17β-Estradiol inhibits HIV-1 by inducing a complex formation between β-catenin and estrogen receptor α on the HIV promoter to suppress HIV transcription

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**ABSTRACT**

Human Immunodeficiency virus type 1 (HIV-1) disproportionately affects women, accounting for > 50% of new HIV infections in adults worldwide. While multiple mechanisms may contribute to a greater degree of HIV infection in women than men, we evaluated the direct effect of 17β-estradiol, the most bioactive form of estrogen in women, on HIV replication in peripheral blood mononuclear cells (PBMCs). We demonstrate that 17β-estradiol, in an ERα dependent manner, inhibits HIV replication by activating β-catenin signaling. Specifically, we show for the first time that 17β-estradiol induces a complex formation between ERα and β-catenin which tether on the HIV LTR at ~143 nt site from +1 start site of HIV transcription to repress HIV promoter activity. These studies define a role of 17β-estradiol in inhibiting HIV replication which may impact HIV pathogenesis in women and add to a growing list of viruses that are inhibited by 17β-estradiol through ERα engagement.

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Background

The current HIV pandemic is heavily skewed against women. HIV infection in women accounts for > 50% of new HIV infections in adults worldwide. Once infected, emerging data points to differences in HIV pathogenesis and anti-HIV immune responses in women in comparison to men (Gandhi et al., 2002; Meier et al., 2009; Sterling et al., 2005; Weinberg et al., 2011). Female sex hormones, at least in part, may contribute to gender-based differences in HIV acquisition and pathogenesis. Particularly, female sex hormones regulate immune responses both systemically and in the female genital tract by impacting homing of HIV-susceptible cells to mucosal tissue, regulating cytokine production and antimicrobial peptides induction, and influencing lymphocyte responses (Fahey et al., 1999; Fahey and Wira, 2002; Fung et al., 2013; Kaushic et al., 1998; Lu et al., 2002; Quayle et al., 1998; Rodriguez-Garcia et al., 2013). This intricate balance between host factors under female sex hormone regulation and HIV is likely to impact HIV transmission and pathogenesis in women.

Some clinical data suggest that lower estrogen level (e.g. progesterone dominant stage) is correlated with higher rate of HIV infection and higher plasma viral load. Specifically, during the periovulatory stage of the menstrual cycle, when estrogen dominates, HIV plasma viral load is decreased (Greenblatt et al., 2000; Hanna, 1999; Money et al., 2003). Further, HIV productive infection of human cervical explant tissue predominately occurs in tissue obtained from women in their secretory phase of the menstrual cycle, a stage where progesterone dominates and counteracts estrogen effects (Saba et al., In press). Lastly, women on progesterone-based injectable hormonal contraceptives are more likely to become HIV infected and to transmit the virus to their male partners (Heffron et al., 2012). Taken together, these clinical observations suggest that estrogen dominance may be linked to lower susceptibility of cells to HIV infection.

17β-Estradiol, the most potent estrogen found in humans, exerts an anti-viral effect for a number of viruses. It inhibits hepatitis C virus (HCV) infection at multiple steps including attachment, entry, replication, and post-replication (Hayashida et al., 2010; Murakami et al., 2013). It also inhibits hepatitis B virus (HBV) transcription (Wang et al., 2012), limits herpes simplex virus (HSV) primary infection and reactivation (Gillgrass et al., 2010; Vicetti Miguel et al., 2010), and inhibits rubella virus replication (Robrig et al., 1979). 17β-Estradiol, on the other hand, promotes the replication of adenovirus type 12 (James et al., 1992).

The signal transduction cascade of estrogen is complex and can be engaged by multiple pathways. Canonical estrogen signaling involves...
the activation of its nuclear receptors, estrogen receptor α (ERα) or estrogen receptor β (ERβ), which in turn bind to estrogen responsive elements (ERE) located near or within gene promoters to regulate the transcriptional activity of cognate target genes (Marino et al., 2006). Estrogen also regulates gene expression via noncanonical signaling whereby (Marino et al., 2006) estrogen receptors bind to other transcriptional factors such as Sp1 or Ap-1, leading to either their stabilization of recruitment of transcriptional co-regulators, which in turn bind to their cognate promoters to regulate gene expression. Also, estrogen can bind to either its nuclear receptors or other receptors to initiate second messenger signaling leading to physiologic effects. These signal transduction pathways include ERK/MAPK, p38/MAPK, or PI3K/AKT, to name a few (Marino et al., 2006).

