Progesterone augments cell susceptibility to HIV-1 and HIV-1/HSV-2 co-infections

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Abstract

In human immunodeficiency virus type 1 (HIV-1)-infected women, oral or injectable progesterone containing contraceptive pills may enhance HIV-1 acquisition *in vivo*, and the mechanism by which this occurs is not fully understood. In developing countries, Herpes simplex virus type-2 (HSV-2) co-infection has been shown to be a risk for increase of HIV-1 acquisition and, if co-infected women use progesterone pills, infections may increase several fold. In this study, we used an *in vitro* cell culture system to study the effects of progesterone on HIV-1 replication and to explore the molecular mechanism of progesterone effects on infected cells. In our *in vitro* model, CEMss cells (*lymphoblastoid cell line*) were infected with either HIV-1 alone or co-infected with HSV-2. HIV-1 viral load was measured with and without sex hormone treatment. Progesterone-treated cells showed an increase in HIV-1 viral load (1411.2 pg/mL) compared with cells without progesterone treatment (993.1 pg/mL). Increased cell death was noted with HSV-2 co-infection and in progesterone-treated cells. Similar observations were noted in peripheral blood mononuclear cells (PBMC) cells derived from three female donors. Progesterone-treated cells also showed reduced antiviral efficacy. Inflammatory cytokines and associations with biomarkers of disease progression were explored. Progesterone upregulated inflammatory cytokines and chemokines conversely and downregulated anti-apoptotic Bcl-2 expression. Nuclear protein analysis by electrophoretic mobility shift assay showed the association of progesterone with progesterone response element (PRE), which may lead to downregulation of Bcl-2. These data indicate that progesterone treatment enhances HIV-1 replication in infected cells and co-infection with HSV-2 may further fuel this process.

Key Words

- ▶ HIV/HSV-2
- ▶ infectivity
- ▶ apoptosis
- ▶ progesterone
- ▶ hormonal contraception

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Introduction

Women account for one half of new HIV-1 infections worldwide ([Joint United Nations Programme on HIV/AIDS 2013](#)). The use of contraceptive pills by

HIV-1-seropositive women is considered to be important for preventing unintended pregnancies and reducing mother-to-child transmissions. Although these

hormones help to prevent unintended pregnancies, they also enhance HIV-1 acquisition (Ramjee & Wand 2012, Morrison *et al.* 2015). Several epidemiological reports are inconsistent for progesterone-containing contraceptive use and risk of HIV-1 acquisition. A prospective study from seven African countries followed 2269 chronically HIV-1-infected women and showed no increased risk of HIV-1 disease progression for women on contraceptives compared with women who did not receive any contraceptive. In addition, this study observed no statistical difference in oral or injectable contraceptive use (Heffron *et al.* 2012, 2013). However, another study reported that injectable depot medroxyprogesterone acetate (DMPA) use had a moderate risk of HIV-1 acquisition, but not the oral hormonal contraception (Morrison *et al.* 2012). Based on these available data, in 2012, the World Health Organization released a technical statement noting that the data on hormonal contraceptives were inconclusive and recommending those women who are either at high risk or living with HIV-1 to continue the use of hormonal contraceptives. After the WHO report, several research studies indicated the risk associated with hormonal contraceptives. The study from South African population-based data on the use of hormonal contraceptive indicated a 12% increase in HIV-1 seroconversions after correcting for other HIV-1 risk variables (Ramjee & Wand 2012). A pigtail model indicates higher susceptibility of HIV or SHIV during luteal phase (high progesterone levels) when macaques are intravaginally challenged with SHIV (Vishwanathan *et al.* 2011). Recently, a study on hormonal contraception and HIV acquisition reports individual patient data of several studies. Meta-analysis indicates that DMPA may increase HIV acquisition (controlling confounders and adjusted odds ratio of 1.5) compared with other methods of contraception (Morrison *et al.* 2015). *In vitro* studies on hormone effects support the clinical studies discussed earlier. A study showed progesterone decreased CCR5 expression and increased CXCR4 expression in PBMCs of HIV-uninfected and -infected patient samples cultured with progesterone suggesting progesterone may modulate HIV infection at receptor level (Cabrera-Munoz *et al.* 2012). We also observed in our previous *in vitro* study that progesterone augments replication and transmission of major HIV-1 subtypes (Ragupathy *et al.* 2013).

It has been shown that among HIV-1-infected women, HSV-2 co-infection is the most common sexually transmitted infection (STI). HSV-2 is the

causative agent for genital herpes in humans and a major co-factor for HIV-1 acquisition (Wald & Link 2002). HIV-1 and HSV-2 are considered to be synergistic pathogens, and genital herpes increases susceptibility to HIV-1. HIV-1 co-infection is known to affect HSV-2 shedding, reactivation rates and HSV-2 infectivity (Foss *et al.* 2011). In women, HSV-2 co-infection increases the risk of HIV-1 infection (Benjamin *et al.* 2008, Thurman & Doncel 2012). The role of progesterone and HSV-2 co-infection was reported from a study from Kenya where both HSV-2 and oral contraceptives increase HIV-1 acquisition. In addition, contraceptive use and risk of HIV-1 acquisition are not associated with HSV-2 seropositivity (Baeten *et al.* 2007).

It is well known that susceptible cells infected with HIV-1 undergo apoptosis-mediated cell death. We had previously shown that an anti-apoptotic Bcl-2 family protein, Bcl-XL, and other proteins are able to decrease HIV-1 replication significantly suggesting that change in expression of this protein may affect HIV-1 replication in susceptible cells (Wang *et al.* 2010). Several other studies indicated that the Bcl-2 molecule inhibits the normal apoptotic pathway to cell death and extends the life span of certain cells (Tsujimoto 1998, Czabotar *et al.* 2013). Similarly, HSV-2 can infect a variety of cell types, and productive infection is characterized by apoptosis and downregulation of Bcl-2 (Sciortino *et al.* 2006). Previous studies focusing the effects of progesterone on the female reproductive tract indicated that it regulates the activities of endometrial epithelial, stromal cells and spiral arteries to modulate blood flow during pregnancy (Perrot-Appianat *et al.* 1994). It has also been shown to exert immunomodulatory effects on T- and B-cells (Kyurkchiev *et al.* 2014). Studies on uterine leiomyoma tissues reported that Bcl-2 mRNA and protein levels might be regulated by progesterone (Yin *et al.* 2007). These studies indicate that progesterone levels affect the metabolism of cells. However, limited knowledge is available regarding the effects of progesterone on replication of pathogens.

In this study, we demonstrate that progesterone increases the replication of HIV-1 or HSV-2 by changing cellular proteins. Understanding the mechanisms behind progesterone use, HSV-2 co-infection and HIV-1 susceptibility and identifying new biomarkers that may be useful for assessing the risk of increased HIV-1 acquisition are important public health issues that may be critical for the development of successful HIV-1 prevention efforts.

