Epub ahead of print December 30, 2009 - doi:10.1189/jlb.0809556

JLB

Brief Conclusive Report

Restricted HIV-1 replication in placental macrophages is caused by inefficient viral transcription

K. García-Crespo, C. Cadilla,† R. Skolasky,‡ and L. M. Meléndez*,1*

Departments of *Microbiology and Medical Zoology and †Biochemistry, University of Puerto Rico, Medical Sciences Campus, San Juan, Puerto Rico; and ‡Department of Orthopaedic Surgery, Johns Hopkins University, Baltimore, Maryland, USA

RECEIVED AUGUST 18, 2009; REVISED NOVEMBER 12, 2009; ACCEPTED DECEMBER 7, 2009. DOI: 10.1189/jlb.0809556

ABSTRACT

HIV-infected PM show restricted replication as compared with MDM. We aimed to determine at what point in the viral replication cycle this restriction occurs in PM as compared with MDM. We performed Alu-LTR PCR for proviral DNA to detect differences in HIV integration, real-time RT-PCR to measure *env* and *gag* mRNA levels, and Western blot analysis to detect differences in viral protein expression. PM and MDM were infected with HIV-1 BaL, and DNA was extracted after 24 h and at 6 days p.i. for real-time PCR studies. At 6 and 12 days p.i., cells were lysed for Western blot analyses. We found no difference in viral integration between PM and MDM but significantly lower levels of viral protein gp120 in PM than in MDM. Real-time RT-PCR analyses revealed 24-fold less *env* mRNA and tenfold less *gag* mRNA in PM. These results suggest that HIV-1 restriction in PM occurs at the level of transcription. This study is significant, as it advances our understanding of HIV-1 infection in PM and its contribution to decreased in utero vertical transmission. *J. Leukoc. Biol.* 87: 000–000; 2010.

Introduction

The World Health Organization estimates that 17.7 million women worldwide have been infected with HIV-1, as have ~2.3 million of the children born to them [1]. Mother-to-child transmission of HIV is an important public health concern, but the mechanism underlying this transmission is not understood completely. Despite the large number of infected children, perinatal transmission of HIV-1 is relatively low, ranging from 10% to 25% without AZT treatment [2]. From studies done on infants born to HIV-positive mothers, it is believed that as many as 80% of transmissions occur at the moment of birth [3]. This suggests that the placenta is a natural barrier to the virus.

Inside the placenta, the main targets for HIV-1 infection are the PM. These cells can be infected productively with laboratory strains and primary isolates. Despite this susceptibility, our laboratory and others [4–8] have demonstrated that HIV-1 production by PM is significantly lower than that observed in MDM. PM express lower levels of the CD4, CXCR4, and CCR5 receptors than do MDM [9, 10]. LPS-stimulated PM produce lower levels of proinflammatory cytokines than do LPS-stimulated MDM, but there is no correlation of cytokine secretion with HIV-1 infection [8]. PM also produce the same levels of β-chemokines as MDM [11] after LPS stimulation. This low susceptibility to infection could be a result of decreased HIV entry caused by low coreceptor expression or to restriction at other stages in the viral replication cycle in these cells. In the current study, we compared viral integration, transcription, and protein expression in HIV-infected PM and MDM to determine at what stage(s) HIV-1 restriction occurs in PM.

