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Brief report

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Abstract

Simian foamy viruses (SFVs) are complex retroviruses widespread throughout nonhuman primates. SFVs can also be transmitted to humans, mostly through bites. We previously observed that the primary zoonotic gorilla SFV strains much more slowly than laboratory-adapted chimpanzee strains. Here, we tested the hypothesis that SFV growth is limited by interferon (IFN)-induced restriction factors using inhibitors of cellular signaling pathways involved in type I IFN induction or action. Inhibitors of JAK1/2 (Ruxolitinib) and TBK-1 (BMX795) led to a 2 to > 20-fold higher percentage of infected BHK-1 and HT1080 cells. However, replication of the laboratory-adapted prototype foamy virus was not sensitive to these molecules, and IKK2 inhibitors had no effect on any of the SFV strains. In conclusion, the addition of small molecules that inhibit type I IFN response to the culture medium can be used as a simple and efficient method to enhance the replication of zoonotic gorilla SFVs.

Keywords

foamy virus; spumaretroviruses; zoonosis; immune response; interferon; virus replication

Introduction

Simian foamy viruses (SFVs) are complex retroviruses widespread throughout nonhuman primates (NHPs) (reviewed in [1]). SFVs can also be transmitted to humans through bites, establishing a persistent infection [1-5]. To date, neither pathogenic effects nor secondary transmission between humans have been reported [5, 6]. Replication-competent SFVs have been isolated from blood cells years or even decades after infection [7-9] and cell-associated SFV DNA has been detected in human blood and buccal samples [2, 8, 10-14]. An understanding of the persistence of SFV in humans and its consequences requires *in vitro* experiments performed with primary strains. Indeed, strains may differ in cell tropism and susceptibility to inhibition by innate and adaptive immune effector molecules and cells [7, 9, 15, 16]. However, repeated cycles of *in vitro* production may lead to the selection of the fittest viral variants in specific cell-culture conditions and/or the expansion of strains that would have been efficiently repressed *in vivo* by immune responses. Thus, tropism and susceptibility to IFNs or antibodies frequently differ between laboratory-adapted and primary strains of retroviruses [17-20].

Central African villagers from East and South Cameroon and North Gabon are at high risk for SFV acquisition and approximately 75% of infected individuals carry strains from gorillas [2, 6, 10, 21]. The only gorilla SFV isolated directly from an animal has limited *in vitro* replication capacity [22]. We previously isolated two zoonotic gorilla SFV strains and demonstrated their high genetic similarity with their NHP counterparts [9, 23]. The sequences of these primary viral strains are identical to those of gene fragments amplified from human PBMC-associated viral DNA [9]. Next, we performed *in vitro* infections, using viral stocks produced after inoculation with original or first passage cell lysates which are referred to as primary strains [15, 16, 24]. We observed that primary

gorilla SFV grow more slowly than laboratory-adapted chimpanzee SFV on hamster kidney (BHK-21) cells, which are used for their isolation and production [24]. Furthermore, primary gorilla SFV stocks collected from cell cultures displaying an extensive cytopathic effect have 100-fold lower cell-free titers on the GFAB indicator cells in which their autologous LTR directs the expression of a reporter molecule [24].

Among host factors that affect replication efficacy, the induction and action of IFN are of outmost importance [25, 26]. SFVs are sensitive to the action of type I IFNs [15, 27, 28]. Tripartite motif protein 5α, APOBEC3 cytidine deaminase, N-myc interactor, IFN-induced protein 35, and tetherin all inhibit SFV replication [3, 29-31]. Systematic screening of IFN-stimulated genes (ISGs) has identified additional SFV inhibitors [32]. In plasmacytoid dendritic cells, SFVs trigger type I IFN production after sensing through TLR7 [15]. Cytosolic pattern-recognition receptors recognize viral nucleic acids and initiate a molecular cascade, resulting in IRF3, IRF7 and/or NF-κB translocation to the nucleus and the triggering of IFN gene and ISG transcription [25]. Cytoplasmic sensing of SFV occurs in monocytic cells [33]. However, SFV sensing has not yet been demonstrated in nonhematopoietic cells [34, 35]. Secreted IFN binds to its cellular receptor and initiates a signaling cascade through the Janus kinase signal transducer and activator of transcription (JAK-STAT) pathway [25, 26].

