Pathogenic Features Associated with Increased Virulence upon Simian Immunodeficiency Virus Cross-Species Transmission from Natural Hosts

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ABSTRACT

While simian immunodeficiency viruses (SIVs) are generally nonpathogenic in their natural hosts, dramatic increases in pathogenicity may occur upon cross-species transmission to new hosts. Deciphering the drivers of these increases in virulence is of major interest for understanding the emergence of new human immunodeficiency viruses (HIVs). We transmitted SIV_{sab} from the *sabaeus* species of African green monkeys (AGMs) to pigtailed macaques (PTMs). High acute viral replication occurred in all SIV_{sab}-infected PTMs, yet the outcome of chronic infection was highly variable, ranging from rapid progression to controlled infection, which was independent of the dynamics of acute viral replication, CD4⁺ T cell depletion, or preinfection levels of microbial translocation. Infection of seven PTMs with plasma collected at necropsy from a rapid-progressor PTM was consistently highly pathogenic, with high acute and chronic viral replication, massive depletion of memory CD4⁺ T cells, and disease progression in all PTMs. The plasma inoculum used for the serial passage did not contain adventitious bacterial or viral contaminants. Single-genome amplification showed that this inoculum was significantly more homogenous than the inoculum directly derived from AGMs, pointing to a strain selection in PTMs. In spite of similar peak plasma viral loads between the monkeys in the two passages, immune activation/inflammation levels dramatically increased in PTMs infected with the passed virus. These results suggest that strain selection and a massive cytokine storm are major factors behind increased pathogenicity of SIV upon serial passage and adaptation of SIVs to new hosts following cross-species transmission.

IMPORTANCE

We report here that upon cross-species transmission and serial passage of SIV_{sab} from its natural host, the *sabaeus* African green monkey (AGM), to a new host, the pigtailed macaque (PTM), viral adaptation and increased pathogenicity involve strain selection and a massive cytokine storm. These results permit the design of strategies aimed at preventing cross-species transmission from natural hosts of SIVs to humans in areas of endemicity. Furthermore, our study describes a new animal model for SIV infection. As the outcomes of SIV_{sab} infection in PTMs, African green monkeys, and rhesus macaques are different, the use of these systems enables comparative studies between pathogenic, nonpathogenic, and elite-controlled infections, to gain insight into the mechanisms of SIV immunodeficiency and comorbidities.

Simian immunodeficiency viruses (SIVs) naturally infect African nonhuman primates (NHPs) with virtually no deleterious pathogenic consequences and have a relatively high propensity for cross-species transmission in the wild and in research settings (1–7). Both human immunodeficiency virus type 1 (HIV-1) and HIV-2, the viruses responsible for the AIDS pandemic, originated by cross-species transmission of SIVs infecting chimpanzees/gorillas (SIV_{cpz/gor}) and sooty mangabeys (SIV_{smm}), respectively (8). While multiple cross-species transmission events from NHPs to humans have been documented to occur over the last century (8), only a fraction of these resulted in the emergence of pandemic (HIV-1 group M) or epidemic (HIV-1 group O and HIV-2 groups A and B) viruses. The remaining viral forms were apparently contained, with limited spread restricted to the area of emergence (8). Likewise, accidental or experimental SIV cross-species transmission from multiple African NHP hosts to rhesus macaques (RMs) may have a very diverse pattern of pathogenic outcomes, ranging

from progression to AIDS (9) to controlled infections (10–14). The mechanism(s) driving the increases in pathogenicity of SIVs in new hosts upon cross-species transmission is unclear, but serial passage appears to be a requirement (9, 15). Altogether, these observations suggest that in addition to exposure to new species, viral adaptation is required for the successful emergence of the

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virus as a highly virulent pandemic pathogen in the new host species. Identification of factors driving the efficacy of SIV cross-species transmission is paramount for assessing the risks for emergence of new HIV strains in human populations and for designing strategies to effectively control cross-species transmissions, especially considering the massive exposure of humans in sub-Saharan Africa to a plethora of SIVs naturally infecting numerous species of monkeys (8, 16).

The RM, which is a widely used model for AIDS pathogenesis and vaccine studies, is not appropriate for the study of the mechanisms of SIV adaptation to a new host upon cross-species transmission. With the exception of SIVsmm, which induces progressive infection upon its direct transmission from the sooty mangabey (*Cercocebus torquatus atys*) (17, 18), cross-species-transmitted SIVs are controlled in RMs (19) due to numerous host restriction factors (20). Conversely, pigtailed macaques (PTMs) appear to be a more appropriate animal model for the study of SIV adaptation following cross-species transmission. In most cases, SIV cross-species transmissions to PTMs result in persistent infection and progressive disease, as reported after experimental PTM exposure to SIVsmm (17, 21), SIVsun from *Cercopithecus lhoesti solatus* (22), SIVlho from *C. lhoesti lhoesti* (22), SIVver from *Chlorocebus pygerythrus* (23, 24), and SIVrcm from *Cercocebus torquatus torquatus* (25). A reason for increased susceptibility to cross-species-transmitted SIV is that TRIM5 α restriction (26) is not effective in PTMs due to aberrant mRNA splicing, resulting in TRIM5 α isoforms unable to restrict either HIV-1 or SIV infection (27).

Here, we report that experimental infection of PTMs with SIVsab, the virus that naturally infects Western African green monkey (AGM) species (the sabaeus monkey) (28), has a highly variable outcome, ranging from rapid progression to controlled infection. A single serial passage of the virus, however, leads to a consistently pathogenic infection, with all PTMs infected with the passaged virus replicating SIVsab at high levels during chronic infection and progressing to AIDS within 1 to 2 years postinfection (p.i.).

MATERIALS AND METHODS

Animals and infection. Twelve PTMs (*Macaca nemestrina*) 4 to 8 years of age were included in this study. All animals were negative for simian T-cell lymphotropic virus (STLV) (Vironostika HTLV-I/II enzyme-linked immunosorbent assay [ELISA]; bioMérieux, Durham, NC) and SIVsab (29) and were housed at the Tulane National Primate Research Center (TNPRC) and the Regional Industrial Development Corporation (RIDC) Park campus of the University of Pittsburgh. Both sites are Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) international accredited facilities. The animals were fed and housed according to regulations set forth by the *Guide for the Care and Use of Laboratory Animals* (30) and the Animal Welfare Act. Animal experiments were approved by the Tulane University and University of Pittsburgh Institutional Animal Care and Use Committees (IACUCs).