Interestingly, many of the reported 17β-estradiol reported anti-viral effects are mediated by ERα and not ERβ (Hayashida et al., 2010; Murakami et al., 2013; Wang et al., 2012; Gillgrass et al., 2010; Vicetti Miguel et al., 2010; Roehrig et al., 1979).

β-Catenin signaling represses HIV replication in multiple targets, including PBMCs at the level of transcription (Henderson et al., 2012; Kumar et al., 2008; Li et al., 2011). β-Catenin is a transcriptional co-regulator and is the central mediator of the canonical Wnt/β-catenin signaling pathway (Al-Harthi, 2012). β-catenin binds to members of the TCF/LEF family of transcription factors (TCF1, TCF3, TCF-4, and LEF) to modulate hundreds of genes, including those that regulate cell proliferation and survival. β-catenin expression is regulated at the protein level by a multi-protein destruction complex (GSK-3β, APC, Axin, and casein kinase 1). This multi-protein complex leads to the phosphorylation, ubiquitination, and proteasomal degradation of β-catenin. When this destruction complex is destabilized, β-catenin is hypophosphorylated and translocates to the nucleus where it binds to TCF/LEF transcription factors, tethering on their cognate DNA binding sites to regulate genes such as CyclinD, C-Myc, Matrix metalloproteinase 7 and 9, and Axin, to name a few (Al-Harthi, 2012).

At least four TCF-4 binding sites have been identified within the HIV promoter at −143 to −136 nt; −336 to −329 nt; +66 to +73 nt; and +186 to +195 nt from the transcription initiation site (Henderson et al., 2012). While all sites have > 70% homology to the TCF-4 binding sequence, the −143 site has 100% homology to the TCF-4 core (5′-([A/G][A/T][A/T]CAAAG)-3′) and is present in approximately one-third of 500 HIV LTR sequences reported in the Los Alamos gene bank (Henderson et al., 2012). TCF-4 binds at a higher affinity at −143 than at any other site (Henderson et al., 2012). Further, β-catenin is tethered on the HIV LTR at the −143 site and knockdown of either TCF-4 or β-catenin enhances HIV transcription (Henderson et al., 2012; Narasipura et al., 2012). Dual knockdown of β-catenin and TCF4 does not further enhance HIV LTR activity, indicating that these factors work together to repress HIV transcription.

Given that HIV replication is regulated by a complex interplay between virus and host factors and some epidemiologic data linking an estrogen dominant state to lower plasma viral load and infection rate, we directly assessed the role of estrogen (17β-estradiol) on HIV replication in peripheral blood mononuclear cells (PBMCs). We demonstrate that estrogen through engaging ERα leads to association between ERα and β-catenin that tethers on the HIV LTR at −143 nt from the transcription initiation site to repress HIV transcription. These findings provide the first direct association between ERα and β-catenin on the HIV LTR, impacting HIV transcription. Further, the mechanism revealed here may contribute to some clinical observations linking lower HIV load under an estrogen dominant state (Asin et al., 2008; Greenblatt et al., 2000; Money et al., 2003). Most importantly, these studies add to a growing list of viruses (HCV, HBV,HSV, rubella) that are inhibited by ER through ERα engagement (Gillgrass et al., 2010; Hayashida et al., 2010; Murakami et al., 2013; Roehrig et al., 1979; Vicetti Miguel et al., 2010; Wang et al., 2012).

Results

17β-Estradiol inhibits HIV replication in PBMCs

PBMCs were stimulated with anti-CD3/CD28 prior to HIV1ad infection then treated with 17β-estradiol (0–3 nM) or AZT (1 µM). At day seven post infection, HIV replication was monitored by conventional HIV core antigen (p24) ELISA. We demonstrate that 17β-estradiol at 1.5 and 3 nM inhibited HIV replication by approximately 40% (Fig. 1a). 17β-Estradiol at these two doses also inhibited HIV strain (IIIB), a T-tropic/CXCR-4 utilizing strain (Fig. 1b). None of these 17β-estradiol doses (0.001–3 nM) were cytotoxic to PBMCs, as evaluated by the MTS assay (data not shown).