The titers of primary gorilla SFV stocks produced on BHK-21 cells are usually low and sometimes insufficient to carry out *in vitro* infections. Therefore, we tested three strategies to enhance the level and/or speed of primary gorilla SFV replication: the search for sensitive human cell lines, improvement of viral entry, and blockade of the type I IFN response. The third approach

71 was successful, as small molecules that inhibit IFN signaling or the IFN response enhanced the
72 replication of zoonotic gorilla SFVs.
73

Results

Primary gorilla SFV strains replicate more slowly than laboratory-adapted chimpanzee SFV strains

BHK-21 and HT1080 cells were infected at the same moi with primary zoonotic gorilla SFV strains GI-D468 and GII-K74 and laboratory-adapted chimpanzee SFV strains CI-PFV and CII-SFV7 [9, 24, 36]. Infection was monitored by flow cytometry. GI-D468 and GII-K74 replicated at the same rate in both BHK-21 and HT1080 cells and massive cell lysis was observed at day 25 (Figures 1a and 1b). CI-PFV and CII-SFV-7 spread rapidly in BHK-21 cells reaching 90% of infected cells by day 14, before the destruction of the cell monolayer (Figure 1a). The growth kinetics of the two chimpanzee strains were even more rapid in HT-1080 cells, infecting approximately 80% of the cells by day 6 (Figure 1b).

We then tested the infection of several human hematopoietic cell lines. In preliminary experiments, gorilla SFV appeared to grow at a very slow rate: only 11% of K562 cells infected with undiluted gorilla SFV expressed Env after 41 days in culture. We therefore tested the susceptibility of four additional cell lines using the fast replicating CI-PFV. K562 cells were productively infected using a moi of 0.6, with an infection level of 75% at day 23, before cell lysis (Figure 1c). Raji, Jurkat, THP-1, and HEL 92.1.7 cells did not express SFV Env during a 41 day-long culture. We used a moi of 0.6 which is 10-fold higher that the maximum reachable with our primary SFV stocks. Unfortunately, concentration of gorilla SFV particles by *iodixanol*-gradient ultracentrifugation or centrifugal filters resulted in reduced infectivity and lower infectious titers on GFAB cells (data not shown). Therefore, we did not further investigate the susceptibility of various cell lines to gorilla SFV. We monitored the appearance of CPE in human fibroblasts (MRC5), U-87MG, murine *mus dunni*, and simian IFN-deficient VERO cells before constructing the GFAB indicator cells and receiving the anti-Env

96 monoclonal antibody. All showed similar or even lower susceptibility to gorilla SFV than BHK-21 cells
97 (Lambert, Gouzil and Buseyne, data not shown). In conclusion, human adherent HT1080 cells were
98 susceptible to infection with primary gorilla SFV strains which spread more slowly than the
99 chimpanzee SFV strains.

100 ## Procedures targeting the early steps of the replication cycle do not enhance infection
101 with gorilla SFV

102 When expressed at the cell surface, SFV Env-mediated fusion is inducible by short exposure
103 to acidic pH [37] and the fusion process is much slower for macaque SFV Env than CI-PFV Env [38].
104 We therefore tested whether changing the pH could enhance infection with viral particles. GFAB
105 cells were infected for 2 h, exposed to pH 7 or pH 5.5 for 1 or 15 min, and cultured for 72 h. Exposure
106 to acidic pH did not significantly increase the number of cells infected with any of the four SFV strains
107 (Figure 2a), whereas it increased cell fusion by PFV Env [37] and gorilla SFV Env (data not shown)
108 after transfection with plasmids expressing *env* gene.

109 Cationic polymers can increase the adsorption of virions on target cell membranes, leading to
110 the enhancement of infection of some retroviruses [39]. We infected GFAB cells with CI-PFV or GI-
111 D468 in the presence of polyethylenimine, polybrene, or DEAE dextran at concentrations ranging
112 from 0.5 to 8 µg/mL. Cationic polymers did not increase infection (Figure 2b).

113 ## IFN inhibitors enhance infection with gorilla SFVs

114 We addressed whether SFV replication is sensitive to type I IFN-mediated restriction by adding
115 small molecules that inhibit either kinases involved in IFN induction (TBK-1and IKK2) or the JAK-STAT
116 pathway [40-42]. Inhibitors of JAK1/2 (Ruxolitinib), TBK-1 (BX795), or IKK2 (TPCA-1 and BMS345541)
117 were added for 4 h before GFAB cell infection and during the 72 h-long culture. Ruxolitinib and

BX795 increased the number of GFAB cells infected with GII-BAK74 by a factor of two (Figure 3a). Neither molecule increased infection with CI-PFV. No additive effect was observed if both molecules were mixed. At the highest concentrations tested, we observed a cytotoxic effect that was more obvious for BX795.

Neither of two IKK2 inhibitors affected GFAB-cell infection with either CI-PFV or GII-K74 (Figure 3b). Thus, blocking IFN signaling through JAK1/2 or the TBK-1-dependent IFN response increased GFAB-cell infection with GII-K74, whereas it had no effect on infection with CI-PFV.