Five PTMs (passage 1 [P1]) received an intravenous (i.v.) inoculation of a plasma virus inoculum obtained from an acutely SIVsab92018-infected sabaeus AGM. The inoculum was adjusted to contain 10^6 viral RNA (vRNA) copies, corresponding to 150 50% tissue culture infectious doses (TCID₅₀) (31). To increase SIVsab pathogenicity in PTMs and to obtain more-reproducible infection patterns, seven PTMs (passage 2 [P2]) received an inoculum of one ml of plasma adjusted to 10^6 SIVsab RNA copies/ml harvested from the terminally infected P1 rapid progressor PTM BH66.

Sampling and cell separation. Blood was collected (10, 31) twice pre-infection, twice weekly for 3 weeks p.i., weekly thereafter up to the set point, and every 2 months during chronic infection (2 to 36 months p.i.). Axillary lymph nodes (LNs) were collected by excisional biopsy (31) at 0, 21, 180, and 360 days postinfection (dpi). Duodenal biopsy specimens were collected by endoscopic guided biopsy prior to infection, during acute infection (10, 21, and 28 dpi), and during chronic SIVsab infection (monthly for 6 months p.i. and every 3 months thereafter). Intestinal resections (5 to 10 cm) were removed surgically (31) at −35, 8, and 225 dpi. Additional LNs and intestinal tissues were obtained at necropsy.

Plasma, peripheral blood mononuclear cells (PBMCs), and mononuclear cells from LN and intestinal biopsy specimens, as well as from multiple tissues collected at the necropsy, were isolated as described previously (31–33).

Viral quantification. Plasma viral loads (VLs) were quantified by SIVsab92018/BH66-specific real-time PCR (31). vRNA loads were also quantified in mononuclear cells isolated from blood, LNs, and intestinal biopsy specimens, as well as from tissues collected at necropsy (33, 34). Simultaneous quantification of RNase P (RNase P detection kit; Applied Biosystems, CA), a single-copy gene with 2 copies per diploid cell, was performed to normalize sample variability and allow accurate quantification of cell equivalents (25). Assay sensitivity was 10 vRNA copies/ 10^5 cells.

Antibodies and flow cytometry. To assess changes in numbers of major T cell populations and their immune activation status, whole blood, LN, and intestinal mononuclear cells were stained for flow cytometry (10) with the following monoclonal antibodies (MAbs): CD3 fluorescein isothiocyanate (FITC); peridinin chlorophyll A protein (PerCP) or phycoerythrin (PE); CD4 allophycocyanin (APC) or PerCP; CD8 β PE (Beckman Coulter) or PerCP; CD20 PE; CD95 FITC; CD28 APC; HLADR-PerCP; and Ki67 FITC. All MAbs were from BD Bioscience Pharmingen, San Diego, CA, unless otherwise indicated. All antibodies were previously tested and calibrated for use in PTMs (25, 35, 36). Cells were stained (10, 31, 33) and analyzed with a FACSCalibur flow cytometer (BD Immunocytometry Systems) using CellQuest software (BD). The relative proportion of CD4 $^{+}$ and CD8 $^{+}$ T cells in LN and intestine samples was calculated as the “index” of these cell populations (i.e., the product of the percentage of CD3 $^{+}$ T cells gated on lymphocytes multiplied by the percentage of CD4 $^{+}$ or CD8 $^{+}$ T cells, divided by 100). The index is a function of both total CD3 $^{+}$ T cells and CD4 $^{+}$ or CD8 $^{+}$ T cells and provides a more accurate reflection of target cells (10).

IHC. Immunohistochemical staining (IHC) was performed as described previously (10, 33) on formalin-fixed, paraffin-embedded tissues using an avidin-biotin horseradish peroxidase technique (Vectastain Elite ABC kit; Vector Laboratories) and a mouse monoclonal anti-human CD4 primary antibody (NCL-CD4-1F6; Novocastra, Newcastle, United Kingdom). Sections were visualized with 3,3'-diamidino-benzidine (Dako Corporation, Carpinteria, CA) and counterstained with hematoxylin.

Dynamics of inflammatory cytokines and chemokines. Cytokine testing in plasma was done using the Cytokine Monkey Magnetic 28-Plex Panel (Invitrogen, Camarillo, CA), as per the manufacturer's instruction. Results were read by a Bio-Plex reader (Bio-Rad Laboratories, Hercules, CA), using Luminex technology (Luminex Corporation, Austin, TX).

Assessment of microbial translocation. The levels of microbial translocation were assessed as follows: plasma LPS levels were measured with the Limulus Amebocyte lysate assay (Lonza, Walkersville, MD) (10). Soluble CD14 (sCD14) levels in plasma were measured by ELISA (Quantikine Human sCD14 immunoassay; R&D Systems, Minneapolis, MN).

Trim5 α genotyping. Total genomic DNA was isolated from PTM PBMCs using the DNeasy blood and tissue kit (Qiagen, Valencia CA), and PCR was used for TRIM5 PCR genotyping (26).

Stock testing. The plasma viral stock collected from the rapid progressor PTM BH66 was tested by Bactec for Gram-positive and Gram-negative bacteria and for mycobacteria. Serological and PCR testing of the stock was done for the following viral pathogens: macaque cytomegalovi-

rus, simian cytomegalovirus, rhesus rhadinovirus, simian agent 8 (SA8), herpesvirus papio 1, simian parvovirus, RM parvovirus, and PTM parvovirus (Zoologix, Chatsworth, CA).

SGA of SIVsab strains from PTMs. The viral diversity of the two stocks and the strain selection in PTMs from both the cross-species-transmitted virus group and the serially passaged group were characterized by single-genome amplification (SGA), sequencing, and phylogenetic analyses (32, 37–39). All animals were sampled during acute infection, and PTM BH66 was also sampled at necropsy as challenge stock for passage infected animals.