Material and methods

Cells and viruses

The T-cell line (lymphoblastoid leukemia cell) CEMss was obtained from NIH AIDS Reagent Program (Cat No: 776) and cultured in RPMI-1640 medium supplemented with 10% FBS (charcoal stripped), 2 mM glutamine, 100 U/mL of penicillin and 100 U/mL streptomycin. Peripheral blood mononuclear cells (PBMC) from three HIV-1 seronegative female blood donors were isolated from buffy coat received from NIH Blood Bank. Before blood draw, informed consent was obtained from all of the healthy, normal blood donors according to the ethical principles of international ethical guidelines for biomedical research involving human subjects. We used our standard laboratory protocol for PBMC isolation from buffy coat (Zhang *et al.* 2008). Briefly, PBMCs were isolated by Ficoll/Hypaque density-gradient centrifugation. Monocytes (>95%) were removed by adherence to the culture flasks, and the remaining peripheral blood lymphocytes (PBL) were stimulated with 2 µg/mL PHA for 3 days to activate T-cells before infection. The PBL or PBMC was cultured in RPMI-1640 supplemented with 15% FBS, 2 mM glutamine, 100 U/mL of penicillin, 100 U/mL of streptomycin and 5 U/mL of human interleukin-2 (Roche). Cell numbers were determined using Invitrogen Countess Automated Cell Counter, and cells stained with trypan blue were considered as dead cells.

The HIV-1IIIB strain was obtained from NIH AIDS Reagent Program and propagated in CEMss cells. HSV-2 strain MS was kindly contributed by Dr Jerry Weir, (FDA/CBER, Silver Spring, MD, USA) and propagated in the mammalian Vero cell line (derived from the kidney of a normal African green monkey) obtained from the American Type Culture Collection (ATCC). HSV-2 standard (Cat No: VR540D) for the TaqMan assay was purchased from ATCC. Serial dilutions were prepared in the ranges of 10–10⁵ pg/mL of dsDNA.

Sex steroid hormones

Two hours before infection, physiological levels of sex steroid hormones were added to cells. Steroid hormones were prepared as per manufacturer instructions, and the solvents were dried and diluted in the culture medium. For all hormone experiments, final concentrations are used as follows: progesterone (Sigma, P7556) 500 pM, β-estradiol (Sigma, E2257) 20 nM and testosterone (Sigma, T5411) 4 nM. No hormone controls has equal amount of either air-dried 100% ethanol (without E₂) dissolved

in culture medium or a culture medium (without P₄). Further, hormone concentrations were maintained throughout the culture by supplementing hormone-treated media at each experimental time point after initial infection. However, hormones fluctuate *in vivo* or between the individuals which cannot be depicted in *ex vivo* studies. The focus here is to demonstrate the effects of selected physiological levels hormones on virus infection kinetics.

Antivirals

Antivirals AZT (3'-azido-2',3'-dideoxythymidine) (Cat: A7803) and Valacyclovir hydrochloride hydrate (Cat: V9889) were purchased from Sigma. For inhibition studies, we used AZT IC₅₀ 5 µg/mL and valacyclovir EC₅₀ 0.9 µg/mL of culture media.

Antibodies and Western blot analysis

Rabbit monoclonal antibodies against BCL2L1 (Clone E18) and CASP3 (Clone E87) were purchased from OriGene Technologies, Inc, Rockville, MD, USA. Western Blot analysis was carried out as described previously (Wang *et al.* 2012). After blocking, the blot was incubated for 2 h with mAb to rabbit BCL2L1 or CASP3 and horseradish peroxidase conjugated. Antibody binding was detected by chemiluminescence staining using the ECL detection kit (Amersham Biosciences).

Experimental design

CEMss cells are susceptible to infection with HIV-1 and HSV-2 as described previously (Legoff *et al.* 2007). These cells were seeded at 1 × 10⁵/mL of culture medium in five 75 mL flasks. One flask served as the uninfected control, and the remaining four were infected with HIV-1 (5 or 10 ng/mL of HIV-1 p24) or HSV-2 (1 or 2 pfu/cell) or HIV-1 followed by HSV-2 or HSV-2 followed by HIV-1. Cells were infected for 4 h total at 37°C and 5% CO₂, washed and cultured. A total of 200 µL culture supernatant were collected from each flask and marked as day 0. Similar set of experiments were performed for progesterone (hereafter called as P₄), estradiol (hereafter called as E₂), progesterone–estradiol mix (hereafter called as E₂/P₄) and testosterone hormone treatment. To monitor infectivity, 1 mL culture supernatant was collected at 4, 7, 14 or 21 days after infection to measure HIV-1 p24 antigen levels or tested by a multiplex in-house HIV-1-1/HSV-2 TaqMan assay.

HIV-1 p24 determinations

We used the Perkin–Elmer HIV-1 p24 Elisa Kit, Cat No: NEK050B001 for p24 measurements. All procedures were followed as per the manufacturer's instructions. Because p24 test kits were optimized for quantification in a narrow range of 200–12.5 pg/mL, most of the culture supernatants were diluted in culture media to 1:10, 1:100 and 1:1000 before quantification.

Real-time TaqMan

Real-time quantitative PCR was performed on a 7500 Applied Biosystems Thermal Cycler in a 25 µL reaction mix containing 1 µL cDNA, 1× TaqMan QuantiTect Probe PCR Master Mix and 1X K15 or BAX or GAPDH assay. Thermal cycling was carried out at 50°C for 2 min, 95°C for 10 min, followed by 40 cycles at 95°C for 15 s, and 60°C for 1 min. All samples were run in duplicate. To determine the viral load of the two viruses in a single assay, we developed a multiplex quantitative TaqMan assay. For standard curve, HIV-1 (Cat No: 3443) was obtained from NIH AIDS Reagent and HSV-2 (Cat. No: VR-540D) from ATCC. Our multiplex TaqMan PCR was designed using the following primers and probes HSV-2 and HIV-1. Primers for HIV-1 included: (114bp) gag gene F-ACCCATGTTTACAGCATTATCAGAAG, R-GCTTGATGTCCCCCTACTGTATT; probe FAM, AGCC-ACCCACAAGATCTAAACACCATGT-MGB; for HSV-2 (118bp), HSV-2 (333) glycoprotein B gene Acc. No: M15118, F-TGCAGTTTACGTATAACCACATACAGCR-AGCTTGCGGGCCTCGTT; VIC-CGCCCCAGCATGTCTGTTACGT-MGB. The formula for measuring the concentration of HSV-2 DNA is 6.02×10^{23} (copies/mol) $\times$ A260 (ng/mL)/(DNA length $\times$ 650) = copies/mL. With several repeated experiments, we optimized the assay conditions to quantify both these viruses through derivation of a standard graph.