We further characterized the effect of ruxolitinib on several cycles of SFV replication in GFAB and HT1080 cells and followed Env expression by flow cytometry. GI-D468 and GII-K74 efficiently spread in GFAB and HT1080 cells treated with ruxolitinib (Figures 4a and 4b). At day 8 post-infection, 23% of the ruxolitinib-treated GFAB cells were infected with GI-D468 *versus* 0.6% of the untreated cells (Figure 4a). In addition, ruxolitinib increased GI-D468 infection of HT1080 cells (16.2% *vs.* 3.4% infected cells, Figure 4b). We observed a similar effect for GII-K74, although it was less efficient than for HT1080 cells (Figure 4a-b). Ruxolitinib did not affect the intensity of envelope protein staining. It also had no effect on infection of HT1080 cells with CI-PFV (Figure 4c), in accordance with the results on GFAB cells (Figure 3a). These data show that treatment with ruxolitinib significantly enhanced the number of cells infected with primary gorilla SFVs but not those infected with CI-PFV.

Discussion

Here, we sought to increase the *in vitro* replication of primary gorilla SFV strains and found that inhibition of the type I IFN response is an efficient way to achieve such a goal. Indeed, the supplementation of culture medium with JAK1/2 or TBK-1 inhibitors is a simple and broadly applicable method, efficient for a variety of viruses [41-43], including retroviruses [44]. While the growth of primary gorilla SFV was sensitive to inhibition of the IFN response, the growth of laboratory-adapted CI-PFV was not.

The blockade of IFN signaling by JAK1/2 inhibitors and the TBK-1-mediated IFN response increased gorilla SFV replication. In contrast, blocking IKK2, which is another component of the IFN response, had no effect on *in vitro* SFV growth. Our data indirectly suggest that gorilla SFV activates the IRF3 but not the classical NF-κB pathway. Indeed, CI-PFV induces IRF3-dependent ISGs in human monocytes [33]. However, its replication was not enhanced by TBK-1 inhibition in our hands. This discrepancy may be explained by differences in innate sensing between hematopoietic and nonhematopoietic cells, as CI-PFV is unable to induce IFN production in nonhematopoietic cells [34, 35]. Alternatively, the rapid and high level of PFV replication may saturate antiviral molecules targeted by the drugs we used.

We tested the susceptibility of several human cell lines to primary gorilla SFVs. No gorilla cell lines or samples are available to test the replication kinetics of gorilla SFV in their natural host cells. Here, we focused on human cells because SFV infection in humans is our major research theme and because more cell biology and immunology reagents are available for their study. We observed that CI-PFV replicates faster in HT1080 than in BHK-21 cells, as reported by others [45]. HT1080 cells were susceptible to both GI-D468 and GII-K74, but both gorilla SFVs had similar replication kinetics

158 in HT1080 and BHK-21 cells. Overall, we observed the slow/low replication kinetics of gorilla SFV in
159 both the hamster cells used for their isolation and human fibroblasts, described to have the highest
160 susceptibility to CI-PFV [45].

161 CI-PFV infects a wide range of adherent and nonadherent cells [46-48]. Here, we tested the
162 infection of five myeloid and lymphoid human cell lines with the fast-growing CI-PFV. We used a low
163 moi for our study, *i.e.* a screen of cells that could be infected at an moi achievable with primary
164 gorilla SFV stocks. Under these conditions, only K562 cells were susceptible to PFV infection. The
165 low moi is a likely explanation for the low cell susceptibility to CI-PFV in this study relative to that
166 reported in previous publications [47, 49, 50].

167 We tested several classical methods to enhance the early steps of SFV infection. The use of
168 polycations to reduce surface charge had no impact on gorilla SFV, as reported by others for PFV
169 [48, 51]. The process regulating SFV Env fusogenic activity and variation across various viral species
170 is not fully defined [37]. Although exposure to a pulse of acidic pH induced fusion of gorilla SFV Env
171 expressed at the cell surface, as described in [37] we observed no effect on SFV Env exposed at the
172 surface of viral particles. We hypothesized that gorilla SFV Env fusion is a slow process, as described
173 for macaque SFV Env [38], and exposed cells to low pH after incubation with the viral inoculum. This
174 timing might be suboptimal if most of the particles were already internalized and unaffected by the
175 change in the pH of the culture medium. Of note, we recently produced chimeric foamy virus vectors
176 expressing gorilla SFV Env and CI-PFV Gag and Pol, for which the titers were equal to or marginally
177 lower (< 5-fold) than those of vectors expressing CI-PFV Env [16]. Overall, our data argue against a
178 restriction of gorilla SFV replication at the Env-mediated early steps of the viral cycle.

