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 (SIV_{MAC} and SIV_{MNE}) 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 SIV_{MAC}/SIV_{MNE} 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 SIV_{MAC} 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 (SIV_{MAC}), 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 SIV_{MAC} 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 SIV_{MAC} is sensitive to inhibition by human tetherin (13). In contrast, SIV_{MAC} 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 SIV_{MAC} infection. Especially potent blocks to HIV-1 replication in macaque cells are imposed by TRIM5 α , 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 SIV_{MAC} 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 TRIM5 α - and APOBEC3-based restrictions by engineering HIV-1 strains that encode the SIV_{MAC} 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

Received 19 November 2012 Accepted 7 January 2013

Published ahead of print 16 January 2013

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doi:10.1128/JVI.03219-12

not active against HIV-1 (21–23) enabled the construction of an stHIV strain in which the only alteration was the inclusion of SIV_{MAC} 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 are. 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 SIV_{MAC239} and the Env coding sequences have been replaced with those of the macaque-adapted HIV-1 envelope from SHIV/KB9. In stHIV(SCA), the HIV-1 CA-encoding sequences have also been replaced with those of SIV_{MAC239}.

The proviral plasmid stHIV Δ Vpu 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 stHIVdenU and stHIVgsnU, overlap extension PCR was used to replace stHIV Vpu coding sequences with those from SIVdenVpu or SIVgsnVpu. Because the Vpu and Env sequences overlap, this manipulation resulted in the replacement of the KB9 Env amino terminus with that of SIVden Env or SIVgsn 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 stHIV Δ envGFP has been described previously (20). Derivatives thereof (stHIV Δ vpu Δ envGFP, stHIVdenU Δ envGFP, and stHIVgsnU Δ envGFP) were generated by combining fragments of stHIV Δ envGFP with stHIV Δ Vpu stHIVdenU or stHIVgsnU.

To generate stHIVVpx, overlap extension PCR was used to insert SIV_{MAC239} Vif and Vpx sequences, rather than Vif alone, between 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 SIV_{MAC239} 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 SIV_{MAC239} proviral DNA was generated on the basis of a pXf3-derived low-copy-number vector termed V1 and using p239SpSp5' and p239SpE3' obtained from the NIH AIDS Research and Reference Reagent Program and contributed by R. C. Desrosiers, J. S. Gibbs, and D. Regier (26). A SIV_{MAC239} Δ Nef proviral plasmid was similarly derived. For the generation of SIV_{MAC239} Δ Vpx, the Vpx initiation codon was mutated without changing the amino acid sequence in the overlapping Vif protein. The SIV_{MNE027} proviral plasmid has been described elsewhere (27).

A proviral plasmid, SIV-GFP, encoding green fluorescent protein

(GFP) in place of SIV_{MAC239}Nef has been described previously (28). To obtain stHIV-GFP and stHIV(SCA)-GFP, an stHIV fragment containing SIV_{MAC} 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 (pgtPBMCs) were kindly provided by Jeff Lifson. After thawing, cells were activated with 3 μ g/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 6×10^6 /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 μ g 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 μ m), and stored at -80°C . To generate vesicular stomatitis virus glycoprotein G (VSV-G)-pseudotyped virus stocks, 1 μ g 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 3×10^3 /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 *in vitro* adaptation) in huPBMCs.

rhAPOBEC3G antagonism. To determine activity against rhAPOBEC3G, 350 ng of HIV-1, stHIV, or SIV_{MAC} 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 μ m) and titers were determined by infecting CEMx174 cells.

Western blot analyses. Cell lysates and virions pelleted through 20% sucrose (20,000 $\times$ 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 183-H12-5C (37) from Bruce Chesebro and Hardy Chen. SIV_{MAC239} 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 Andrew. 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 13 to 15 days. The amount of virus particles released into the cell culture supernatant was determined by measuring RT activity (Lenti RT Activity kit; Cavid).