In vitro replication studies. The two plasma stocks employed to infect the P1 and P2 PTMs were assessed for *in vitro* replication, as described previously (40). Briefly, PBMCs collected from four uninfected PTMs were depleted of CD8⁺ cells by using a positive selection of CD8⁺ T cells (CD8 microbead kit; Miltenyi Biotech, Auburn, CA). CD8⁺-depleted cells were then stimulated with 10 µg/ml phytohemagglutinin (PHA) for 2 days, followed by overnight incubation in interleukin-2 (IL-2) medium. Five hundred thousand CD8⁺-depleted cells were incubated with one ml of plasma adjusted to 10⁶ vRNA copies collected from either an acutely infected AGM (EI45) (i.e., the viral stock used for the P1 monkeys) or from the terminally infected, rapid progressor PTM BH66 (i.e., the viral stock used for the P2 monkeys). Cells were incubated with plasma for 4 h, followed by two washes to remove cell-free virus and addition of fresh medium. For 4 weeks, one-half of the supernatant was collected every third day and replaced with fresh IL-2-containing medium. Virus production in culture supernatants was monitored by SIV P27 antigen capture assay, as previously described (40).

Induction of cytokine production *in vitro*. The two plasma stocks used for the infection of the P1 and P2 PTMs were adjusted to 10⁶ vRNA copies and used to stimulate cryopreserved PBMCs collected from the PTMs included in this study prior to SIVsab infection. After a 4-h incubation, cells were washed twice to remove cell-free virus and then cultured for 24 h. Culture supernatants were harvested at 6 and 24 h poststimulation without replacement of the medium. Control cultures included the same *in vitro* conditions without virus stimulation. Cytokine and chemokine concentrations in the supernatants were measured using the Cytokine Monkey Magnetic 28-Plex panel (Life Technologies, Carlsbad, CA) according to the manufacturer's instructions. Mean fluorescence of samples was quantified and analyzed using the Luminex detection system.

Statistical analysis of data. Data comparisons were done using two-tailed nonparametric tests (Mann-Whitney). Where needed, the Gehan-Breslow-Wilcoxon test was performed. Significance was assessed at *P* levels of 0.05. The area under the curve of VL was done by numerical integration of a spline interpolation of the logarithm of the data, to avoid overemphasizing the peak of viral infection (Mathematica 6.0; Wolfram Research Inc., IL). Horizontal bars in figures reflect medians. Vertical bars in figures reflect standard errors of means. All statistics were performed using Prism 5.0 software.

Nucleotide sequence accession numbers. All 146 viral sequences from all animals were deposited in GenBank under accession numbers [KJ362078](#) to [KJ362223](#).

RESULTS

Clinical follow-up. To ascertain the factors associated with viral adaptation to a new host, we used two PTM groups. The cross-species-transmitted virus group received SIVsab92018 directly derived from an acutely infected Caribbean sabaes monkey. The derivation of this plasma virus stock is described elsewhere (31). The serially passaged virus group received terminal virus collected from a rapid progressor PTM (BH66) from the first group of infected animals. All SIVsab-inoculated PTMs became infected, as documented by plasma vRNA quantification. Infection outcome was highly variable in the cross-species-transmitted virus group: one PTM (BH66) experienced rapid disease progression and died

FIG 1 More-rapid and -consistent progression to AIDS in pigtailed macaques infected with a serially passaged virus than in those infected with a directly cross-species-transmitted virus. Survival was measured in days until AIDS-defining illnesses and death in 5 PTMs infected with an SIVsab directly derived from an African green monkey (black), compared to that in 7 PTMs infected with the virus collected from the rapid progressor from the first group (red). The *P* value was determined using the Gehan-Breslow-Wilcoxon test.

with AIDS (weight loss and diarrhea) within 180 dpi; two PTMs progressed to AIDS (weight loss, diarrhea, pneumonia, and lymphadenopathy) after 900 dpi (EC10) and 1,080 dpi (CF76); PTM CC35 was still alive at the end of the follow-up (1,250 dpi), and PTM BK42 died 186 dpi after an accidental surgical perforation of the intestine with no signs of viral pathogenesis (Fig. 1). Conversely, the PTMs from the serially passaged virus group experienced a significantly more rapid disease progression (*P* = 0.0441), with 3/7 rapid progressor PTMs developing AIDS within 300 dpi and the remaining 4/7 PTMs progressing to AIDS within 500 dpi (Fig. 1).

Natural history of SIVsab infection in PTMs upon cross-species transmission and serial passage. To assess SIVsab adaptation to the new PTM host, we closely monitored viral replication in plasma and PBMCs throughout infection, as well as in numerous intestine and LN samples collected at necropsy from selected animals (Fig. 2). Acute viral replication was similar for the two groups, with plasma VLs peaking at 9 to 10 dpi, at very high levels ($1 \times 10^9 \pm 2.27 \times 10^8$ vRNA copies/ml versus $1.16 \times 10^9 \pm 3.76 \times 10^8$ copies/ml, for the PTMs infected with the cross-species-transmitted virus and those infected with the serially passaged virus, respectively; *P* = 0.2043) (Fig. 2a). However, postpeak decline of plasma VL was drastically different for the two groups. In the cross-species-transmitted group, VLs showed a sharp decline in all but one (BH66) PTM. Conversely, in the serially passaged virus group, control of viral replication was very poor (Fig. 2a). Viral set points were reached in both groups by day 42 p.i., being low and highly variable in the PTMs from the first group (average, $1.44 \times 10^6 \pm 1.2 \times 10^6$ vRNA copies/ml) and homogeneously high in the animals infected with the PTM-passaged virus (average $4.2 \times 10^7 \pm 3.3 \times 10^7$ vRNA copies/ml) (Fig. 2a; *P* < 0.0001). In the cross-species-transmitted virus group, the rapid progressor PTM BH66 experienced very limited control of viral replication throughout infection (Fig. 2b). PTM EC10 showed a relatively well contained persistent chronic viral replication and no significant VL increase during progression to AIDS (Fig. 2b). PTMs CC35, BK42, and CF76 experienced a decline of viremia to undetectable levels by 100 to 150 dpi; yet, residual replication was documented in these PTMs, initially as VL blips and then as persistent replication (Fig. 2b), with PTM CF76 eventually progressing to AIDS.