MATERIALS AND METHODS

Isolation and culture of PM and MDM

To isolate PM, placentas were collected from HIV-seronegative women and processed as described before [7, 8, 10–12]. To isolate MDM, PBMC were obtained from the blood of HIV-1-seronegative donors by Ficoll density gradient centrifugation and purified by adherence. Alternatively, when large quantities of cells were needed, MDM were cultured from elutriated monocytes by adherence, as described previously [13]. MDM were then seeded in 25 cm^2 flasks (15×10^6 cells) and in six-well plates (3.0×10^6 cells/well) and supplemented with monocyte medium [70% RPMI, 10% human serum, 20% FBS (Sigma Chemical Co., St. Louis, MO, USA)]. Cells obtained by leukopheresis were supplemented with cell medium (87% DMEM, 10% human serum, 2% L-glutamine, 0.1% M-CSF, 0.2% gentamycin, 0.04% Cipro). At Day 7 of culture, cells were incubated for 1 h at 37°C with HIV-BAL (25 ng p24/2.0×10^5 cells) and cultured for 1, 6, and 12 days. To monitor virus production, cell supernatants were collected at Days 3, 6, 9, and 12 p.i., and HIV p24 levels were measured using an ELISA assay (XpressBio, West Thurmont, MD, USA).

Abbreviations: Ct=comparative threshold, DC=dendritic cell, LTR=long-terminal repeat, MDM=monocyte-derived macrophage(s), NIAID=National Institute of Allergy and Infectious Diseases, p.i.=postinfection, PM=placental macrophage(s)

1. Correspondence: University of Puerto Rico Medical Sciences, Microbiology, Biomedical Building I, Rm. 310 Medical Center, San Juan, PR 00935, Puerto Rico. E-mail: loyda.melendez@upr.edu

Real-time PCR for detection of proviral DNA

Cells were detached 24 h after infection, and DNA was extracted from the cells using the QIAamp blood kit (Qiagen, Valencia, CA, USA). After extraction, a nested Alu-LTR PCR was done to detect the proviral DNA as described [14]. The primer sequences for the first amplification step were as follows: Alu-LTR (sense) 5′-TCCCAGCTACTCGGGAGGCTGAGG-3′ and Alu-LTR (antisense) 5′-AGGCAAGCTTTATTGAGGCTTAAGC-3′. Real-time PCR was done for the second amplification step using the Qiagen QuantiFast SYBR Green PCR kit. Primers used for the second step were as follows: (sense) 5′-CACACACAAGGCTACTTCCCT-3′ and (antisense) 5′-GCCACTCCCCIGTCCCGCCC-3′. Samples were run and analyzed using the iCycler system (Bio-Rad, Hercules, CA, USA). A standard curve was done using the OM10.1 cell line (AIDS Reference and Reagent Program, Germantown, MD, USA) to calculate copy number of integrated provirus in PM and MDM.

Western blot analysis of gp120 expression

PM and MDM cultures were lysed at 6 and 12 days p.i. for Western blot analysis of HIV envelope glycoprotein gp120 expression. Cell lysates (20 μg) were separated on a 4–20% Tris-HCl Ready Gel and then transferred to a nitrocellulose membrane (Bio-Rad). Membranes were incubated overnight at 4°C with the following primary antibodies: polyclonal goat anti-HIV gp120 (Abcam, Cambridge, MA, USA; 1:5000) and monoclonal mouse anti-human α-tubulin (1:5000). Thereafter, membranes were incubated for 1 h at room temperature with HRP enzyme-conjugated donkey anti-goat and goat anti-mouse secondary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at a 1:40,000 and a 1:5000 dilution, respectively. Proteins were detected using the Supersignal West Femto system (Pierce, Rockford, IL, USA).

Real-time PCR for detection of env and gag mRNA

Cells were detached at 6 days p.i., and total RNA was extracted using the RNAeasy kit (Qiagen). The cDNA was obtained using the QuantiTect RT kit (Qiagen). The relative amount of product was determined by the Ct method. Each PM sample was normalized to β-actin and expressed as fold-change based on comparison with control MDM. Primers used were as follows: *env* (forward) 5′-GGGGACCAGGGAGAGCATT-3′ and *env* (reverse) 5′-TGGGTCCCCTCCTGAGGA-3; *gag* (forward) 5′-CCAGATGAGAGAACCAAGGG-3′ and *gag* (reverse) 5′-TTGTGAAGCTTGCTCGGCTCT-3′.