Virus release assays. Proviral plasmids (500 μ g) were cotransfected into 293T cells with increasing amounts of plasmids expressing human or pigtailed macaque tetherin (0 ng, 11 ng, 33 ng, and 100 μ g). 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 8×10^3 cells/well, with 50 μ l of serially diluted supernatants. At 48 h after infection, β -galactosidase activity was determined with GalactoStar reagent (PerkinElmer). The remainder of the virion-containing supernatant (750 μ l) was layered onto 400 μ l of 20% sucrose in phosphate-buffered saline and centrifuged at $20,000 \times g$ 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 5×10^4 /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 Δ env 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 LSRII 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 SIV_{MAC239}, SIV_{MNE027}, and stHIV in huPBMCs and pgtPBMCs in the presence or absence of IFN- α . The stHIV strain used was based on NL4-3 but encoded a dual-tropic envelope protein (from SHIV/KB9), as well as the SIV_{MAC239} Vif protein (24) (Fig. 1A). In contrast to HIV-1, expressing HIV-1 Vif, stHIV antagonized rhAPOBEC3G as efficiently as SIV_{MAC} (Fig. 1B), suggesting that both viruses express SIV_{MAC} Vif at approximately equivalent levels. As ex-

FIG 1 Differential sensitivities of stHIV and SIV_{MAC239} to IFN- α in huPBMCs and pgtPBMCs. (A) Schematic representation of the stHIV genome. The Vif protein is from SIV_{MAC239}, and the Env protein is from SHIV/KB9. All other sequences are from NL4-3. (B) Equivalent amounts of HIV-1, stHIV, and SIV_{MAC} 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 (C) or pgtPBMCs (D) were infected with stHIV or SIV_{MAC239}/SIV_{MNE} 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 (Cavid Tech). LTR, long terminal repeat; p.i., postinfection.

pected, both stHIV and SIV_{MAC} replicated efficiently in both huPBMCs and pgtPBMCs (Fig. 1C and D). However, addition of IFN- α completely suppressed the replication of SIV_{MAC239} and SIV_{MNE027} in huPBMCs, while stHIV was clearly less IFN- α sensitive (Fig. 1C). Conversely, in pgtPBMCs, IFN- α completely suppressed stHIV replication while SIV_{MAC239} and SIV_{MNE027} were less sensitive (Fig. 1D). Of note, the doses of

FIG 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, stHIV Δ vpu, 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 stHIV Δ envGFP 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.

FIG 3 Resistance of stHIVdenU and stHIVgsnU to pigtailed macaque tetherin. (A) Western blot analysis of 293T cells cotransfected with the indicated proviral plasmid and increasing amounts (0, 11, 33, or 100 ng) of a plasmid expressing either human or pigtailed macaque tetherin. Cell and virion lysates were probed with anti-CA monoclonal and/or rabbit anti-HA antibodies, and signals were detected with fluorescent secondary antibodies. (B) Virion-containing supernatants harvested at 48 h postinfection were titrated on TZM reporter cells, and the infectious virion yield was determined with a chemiluminescent β -galactosidase assay. RLU, relative light units.

IFN- α (100 or 1,000 U/ml) used in Fig. 1 are within the range of those induced by HIV-1 or SIV_{MAC239} 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_{MAC239} is sensitive to inhibition by the human or pigtailed macaque forms of these factors (41). Moreover, since stHIV and SIV_{MAC239} 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, stHIVdenU and stHIVgsnU, that express the Vpu proteins from SIVden (43) and SIVgsn71 (44), respectively (Fig. 2B). We also generated an stHIV variant that does not express a Vpu protein (stHIV Δ U). When 293T cells were transfected with stHIV, stHIV Δ U, stHIVdenU, or stHIVgsnU 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 stHIVdenU and stHIVgsnU 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 Δ U, stHIVdenU, and

FIG 4 Addition or removal of functional tetherin antagonists does not confer stHIV or SIV_{MAC239} resistance or sensitivity to IFN- α . (A to D) huPBMCs (A and C) or pigtailed PBMCs (B and D) were infected with stHIV, stHIV Δ vpu, stHIVdenU, stHIVgsnU (A and B), SIV_{MAC239}, or SIV_{MAC239} Δ nef (C and D) 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-based assay (Cavidi Tech). Results from two huPBMC and pigtailed PBMC donors are shown. Note that the virus replication curves in the presence of 100 or 1,000 U/ml IFN- α (gray and open circles) are superimposed in panel B. p.i., postinfection.