PCR arrays

RNA was prepared from cell pellets using the RNeasy Mini Kit (Cat: 74104) from Qiagen after homogenization. The RNA samples were DNase I (Roche Laboratories) treated and further purified on a column from an RNeasy kit (Qiagen). Reverse transcription was carried out using a QIAGEN RT2 first-strand kit (Cat No: 330401) using 1 µg RNA. The inflammatory cytokine–chemokine 'RT2 profiler array' from SA Biosciences was used to measure the transcript levels of various cytokines. The array contains primer sets for 84 cytokines/chemokines

(PAHS-011A-24) and appropriate housekeeping controls. We also performed similar analysis for HIV-1 infection and host responses (PAHS-012A). Real-time PCR was carried out as described by the manufacturer, using an Applied Biosystems T7500 Thermal Cycler.

The mRNA expression level for each gene was normalized using the expression of HPRT as a control housekeeping gene and compared with the data obtained with the negative control (RNA from uninfected cells) according to the $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen 2001). Results were considered significant when relative mRNA expression was twofold higher or lower than that of the uninfected cells. The results were confirmed by quantitative real-time PCR on individual RNA samples using the QuantiTect SYBR Green PCR Kit (Qiagen) and the same sets of primers as used in the PCR cytokine array (S A Bioscience Corporation, Frederick, MD, USA).

Electrophoretic mobility shift assay

CEMss cells were treated for 2 h with P_4 as indicated earlier. Nuclear protein was extracted from whole cells using NE-PER nuclear and cytoplasmic extraction reagents (Pierce). EMSA was performed using a LightShift Chemiluminescent EMSA kit (Pierce). In EMSA, we used previously published (Yin *et al.* 2007) sequence of the double-stranded oligonucleotide 5-GACAGAGGATCATGCTGTACTTAAA-3, which is identical to the 25-bp-long sequence (–559/–535 bp) within the promoter region of the Bcl-2 gene. This sequence contains an imperfect 15-bp palindromic PRE (553/539). The mutant probe is an unlabeled 25-bp oligonucleotide, in which 3 bp of the 15-bp PRE at –549, –543 and –540 positions were replaced by As (GACAAAGGATAATACTGTACTTAAA). Biotin end-labeled oligonucleotide probes were synthesized from the CBER core facility and annealed to the complementary oligonucleotides. Hundred pico moles labeled probe and 5 µg nuclear extract were incubated for 20 min at room temperature in a reaction mix containing 5% (vol/vol) glycerol, 10 mM Tris–HCl, 50 mM NaCl, 1 mM $MgCl_2$, 0.5 mM dithiothreitol and 2 g poly (dI–dC). For the EMSA, unlabeled wild-type or mutant probe (4 pmol) was added simultaneously with the labeled probe. Nondenaturing polyacrylamide gels (5%) were used to resolve protein–DNA complexes. Protein–DNA samples were transferred to the Bodine B pre-cut modified nylon membrane (Pierce). Protein–DNA complexes were detected using a LightShift Chemiluminescent EMSA Kit (Pierce) and exposed to the CL-XPosure film (Pierce).

Statistical analysis

The statistical module Prism, version 5.0 (GraphPad Software) was used to perform unpaired two-tailed Student's *t*-test with group sizes of two or analysis of variance (ANOVA) with Bonferroni post hoc *t*-test for greater than two group sizes.

Results

Infectivity of HIV-1 and HSV-2 and multiplex TaqMan

To determine the HIV-1 viral load from CEMss cells in the presence of hormone or HSV-2, cells were exposed to HIV-1 (10 ng p24/mL) and HSV (2 pfu) either alone or together. Two hours before infection, an aliquot of cells were exposed to physiological levels of hormone also. HIV-1 viral load quantification was carried out using two different assays, the HIV-1 p24 Ag ELISA and Real-time TaqMan Assay. At 7 d.p.i (days post infection) the means of HIV-1 p24 levels were higher (Fig. 1A) for cells treated with E₂ (1229.1 pg/mL) and significantly ($P < 0.05$) higher P₄ (1411.2 pg/mL) relative to NH (993.1 pg/mL). However, no significant differences were noted for cultures treated with E₂/P₄ mix (1076.7 pg/mL) and testosterone (876.5 pg/mL). HSV-2 co-infection commonly occurs with HIV-1 and shares the same sexual route of transmission. We investigated when the HSV-2-infected cells were superinfected with HIV-1 and measured the HIV-1 viral load at 7 d.p.i. HIV-1 p24 level means were significantly ($P < 0.05$) lower (Fig. 1A) for cells treated with E₂ (508 pg/mL), E₂/P₄ mix (346.8 pg/mL), testosterone (350.5 pg/mL) and very significantly lower ($P < 0.01$) for P₄ (190.9 pg/mL), relative to NH (770.4 pg/mL).

In this study, we used two virus types; to quantify both these viruses simultaneously, we used multiplex TaqMan Assay. Nucleic acids were extracted from culture supernatants to quantify both HIV-1 and HSV-2. Like HIV-1 p24 assay, similar trend was observed with TaqMan Assay although TaqMan is not true measure of infectivity. At 7 d.p.i increased levels of HIV-1 RNA (Fig. 1B) for cells treated with E₂ (2.81 log₁₀ cps/mL), P₄ (3.22 log₁₀ cps/mL), E₂/P₄ mix (3.06 log₁₀ cps/mL) and testosterone (2.78 log₁₀ cps/mL) relative to NH (2.72 log₁₀ cps/mL). Although no significant differences were noted for E₂ and testosterone, P₄ viral load was significantly higher ($P < 0.05$). In HSV-2 co-infection, HIV-1 viral load was lower (Fig. 1B) for cells treated with P₄ (1.86 log₁₀ cps/mL), E₂/P₄ mix (2.39 log₁₀ cps/mL) and testosterone (2.38 log₁₀ cps/mL) relative to NH (2.66 log₁₀ cps/mL). However, for E₂ (2.50 log₁₀ cps/mL), no difference in viral load was noted in comparison with NH.