Statistical analysis

Laboratory analyses on detection of proviral DNA, Western blot analysis of gp120 expression, and expression of p24 antigen were assessed to address the main objectives of the current study, namely comparison between MDM and PM. Each laboratory analysis was performed in multiple replicates. The results of these replicates were averaged across samples. These average values were used in subsequent analyses. All measures were tested for normality using the Shapiro-Wilk test. Given the small sample size, nonparametric Kruskal-Wallis test statistics were used to compare median expression across cell type and/or days of infection. All analyses were performed using Statistical Analysis Software, Version 9.2 (SAS Institute, Cary, NC, USA).

RESULTS AND DISCUSSION

To determine if the HIV-1 restriction observed in PM was a result of a difference in integration, we measured the copy number of integrated proviruses in PM and MDM by real-time Alu-LTR-nested PCR using DNA from OM10.1 cells as a standard for copy number. Samples from three PM and three MDM infections were analyzed using the standard curve method. No significant difference was found in integrated provirus copy number between the two cell types (P=.513; **Fig. 1**).

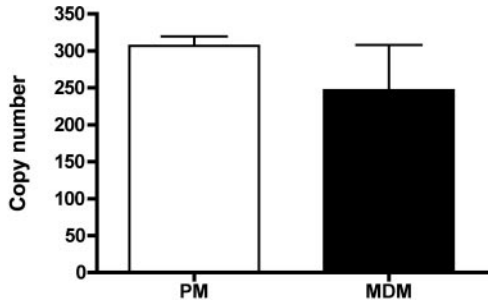

Figure 1. Proviral copy number. PM and MDM cultures were infected with HIV-BaL. After 24 h, DNA was isolated, and real-time Alu-LTR PCR was done to measure integrated provirus. Values are the average of three PM and three MDM samples run in duplicate in four independent PCR experiments. P = 0.513, Kruskal-Wallis test.

These results indicate that the restriction does not occur at or prior to viral integration.

Intracellular viral protein expression levels were compared between PM and MDM to determine if HIV-1 replication in PM was decreased at this level. Cells were lysed at 6 and 12 days p.i., and expression of the viral protein gp120 was determined by Western blot analysis. This analysis demonstrated that gp120 is produced at lower levels by PM than by MDM, with significance on Day 6 p.i. (P=.004 at Day 6; P=.150 at Day 12; **Fig. 2**). Decreased gp120 levels in PM may indicate restriction of viral proteins in PM and suggest a decreased viral gene transcription or protein synthesis and processing in these cells.

We also measured p24 protein levels in the supernatant of PM and MDM cultures at different time-points by ELISA to monitor the progress of the infection. A rise in p24 levels was observed over time in both cell types, thus indicating a productive infection (PM: P=.043; MDM: P=.045; **Fig. 3**). After day 3 p.i., p24 levels were generally lower in supernatants from PM cultures, with significant differences seen at 9 and 12 days p.i. (P=.049 for Days 9 and 12; Fig. 3). This result confirms previous work from our group that found lower p24 levels in PM cultures [8]. Such results further confirm a defect in viral production in PM.

As viral integration was not affected in PM, but expression of gp120 and p24 was significantly lower than in MDM, we proceeded to determine if the expression of *env* and *gag* mRNA was decreased. Total RNA from PM and MDM was isolated after 6 days p.i., and mRNA levels were determined by real-time PCR. Indeed, PM showed a 24-fold decrease in *env* mRNA levels [average Ct values; experimental, PM (28.0); control, MDM (25.0)]. For *gag* mRNA, a tenfold decrease, as compared with MDM, was observed [average Ct values; experimental, PM (30.0); control, MDM (27.0; **Fig. 4**)]. This marked reduction in viral gene expression suggests that viral restriction in PM occurs at the level of transcription.