We aimed to avoid the selection of fast-growing laboratory-adapted gorilla SFV strains for the realization of *in vitro* infections. We show here that the presence of ruxolitinib during the production of gorilla SFV stocks is a simple way to overcome the slow/low *in vitro* replication of these strains. At first glance, blocking restriction mechanisms to enhance SFV growth does not appear to fit our initial goal, because viral particles produced in ruxolitinib-treated cells may differ from those produced in untreated cells, with active innate sensing and an IFN response. It will thus be critical to consider the final experimental outcome before producing viral stocks with IFN blockade. In addition, we propose the use of ruxolitinib for a single round of virus amplification, starting from original material for each new round of production to limit the selection of fitter viral variants over serial passages.

Although we focused on the production of primary SFV stocks, our results may be relevant for the isolation of new replicating SFV strains. Our current procedure relies on the stimulation of human peripheral blood cells to induce viral replication and coculture with susceptible cells from a nonhuman species to avoid inhibition by human IFN-γ and other soluble mediators produced by activated PBMCs [9, 10, 52]. The use of small-molecule inhibitors of the type I IFN response may be useful for improving the isolation of new primary SFV, as demonstrated by the inhibition of IFN-γ [52].

In conclusion, we show that the addition of JAK1/2 or TBK-1 inhibitors to culture medium is a simple and efficient procedure to enhance the growth of slow/low primary gorilla SFV isolates. The same inhibitors did not enhance replication of the laboratory-adapted CI-PFV, showing the utility of primary SFV strains for the study of innate sensing. Our results should be useful for researchers in the field of foamy viruses.

Materials and Methods

Cells

K562 (ECACC 89121407, human erythroleukemia cells) and BHK-21 (ATCC-CLL-10, hamster kidney fibroblasts) cells were obtained from colleagues at the Institut Pasteur. BHK-21-derived gorilla foamy virus-activated β-galactosidase (GFAB) cells were generated in our laboratory [24]. Other cell lines were purchased from LGC standards or Sigma-Aldrich and stocks were produced after less than 10 passages. The cells were screened for mycoplasma infection every 10 passages with a bioluminescent assay (#LT07-418, Lonza). The HT1080 cells (ECACC 85111505, human fibrosarcoma) were cultivated in Eagle's Minimum Essential Medium with Earle's Balanced Salts and L-Glutamine (EMEM-EBSS, Lonza) supplemented with 10% fetal bovine serum (FBS, PAA Laboratories) and 1% nonessential amino acids (NEAA, Invitrogen). K562, HEL 92.1.7 (ATCC-TIB-180, human erythroleukemia cells), THP-1 (ECACC 88081201, human monocytic leukemia cells), Raji (ATCC-CCL-86, human B lymphoma cells), and Jurkat (ATCC-TIB-152, human T leukemia cells) were grown in RPMI medium containing GlutaMAX I (Invitrogen) supplemented with 10% FBS. BHK-21 and GFAB cells were cultivated in Dulbeco's modified Eagle's medium (DMEM-GlutaMAX I, Invitrogen) supplemented with 5% FBS. G418 (300 µg/ml, Sigma-Aldrich) was added to the GFAB cultures.

Viruses

SFV strains consisted of the primary zoonotic gorilla SFVs, SFVggo_huBAD468 (GI-D468) and SFVggo_huBAK74 (GII-K74) [9], and the laboratory-adapted chimpanzee SFVs, SFVpsc_huPFV (CI-PFV) and SFVpve_Pan2 (CII-SFV7) [33]. GI-D468 and GII-K74 viral stocks were produced by infecting BHK-21 cells with original or first passage cell lysates. CI-PFV and CII-SFV7 viral stocks were obtained

223 by a single round of infection with aliquots obtained from A. Saib and A. Rethwilm, respectively.
224 Infected cultures were passaged twice a week and uninfected cells added after the appearance of
225 the first syncytia to amplify the virus. Once the cytopathic effect (CPE) had destroyed > 70% of the
226 cell layer, infected cells and supernatants were treated by three cycles of freezing and thawing (-
227 80°C; +37°C) to enable the release of viral particles. The lysate was cleared by centrifugation (1500
228 x g for 10 min), filtered through a 0.45 µm pore-size filter, and stored as single-use aliquots at -80°C.
229 Both chimpanzee and gorilla SFV are efficiently detected by GFAB cells [24]. Virus titers were
230 determined by infecting GFAB cells at 30 to 40% confluence in flat-bottom 96-well plates with 30
231 µl/well of serially-diluted viral solutions prepared in DMEM. After a 2-h incubation, 170 µl DMEM-
232 5% FBS was added to each well. Tests were performed in triplicate. The cells were fixed after 72 h
233 with 0.5% glutaraldehyde in a phosphate-buffered saline solution (PBS) for 10 min at room
234 temperature (RT). Cells were washed with PBS and incubated 1 h at 37°C with an X-Gal staining
235 solution (2 mM $MgCl_2$; 10 mM Potassium ferricyanide, 10 mM Potassium ferrocyanide; and 0.5
236 mg/mL 5-Bromo-4-chloro-3-indolyl-B-D-galactopyranoside in PBS). An Ultimate UV Image analyzer
237 (CTL Europe, Bonn, Germany) was used to count X-Gal stained cells. One infectious unit was defined
238 as a blue cell or syncytia.

