-α occurred early in the life cycle prompted us to examine further whether this or any other CA-targeting restriction factor might contribute to the aforementioned effects. To this end, we examined whether an stHIV strain in which the HIV-1 CA domain of Gag was replaced with that of SIVMAC239 [stHIV(SCA)] (20) would behave like stHIV or SIVMAC239. In fact, during single-cycle infection experiments in which huPBMCs or pgtPBMCs were treated with IFN, stHIV(SCA) displayed the same degree of sensitivity or resistance to inhibition by IFN-α as did stHIV, irrespective of the species from which the PBMCs were derived (Fig. 7A and B). Thus, this experiment effectively excludes TRIM5 proteins and any other CA-targeting factors as mediators of the target cell species-dependent inhibitory activity of IFN-α.

**Effects of IFN-α on naturally occurring HIV-1 and HIV-2 strains.** A caveat of these studies is that stHIV is an engineered derivative of a laboratory-adapted HIV-1 strain (NL4-3). It was necessary to use stHIV in these studies because unmanipulated HIV-1 strains cannot replicate in pgtPBMCs because of blocks imposed by APOBEC3 proteins (24). Nevertheless, it was possible to compare the sensitivity of stHIV to that of more authentic viral strains in huPBMCs. Therefore, we analyzed the effect of IFN-α on the replication of a panel of transmitted founder HIV-1 strains (30–34) in huPBMCs. These naturally occurring HIV-1 strains replicated to levels similar to that of stHIV in untreated huPBMCs (Fig. 8A). Moreover, although there was some PBMC donor-to-donor variation, natural HIV-1 strains were clearly more resistant to IFN-α in huPBMCs than were SIV strains (compare Fig. 8A with Fig. 1, 4A, and 6A).

Given that SIVMAC239 and SIVMNE027 were both hypersensitive to IFN-α in huPBMCs (Fig. 1), we also tested whether HIV-2 strains shared this property. HIV-2s are derived from the same lineage as SIVMAC239 and SIVMNE027, namely, the SIVsm group of viruses found in sooty mangabeys (53), but HIV-2 strains have obviously had a greater opportunity to adapt to replication in human lymphocytes and perhaps might be comparatively IFN-α resistant therein. Therefore, we determined the sensitivities of a panel of six HIV-2 strains (35, 36) to IFN-α in huPBMCs. In contrast to SIVMAC239, primary HIV-2 strains appeared relatively resistant to IFN-α in huPBMCs (Fig. 8B), although two primary HIV-2 strains (HIV-27112A and HIV-2310072) appeared sensitive, particularly in the presence of higher IFN-α concentrations (1,000 U/ml) and depending on the PBMC donor (Fig. 8B; data not shown). Thus, there appeared to be significant differences in IFN-α sensitivity among primary HIV-2 strains that might indicate that certain HIV-2 strains are less well adapted to the IFN-α-induced antiviral state in huPBMCs than is HIV-1.

**DISCUSSION**

HIV-1 and SIVMAC replication persists in human and macaque hosts despite a chronic state of immune activation that includes the expression of ISGs and a presumptive “antiviral state” (7). This finding suggests that primate lentiviruses have adapted to replicate in the presence of proteins that might ordinarily inhibit their replication. Indeed, there are clear examples, namely, the APOBEC3 proteins, TRIM5, tetherin, and SAMHD1, where adaption of primate lentiviruses to the presence of antiretroviral ISGs is demonstrable (8, 9).

Nevertheless, previous work has shown that HIV-1 replication can be inhibited in human cells, to at least some degree, by IFN-α (9, 54). Indeed, consistent with these observations, we found that HIV-1, SIVMAC239, and SIVMNE027 replication was inhibited by IFN-α. However, the degree to which replication was inhibited by IFN-α in PBMCs from their usual hosts was comparatively modest, while each of these viruses was hypersensitive to IFN-α in unnatural host PBMCs. These findings strongly suggest that HIV-1, SIVMAC239, and SIVMNE027 have adapted, at least to some extent, to replicate in the face of the antiviral state in cells from their usual hosts but not in cells from species outside their usual host range. Although this conclusion held for the most part for HIV-2, we found that two of the six HIV-2 strains tested were...
quite sensitive to IFN-α in huPBMCs. Although the number of strains we analyzed was relatively small, these findings demonstrate strain-to-strain variability in IFN-α sensitivity among HIV-2 strains. Whether this reflects differences among HIV-2 strains in passage history in humans is unclear at present, but it will be interesting to determine IFN-α sensitivity in larger numbers of HIV-2 strains. It is also possible that IFN-α sensitivity varies among HIV-1 and SIV strains, although there is, as yet, no evidence that this is the case.