Figure 1

HIV-1 viral load determination by a quantitative p24 ELISA and TaqMan Assay. Comparisons of two virus quantification measurements. Cells were infected with HIV (dark bar) or co-infected with HSV (light bar), and culture supernatant collected at 7 d.p.i. for viral load measurements. Infected cells were either not hormone treatment (NH) or treated with hormones estradiol (E₂), progesterone (P₄), a mix of estradiol with progesterone (E₂/P₄ Mix) and testosterone. Statistical significance was derived by comparing NH with hormone-treated groups. (A) Measurement of HIV-1 by quantitative enzyme immuno p24 assay. HIV-1 viral loads of cells treated with different hormone types were compared with cells treated with no hormone. Although differences in HIV viral load noted comparing NH with hormone treatment only P₄ treatment had significantly ($*P < 0.05$) higher viral load. Similar experiments were repeated for HSV-2/HIV-1 co-infected cells. Significant ($*P < 0.05$) low viral load was noted for hormone-treated groups and very significant ($**P < 0.01$) reduction in viral load was noted for P₄-treated group. But for E₂-treated cells, there were no significant differences in viral load when compared with NH. (B) Measurement of HIV-1 by quantitative TaqMan assay indicating similar trends of viral load differences for P₄- and E₂/P₄ mix-treated cells with HIV or HSV-2/HIV-1 co-infection. However, no statistical significance noted for HIV-infected cells treated with E₂ or testosterone. In co-infected cells, P₄ ($**P < 0.01$), mix and testosterone treatment showed significant ($*P < 0.05$) viral load differences. No significant differences were noted for E₂ compared with NH. The error bars represent standard errors of the means (s.e.m.) for triplicate measurements of at least three experiments.

Once we determined that viral load was quantifiable with or without hormone treatment using multiplex TaqMan, we focused on specifically quantifying each of these viruses in the presence and absence of hormones. In addition, due to assay limitations and high viral load at day 7, in rest of the long-term experiments, virus infectivity dose was reduced to half for optimal quantifications.

Effects of hormone-treated cells and virus replication

Cells exposed to HIV-1 and HSV-2 was cultured up to 14 days, and viral load was quantified at two intervals to study the hormone effects on replication kinetics. HIV-1 viral load in terms of RNA cps/mL were represented in $\log_{10}$ values. Although two time points were measured, infectivity was delayed for certain culture conditions; hence, data were shown only for measurements at day 14. As noted earlier, HIV-1 RNA cps/mL means were significantly higher (Fig. 2A) at 14d.p.i ($P < 0.01$) for cells treated with E_2 ($3.9 \log_{10}$ cps/mL), P_4 ($3.61 \log_{10}$ cps/mL), E_2/P_4 mix ($3.52 \log_{10}$ cps/mL) and testosterone ($3.51 \log_{10}$ cps/mL) relative to NH ($2.39 \log_{10}$ cps/mL). Similarly, HSV-2 DNA level means were significantly higher ($P < 0.05$) (Fig. 2B) for cells treated with E_2 ($12.24 \log_{10}$ cps/mL), E_2/P_4 mix ($12.41 \log_{10}$ cps/mL) and nonsignificant levels for P_4 ($11.1 \log_{10}$ cps/mL) and testosterone ($10.76 \log_{10}$ cps/mL) relative to NH ($10.74 \log_{10}$ cps/mL).

Next, we performed co-infection studies with HIV-1 infection followed by HSV-2 or vice versa to determine

which co-infection augments the pathogenesis of HIV-1. The experiments were performed with and without hormone treatment to study viral replication kinetics.

As described earlier, the mean HIV-1 RNA was significantly higher (Fig. 2C) at 14 d.p.i ($P < 0.05$) with P_4 ($3.42 \log_{10}$ cps/mL), E_2/P_4 mix ($3.57 \log_{10}$ cps/mL) and testosterone ($3.47 \log_{10}$ cps/mL) relative to NH ($2.47 \log_{10}$ cps/mL) but nonsignificant with E_2 ($2.85 \log_{10}$ pg/mL). When HIV-1 infected cells were superinfected with HSV-2, HIV-1 viral load was significantly higher (Fig. 2D) for E_2 ($3.66 \log_{10}$ cps/mL), P_4 ($3.68 \log_{10}$ cps/mL), E_2/P_4 mix ($3.45 \log_{10}$ cps/mL) and testosterone ($3.45 \log_{10}$ cps/mL) relative to NH ($2.23 \log_{10}$ cps/mL). It was observed that there were no significant differences ($P > 0.05$; 0.93) in HIV-1 viral load in cells infected with HSV-2 first and later infected with HIV-1 or vice versa. HIV-1 RNA was little higher ($2.74 \log_{10}$ cps/mL) in HIV-1 superinfection of HSV-2-infected cells relative to NH ($2.39 \log_{10}$ cps/mL). In all subsequent co-infection experiments, cells were first infected with HSV-2 followed by HIV-1. In co-infected cells treated with P_4 , mean HIV-1 viral load ($3.68 \log_{10}$ pg/mL)

Figure 2

Screening of hormone effects on HIV-1 or HSV-2 replication kinetics. (A) Virus infection of cells and hormone treatment are similar to Fig. 1. At 7 d.p.i, no significant HIV-1 viral load changes were noted and hence not presented here. Bar chart indicates viral replication with and without hormone treatment measured at 14 d.p.i. Error bars represent standard errors of the means (S.E.M.). Significance was evaluated by two-way analysis of variance (ANOVA) with Bonferroni posttests. * $P < 0.05$. (B) Similarly, cells were infected with HSV-2 virus, and its replication was measured and compared for hormone-treated and -untreated cells. HSV-2 replication was significantly ($P < 0.05$) higher at 14 d.p.i in cells treated with E_2 or E_2/P_4 mix but nonsignificant with P_4 or testosterone. (C) Cells were first infected with HSV-2 (2 h) and co-infected with HIV-1 (2 h) or vice versa (D). In (C) compared with cells treated with no hormone, significant ($P < 0.05$) increase in HIV-1 titer was seen with cells treated with P_4 , testosterone and E_2/P_4 mix but nonsignificant (ns) for E_2 -treated cells. (D) All hormone-treated cells had significant increase ($P < 0.01$) of HIV-1 viral load compared with NH.

Figure 3

HIV-1 p24 levels were measured in cells untreated (NH) or treated with P₄ or E₂/P₄ mix and compared with cell viability. These cells were co-infected with HSV-2, and experiments were performed at similar conditions. HIV-1 virus titer was measured at two time points, days 7 and 14, and data are shown as bar graph (primary axis). Cell viability (trypan dye exclusion) was measured using automated cell counter and plotted as percentages (in right y-axis). At day 7, >80% of cells were viable in untreated or hormone-treated cells, whereas HIV-1 viral load was little higher in P₄-treated cells (802 pg/mL) compared with NH (525 pg/mL) or E₂/P₄ (410 pg/mL) mix. At day 14, spontaneous loss of viable cells was observed ($P < 0.05$) with P₄- and E₂/P₄ mix-treated cells (line graph) and no significant rise in HIV-1 p24 levels (bar graph). Results shown are the mean (S.E.M.) from three different replicates of at least three experiments.

at day 14 was relatively higher compared with NH (2.74 log₁₀ pg/mL). In addition, co-infection experiments with P₄-treated cultures showed extensive cell death.