239 ## SFV infections

240 Adherent cells were infected at 30 to 40% confluence. On the day before the infection, BHK-
241 21 and GFAB cells were seeded at 5 10^3 cells/P96-well and HT1080 were seeded at 2.5 10^3 cells/P96
242 well. Adherent cells were infected at 30 to 40% confluence with 25µl of undiluted GI-D468 and GII-
243 K74 (5 10^3 infectious unit (IU)/ml). CI-PFV (2 10^6 IU/ml) and CII-SFV7 (3 10^5 IU/ml) stocks were
244 diluted to achieve the same moi as the gorilla SFV strains in experiments comparing the four strains.

The corresponding moi were 0.01 for BHK-21 and GFAB cells, 0.02 for HT1080. Certain CI-PFV infections were carried out at a 10-fold higher moi to reduce the length of the experiments. Non-adherent cells (10^6 cells) were centrifuged at 500 x g before the addition of the 500 µl of CI-PFV to the cell pellet. Infected cultures were passaged twice a week by dilution into fresh culture medium at the same cell density as for the propagation of uninfected cells and maintained up to the occurrence of a massive CPE. GFAB cells were infected in triplicate using the titration protocol and stained after 72 h. The corresponding means and standard deviations (SD) were calculated.

Exposure to acidic pH was performed 2 h post-infection by removing the medium, adding PBS at pH 7 or 5.5, and incubation for 1 or 15 min. The PBS was discarded and DMEM with 5% FBS added to the wells. Polyethyleneimine (jetPEI, #101-10, Polyplus), 1,5-dimethyl-1,5-diazaundecamethylene polymethobromide (Polybrene, # TR-1003, Sigma-Aldrich), and diethylaminoethyl dextran (DEAE Dextran, wt 500.000, #D9885 Sigma-Aldrich) were added to the viral inoculum immediately before the infection of GFAB cells. Inhibitors of JAK1/2 (Ruxolitinib, #S1378, Euromedex), TBK-1 (BX795, #S1274, Euromedex), and IKK2 (TPCA-1, #T1452 and BMS345541, #B9935, Sigma-Aldrich) were added to the culture medium after a 2-h incubation with the viral inoculum.

Flow cytometry

A murine monoclonal antibody specific for the leader peptide of the SFV envelope (clone P6G11G11 generated by M.L. Linial) was conjugated to Alexa Fluor 647 dye (anti-Env-AF647). Antibody production, coupling, and purification were performed by RD Biotech, Besançon, France. Cells were fixed with 2% paraformaldehyde (PFA) diluted in PBS for 10 min at RT, washed in PBS supplemented with 0.1% bovine serum albumin (BSA), permeabilized with 0.5% Triton X-100 in PBS-0.1% BSA for 10 min at RT, washed before addition of anti-Env-AF647 at 20 ng/mL and viability dye

267 (0.5 µl/tube, Live Dead Aqua, #L34957, Life Technologies), and incubated for 30 min at RT. Cells were
268 washed with PBS-0.1%BSA and resuspended in 300 µL PBS-2%PFA. Data was acquired on a Gallios
269 cytometer (Beckman Coulter) and analyzed with Kaluza software. Results are expressed as the
270 percentage among viable cells.
271

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Conflicts of Interest

The authors had no conflicting interests relevant to the study.

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Figure legends

Figure 1. Replication kinetics of SFVs in several cell lines. (**a**) BHK-21 and (**b**) HT1080 cells were infected with CI-PFV (black), CII-SFV7 (green), GI-D468 (blue), or GII-K74 (red) at a moi of 0.01 for BHK-21 cells and 0.02 for HT1080. (**c**) K562, Jurkat, Raji, THP-1, and HEL 92.1.7 cells were infected with CI-PFV at a moi of 0.6. Infected cultures were split twice a week and a fraction of the cells stained with a viability marker and an anti-Env-AF647 antibody. Cultures were maintained until their death due to a massive cytopathogenic effect. The percentage of Env^+ cells among live cells is presented as a function of time. The lack of symbols at day 25 in panels a and b indicate that most cells expressed Env but that their accurate quantification was not possible because most were already dead. The data correspond to a representative test of two (panel c) or three independent experiments (panel a and b).