stHIVgsnU (Fig. 2D). 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 Δ U 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 Δ U) 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 Δ U 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

FIG 5 Insertion of SIV_{MAC239} Vpx into the stHIV genome and incorporation into infectious virions. (A) Schematic representation of the stHIVSp6Vpx genome. The Vpx proteins and the Vpx packaging signal within p6 are from SIV_{MAC239}. All other sequences are from the stHIV construct described in Fig. 1A. (B) Western blot analysis of 293T cells and virions transfected with stHIV, stHIVSp6, stHIVVpx, and stHIVSp6Vpx that were harvested at 48 h postinfection. Cell lysates and virions were probed with anti-CA and anti-Vpx monoclonal antibodies, and signals were detected with fluorescent secondary antibodies. (C) Virion-containing supernatants were titrated on TZM reporter cells, and infected foci were revealed by X-Gal staining and counted. LTR, long terminal repeat.

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 -induced block to stHIV replication, then stHIVdenU and stHIVgsnU should be less sensitive than stHIV and stHIV Δ U to IFN- α in pgtPBMCs and more sensitive than stHIV to IFN- α in huPBMCs. As a complementary approach, we also used an SIV_{MAC239} Δ Nef construct. Nef is the only SIV_{MAC239} protein that can antagonize tetherin (13, 14). Therefore, SIV_{MAC239} Δ Nef should be more sensitive to IFN- α 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 stHIV Δ Vpu, 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, stHIV Δ Vpu, stHIVdenU, and stHIVgsnU were equally sensitive to IFN- α in pgtPBMCs, which abolished 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, SIV_{MAC239} Δ Nef did not exhibit increased sensitivity to IFN- α in pgtPBMCs, while both wild-type SIV_{MAC239} and SIV_{MAC239} Δ Nef 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 SIV_{MAC} 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 SIV_{MAC} 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 SIV_{MAC239} 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 SIV_{MAC239} Vpx into particles (51, 52). However, introduction of a Vpx packaging signal from SIV_{MAC239} p6 into HIV-1 p6 allows the efficient incorporation of SIV_{MAC239} Vpx into HIV-1 particles (25). On the basis of this finding, we generated three stHIV-based proviral plasmids that included the minimal SIV_{MAC239} Vpx packaging signal within p6 (25) (stHIVSp6), the SIV_{MAC239} 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-

FIG 6 Insertion of Vpx into or its removal from the stHIV or SIV_{MAC239} 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 SIV_{MAC239} or SIV_{MAC239}ΔVpx (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.

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 (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 (Fig. 6B). These results indicate that the putative IFN- α -induced inhibitors of HIV-1 replication in pgtPBMCs are not antagonized by SIV_{MAC239} Vpx. To corroborate these findings, the replication of SIV_{MAC239} and SIV_{MAC239}ΔVpx was analyzed in huPBMCs and pgtPBMCs. In the absence of IFN- α , the replication of SIV_{MAC239}ΔVpx was marginally reduced in both huPBMCs and

pgtPBMCs compared to that of SIV_{MAC239} (Fig. 6C and D). However, SIV_{MAC239}ΔVpx was able to replicate in IFN- α -treated pgtPBMCs but both viruses were profoundly inhibited by IFN- α treatment in huPBMCs (Fig. 6C and D). Thus, for both stHIV and SIV_{MAC239}, the addition of Vpx to or its removal from the viral genome did not affect the host species-dependent IFN- α -induced restriction. These results suggest that neither SAMHD1 nor any other potential Vpx target protein is responsible for the IFN- α -induced restriction of HIV-1 and SIV_{MAC239} replication in unnatural host cells.