FIG 2 Dynamics of viral replication in SIVsab-infected PTMs. Viral replication was assessed by quantifying SIVsab RNA loads in plasma. Shown are the geometric means of viral loads (a), individual viral loads (b), and SIVsab RNA loads in PBMCs (c). Animals infected with the SIVsab92018 strain directly derived from an acutely infected AGM are shown as black lines and symbols. Animals infected with SIVsabBH66, collected from an SIVsab92018-infected PTM (BH66) rapid progressor (passaged virus), are shown as red lines and symbols. In panel b, rapid progressors from both P1 and P2 groups are illustrated with green symbols. (d) Viral replication in different tissues was assessed in cells separated from necropsy samples collected from 4 animals. BH66 (P1) and ET30 (P2) progressed to AIDS within 1 year postinfection. EC10 (P1) progressed to AIDS after 2.5 years of follow-up. The remaining PTM, BK42 (P1), died from complications of surgery. Abbreviations: PW, peritoneal wash; BAL, bronchoalveolar lavage fluid; BM, bone marrow; ING, inguinal; AX, axillary; Mes, mesenteric; LN, lymph node; Jej, jejunum; Ile, ileum; IEL, intraepithelial lymphocytes; LPL, lamina propria lymphocytes. Plasma viral loads are expressed as SIVsab RNA copies/ml of plasma; the detection limit of the assay is 10^2 copies/ml. SIVsab RNA levels in tissues are expressed per 10^5 cells with a detection limit of 10 copies.

In contrast to this highly variable pattern of viral replication, a uniform pattern of high chronic viral replication (range, 9×10^5 to 3×10^7 vRNA copies/ml) was observed throughout the follow-up in the PTMs infected with the passaged SIVsabBH66 strain (Fig. 2b). The highest levels of chronic VLs were observed in the three rapid progressors (Fig. 2b). Thus, in both groups, the levels of chronic plasma VLs in SIVsab-infected PTMs were predictive of progression to AIDS, similar to HIV-infected humans and SIVmac/smm-infected macaques (41–45). SIVsab replication in PBMCs mirrored plasma VLs, with similar peak vRNA levels in both groups (Fig. 2c), followed by variable levels of chronic replication in the cross-species-transmitted group and a consistent high level of chronic replication in the serially passaged group (Fig. 2c). These results were also confirmed by the levels of viral replication measured at selected time points in the LNs and intestine (data not shown).

Mononuclear cells purified from multiple tissues collected

from selected PTMs at necropsy were used to assess the overall SIVsab viral burden (Fig. 2d). We found a good correlation between plasma VLs and vRNA loads in tissues, with the necropsy samples from the two rapid progressors (BH66–P1 and ET30–P2) exhibiting the highest VLs (Fig. 2d). Interestingly, tissue VLs from the normal progressor EC10 were in the same range as for controller BK42 (Fig. 2d), indicating ongoing viral replication even in the PTMs with undetectable plasma VLs (Fig. 2d). In the rapid progressor PTMs, the highest viral replication levels were found in the intestine, the major site of SIV replication and CD4⁺ T cell depletion (46–52), and in the LNs (Fig. 2d). High levels of virus replication were also observed in thymus, peritoneal washes, bronchoalveolar lavage fluids, and lung tissues (Fig. 2d).

High acute viral replication resulted in a massive pandepletion of major blood cell populations in PTMs, similar to that reported in acutely SIV-infected RMs (53). CD4⁺ T cell depletion (Fig. 3) exceeded that of any other cell type and lasted longer, as a result of

FIG 3 CD4⁺ T cell dynamics in blood and tissues of SIVsab-infected PTMs. (a) Significant acute depletion followed by a limited and transient restoration of circulating CD4⁺ T cells. (b) CD4⁺ T cell depletion in the lymph nodes. (c) Catastrophic acute CD4⁺ T cell depletion in the intestine of SIVsab-infected PTMs from both groups, followed by only modest restoration in animals infected with the cross-species-transmitted virus and virtually no restoration in PTMs infected with the passaged virus. Immunohistochemical assessment of CD4⁺ T cell depletion in the lymph nodes (d) and intestine (e). Massive depletion of CD4⁺ T cells can be observed at both sites at the chronic time points (42 and 60 dpi) compared to baseline levels (0 dpi). The CD4 staining pattern

direct targeting by the virus of this particular subset; at the end of acute SIVsab infection, CD4⁺ T cells were massively depleted: more than 70% in circulation (Fig. 3a), 50% in the LNs (Fig. 3b), and over 95% (range, 90 to 99%) at the mucosal sites (Fig. 3c). This catastrophic CD4⁺ T cell depletion was confirmed by IHC both in LNs (Fig. 3d) and in intestine (Fig. 3e). Interestingly, in spite of the catastrophic depletion of CD4⁺ T cells, set-point levels of viral replication were very high, which is consistent with studies of experimental CD4 depletion in rhesus macaques, in which very low levels of CD4⁺ T cells were associated with higher levels of VL and rapid disease progression (54, 55). It is thus possible that in the context of the CD4⁺ T cell depletion, virus production was supported by alternative cell targets, as reported by previous studies (55, 56).

During the chronic infection, a transient partial restoration of CD4⁺ T cells occurred in PTMs and controlled viral replication (in BK42, CC35, and CF76), which was followed by a second progressive CD4⁺ T cell depletion when viral replication became consistently detectable. Restoration was minimal in the PTMs with persistent viral replication, while in the rapid progressor PTMs, no CD4⁺ T cell restoration was observed, and depletion was further consolidated during the follow-up (Fig. 3). As such, our data confirm our previous reports that chronic CD4⁺ T cell recovery rather than the degree of acute mucosal CD4⁺ T cell depletion is predictive of SIV disease progression (10, 19).