Figure 2. Factors affecting early steps of the replication cycle have no effect on infection with SFV. (**a**) Effect of reducing pH treatment on SFV replication. GFAB cells were infected with CI-PFV, CII-SFV7, GI-D468, or GII-K74 at a moi of 0.01. Two hours post-infection, medium was removed and PBS at pH 7 or 5.5 added and the cultures incubated for 1 or 15 min. After 72 h of infection, β-galactosidase expression was detected by X-gal staining. The number of infected cells/well is shown as the mean and SD of triplicates. (**b**) Effect of cationic polymers on SFV replication. GFAB cells were infected with CI-PFV (black) or GI-D468 (blue) viruses at a moi of 0.01 in the presence of polyethyleneimine (PEI), polybrene (PB), or DEAE Dextran (DEXT) at various concentrations. After 72 h of infection, β-galactosidase expression was detected by X-gal staining. The number of infected cells/well is shown as the mean and SD of triplicates. All the data correspond to a representative experiment of three independent experiments.

418 **Figure 3. Effect of inhibitors of the IFN response on SFV replication.** GFAB cells were treated
419 with IFN inhibitors for 4 h at various concentrations prior to infection. After 72 h of infection, β-
420 galactosidase expression was detected by X-gal staining. The number of infected cells/well is shown
421 as the mean and SD of triplicates. (**a**) GFAB cells were treated with ruxolitinib, BX795, or a mix of the
422 two molecules and infected with CI-PFV (black, moi 0.1) or GII-K74 (red, moi 0.02). (**b**) GFAB cells
423 were treated with TPCA-1 or BMS345541 and infected with CI-PFV (black, moi 0.1) or GII-K74 (red,
424 moi 0.02). All the data correspond to a representative experiment of three independent
425 experiments.

426 **Figure 4. Ruxolitinib enhances replication of gorilla SFV but has no effect on CI-PFV.** GFAB
427 (**a**) and HT1080 (**b** and **c**) cells were treated with ruxolitinib at 10 µM for 4 h and infected with SFV
428 strains at a moi of 0.02. Infected cultures were stained twice a week with a viability marker and an
429 anti-Env-AF647 antibody. Data are presented at the peak of infection on a FSC/anti-Env dot-plot of
430 viable cells. Percentages of Env+ cells are indicated. (**a**) GI-D468, day 8 post-infection; (**b**) GII-K74,
431 day 8 post-infection; (**c**) CI-PFV, day 5 post-infection.
432

433 Figure 1

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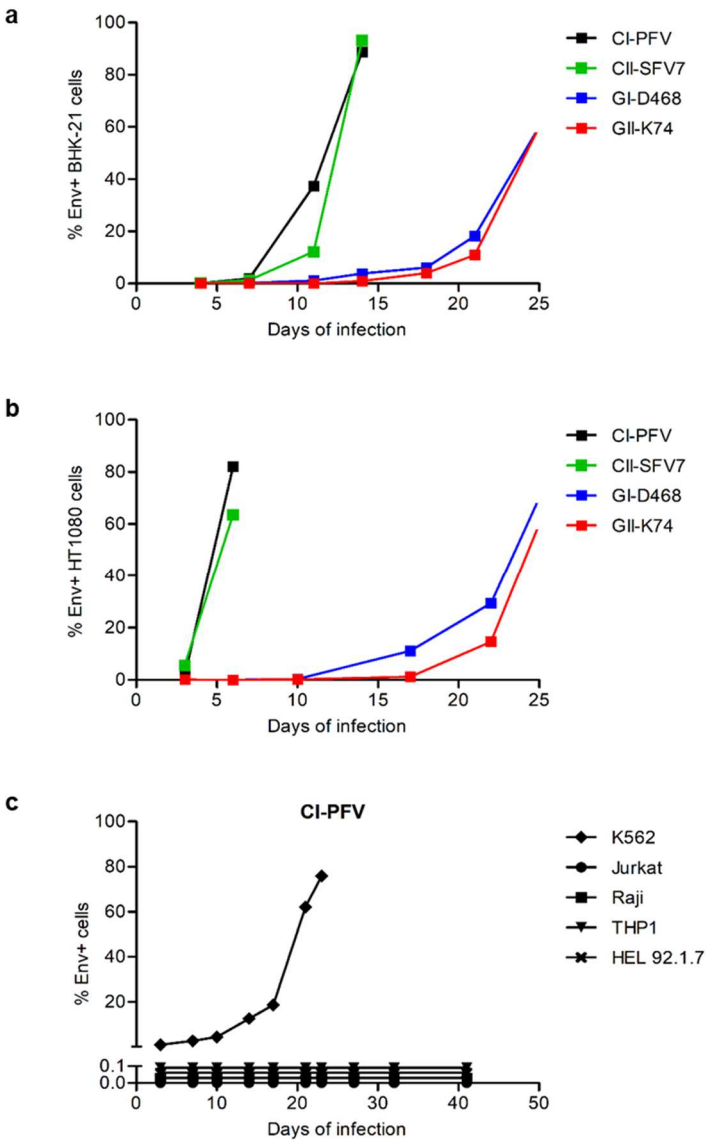