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

FIG 7 An early, species-dependent but TRIM5-independent block to stHIV and SIV_{MAC239} infection is induced by IFN- α . (A and B) huPBMCs (A) or pgtPBMCs (B) were treated with the indicated concentrations of IFN- α for 24 h and then infected with SIV-GFP, stHIV-GFP, or stHIV(SCA)-GFP reporter virus in the presence of IFN- α . At 24 h postinfection, the medium was changed and 100 ng/ml 3TC was added. At 48 h postinfection, the cells were fixed and the percentage of GFP-positive cells was determined by flow cytometry. The data represent the mean $\pm$ standard deviation of three huPBMC or pgtPBMC donors and are plotted relative to the level of infection in the absence of IFN- α (which was set at 100%).

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 SIV_{MAC239}, 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 SIV_{MAC239}/GFP infectivity in a dose-dependent manner up to $\sim$ 8- to 10-fold (Fig. 7A). The same treatment inhibited single-cycle stHIV/GFP infectivity by only $\sim$ 2- to 3-fold. In contrast, IFN- α treatment of pgtPBMC targets inhibited single-cycle SIV_{MAC239}/GFP infectivity by $\sim$ 3-fold but inhibited single-cycle stHIV/GFP infectivity by $\sim$ 8 to 10-fold (Fig. 7B). The greater potency with which IFN- α inhibited single-cycle SIV_{MAC239}/GFP and stHIV/GFP infectivity in PBMCs from 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 SIV_{MAC239} (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 SIV_{MAC239} [stHIV(SCA)] (20) would behave like stHIV or SIV_{MAC239}. 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 SIV_{MAC239} and SIV_{MNE027} 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 SIV_{MAC239} and SIV_{MNE027}, namely, the SIV_{SM} 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 SIV_{MAC239}, primary HIV-2 strains appeared relatively resistant to IFN- α in huPBMCs (Fig. 8B), although two primary HIV-2 strains (HIV-2_{7312A} 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 SIV_{MAC} 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 adaptation 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, SIV_{MAC239}, and SIV_{MNE027} 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, SIV_{MAC239}, and SIV_{MNE027} 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

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.

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 SIV_{MAC239} Vif, effectively excluding the range of APOBEC3 proteins that

are targeted by SIV_{MAC239} Vif (APOBEC3B, -C, -D, -F, -G, and -H) (19) as mediators of the differential sensitivity of stHIV and SIV_{MAC239} to IFN- α . Additionally, endowing stHIV with Vpu proteins that could antagonize macaque tetherin or removing the tetherin antagonist (Nef) from SIV_{MAC239} 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 SIV_{MAC239} Vpx protein to stHIV or the removal of Vpx from SIV_{MAC239} 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 (55). Moreover, SIV_{MAC239} encodes a Vpx protein

that can antagonize human SAMHD1 (45, 48), while HIV-1 does not, yet SIV_{MAC239} was more sensitive to IFN- α in huPBMCs than was HIV-1.

In the case of TRIM5 proteins, neither huTRIM5 α nor pgpTRIMCyp is active against HIV-1 or SIV_{MAC} (41, 56). Furthermore, by using single-cycle infectivity experiments, we could demonstrate that the relative resistance of IFN- α -treated huPBMCs to SIV_{MAC239} and the relative resistance of IFN- α -treated pgpPBMCs to stHIV were not determined by the CA protein. Again this excludes TRIM5 α as the determinant of the differential susceptibility of IFN- α -treated PBMCs to infection with stHIV and SIV_{MAC239}.

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, overexpression 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.

ACKNOWLEDGMENTS

We thank Jeffrey D. Lifson, Gregory Del Prete, and colleagues for providing pgpPBMCs. We are grateful to Frank Kirchhoff for the SIVden and SIVgsn Vpu plasmids. The following reagents were obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH: p239Sp5p' and p239SpE3' from R. C. Desrosiers, J. S. Gibbs, and D. Regier; a panel of infectious HIV-1 molecular clones (catalog number 11919) from John Kappes; HIV-2 stocks 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. We also thank Vineet KewalRamani and members of the Bieniasz and Hatzioannou labs for helpful discussions.

J.B. is funded by a DFG research fellowship (BI 1540/1-1), P.D.B. is funded by the National Institutes of Health (grant R37AI64003) and the Howard Hughes Medical Institute, and T.H. is funded by the National Institutes of Health (grants R01AI078788 and R21AI093255).

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