We further analyzed the effects of P₄ either alone or in combination with estradiol and simultaneously measured the cell viability. Analysis was performed for two time points, day 7 and 14. At day 7, the mean HIV-1 p24 of NH was 563.2 pg/mL, P₄, 776 pg/mL; E₂/P₄ mix, 427 pg/mL and cell viability for each experimental condition was 81–84% (Fig. 3). At day 14, mean HIV-1 p24 of NH was 1119.5 pg/mL, P₄, 721 pg/mL; E₂/P₄ mix, 593 pg/mL and cell viability was significantly ($P < 0.05$)

lower for P₄ (42%) and E₂/P₄ mix (35%) than for NH (66%).

Next, we determined the cell viability using an automated cell counter at three different time points of multiple experimental conditions (Fig. 4). Equal numbers of cells were either infected with HIV-1 or HSV-2 or uninfected and treated with P₄ alone or together with estradiol. At days 7 and 9, cell viability was 80–92% in all experimental conditions. However, at day 14, the viability dropped to 35–88% and more specifically HSV-2/HIV-1 cultures treated with P₄ had significantly ($P < 0.05$) lower percent of viable cells compared with uninfected P₄-treated cells. The extensive cell death of cultures with co-infection and P₄ treatment may likely be due to the involvement of cellular factors.

Reduced efficacy of antivirals for virus-infected cells treated with P₄

We further determined the effect of treatment with antivirals on P₄-exposed cells infected with HIV-1 or HSV-2/HIV-1. Infected cells were cultured with and without P₄ treatment for 7 days, and a subset of cells were treated with AZT for HIV-1 and AZT/Valacyclovir for co-infections of HSV-2/HIV-1. The baseline HIV-1 viral load (without ARVs) at day 7 was 0.21 ng/mL for P₄-untreated cells and 0.27 ng/mL of p24 for P₄-treated cells and in co-infection, 0.55 ng/mL for cells not treated with P₄ and 0.645 ng/mL of p24 for P₄-treated cells confirming that there was productive HIV-1 infection.

The cultures were split into two for antiviral treatment and no treatment. The HIV-1 p24 levels for AZT-treated cultures had significantly lower HIV-1 p24 levels (means of: day 10=0.29; day 14=0.29; day 21=0.48 ng/mL) than cultures without AZT (means of HIV-1 p24: day 10=0.29; day 14=2.48; day 21=5.92 ng/mL) (Fig. 5A). When similar experiments were repeated for P₄-treated cells (Fig. 5A), HIV-1 viral load was 5-fold higher ($P < 0.01$) at day 21 (29.3 ng/mL of p24) compared

Figure 4

Cell viability under different experimental conditions. Cells were either uninfected (negative control) or infected and hormones treated as indicated. Cells viability (trypan dye exclusion) was measured at three time points, day 7, 9 and 14 using automated cell counter. Significant cell death ($P < 0.05$) was noted with P₄-treated cells and co-infected with HSV/HIV. Error bars represent the standard errors of the means, and asterisks indicate cells that are significantly low viable cells as determined by ANOVA.

Figure 5

Progesterone effects on antiviral efficacy. (A) HIV-1-infected cells were cultured with and without P_4 for 21 days. Similarly, another P_4 -treated HIV-infected cells are cultured in the presence of HIV-1 replication inhibitor AZT. In both, HIV titer was measured at day zero to day 21 (5 intervals) using PerkinElmer Quantitative Enzyme Immuno p24 Assay. At each time point, three replicates were measured and means were plotted in the bar graph. P_4 -treated cells had significant ($P<0.01$) higher viral load compared with P_4 -untreated cells. AZT inhibits HIV replication in the absence of P_4 but significantly ($***P<0.001$) increased virus titer as observed in P_4 -treated cells. (B) Similar experimental conditions of (A) were repeated for HSV/HIV co-infections. For inhibition studies in addition to AZT, valacyclovir (to inhibit HSV-2) was used. Similar trend was observed. P_4 -treated cells had significantly ($***P<0.001$) higher viral load compared with no P_4 cells. AZT and valacyclovir inhibit HIV/HSV-2 replication in no P_4 , but significantly ($***P<0.001$) increased virus titer observed in P_4 -treated cells.

with that of cultures without P_4 (5.92 ng/mL of p24). In addition, HIV-1 p24 levels did not decrease completely with AZT treatment (day 10=0.44; day 14=1.67; day 21=2.26 ng/mL) compared with those in cultures without AZT (means of HIV-1 p24: day 10=3.70; day 14=12.23; day 21=29.30 ng/mL). Moreover, in P_4 -treated cells, AZT efficacy was lower (means of HIV-1 p24: day 14=1.67; day 21=2.26 ng/mL) than in cultures without P_4 ($P<0.001$) (means of HIV-1 p24: day 14=0.29; day 21=0.48 ng/mL).

Similarly, in co-infections, the HIV-1 p24 levels for AZT/valacyclovir-treated cultures were significantly lower (means of HIV-1 p24: day 10=0.22; day 14=0.38; day 21=0.65 ng/mL) than those for cultures without AZT/valacyclovir (means of HIV-1

p24: day 10=0.55 ng/mL; day 14=0.57; day 21=13.1 ng/mL) (Fig. 5B). When similar experiments were repeated for P_4 -treated cells (Fig. 5B), HIV-1 viral load was 9-fold higher ($P<0.001$) at day 21 (137.1 ng/mL of p24) compared with that in cultures without P_4 (15.11 ng/mL of p24). In addition, HIV-1 p24 levels did not decrease completely with AZT/valacyclovir treatment (day 10=0.55; day 14=0.57; day 21=13.12 ng/mL) compared with cultures without AZT/valacyclovir (means of HIV-1 p24: day 10=3.60; day 14=12.48; day 21=137.12 ng/mL). Moreover, in P_4 -treated cells, AZT/valacyclovir efficacy was lower (means of HIV-1 p24: day 14=0.57; day 21=13.12 ng/mL) than that in cultures without P_4 ($P<0.001$) (means of HIV-1 p24: day 14=0.38; day 21=0.65 ng/mL).