At mucosal sites, the catastrophic depletion of CD4⁺ T cells prevented any conclusion with regard to the preferential targeting of a particular CD4⁺ T cell subset by the virus. Conversely, memory CD4⁺ T cells expressing CCR5 were preferentially wiped out from circulation in SIVagm-infected PTMs within a few weeks p.i. (i.e., 14 to 21 dpi) and only minimally recovered during the follow-up in those PTMs that controlled viral replication (data not shown), supporting the *in vivo* usage of the CCR5 coreceptor by SIVsab in PTMs. This observation is in agreement with our previous results showing that, in spite of being dually tropic (CCR5 and CXCR4) *in vitro* (57), SIVsab targets only CCR5-expressing cells *in vivo* (10, 19). We therefore concluded that the different patterns of infection between the two PTM groups were not due to expansion of coreceptor usage by the serially passaged SIVsab.

Thus, the study of the natural history of SIVsab infection in PTMs identified major clinical and biological differences between infection with the virus directly cross-species transmitted from the sabaeus monkey and infection with the virus serially passaged in PTMs. These different outcomes could be due either to major differences in susceptibility to SIVsab between the PTMs included in the two groups or, more likely, to SIV adaptation in the first group as a necessary step to induce persistent progressive infection in the PTM host. We therefore next focused on the identification of the factors associated with the observed increase in pathogenicity.

was different between LNs collected at the baseline (d), in which CD4⁺ cells were small, round cells, and those collected after the peak of viral replication, in which the stained cells were larger, sometimes multinucleated cells (most probably macrophages). Animals infected with the SIVsab92018 strain directly derived from an acutely infected AGM are shown as black lines and symbols. Animals infected with SIVsabBH66, collected from an SIVsab92018-infected rapid progressor PTM (BH66) (passaged virus) are shown as red lines and symbols. The graphs show the average levels of immune cells $\pm$ standard errors of the means.

Assessment of Trim5 α diversity and its potential impact on SIVsab replication in PTMs. Host restriction factors are dominant-acting blocks to retroviral infection (27) that have a major influence on retrovirus distribution, cross-species transmission, and emergence of new viral pathogens. Of these, the TRIM5 gene, which encodes TRIM5 α , a protein that blocks target cell infection by retroviruses, was reported to be the main factor behind the reported high susceptibility of PTMs to cross-species-transmitted infections (58). The high susceptibility of PTMs to cross-species-transmitted viruses was related to the expression of a TRIMCyp fusion protein by PTMs (27), for which two isoforms (TRIM5 η or TRIM5 θ) were described (59). While both are considered incapable of restricting either HIV-1 or SIV infection (59), we reasoned that TRIMCyp isoforms may have a discrete impact upon SIV cross-species transmission, similar to the role described for TRIM5 genotypes on viral replication levels in RMs (26). TRIM genes from all of the PTMs were sequenced and analyzed; no difference that might have impacted the different levels of SIVsab replication in PTMs was observed (data not shown).

No correlation between preinfection and postinfection levels of microbial translocation and pathogenicity of SIVsab infection in PTMs. It was also reported that a fraction of SIV-uninfected PTMs have intrinsic damage to the gastrointestinal tract, resulting in increased microbial translocation and higher levels of immune activation compared to SIV-uninfected RMs (60). Immune activation being the driving factor of SIV disease progression and death (61), we reasoned that differences in the intrinsic integrity of the gastrointestinal mucosa may explain the different pathogenic outcomes. We therefore assessed preinfection microbial translocation levels and performed a prospective comparative assessment of microbial translocation at critical time points of SIV infection between PTMs included in the two groups and between rapid progressor, normal progressor, and long-term progressor PTMs (Fig. 4). No significant difference in the baseline levels of lipopolysaccharide (LPS), endotoxin core antibody (endocab), and sCD14 between PTMs in the two groups (Fig. 4a) that may have predicted the outcome of SIVsab infection was observed. Furthermore, no significant differences in MT were observed between the two groups in the initial stages of infection (Fig. 4b and c). Such differences became discernible only with disease progression, when both the levels of LPS (Fig. 4b) and the levels of sCD14 (Fig. 4c) showed a more prominent increase in the animals progressing to AIDS.

T cell activation and proliferation correlated with pathogenicity of SIVsab infection during serial passage in PTMs. Massive, nonspecific T cell immune activation is a key determinant of disease progression in pathogenic SIV/HIV infections. Immune activation is a multifactorial process driven by different factors at different stages of infection: mainly by virus replication during acute infection and preponderantly by microbial translocation during the chronic infection (62). We therefore assessed the levels of immune activation in SIVsab-infected PTMs and their potential impact on the virus's ability to induce persistent progressive infection in the new host upon cross-species transmission, by measuring HLA-DR and CD69 expression on CD4⁺ and CD8⁺ T cells. As shown in Fig. 5a and b, significant increases in HLA-DR expression on both CD4⁺ and CD8⁺ T cells were observed in all PTMs at the peak of viral replication, with a 4-fold-higher increase in HLA-DR expression in PTMs infected with the passaged virus than in those that were infected with the cross-transmitted virus

FIG 4 Assessment of microbial translocation in pigtailed macaques infected with SIVsab. (a) The baseline levels of microbial translocation (LPS, endocab, and sCD14) of the PTMs used for the two virus passages and those of the controllers, normal progressors, and rapid progressors were similar. Shown are PTMs infected with the unpassaged (P1) virus (illustrated as black and white circles) and with the passaged (P2) virus (illustrated as red circles). RP, rapid progressors; NP, normal progressors; C, controllers. (b and c) Changes in the levels of LPS (b) and sCD14 (c) between PTMs infected with the unpassaged (P1) virus and those infected with the passaged (P2) virus. Shown are the average fold increases from the baseline $\pm$ standard errors of the means. SP, set point; EC, early chronic infection; LC, late chronic infection.