In the current study, we determined the stages at which restricted HIV-1 replication in placental macrophages could occur to better understand the mechanisms involved in their low susceptibility to infection. We demonstrated that PM harbor

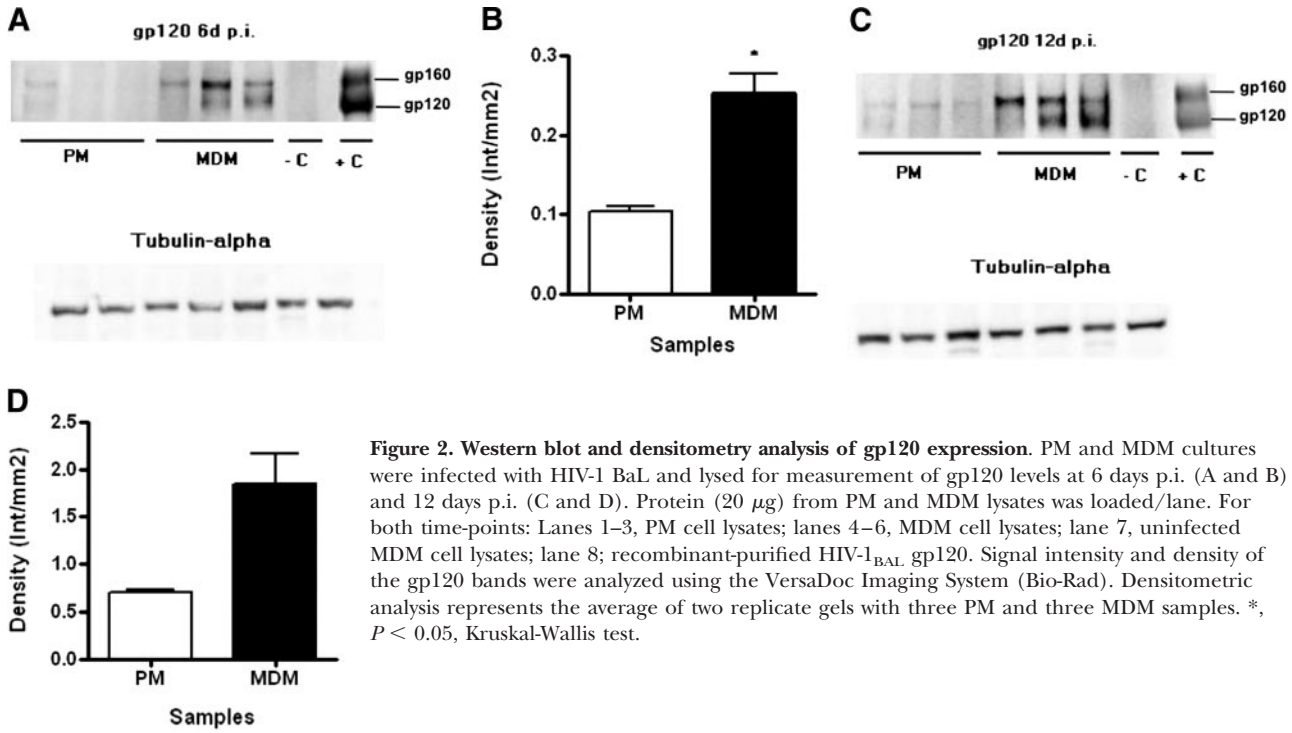

Figure 2. Western blot and densitometry analysis of gp120 expression. PM and MDM cultures were infected with HIV-1 BaL and lysed for measurement of gp120 levels at 6 days p.i. (A and B) and 12 days p.i. (C and D). Protein (20 μg) from PM and MDM lysates was loaded/lane. For both time-points: Lanes 1–3, PM cell lysates; lanes 4–6, MDM cell lysates; lane 7, uninfected MDM cell lysates; lane 8; recombinant-purified HIV-1_{BAL} gp120. Signal intensity and density of the gp120 bands were analyzed using the VersaDoc Imaging System (Bio-Rad). Densitometric analysis represents the average of two replicate gels with three PM and three MDM samples. *, $P < 0.05$, Kruskal-Wallis test.