Robust expression of ER α in PBMCs

To determine which estrogen receptor (ERα or ERβ) is involved in 17β-estradiol-mediated inhibition of HIV replication, we evaluated ERα and ERβ expression in PBMCs. Using an antibody that recognizes both ERα and ERβ we show that ER expression is > 80% in all lymphocyte subsets and that T cell activation through anti-CD3/CD28 stimulation did not significantly increase ER expression in T cells (Fig. 2a and b). Using an antibody that is specific for either ERα or ERβ, we demonstrate robust expression of ERα in PBMCs and low level of ERβ expression (Fig. 2c). 17β-Estradiol treatment of PBMCs at doses that inhibited HIV replication (1.5 and 3 nM) did not significantly induce ERα or ERβ expression (Fig. 2c).

17β-Estradiol inhibition of HIV replication is dependent on ERα and is at the level of HIV transcription

To determine the role of ERα or ERβ in estrogen mediated inhibition of HIV, we used an ERα agonist and an ERβ antagonist. We were unable to use an ERα antagonists in PBMCs because ERα antagonists are highly context dependent and in some cases they function as agonist instead of antagonist. For example, 1,3-bis(4-hydroxyphenyl)-4-methyl-5-[4-(2-piperidinylethoxy)phenol]-1H-pyrazole dihydrochloride (known as MPP) depending on the cell type can be an ERα agonist or antagonist (Shang and Brown, 2002).

In PBMCs, ER agonists functioned as antagonist (data not shown). For this reason, we used an ERα agonist (4,4′,4″-4′-Propyl-[1H]-pyrazole-1,3,5-triyl)[trisphenol, PPT] to assess if engaging ERα leads to HIV inhibition. PPT inhibited HIV replication by approximately 40%, a magnitude that was similar to that mediated by 17β-estradiol (Fig. 3a). The ERβ antagonist (2-Phenyl-5,7-bis( trifluoromethyl) pyrazolol[1,5-a]pyrimidin-3-yl]phenol, PHTPP) did not abrogate the ability of 17β-estradiol to inhibit HIV replication in PBMCs (Fig. 3b).

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17β-Estradiol induces β-catenin signaling

Given that 17β-estradiol inhibited HIV infection and we defined β-catenin through its interaction with TCF-4 as a potent repressor of HIV transcription (Henderson et al., 2012; Kumar et al., 2008; Narasipura et al., 2012), we evaluated the relationship between 17β-estradiol and β-catenin signaling. 17β-Estradiol induced active β-catenin expression in PBMCs as evaluated by Western blot (Fig. 5a) and intracellular staining for β-catenin (Fig. 5b). 17β-estradiol also
induced classical target genes of β-catenin-mediated transcription such as axin-2, c-Myc, and cyclinD1 (Fig. 5c). Inhibition of TCF-4 through transfection with a dominant negative TCF-4 construct abrogated the ability of 17β-estradiol to induce axin-2 mRNA, indicating that 17β-estradiol-mediated induction of axin-2 is β-catenin specific (Fig. 5d). Collectively, these data demonstrate that 17β-estradiol activates β-catenin signaling. This is the first study, to our knowledge, to demonstrate 17β-estradiol activation of β-catenin signaling in PBMCs.

**17β-Estradiol induces the stable association of β-catenin and ERα**

We assessed the interaction between ERα and β-catenin in PBMCs by immunoprecipitation (IP) and western blot (WB). While β-catenin was pulled down by ERα IP, the level of β-catenin pulled down by ERα IP was significantly higher when the cells were treated with 17β-estradiol (Fig. 6a). These data indicate that 17β-estradiol potentiates the interaction between ERα and β-catenin, which maybe driven by higher level of β-catenin in response to 17β-estradiol or stabilization of β-catenin in presence of 17β-estradiol to promote its interaction with ERα.

**Inhibiting β-catenin abrogates the ability of 17β-estradiol to inhibit HIV replication**

XAV and IWR-1 Endo are two small molecules described to inhibit β-catenin signaling by stabilizing axin-2, a scaffolding protein that is part of the β-catenin destruction complex (Huang...
et al., 2009). By stabilizing axin-2, β-catenin is phosphorylated, ubiquitinated, and tagged for proteasomal degradation. Treating HIV infected PBMCs in the presence of 17β-estradiol and XAV or IWR-1endo abrogated 17β-estradiol inhibition of HIV replication (Fig. 6b). Collectively, these data indicate that the mechanism by which 17β-estradiol mediates HIV inhibition is both ERα dependent and β-catenin dependent.