By using a variety of chimeric viruses encoding nonnative CA, Vif, Vpu, and Vpx proteins, we could demonstrate that IFN-α hypersensitivity in unnatural host cells was unlikely to be attributable to the known restriction factors that target, or are antagonized by, these viral proteins. Indeed, stHIV expresses SIVMAC239 Vif, effectively excluding the range of APOBEC3 proteins that are targeted by SIVMAC239 Vif (APOBEC3B, -C, -D, -F, -G, and -H) (19) as mediators of the differential sensitivity of stHIV and SIVMAC239 to IFN-α. Additionally, endowing stHIV with Vpu proteins that could antagonize macaque tetherin or removing the tetherin antagonist (Nef) from SIVMAC239 did not dramatically alter their sensitivity or resistance to IFN-α in PBMCs from their usual host or those from an unnatural host. In a similar approach, the addition of a SIVMAC239 Vpx protein to stHIV or the removal of Vpx from SIVMAC239 did not alter their sensitivity or resistance to IFN-α in usual or unnatural host PBMCs, effectively excluding SAMHD1 as the relevant antiviral protein. Indeed, analysis of HIV-1 replication in cells from Aicardi-Goutières syndrome patients has indicated that the inhibitory effects of SAMHD1 are not evident in activated lymphocytes (35). Moreover, SIVMAC239 encodes a Vpx protein

FIG 8 Effects of IFN-α on primary HIV-1 and HIV-2 strain replication in huPBMCs. (A and B) huPBMCs were infected with the indicated HIV-1 (A) or HIV-2 (B) strains at an MOI of 0.001. The next day, cells were washed and divided among three wells that were treated with 0, 100, or 1,000 U/ml IFN-α. Supernatants were collected every 48 h for the following 15 days, and RT activity was measured with an enzyme-linked immunosorbent assay (Cavidi Tech). Results from two huPBMC donors are shown.
that can antagonize human SAMHD1 (45, 48), while HIV-1 does not, yet SIVmac239 was more sensitive to IFN-α in huPBMCs than was HIV-1.

In the case of TRIM5 proteins, neither huTRIM5α nor pgTTRIM5α is active against HIV-1 or SIVmac (41, 56). Furthermore, by using single-cycle infectivity experiments, we could demonstrate that the relative resistance of IFN-α-α-treated huPBMCs to SIVmac239, and the relative resistance of IFN-α-α-treated pgTPBMCs to stHIV were not determined by the CA protein. Again this excludes TRIM5α as the determinant of the differential susceptibility of IFN-α-α-treated PBMCs to infection with stHIV and SIVmac239.

These findings lead to the conclusion that at least one undiscovered ISG is present in cells of macaque origin that can inhibit HIV-1 replication therein. They also suggest that at least one undiscovered ISG is present in huPBMCs that can inhibit SIV replication. Although it is possible that a single polymorphic and relatively potent antiretroviral factor is responsible for all of the species-dependent antiretroviral activities described herein, there is as yet no evidence that this is the case. It is equally possible that several, perhaps many, factors, each with modest antiretroviral activity, contribute to the overall activity of IFN-α. Indeed, over-expression screens of hundreds of ISGs for activity against several viruses reveal numerous factors with modest antiviral activities that are additive when combinations of ISGs are coexpressed (57). The identification of such factors will aid both our understanding of how hosts defend against lentivirus infections and the development of HIV-1-based strains that can replicate in unnatural hosts.

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Institutes of Health (grants R01AI078788 and R21AI093255). Howard Hughes Medical Institute, and T.H. is funded by the National Institutes of Health (grant R37AI64003) and the Procter & Gamble TRIMCyp is active against HIV-1 or SIVMAC (41, 56). Further-

ment of HIV-1-based strains that can replicate in unnatural hosts.

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Correction for Bitzegeio et al., Adaptation to the Interferon-Induced Antiviral State by Human and Simian Immunodeficiency Viruses

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Volume 87, no. 6, p. 3549–3560, 2013. Page 3551, Fig. 1C: This panel depicts the replication of three different viruses in human peripheral blood mononuclear cells (PBMCs) from a particular donor. The right panel was erroneously labeled “SIVMNE027.” In fact, the replication of SIVMNE027 in this PBMC donor was not tested. Figure 1C should have depicted replication in PBMCs from a different human donor in which the replication of all three viruses was measured.

Fig. 1C should appear as shown below.

Page 3557, Fig. 7: Incorrect values were used to plot the graphs. Values were manipulated by Julia Bitzegeio without the knowledge of the other authors. This matter has been investigated through an institutional inquiry resulting in a misconduct finding against Dr. Bitzegeio by the Office of Research Integrity.

Fig. 7 should appear as shown below.

These corrections do not change the conclusions of the paper.