the same levels of integrated HIV provirus as do MDM, which suggests that there is no difference in RT or integration events in PM as compared with MDM and also that the reported low expression of CCR5 by PM [10] does not account for the low viral production by these cells. We found that PM express significantly lower levels of intracellular envelope protein, in addition to p24, already measured from cell supernatants, as a result of an apparent restriction of viral gene transcription, as demonstrated by a 24-fold and a tenfold decrease in *env* and *gag* mRNA, respectively.

Other tissue macrophages, such as nonactivated microglia and lamina propia macrophages, have also been shown to have low virus production. Although these cell types show low permissiveness to HIV-1, the point of viral restriction is different from PM. Low infection of intestinal macrophages appears to be a result of a low expression of CCR5 on the cell surface [15]. However, for PM, which also express low CCR5 levels, we did not detect lower copies of integrated provirus, as was observed in a study by Lin et.al. [16] in 2002, where low CCR5 levels resulted in low levels of integrated provirus as compared with cells expressing high CCR5. In the case of nonactivated microglia, restriction has been identified as a defect in *gag* protein processing and virus release, as no defect was detected in *gag* mRNA production [17]. These findings suggest that PM differentially express unique cellular factors, as compared with other macrophages that prevent efficient transcription of viral genes. Previous reports from our group examining the pro-

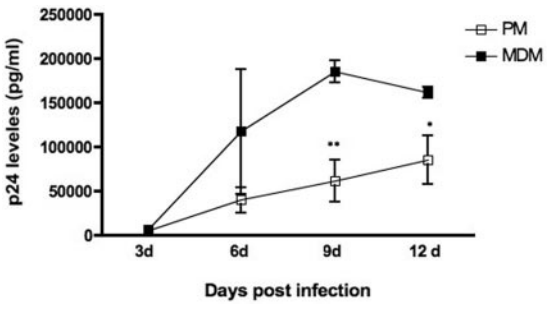

Figure 3. HIV-1 infection of placental and MDM. Three million cells were cultured in six-well plates infected with HIV-1 BaL and kept in culture for 12 days. p24 levels were measured by an ELISA. Experiments are the average of three PM and three MDM samples. *, $P = 0.05$; **, $P < 0.01$, Kruskal-Wallis test.

Figure 4. Fold change in gag and env mRNA levels. PM and MDM cultures were infected with HIV-1 BaL, and total RNA was extracted at 6 days p.i. mRNA levels were measured by real-time PCR. Three PM and three MDM samples were run in duplicate wells in three independent real-time PCR experiments. Values presented are the fold-changes of each PM sample as compared with the average of the three MDM samples. For *gag*, SEM = ±0.409; for *env*, SEM = ±0.909.

teome and secretome of PM identified differentially expressed proteins in these cells as compared with MDM. Of special interest were cystatin B and peroxiredoxin 5, both of which could interfere with viral transcription [18, 19]. A recent study found that cystatin B interacts with STAT-1, and tyrosine phosphorylation of this protein was higher in PM. This type of phosphorylation in STAT-1 is associated with decreased HIV transcription [20]. Further studies are needed to elucidate the signaling pathways for the observed restriction in PM.

Other related cell types, such as monocytes and mature DCs, also present restriction to HIV-1 replication. In the case of mature DCs, the inhibition of virus replication has been shown to occur mainly at the transcription level [21], but the cellular factors that might be responsible for this block have not been identified. Restriction in monocytes has been identified at two points in the viral life cycle: before RT [22] and at the transcriptional level [23]. Restrictions in the early viral life cycle have been related to the existence of enzymatically active APOBEC3G in monocytes, which later becomes inactive, as these cells differentiate into macrophages; the latter restriction has been associated with differentiation-dependent expression of CycT1. As differential expression and activity of both of these cellular factors are related specifically to differentiation, it is unlikely that they play a role in the restriction that we have observed in PM.