**Discussion**

17β-Estradiol levels greatly fluctuate during the menstrual cycle, reaching peak levels in the preovulatory phase ( > 690 to < 2,120 pmol/l) and declining in the luteal phase ( > 300 to < 710 pmol/l) (Harlow and Ephross, 1995). The level of 17β-estradiol is also highly variable among women and within the same woman from one menstrual cycle to the next. For this reason, we evaluated a wide dose of 17β-estradiol effects on HIV replication in PBMCs. We show here that 17β-estradiol at a physiologic dose reported during the preovulatory phase of women of reproductive age (1.5 nM equivalent to 1500 pmol/l) (Harlow and Ephross, 1995) inhibits HIV replication in PBMCs at the level of transcription by engaging the association between β-catenin, TCF-4 and ERα at ~143 nt site of the HIV LTR. The ~143 nt site of the HIV LTR is of a particular importance because it was recently demonstrated to be associated with β-catenin/TCF-4 and SMAR protein to inhibit POLII docking and repress HIV transcription (Henderson et al., 2012). Based on our data we propose that under low level of 17β-estradiol minimal interaction between ERα and β-catenin occurs, which does not protect β-catenin from degradation. However, under a 17β-estradiol dominant state, the interaction between ERα and β-catenin is induced leading to stabilization of β-catenin, its subsequent translocation into the nucleus, and its association with TCF-4 to repress HIV LTR activity. Although the LTR has estrogen responsive elements (EREs) at 343 and 231 nt from transcription initiation site, those sites are not relevant to the association between β-catenin and ERα on the HIV LTR, as deletion of ~143 nt site alone abrogated the tethering of β-catenin, TCF-4, and ERα on the HIV LTR.

Our findings suggest that fluctuations in 17β-estradiol levels during the menstrual cycle and across the reproductive stage of women may impact HIV load and in turn HIV pathogenesis and transmission. Indeed, some studies reported that HIV genital shedding is highest during menses (low estrogen) and declines during the follicular stage (high estrogen). Other studies found that the menstrual cycle leads to a decline in plasma HIV load in the follicular to mid-luteal stage (high estrogen) without impacting HIV vaginal shedding (Greenblatt et al., 2000; Hanna, 1999; Reichelderfer et al., 2000, 2002; Villanueva et al., 2002). Mid-secretory levels of estrogen and progesterone, together, were also found to inhibit HIV replication in vitro in PBMCs (Asin et al., 2008). However, depending on the estrogen dose, there was either no effect or negligible effect on HIV...
replication in this study (Asin et al., 2008). This may be reflection of several experimental factors, the most important of which is the use of charcoal stripping in these studies. In our study, the ability of 17β-estradiol to inhibit HIV replication in PBMCs was conducted without charcoal stripping. Historically, charcoal stripping of FBS was performed to remove endogenous steroid hormones, especially in breast and cervical cancer cell lines that are highly enriched in steroid production. However, emerging evidence indicates that charcoal stripping eliminates other metabolites essential for cellular health (Cao et al., 2009). In our experience, charcoal stripping of FBS compromises the survival of PBMCs (data not shown). For this reason and because untreated cultures serve as background for endogenous 17β-estradiol/steroids levels, we have elected not to use charcoal stripping in our cultures and showed that 17β-estradiol at preovulatory doses inhibits HIV replication and revealed the mechanism by which it does so.

Cross-talk between 17β-estradiol and Wnt/β-catenin has been described in mouse and human epithelial cells (Hou et al., 2004; Kouzmenko et al., 2004). We provide the first evidence in human PBMCs that 17β-estradiol induce ERα and β-catenin interaction to potentiate HIV repression. In human and mouse uterine epithelial cells, 17β-estradiol induces β-catenin gene expression and activates a number of Wnt proteins, including Wnt4, Wnt5a, and frizzled receptor 2 (Hou et al., 2004). Further, ERα and β-catenin in human colon and breast cancer cells precipitate within the same immune complexes (Kouzmenko et al., 2004), demonstrating that ERα and β-catenin are physically associated with each other. Exogenous 17β-estradiol further enhances this physical interaction of ERα and β-catenin. The result of ERα and β-catenin physical association is a reciprocal regulation of cognate target genes (Kouzmenko et al., 2004).