($P = 0.0012$ and $P = 0.0008$, for CD4⁺ and CD8⁺ T cells, respectively) (Fig. 5a and b). Following the VL peak, HLA-DR expression on CD4⁺ and CD8⁺ T cells remained significantly higher ($P = 0.0009$ and $P = 0.0008$, respectively) in monkeys infected with the passaged virus than in PTMs infected with the cross-transmitted virus, in which HLA-DR expression returned to near-baseline levels during early chronic infection (Fig. 5a). Increased chronic HLA-DR expression on T cells was associated with disease progression in both groups (Fig. 5a and b). A similar pattern of more-prominent increases in the levels of immune activation in

FIG 5 Kinetic expression of immune activation and T cell proliferation in SIVsab-infected PTMs. (a and b) Dynamics of CD4⁺ and CD8⁺ T cell immune activation (as defined by changes in the expression of DR markers) in blood. (c and d) Dynamics of CD4⁺ and CD8⁺ T cell proliferation (as defined by changes in the expression of Ki-67) in blood. Animals infected with the SIVsab92018 strain directly derived from an acutely infected AGM are shown

animals infected with the passed virus was observed after assessing immune activation by measuring CD69 expression on T cells (data not shown).

Conversely, the levels of acute CD4⁺ and CD8⁺ T cell proliferation (assessed using Ki-67) did not differ significantly between the two groups (Fig. 5c and d). During chronic SIVsab infection, the increases in Ki-67 expression on CD4⁺ and CD8⁺ T cells were significantly higher ($P = 0.0024$ and $P = 0.0018$ for CD4⁺ and CD8⁺ T cells, respectively) in PTMs infected with the passed virus (Fig. 5c and d), the greatest increases being observed in the animals that progressed to AIDS in both groups.

These differences in the levels of acute immune activation and proliferation between the cross-species-transmitted virus group and the passed virus group occurred in the context of similar levels of acute virus replication in both groups.

Immune activation and inflammation correlated with the pathogenicity of SIVsab during its serial passage in PTMs. We next confirmed the higher level of acute immune activation and inflammation in PTMs receiving the passed virus by assessing cytokine/chemokine production in the two groups. Proinflammatory cytokines peaked at significantly higher levels in PTMs infected with the passed virus than in those infected with the cross-species-transmitted virus: IL-1 β (Fig. 6a), IL-2 (Fig. 6b), IL-4 (Fig. 6c), IL-5 (not shown), IL-6 (Fig. 6d), IL-12 (Fig. 6e), IL-15 (data not shown), and tumor necrosis factor alpha (TNF- α) (Fig. 6f) all increased to generate a “cytokine storm” involving Th-1, Th-2, and Treg pathways, as well as numerous other immune cell subsets, including NK cells, B cells, monocytes/macrophages, and dendritic cells. This cytokine storm occurred in the context of comparable levels of acute viral replication between the two PTM groups and indicated a significant association between nonspecific T cell immune activation and increased pathogenicity of serially passed SIV in new hosts.

Lack of detectable adventitious agents in the viral stock used for serial passage infection. Since the virus stock used in the serial passed infection consisted of terminal plasma collected at the AIDS stage from a rapid progressor PTM and infection with this virus produced more-significant immune activation and inflammation, we reasoned that the cause of the observed difference could be that the virus stock contained adventitious opportunistic pathogens responsible for the cytokine storm and the increased pathogenicity. Plasma stock testing for adventitious bacterial and viral agents did not document any detectable pathogen. We therefore concluded that the increase in pathogenicity is intrinsic to the virus and not due to adventitious agents present in the viral stock.

Comparative assessment of the *in vitro* pathogenicity of the two SIVsab stocks used for *in vivo* infection of PTMs identified a higher intrinsic pathogenicity of the serially passed virus. To identify the factors associated with the observed differences in pathogenicity between the two groups, we adjusted the two plasma viral stocks to 10^6 vRNA copies/ml and used 1 ml of each to infect CD4-enriched PBMCs from uninfected PTMs (40). PBMCs originated from 4 PTMs, and both viruses were passaged in PBMCs from the same animals. *In vitro* viral replication was quantified by

as black lines and symbols. Animals infected with SIVsabBH66 collected from an SIVsab92018-infected rapid progressor PTM (BH66) (passed virus) are shown as red lines and symbols. Shown are the average fold increases from the baseline $\pm$ standard errors of the means.

FIG 6 Cytokine storm during acute infection of PTMs infected with serially passed SIVsabBH66. Significantly higher increases in proinflammatory cytokines in PTMs infected with the serially passed SIVsabBH66 (red lines and dots) versus PTMs infected with the AGM-derived SIVsab92018 strain (black lines and dots): IL-1 β (a); IL-2 (b); IL-4 (c); IL-6 (d); IL-12 (e); and TNF- α (f). Shown are the average fold increases from the baseline $\pm$ standard errors of the means.

measuring the P27 Ag (Fig. 7a) and vRNA (data not shown) in the supernatants. The two SIVsab stocks replicated at similar levels in the CD4⁺-enriched PTM PBMCs (Fig. 7a).

We next assessed the ability of each virus inoculum to induce immune activation and inflammation by incubating PBMCs collected prior to infection from the PTMs included in this study with plasma stocks adjusted to 10⁶ vRNA copies. We then measured cytokine production in the supernatants at 6 and 24 h postincubation. This experiment showed that cytokine and chemokine production was significantly higher in cell cultures infected with the passed virus (Fig. 7b), thus confirming the increased virulence of this viral strain.

Relationship between strain selection and pathogenicity of serially passed SIVsab infection. Single-genome amplification and direct sequencing were used to determine the genetic relationship between the original SIVsab92018 and infected animals challenged with this virus (cross-species transmission), as well as the passed SIVsabBH66 strain obtained at necropsy and subsequently used as passed virus to infect additional animals. For the cross-species transmission group, we found consistent genetic overlap between the infected animals and the inoculum, suggesting productive infection with many variants generally representing the overall diversity of the original SIVsab92018 stock (Fig. 8). Within the *env* gene, the maximum nucleotide genetic diversity of the stock was 1.9%, which was completely retained in infected animals, which contained a maximum *env* diversity of 2.3%. Importantly, however, the virus from PTM BH66 accumulated significant changes during the course of infection. At necropsy, the

majority of PTM BH66 virus was genetically diverse but significantly different from the virus collected during acute infection from the same PTM (Fig. 8). The maximum overall genetic diversity of PTM BH66 at necropsy/acutely infected PTMs from P2 was 1.5%. Animals infected with virus from PTM BH66 showed genetic overlap between the inoculum and within the group with a maximum overall diversity of 1.9%, which is consistent with the transmission of multiple variants representing the overall diversity of PTM BH66. These changes are a surrogate for the accumulated genetic mutations occurring over the entire genome, and the exact changes necessary and sufficient for increased pathogenesis cannot be determined by *env* sequences alone. However, single viral passage through PTM BH66 clearly affected both the genetic diversity and the pathogenicity of the original SIVsab92018 inoculum.