The present study has the limitation of small sample size used; nonetheless, we believe that the data presented show encouraging results that warrant further exploration of HIV-1 restriction in these cells. The current study contributes important information that advances our understanding of the HIV-1 infection process in PM. It also identifies a different point of restriction as compared with that of other tissue macrophages. This information provides a unique opportunity to identify new cellular factors needed for efficient transcription of viral genes.

AUTHORSHIP

Katia García-Crespo: experimenal design, performed experiments, and wrote the manuscript; Carmen L. Cadilla: experimental design and manuscript revision; Loyda Meléndez: principal investigator, experimental design, and manuscript revision; Richard Skolasky statistical analysis.

ACKNOWLEDGMENTS

Dr. Meléndez's laboratory staff and the grants from the National Institutes of Health (NIGMS-MBRS-SCORE S06GM0822 and NINDS-SNRP-U54NS430Ms) are acknowledged. Ms. Katia García-Crespo received MBRS-RISE NIGMS R25GM061838 support during her graduate studies. We thank Dr. Howard Gendelman for providing elutriated monocytes. The OM10.1 cell line was obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, from Dr. Salvatore Butera and HIV-1_{BaL} gp120 from the Division of Acquired Immunodeficiency Syndrome, NIAID. We thank Dr. Edna Aquino and Jessica Rentas for technical assistance and helpful suggestions in experimental design and analysis of data of all real-time PCR experiments.