ERα represses the replication of a number of viruses, including HCV, HBV, HSV, and rubella (Gillgrass et al., 2010; Hayashida et al., 2010; Murakami et al., 2013; Roehrig et al., 1979; Vicetti Miguel et al., 2010; Wang et al., 2012). We show here that ERα also inhibits HIV replication. These findings indicate that ERα engages direct anti-viral pathways to limit replication of some viruses, which may mediate gender-specific differences in susceptibility to virus infection and/or spread. In the case of HCV, women are more likely to clear HCV in acute stage than men and female sex hormones have been suggested as a potential factor for better clearance (Bakr et al., 2006). Some studies have also alluded to women having a 2–6 fold lower plasma viral load than men during acute and chronic HIV infection (Delmas et al., 1997; Gandhi et al., 2002; Lemly et al., 2009; Meditz et al., 2011; Sterling et al., 1999, 2001), although additional studies are needed to assess the direct contribution of female sex hormones on HIV replication and progression in the clinical setting suggest.

Materials and methods

Ethics statement

This study was approved by Rush Institutional Review Board (09040706-IRB01) and participants’ written consent was provided.
Reagents

AZT, 17β-estradiol, PHTPP (4-[2-phenyl-5,7-bis(trifluoromethyl)pyrazolo[1,5-a]pyrimidin-3-yl]phenol), PPT (4,4′,4′′-(4-propyl-[1H]-pyrazole-1,3,5-triyl)trisphenol), anti-total β-catenin antibody, amino acids 768-781 (cat. C2206), and anti-GAPDH antibody (cat. G9545) purchased from Sigma Aldrich (St. Louis, MO, USA). Anti-active β-catenin antibody clone 3C196 purchased from US Biologicals (San Antonio, TX, USA). Anti-ERα antibody clone 62A3 purchased from Cell Signaling (Boston, MA). Anti-ERβ antibody clone 14C8 purchased from Abcam (Cambridge, MA, USA). XAV 939 and IWR-1-endo purchased from Tocris Bioscience (Bristol, United Kingdom).

Isolation and treatment of PBMCs

PBMCs were isolated from venous blood of healthy women of reproductive age and not on hormone contraceptives using lymphocyte separation medium (Lonza Biotech, Allendale, NJ, USA) and density centrifugation. The cells were suspended in phenol red free-RPMI complete medium supplemented with 10% fetal bovine serum (FBS), 1% L-glutamine, 1% penicillin, 1% streptomycin, and 20 U/ml IL-2. PBMCs were left unstimulated or stimulated with 1 μg/ml each of anti-CD3/CD28 antibodies (BD Biosciences, Franklin Lake, NJ, USA) for 24 h at 37°C under 5% CO₂.

Fig. 5. 17β-estradiol induces β-catenin signaling. PBMCs were stimulated and treated with 17β-estradiol (1.5 nM) for three days. Western blot and densitometry quantification for active β-catenin measured by Western blot is shown in (a). Flow cytometric analysis of active β-catenin expression in CD4+ and CD8+ T cells is shown in (b). Level of downstream targets of Wnt/β-catenin pathway (axin-2, c-Myc, cyclin D-1) post 17β-estradiol treatment performed by real-time RT-PCR is shown in (c). GAPDH was used as endogenous control and data shown as fold change relative to untreated cells. In (d) PBMCs were stimulated with antiCD3/CD28 for 48 h and nucleofected with a dominant negative plasmid for TCF-4 (dsTCF4) or backbone vector (pcDNA). Post nucleofection the cells were treated with 17β-estradiol (1.5 nM) for three days and axin-2 and c-myc mRNA level was evaluated by real-time RT-PCR. GAPDH was used as endogenous control. Data is representative of a minimum of three experiments and analyzed by Student T-test; *p < 0.05.
Immunofluorescence staining and flow cytometric analysis

Up to nine-color flow cytometric analyses were performed using a FACSCalibur cytometer with CellQuest (BD) and LSR-II cytometer with Diva software (BD). Aqua Live/Dead staining was used. The following antibodies from BD Biosciences were used for cell surface staining as per instructions: For T cell subsets, CD3-alexafluor 700, CD14-pacific blue, CD8-phycocerythrin-cyanine (PerCP-Cy) 5.5, CD4-allophycocyanin (APC), CD19 APC-H7; for dendritic cell subsets, CD3-PerCP-Cy7, CD123-phycocerythrin (PE), HLA-DR PerCP, CD14-pacific blue, CD11c-APC, CD56/CD16-alexafluor 700 and CD19/CD20-APC-H7. Proper isotypes were purchased from same vendor for primary stain. Intracellular flow cytometry staining was performed using CytoPerm/fix and CytoWash reagents, according to recommendations of the supplier (BD Biosciences). For ERs staining, 10 μl of a FITC-conjugated rabbit monoclonal antibody that recognizes both ERα and ERβ (clone SP1, Abcam, Cambridge, MA, USA) was used. For active β-catenin, 200 μl of 0.05 μg dilution in PBS of mouse anti-human active β-catenin un-conjugated antibody (clone 3C196, US Biological, Marblehead, MA) was used followed by 1 μl of a secondary anti-mouse F(ab')2 FITC-conjugated antibody at 2 mg/ml. The cells were subsequently washed and fixed with 2% paraformaldehyde.