All sequences were examined for G-to-A mutations, and no sequences were identified with overt mutations, which would have suggested an apolipoprotein B-editing catalytic polypeptide-like subunit (APOBEC) restriction.

DISCUSSION

We explored the factors associated with SIV adaptation to a new host upon cross-species transmission. This is an important area of AIDS research, because HIV-1 and HIV-2 were generated by multiple SIV cross-species transmissions to humans (8, 63). The assessment of the real risks for the emergence of new HIV lineages into the human population is a priority. We report that viral adaptation to a new host through selection of strains that are able to

FIG 7 *In vitro* assessment of the intrinsic virulence of the plasma SIVsab stocks used to infect PTMs. (a) *In vitro* replication of the SIVsab92018 (P1) and SIVsabBH66 (P2) plasma stocks on CD4⁺ enriched PBMCs from four uninfected PTMs, as assessed by the measurement of p27 production in the supernatants. Shown are the average fold increases from the baseline $\pm$ standard errors of the means. (b) Comparative assessment of induction of cytokine/chemokine production *in vitro* in PBMCs collected prior to infection from the PTMs challenged with SIVsab92018 (P1) and SIVsabBH66 (P2). The levels of cytokines/chemokines in supernatants harvested at 6 and 24 h after incubation with the two viral stocks are shown as gray (P1) or black columns (P2). Controls (cells incubated under the same conditions in the absence of virus exposure) are shown as white columns. Differences between multiple experiments are illustrated as standard errors of the means. Statistical differences ($P < 0.005$) between P1 and P2 are illustrated as asterisks (*).

ensure persistent infection in the new host is paramount for a successful cross-species transmission event.

In recent years, a large body of data has been generated, focusing on host restriction factors, host cell proteins that inhibit lentivirus replication in a species-specific manner and

prevent SIV transmission to humans and to various other species (64, 65). Identification of these host restriction factors (acting mainly through the interferon system) permitted better assessment of the risks for SIV cross-species transmission to humans. Targeting these host restriction factors will likely gen-

FIG 8 Phylogenetic tree of initial SIVsab infection of pigtail macaques and subsequent serial passage. The neighbor-joining phylogenetic tree consists of the original SIV sab92018 (blue) with initial infection of pigtail macaques (green). Genetic analysis of SIVsabBH66 at necropsy (red) revealed significant accumulation of changes and subsequent passage of these strains to new recipients (orange).

erate therapeutic approaches to limit HIV transmission. Meanwhile, engineering HIV-1 to overcome certain restriction factors may enable the construction of simian-tropic HIV (st-HIV) strains for use in macaques for testing new vaccines and therapeutic approaches. Yet, in spite of intensive research, such st-HIVs are not yet available, and those in development induce persistent infection only after depletion of cellular and humoral immune responses (66).

Therefore, we have focused on the viral and immune factors associated with successful cross-species transmission to understand whether or not further requirements in addition to overcoming host restriction factors are necessary for virus adaptation to a new host. Our approach of serially passing the cross-species transmitted virus in the new PTM host was previously employed by others with simian-human immunodeficiency viruses (SHIVs) and SIV (23, 67). We employed SIVsab, the virus that naturally infects the *sabaeus* monkey, an African green monkey species indigenous to West Africa and the Caribbean Islands (31). Unlike previous approaches in which the cross-species-transmitted virus was isolated *in vitro* prior to experimental infection, we did not use an *in vitro*-produced stock, to avoid *in vitro* strain selection or alterations of the viral stock induced by passage in macaque PBMCs or human T cell lines (31, 57). Instead, we used a plasma stock collected from an acutely infected *sabaeus* monkey, in which we previously documented a significant viral diversity and could infer transmitted-founder strains (32, 37).

We have chosen PTMs over RMs because they are more permissive to cross-species-transmitted viruses than RMs (23, 25) and have already been used for the adaptation of SIVver, a strain isolated from another AGM species (23). The high permissivity of PTMs to cross-species-transmitted SIVs is due to expression of a TRIMCyp protein that is ineffective in restricting HIV/SIVs (27); as such, a potent block to HIV-1 replication in RMs is not present in PTMs.

SIVsab infection was pathogenic to PTMs upon direct cross-species transmission, with >50% of the PTMs progressing to AIDS during a 3.5-year follow-up. The remaining PTMs controlled SIVsab during the follow-up. A previous study reported that in SIVsmm-infected RMs the differences in viral replication and disease progression can be attributed to Trim5 α genotypes (26). Therefore, as PTMs were reported to carry two Trim isoforms, we first characterized the Trim genotypes in PTMs. All PTMs in both study groups harbored the same permissive Trim genotype, and thus, the differences in the rates of disease progression were not due to Trim genotypes.

PTM BH66 in the cross-species-transmitted group was a rapid progressor showing virtually no virus control and presenting with AIDS within 6 months of infection. Terminal plasma from this PTM was used to infect a second group of PTMs. All the animals in this group progressed to AIDS in less than 2 years. Set-point VLs were consistently high in this group, and the natural history of SIVsabBH66 was highly consistent between the infected animals, in agreement with previous studies showing that SIVver, which naturally infects the *vervet* monkey, is pathogenic in only a fraction of PTMs upon direct cross-species transmission and consistently pathogenic after serial passage (23, 24).