REFERENCES

1. Joint United Nations Program on HIV/AIDS (2006) *2006 AIDS Epidemic Update,* www.unaids.org.
2. Anderson, V. M. (1997) The placental barrier to maternal HIV infection. *Obstet. Gynecol. Clin. North Am.* **24,** 797–820.
3. Mofenson, L. M. (1997) Mother-child HIV-1 transmission: timing and determinants. *Obstet. Gynecol. Clin. North Am.* **24,** 759–784.
4. Kesson, A. M., Fear, W. R., Kazazi, F., Mathijs, J. M., Chang, J., King, N. J., Cunningham, A. L. (1993) Human immunodeficiency virus type 1 infection of human placental macrophages in vitro. *J. Infect. Dis.* **168,** 571–579.
5. Kesson, A. M., Fear, W. R., Williams, L., Chang, J., King, N. J., Cunningham, A. L. (1994) HIV infection of placental macrophages: their potential role in vertical transmission. *J. Leukoc. Biol.* **56,** 241–246.
6. McGann, K. A., Collman, R., Kolson, D. L., Gonzalez-Scarano, F., Coukos, G., Coutifaris, C., Strauss, J. F., Nathanson, N. (1994) Human immunodeficiency virus type 1 causes productive infection of macrophages in primary placental cell cultures. *J. Infect. Dis.* **169,** 746–753.
7. Melendez-Guerrero, L. M., Holmes, R., Backé, E., Polliotti, B., Ibegbu, C., Lee, F., Panigel, M., Schwartz, D., Huddleston, J., Nahmias, A. (1994) In vitro infection of Hofbauer cells with a monocyte-tropic strain of HIV-1. *Placenta* **15,** 33–45.
8. Plaud-Valentin, M., Delgado, R., Garcia, V., Zorrilla, C., Gandia, J., Melendez-Guerrero, L. M. (1999) HIV infection of placental macrophages: effect on the secretion of HIV stimulatory cytokines. *Cell. Mol. Biol. (Noisy-le-grand)* **45,** 423–431.
9. Fear, W. R., Kesson, A. M., Naif, H., Lynch, G. W., Cunningham, A. L. (1998) Differential tropism and chemokine receptor expression of human immunodeficiency virus type 1 in neonatal monocytes, monocyte-derived macrophages, and placental macrophages. *J. Virol.* **72,** 1334–1344.
10. Torres, G., Garcia, V., Sanchez, E., Segarra, A., Patterson, B. K., Melendez-Guerrero, L. M. (2001) Expression of the HIV-1 co-receptors CCR5 and CXCR4 on placental macrophages and the effect of IL-10 on their expression. *Placenta* **22** (Suppl. A), S29–S33.
11. Melendez, J., Garcia, V., Sanchez, E., Delgado, R., Torres, G., Melendez-Guerrero, L. M. (2001) Is decreased HIV-1 infectivity of placental macrophages caused by high levels of β-chemokines? *Cell. Mol. Biol. (Noisy-le-grand)* **47,** OL51–OL59.
12. Uren, S., Boyle, W. (1985) Isolation of macrophages from human placenta. *J. Immunol. Methods* **78,** 25–34.
13. Gendelman, H. E., Orenstein, J. M., Martin, M. A., Ferrua, C., Mitra, R., Phipps, T., Wahl, L. A., Lane, H. C., Fauci, A. S., Burke, D. S., et al. (1988) Efficient isolation and propagation of human immunodeficiency virus on recombinant colony-stimulating factor 1-treated monocytes. *J. Exp. Med.* **167,** 1428–1441.
14. Chun, T. W., Stuyver, L., Mizell, S. B., Ehler, L. A., Mican, J. A., Baseler, M., Lloyd, A. L., Nowak, M. A., Fauci, A. S. (1997) Presence of an inducible HIV-1 latent reservoir during highly active antiretroviral therapy. *Proc. Natl. Acad. Sci. USA* **94,** 13193–13197.
15. Smith, P. D., Li, L., Meng, G. (1999) Mucosal events in the pathogenesis of human immunodeficiency virus type 1 infection. *J. Infect. Dis.* **179** (Suppl. 3), S436–S440.
16. Lin, Y. L., Mettling, C., Portales, P., Reynes, J., Clot, J., Corbeau, P. (2002) Cell surface CCR5 density determines the postentry efficiency of R5 HIV-1 infection. *Proc. Natl. Acad. Sci. USA* **99,** 15590–15595.
17. Albright, A. V., Vos, R. M., Gonzalez-Scarano, F. (2004) Low-level HIV replication in mixed glial cultures is associated with alterations in the processing of p55(Gag). *Virology* **325,** 328–339.
18. Luciano-Montalvo, C., Ciborowski, P., Duan, F., Gendelman, H. E., Melendez, L. M. (2008) Proteomic analyses associate cystatin B with restricted HIV-1 replication in placental macrophages. *Placenta* **29,** 1016–1023.
19. Garcia, K., Garcia, V., Perez Laspiur, J., Duan, F., Melendez, L. M. (2009) Characterization of the placental macrophage secretome: implications for antiviral activity. *Placenta* **30,** 149–155.
20. Luciano-Montalvo, C., Meléndez, L. M. (2009) Cystatin B associates with signal transducer and activator of transcription 1 in monocyte-derived and placental macrophages. *Placenta* **30,** 464–467.
21. Bakri, Y., Schiffer, C., Zennou, V., Charneau, P., Kahn, E., Benjouad, A., Gluckman, J. C., Canque, B. (2001) The maturation of dendritic cells results in postintegration inhibition of HIV-1 replication. *J. Immunol.* **166,** 3780–3788.
22. Triques, K., Stevenson, M. (2004) Characterization of restrictions to human immunodeficiency virus type 1 infection of monocytes. *J. Virol.* **78,** 5523–5527.
23. Dong, C., Kwas, C., Wu, L. (2009) Transcriptional restriction of human immunodeficiency virus type 1 gene expression in undifferentiated primary monocytes. *J. Virol.* **83,** 3518–3527.

KEYWORDS:
primary cells · monocytes · macrophages · viral mRNA · RT-PCR · Western blots