Cell viability, HIV infection, and p24 assay

Cell viability was assessed by standard MTS assay (Promega, Madison, WI). HIV-1Δ700 or HIVΔ4168 at 2 ng HIV p24/10⁶ cells was added to anti-CD3/CD28 stimulated PBMCs and incubated for 4–6 h at 37 °C under 5% CO₂. Cells were then washed with PBS three times to remove un-bound virus, suspended in phenol red free-RPMI complete medium with 20 units/ml IL-2, treated as indicated per experimental condition, and plated in 96 well U-bottom plate at 200,000 cells/200 μl volume of the media in quadruplicates for 7 days at 37 °C, 5% CO₂. HIV p24 assay was performed using p24 ELISA kit (SAIC-Frederick, MD) according to the manufacturer's protocol.

Western blot and immunoprecipitation (IP)

The cells were lysed using RIPA buffer (Sigma) with 10% protease inhibitors and protein content measured by Pierce BCA assay (Thermo Fisher, Barrington, IL). Western blot was performed as described (Narasipura et al., 2012), using anti-active β-catenin antibody at 1:8000, anti-total β-catenin antibody at 1:8000, anti-ERα at 1:1000, anti ERβ at 1:1000, and/or anti-GAPDH at 1:30,000. Secondary antibodies included goat anti-mouse HRP (Pierce) at 1:8000, anti-total β-catenin antibody at 1:8000, and/or anti-GAPDH at 1:30,000. Secondary antibodies included goat anti-mouse HRP (Pierce) at 1:50,000 and goat anti-rabbit HRP (Cell Signaling) at 1:60,000 for GAPDH. Densitometries were measured by ImageJ software. For IP, cell lysate was pre-cleared with 25 μl of protein A/G magnetic
beads (Thermo Scientific), cross-linked with specific antibodies using Protein A/G MagSepharose and SpinTrap Buffer Kit (GE Healthcare, Piscataway, NJ) as outlined by manufacturer.Denatured proteins were then resolved on a 12% SDS gel. IgG controls were run in parallel.

RNA isolation and real time PCR

Total RNA was isolated from PBMCs using Trizol (Life Technologies, Grand Island, NY). Complimentary DNA synthesis was performed using qScript cDNA Supermix (Quanta BioSciences, Gaithersburg, MD). Real-time PCR was performed employing SsoFast EvaGreen Supermix with low ROX (BioRad, Hercules, CA) in a 7500 real time PCR system (Applied Biosystems, Carlsbad, CA) to quantify target genes. Reaction conditions were: 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s, 60 °C for 1 min and 95 °C for 30 s. A melting curve stage was included to ensure amplification of a single product. Change in binding was calculated by relative quantification using the comparative threshold cycle (Ct) method. Results were reported as fold change relative to IgG control (ΔCt=ΔCt target−ΔCt IgG control; fold change relative to IgG control=2−ΔΔCt).

Statistics

When the data was distributed normally, ANOVA and post-hoc tests were used. When the data was not normally distributed, nonparametric analysis was performed. All tests assumed a two-sided significance level of 0.05. GraphPad Instat 3 software (San Diego, CA) was used for data analysis.

Funding

This work was supported by funding from the National Institute of Allergy and Infectious Diseases [PO A108297] and the National Institute of Neurological Disorders and Stroke [R01NS060632].

Acknowledgments

We thank the participants in this study for donating blood. The following reagents were obtained from NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH, Frederick, MD: Human recombinant interleukin (rIL-2) provided by Dr. Maurice Gately, Hoffman-LaRoche Inc, HIV 1-BalΔΔ provided by Dr. Suzanne Gartner, Dr. Mikulas Popovic and Dr. Robert Gallo, and HIV 1-BalΔΔ provided from Dr. Robert Gallo to the repository.

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