To understand the reasons for the observed differences in pathogenicity between the PTMs infected with the cross-species-transmitted virus and those infected with the passaged SIVsabBH66 strain, we thoroughly compared the viral and immune

parameters between the two groups and showed that SIVsabBH66 infection induced a higher level of acute immune activation and a cytokine storm in the serially passaged group. We reasoned that these are critical factors for SIVsab adaptation to the new PTM host, because increased acute immune activation/inflammation will result in an increased availability of SIV target cells (which have an activated phenotype) and thus favors persistent high viral replication. Therefore, we focused on understanding the reason(s) for which passaged virus infection was associated with increased levels of immune activation/inflammation.

The differences in acute immune activation/inflammation between the two passages were not due to differences in the levels of acute viral replication, which were similar for the two groups.

As PTMs in the second group were infected with a plasma virus collected at the time of AIDS, we further hypothesized that the presence of adventitious agents in this stock may be responsible for the observed differences in levels of immune activation/inflammation between the two groups. Consequently, we tested the plasma stock for multiple bacterial and viral opportunistic agents associated with immunosuppression and AIDS. The stock did not test positive for any of these opportunistic agents.

We next assessed the *in vitro* characteristics of the two viral stocks and showed that, in spite of similar ability to replicate *in vitro* on CD4-enriched PBMCs, the PTM-derived SIVsabBH66 stock induced significantly higher levels of cytokines in cell cultures than did the AGM-derived SIVsab92018 stock. This observation suggests that the increased pathogenicity of infection in the second passage can be directly related to the virus stock. These two sets of experiments were performed under different conditions because, while *in vitro* viral replication is strictly dependent on CD4⁺ T cells, the production of cytokines and chemokines that might shape the outcome of SIV infection involves multiple cellular subsets. One may argue that the observed differences in cytokine/chemokine production between the two stocks may not necessarily rely on intrinsic differences in virus virulence, as the two plasma stocks were collected from monkeys at different stages of SIV infection (acute infection for P1 versus terminal infection for P2). As such, the P2 stock would contain both infectious and soluble factors that could have been responsible for the more aggressive outcome of the *in vitro* stimulation assays. Yet, as discussed above, we could not detect major viral or bacterial contaminants in the stock. Furthermore, while the AIDS stage is associated with dramatic increases in soluble factors, the massive viral replication characteristic of acute SIV infection is also associated with dramatic increases in cytokine/chemokine production (66, 68, 69). Finally, we did not employ large stock volumes that would have induced changes in the soluble markers, and the volumes of plasma used for *in vivo* infections of PTMs were similar for P1 and P2.

We next tested the hypothesis that strain selection associated with the virus passage was responsible for the different outcomes of infection. To this goal, we performed single-genome amplification and sequence analysis of the transmitted/founder viruses in the two groups. We also characterized the viral diversity of the two stocks. This analysis showed that the total diversity of the original stock was retained but was shifted after passage in PTM BH66, and as such, our analysis suggests that adaptation of SIVsab to the new PTM host was associated with, and probably necessary for, increased pathogenicity upon SIV cross-species transmission. Note, however, that sequence analyses were limited to the *env* gene,

which did not permit us to identify the genetic signature(s) of increased pathogenicity, a process which would require dedicated studies to identify all mutations throughout the genome, generate clones of these various combinations, and test each combination *in vitro* and *in vivo* to show which mutations were important for increased pathogenesis. Such experiments were beyond the scope of the current study. In the past, viral determinants of increased pathogenicity, occurring mainly as point mutations, were reported but not always confirmed as being clearly associated with induced pathogenicity (70). Therefore, the viral mechanisms of increased pathogenicity of SIVs in new hosts are still unclear.

Our study demonstrates that the ability of the virus to stimulate the innate immune system is probably the main determinant of increased pathogenicity and virus adaptation to a new host. Identification of the specific motifs/genes associated with increased pathogenicity may contribute to the design of new therapeutic approaches to control HIV.

We conclude that a stringent selection of virus variants inducing high levels of immune activation/inflammation and increasing the availability of target cells in the initial stages of infection are conditions for successful viral adaptation to a new host. One may argue that such conditions are achieved only in experimental conditions and that serial passage is not characteristic of viral adaptation. Yet, similar conditions may be achieved when cross-species SIV transmissions occur in association with concurrent infections that contribute to a coincidental inflammatory response. Furthermore, transmission to humans may involve serial passage through injections with unsterilized material (15). Our study shows that irrespective of the circumstances of successful SIV cross-species transmission, virus adaptation is a prerequisite. These results explain the scarcity of documented cross-species transmissions of SIVs to humans in the context of extensive exposure of humans to a plethora of SIVs in Central Africa (16).

In addition to the characterization of SIV adaptation to a new host, we report here the development of a new animal model for progressive SIV infection. SIVsabBH66 infection in PTMs is very similar to SIVmac infection in both RMs and PTMs (70–72), the reference NHP models for AIDS vaccine and pathogenesis studies. Furthermore, as per previous studies, SIVsab-infected PTM is the model of choice for the study of SIV-associated comorbidities (i.e., cardiovascular disease), due to the similarities of SIVsab infection in PTMs to HIV-1 infection in humans (36).

Finally, this new model completes a new animal system for performing comparative studies between pathogenic, nonpathogenic, and controlled SIV infections. In the only other available system, comparison between the nonpathogenic SIVsmm infection of sooty mangabeys and the pathogenic SIVsmm/mac infection of RMs (73, 74) is considered a major advantage, as it permits meaningful comparative studies to identify the host factors associated with progression to AIDS (75). Our new model also enables similar comparisons between the pathogenic infection of PTMs and natural host (AGM) infection by employing a single SIVsab strain (36), with several major advantages over the conventional RM/sooty mangabey/SIVmac/smm system: AGMs are not endangered and can thus accommodate invasive studies, which are prohibited in sooty mangabeys. Furthermore, since SIVsab infection of RMs is functionally cured (19), this system expands to comparative studies between pathogenic, nonpathogenic, and elite-controlled infections (10, 35), to gain insight into the mechanisms of immunodeficiency associated with lentiviral infection. As such,

this new system is unique and can be used to assess essential aspects of AIDS pathogenesis; among these are identifying the discrete immunopathogenic mechanisms of SIV infection and comparing the genetic evolution of SIV in different host species (76), both of which are needed to develop effective strategies for prevention of AIDS disease progression.

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