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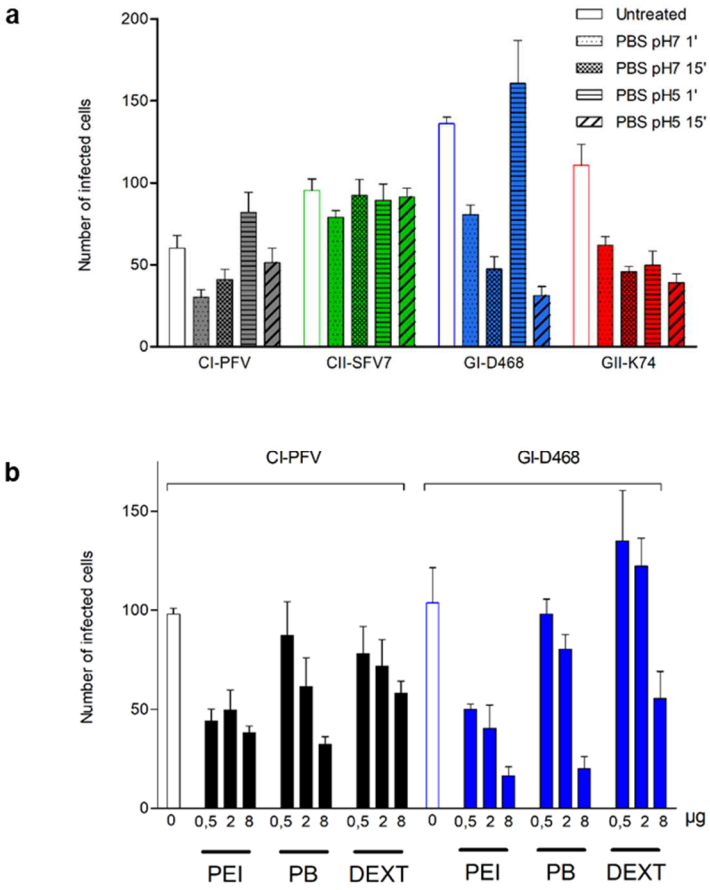
436 Figure 2



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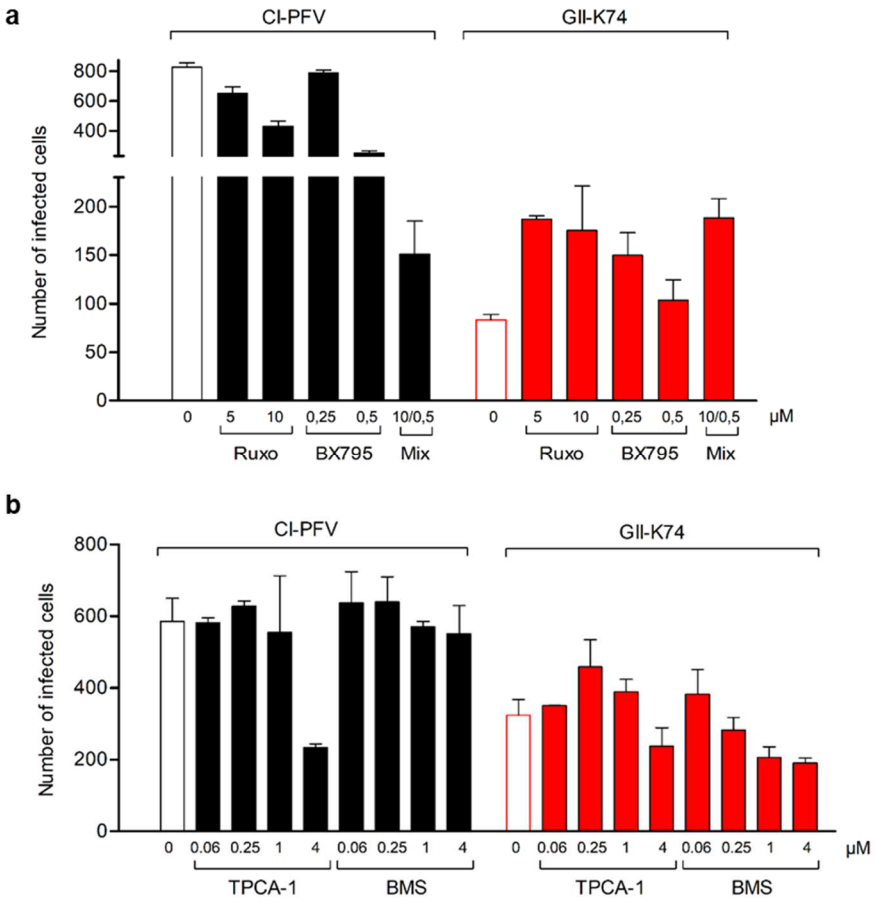
439 Figure 3