P_4 supports HIV-1 replication and cell death

Next, we wanted to study the effects of P_4 treatment on primary cells and determine whether P_4 treatment or HSV-2 co-infection increased HIV-1 replication and augmented cell death. We cultured PBMC cells collected from three different donors and treated them with P_4 at similar concentrations of hormones used in the experiments with cell lines. The PBMC cells not treated with hormones served as a control. At 14 d.p.i. P_4 -treated mean of HIV-1 p24 levels of donor 2 (81.6 ng/mL) and donor 3 (126.9 ng/mL) was significantly ($P<0.05$) higher than with NH donor 2 (62 ng/mL) and donor 3 (61.5 ng/mL). However, no significant differences were observed between P_4 -treated donor 1 (90 ng/mL) and NH donor 1 (80.6 ng/mL) (Fig. 6A), suggesting donor-to-donor variations in infectivity. When similar experiments were repeated for HSV-2 co-infection, at 14 d.p.i. significant ($P<0.05$) cell death and low HIV-1 p24 levels were noted for P_4 -treated donor 1 (990 pg/mL), donor 2 (1160 pg/mL) and donor 3 (1880 pg/mL) compared with NH donor 1 (5000 pg/mL), donor 2 (6500 pg/mL) and donor 3 (7240 pg/mL) (Fig. 6B). These results suggest that P_4 enhances HIV-1/HSV-2 replication and leads to cell death in primary cells.

Host gene expression profiling

To further elucidate the cellular mechanisms in relation to P_4 -treated cells infected with HIV-1 or co-infected with HSV-2, mRNA gene expression profiling of major chemokines, cytokines and apoptosis-specific biological markers were analyzed.

Figure 6

HIV-1 replication kinetics in primary PBMC cells either untreated (NH) or treated with progesterone (P₄). (A) HIV-1 p24 levels are significantly higher ($P < 0.05$) for P₄-treated donors (2,3) compared with NH. For donor 1, there was no significant differences in HIV-1 p24 levels between NH and P₄ treatment. (B) Measure of HIV-1 p24 levels in HSV-2/HIV-1 co-infected donors had significantly low ($P < 0.01$) p24 levels in P₄-treated cells compared with NH. Error bars represent the standard errors of the means, and samples that are significant (*) and very significant (**) as determined by unpaired t-test.

A total of 84 genes encoding inflammatory cytokines/receptors, chemokines/receptors and others involved in inflammation were analyzed using the Human Inflammatory Cytokines and Receptors PCR Array. Cells were either P₄ treated or untreated and infected with HIV-1 or HSV-2/HIV-1 for 7 days. The total nucleic acid

was extracted, normalized and analyzed. Data were pooled from each experimental group (uninfected, NH-HIV-1, P₄-HIV-1, NH-HIV-1/HSV-2 and P₄-HIV-1/HSV-2) and analyzed with Web-based RT² profiler PCR array data, version 3.5. Gene expression was evaluated on the basis of 2-fold upregulation or downregulation compared with uninfected controls. Based on earlier observations, P₄-treated cells infected with HIV-1 and HSV-2 could modulate certain genes in support of pathogenesis. Hence, our analysis was focused on host gene expression profile for P₄-treated cells with HIV-1 or HSV-2 co-infections. Our results indicate that relatively few genes (CCL15, CCL19, CCR6, IL36A and IFNA2) were differentially expressed with HIV infection (NH-HIV or without P₄ treatment). Interestingly, when the expression profile was compared with P₄-treated HIV-1-infected cells, several inflammatory genes were 2- to 15-fold upregulated ($P < 0.05$) (Fig. 7A). Notably, chemokines CCL11, CCL13 and CCL23 and the receptors CCL13, CCR3, CCR6, CX3CR1 and XCR1 were 2- to 6-fold upregulated ($P < 0.05$) compared with HIV-1-infected cells without P₄ treatment or uninfected P₄-treated controls. The cytokines IL36B and receptor IFNA2 were also 6- to 12-fold upregulated for HIV-1-infected P₄-treated cells. In co-infection studies, chemokine and cytokine levels in HIV-1-infected and P₄-treated cells were upregulated 2-410 higher than only HIV-1 infection (Fig. 7B). Notably, several-fold increase of CCL4, CXCL9, CCR5 and CXCR1 expression was observed. The cytokines IL22, IL10RA, IL13, IL13RA1, IL5RA and IL9 were 15- to 166-fold upregulated.

To identify host factors specifically involved in cell death due to infection, similar to the inflammatory response PCR array, an apoptosis PCR array of 84 genes was analyzed. For analysis, data were pooled from each experimental group (uninfected, NH-HIV-1, NH-HSV, NH-HIV-1/HSV-2, P₄-control, P₄-HIV-1 and P₄-HIV-1/HSV-2). To elucidate the expression of apoptotic genes in cells co-infected with HSV-2/HIV-1, differential gene expression was analyzed and compared with the expression profile of P₄-treated cells. Although most of the genes were not differentially expressed in cells infected with HIV-1 or HSV-2 alone, downregulation of anti-apoptotic factor BCL2L10 was consistently observed (>2 fold) with P₄-treated cells for both viruses (Fig. 8). In addition, TNFRSB10B was downregulated (>2 fold) for HSV-2-infected cells with P₄ treatment. In cells co-infected with HIV-1 and HSV-2, few other genes were upregulated, and genes BCL2L10 and TNFRSB10B were downregulated (>2 fold) for HSV-2/HIV-1-infected cells with P₄. The downregulation of Bcl-2 was further

Figure 7

Cytokine and chemokine mRNA expression of HIV-1- and HSV-2-infected cells. The figure represents genes that are differentially expressed by progesterone treatment and HIV-1 or HSV-2/HIV-1 infections of 7 d.p.i. (A) and (B) Genes were evaluated on the basis of the criteria of at least 2-fold upregulation or downregulation compared with uninfected control. Error bars represent the standard errors of the means, and statistical significance of $P < 0.05$ was determined by ANOVA.

Figure 8

mRNA expression of apoptosis-related genes in HIV-1 and HSV-2 or co-infected cells. The figure represents genes that are differentially expressed by progesterone treatment and HIV-1 or HSV-2/HIV-1 infections of 7 d.p.i. (A), (B) and (C). Genes were evaluated on the basis of the criteria of at least a 2-fold upregulation or downregulation compared with uninfected control. Error bars represent the standard errors of the means, and statistical significance of $P < 0.05$ was determined by ANOVA.

Figure 9

Bcl-2 downregulation in response to progesterone (P₄)-treated cells with HSV or HSV/HIV co-infections. (A) Bcl-2 expression was measured in duplicate from duplicate cultures by qRT-PCR at 7 d.p.i. Mean values are shown + S.E.M. The expression was calculated relative to GAPDH mRNA levels (bcl-2/GAPDH). **P* ≤ 0.05 when comparing NH and P₄ from the same time point. Bars represent Bcl-2 expression from uninfected negative control (-ive control), HIV or HSV or HSV/HIV co-infection. (B) Proteins were detected by Western blot analysis using antibodies to Bcl-2, Caspase 3 and GAPDH. (C) Densitometry for Bcl-2 blot was performed using ImageJ software. Arbitrary units represent protein-level analysis with respect to without or with P₄ treatment. Significant reduction in Bcl-2 expression was noted as (*) compared to uninfected cells without P₄ treatment.

confirmed with qPCR and at the protein level by Western blot analysis.

P₄ regulation of Bcl-2 mRNA and protein levels

To confirm the PCR array data of Bcl-2 down-regulation by P₄, we performed qPCR for Bcl-2 mRNA expression in P₄-treated cells infected with HIV-1 or co-infected with HSV-2. At 7 d.p.i, RNA was extracted and quantified by GAPDH normalization. Different patterns of host gene expression were observed (Fig. 9A). In cells not treated with P₄, consistent increase of Bcl-2 was observed for HIV-1- or HSV-2-infected cells. Cells treated with P₄ had significant (*P* < 0.05) down-regulation of Bcl-2 (P₄ uninfected, infected with HIV or HSV-2 and HSV-2/HIV-1). These observations were further confirmed at the protein level using immunoblotting, which showed that P₄-treated uninfected cells and

infected with HSV-2/HIV-1 had lower expression of Bcl-2 than P₄-untreated cells (Fig. 9B), and densitometry was performed on bands using ImageJ software (Fig. 9C). Cells treated with P₄ and infected with HIV-1 or HSV-2 show Bcl-2 expression due to the fact virus–host interaction this phenomenon diminishes at later infection time point. In addition, caspase 3 protein expression was evaluated to confirm extensive cell death of co-infected cells. As expected, increased caspase-3 protein expression (Fig. 9B) was noted with co-infected cells treated with P₄ or untreated.

Increased association of P₄ with PRE region of Bcl-2 promoter

This is the first study to show that P₄ treatment augments HIV-1 replication, which increases several folds in the presence of HSV-2 co-infection by the downregulation of

Figure 10

Electrophoretic mobility shift assay (EMSA) analysis of P₄-binding region of Bcl-2 promoter. (A) EMSA was performed using samples of nuclear extracts (3 µg) from CEMss cells either untreated (1–4) or treated with progesterone for 2 h (5–8) and infected with HIV or HSV as indicated for 2 h. Each lane in no P₄ or P₄-treated cells represents wild-type or mutant probe incubation with nuclear factor. The arrow indicate DNA–protein complex and free DNA. The intensity of DNA–protein complex partially decreased in lane 9 for competition studies with 100-fold excess of cold unlabeled and labeled probe. The figure represents one of the three independently performed experiments. (B) Biotin-labeled oligonucleotide probes containing putative PRE-binding sites in box and its flanking sequence. In mutant probe, bases with (*) were replaced as A's.

the Bcl-2 molecule. We wanted to explore the mechanism of Bcl-2 down-regulation by P_4 treatment. Previously, it was reported that PRE is present in the Bcl-2 promoter thereby modulating Bcl-2 gene expression in P_4 -treated cells. To test whether co-infection in the presence of P_4 increases the association of transcription factors with PRE, nuclear proteins were extracted from cells cultured with or without P_4 and infected with HIV-1, HSV-2 or co-infected for 4 h and analyzed.

The Bcl-2 promoter region of P_4 response element (PRE) was identified through the Web portal Transcription Element Search Site (TESS, 2011). PCR was performed to amplify the 242bp fragment of the Bcl-2 promoter containing the PRE and confirming the presence of the sequence within the amplified product. The biotinylated PRE probes were designed for binding in electrophoretic mobility shift assay (EMSA). Extracted nuclear proteins from P_4 -untreated (uninfected and infected with HIV-1, HSV-2 or co-infected) or P_4 -treated (P_4 uninfected and infected with P_4 -HIV-1, P_4 -HSV-2 or P_4 -co-infected) cells of different experimental conditions (lanes 1–8) were incubated with biotinylated PRE probe or mutant probe (1a–8a) of transcription factor-binding region for determining PRE association. The PRE probe and mutant probe were loaded as pairs for each experimental condition, in which lanes 1–4 represent no P_4 treatment and lanes 5–8 indicate treatment with P_4 . It was inferred from Fig. 10 that DNA–protein complex was formed when the biotin labeled probe was incubated with nuclear extract (4–8). In lane 9, incubation with excess of cold unlabeled probe (100-fold) decreased labeled probe binding, demonstrating specificity. In addition, mutant labeled probe (lanes 1a–8a) increased binding of other nuclear proteins. These data indicate that nuclear factors induced by P_4 bind to Bcl-2 promoter specifically.

Discussion

The use of progestin (P_4 analog) as contraceptive pills for HIV-1-positive women has been extensively debated recently with regard to its ability to increase HIV-1 risk or not (Colvin & Harrison 2015, Morrison *et al.* 2015, Ralph *et al.* 2015). Some studies indicate no link between contraceptive use and risk of HIV-1 (Heffron *et al.* 2013, Polis *et al.* 2014), whereas others reported significant risk with contraceptive use (Heffron *et al.* 2012, Haddad *et al.* 2014). Recently, by examining individual participant data instead of an aggregate of different studies, an increase in risk was found to be associated with the use of P_4 -containing contraceptives (Morrison *et al.* 2015). Considering the

immediate need in the prevention of HIV-1 in women, we investigated the basic mechanism of P_4 effects on HIV-1 pathogenesis and with co-infection of HSV-2. First, we investigated the cell type that could infect HIV-1 and HSV-2. Previously, it was shown that CEMss cell line is capable of infection by these two viruses (Legoff *et al.* 2007). We tested the infectivity of these two viruses at the indicated (5 ng/mL of HIV-1 p24 or 1 p.f.u. HSV-2) concentrations, which were normalized for optimal infectivity after multiple experiments. Infectivity was determined by rise in titer from time zero. To evaluate the effects of steroid hormones on replication of HIV-1 and HSV-2, we tested testosterone and estradiol along with P_4 . The measurable HIV-1 p24 levels at 7 d.p.i in both untreated and hormone-treated cultures indicate no inhibition of hormones for HIV-1 replication. In co-infected cultures, we quantified both viruses simultaneously using an in-house multiplex quantitative TaqMan assay. An increase in virus titer was observed for both viruses from time zero and could be quantitated. In addition, HIV-1 viral load levels for cultures with and without hormone treatment showed a similar trend compared with p24 quantification (Fig. 1A and B). Although our present work focuses on P_4 , E_2 and testosterone hormones significantly increase HIV-1 viral load. Interestingly, E_2 or E_2/P_4 mix increase HSV-2 viral load but not P_4 or testosterone, which may require further functional studies to validate HSV-2 replication kinetics in the presence of hormones.