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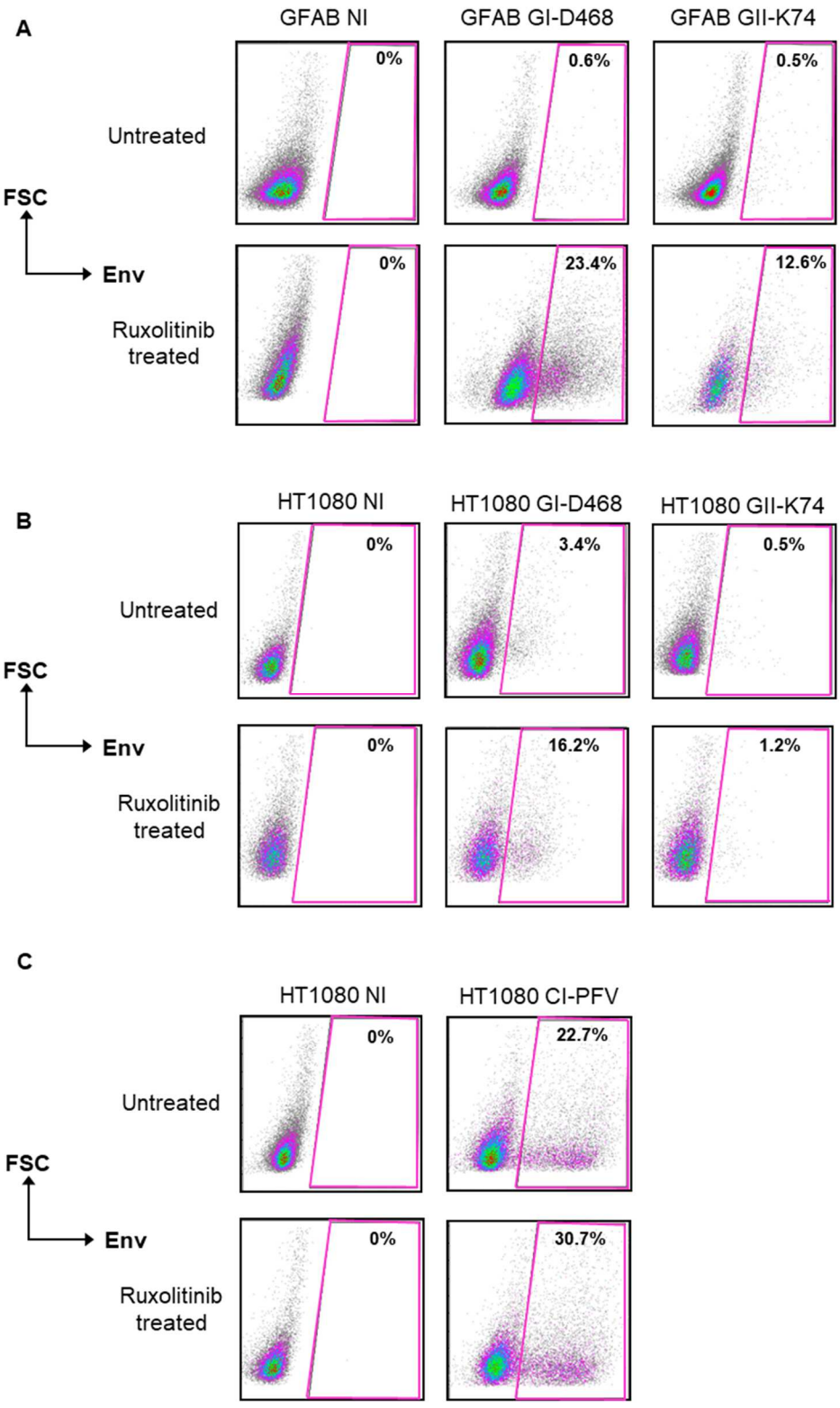
442 Figure 4



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