Previously, several studies had indicated that P_4 or its synthetic form increased HIV-1 susceptibility or risk of infection (Asin *et al.* 2008, Ramjee & Wand 2012, Ferreira *et al.* 2014) in a macaque model using subcutaneous implants of P_4 which increased vaginal SIV transmission several fold (Marx *et al.* 1996). Similar to these earlier studies, we also observed that treatment with steroid hormones increased HIV-1 viral load levels in culture supernatants. Several studies have shown that HSV-2 infection increases HIV-1 acquisition (Schacker *et al.* 1998, Freeman *et al.* 2006) and pathogenesis (Sartori *et al.* 2011, Rollenhagen *et al.* 2014). Hence, HSV-2-infected individuals are at high risk for HIV-1 co-infection and that HSV-2 incidence among HIV-1 sero-discordant couples increases the risk of HIV-1 transmission (Muiru *et al.* 2013). To verify this observation made at the population level, using an *in vitro* cell culture system, we tested several combinations of hormone and virus treatment to determine their effect on HIV-1 viral load. In cells co-infected with HSV-2 and or vice versa, we observed significant differences in viral load between the different combinations of hormone treatment (Fig. 2A,

B, C and D) except cells treated with E₂ (Fig. 2C), P₄ and testosterone (Fig. 2B). It is interesting to note that P₄ supports HIV-1 replication in cells treated with P₄ only (Fig. 2A) or mixed with E₂/P₄ (Fig. 2A, C and D). Conversely, E₂ supports HSV-2 replication in cells treated with E₂ only or mixed with E₂/P₄ (Fig. 2B). Although sex hormones support HIV-1 or HSV-2 replication, significant cell death was noted in co-infected cultures at 14 d.p.i of P₄-treated cells. We ruled out hormone toxicity by cell viability analysis from multiple experiments with and without P₄ treatment and co-infection with HIV-1 or HSV-2. Up to day 9, >85% cells were viable (Figs 3 and 4). Thus, in early part of HIV-1 infection (up to day 9), P₄ did not decrease the cell viability but augments HIV-1 replication. However, significant loss of cells was noted with co-infected cultures treated with P₄. When we compared cell viability with p24 levels of co-infected cells, lower p24 levels were noted with P₄ and E₂/P₄ treatment at 14 d.p.i. (Fig. 3), which may be due to the decrease in cell numbers as the infection progresses.

Previous studies had reported that the use of P₄-only-based contraception may be associated with a small risk of HIV-1 infection for patients on antiviral therapy. Even though recent reports indicate that the contraceptive DMPA increases risk of HIV-1, there is no link between HSV-2 shedding, antiviral therapy and contraceptive use (McClelland *et al.* 2002, Morrison *et al.* 2007). In this study, we investigated the effect of ARVs in P₄-treated cells. In cell cultures, it was observed that p24 levels decreased with AZT (inhibitor of HIV-1) or valacyclovir (inhibitor of HSV-2) treatment. However, in P₄-treated cells, a significant increase in HIV-1 p24 levels were noted (Fig. 5) for HIV-1 or HIV-1/HSV-2 co-infections, which is in agreement with earlier observations that hormonal contraceptive users had lower viral load reduction than non-contraceptive users (Johnson *et al.* 2011).

To further confirm the effects of P₄ on HIV-1, we obtained PBMCs from three blood donors, treated them with P₄ and infected them with HIV-1 alone or co-infected with HSV-2. We found that there were significantly higher p24 levels when the cells were exposed to P₄ (Fig. 6A). Similarly, when PBMCs are co-infected with HSV-2 and exposed to P₄, significant cell death and lower p24 levels were observed (Fig. 6B). This observation with primary cells confirms our earlier finding with CEMss cell line (Fig. 3). It should also be noted that these findings are not consistent for all donors suggesting that there may be donor-to-donor variations.

The P₄-exposed cells infected with HIV-1 or co-infected with HSV-2 upregulate certain inflammatory cytokines

and chemokines. Notably, upregulation of CCL23 was observed with HIV-1/P₄-treated cells, which have not been shown previously in the context of HIV-1 infection. Upregulation of other pro-inflammatory cytokines, such as XCR1 supports HIV-1 replication (Vasilescu *et al.* 2007), and IL-36 β is indicative of the activation of the MAPK and NF- κ B pathway (Tripodi *et al.* 2012). These results suggest that P₄ induces pro-inflammatory responses for HIV-1 infection (Fig. 7A). When cells are co-infected with HSV-2, specific chemokines such as CCL4 and CXCL9 were upregulated (Fig. 7B). Our observations support the previous studies in that CXCL9 upregulation was observed in HSV-2 infection (Huang *et al.* 2012, Rollenhagen *et al.* 2014) and activation of HIV-1 in target cells. In addition, elevation of IL22 and IL5 are indicative of active replication of HIV-1 or HSV-2, which was reported previously. These results suggest that P₄ plays an important role in cellular immunity to infection.

We focused our attention on the extensive cell death in P₄-treated and co-infected cultures. We used the apoptosis array to identify biomolecules involved in cell death. We observed Bcl-2 down-regulation by P₄, which may lead to active replication of HIV-1 or HSV-2 in co-infected cells with subsequent cell death (Fig. 8). Several other studies have reported that Bcl-2 down-regulation by HIV-1 leads to oxidative stress-mediated apoptosis and subsequent depletion of CD4T cells (Strack *et al.* 1996, Cummins & Badley 2010). In addition, previously it was reported that down-regulation of Bcl-2 by HSV-2 leads to low productive infection and apoptosis (Sciortino *et al.* 2006). In this study, we also observed similar effects of lower viral loads and extensive cell death with co-infections. The down-regulation of Bcl-2 by P₄ and co-infection was subsequently confirmed by qPCR and Western blot (Fig. 9A and B). To further support our observation that P₄ treatment leads to cell death, we noted increased caspase-3 expression in co-infected cells, which was reported previously for both HIV-1 and HSV-2 (Cicala *et al.* 1999, Jones *et al.* 2003).

The data presented here clearly show that P₄ supports the replication of two synergistic viruses. Previously, it was shown that Bcl-2 may have a PRE in its promoter sequence (Viegas *et al.* 2004, Yin *et al.* 2007). We show PRE-like sequence in Bcl-2 promoter region of P₄-treated cells (Fig. 10). This observation supports the notion that P₄ has a role in transcriptional regulation of Bcl-2. In HIV-1/HSV-2 co-infected cells, the combined effects of viral and P₄ may be one of the mechanisms that may augment viral

replication. In conclusion, our results provide insights into molecular and cellular mechanisms by which P₄ may enhance the susceptibility to infection of two viruses presented here.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

V R drafted the manuscript and made substantial contributions to conception, design, analysis and interpretation of data; W X and J T performed Western blot; Y G provided technical assistance; K D provided suggestions for the work and reviewed the manuscript; and I H was involved in concept, design, data interpretation and supervision of the project and provided input in revising the manuscript critically for publication.

Disclaimer

The findings and conclusions in this article have not been formally disseminated by the FDA and should not be construed to represent any Agency determination or policy.

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