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Cystatin B and HIV regulate the STAT-1 signaling circuit in HIV-infected and INF-β-treated human macrophages

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Abstract Cystatin B is a cysteine protease inhibitor that induces HIV replication in monocyte-derived macrophages (MDM). This protein interacts with signal transducer and activator of transcription (STAT-1) factor and inhibits the interferon (IFN-β) response in Vero cells by preventing STAT-1 translocation to the nucleus. Cystatin B also decreases the levels of tyrosine-phosphorylated STAT-1 (STAT-1PY). However, the mechanisms of cystatin B regulation on STAT-1 phosphorylation in MDM are unknown. We hypothesized that cystatin B inhibits IFN-β antiviral responses and induces HIV replication in macrophage reservoirs through the inhibition of STAT-1 phosphorylation. Macrophages were transfected with cystatin B siRNA prior to interferon-β treatment or infected with HIV-ADA to determine the effect of cystatin B modulation in STAT-1 localization and activation using immunofluorescence and proximity ligation assays. Cystatin B decreased STAT-1PY and its transportation to the nucleus, while HIV infection retained unphosphorylated STAT (USTAT-1) in the nucleus avoiding its exit to the cytoplasm for eventual phosphorylation. In IFN-β-treated MDM, cystatin B inhibited the nuclear translocation of both, USTAT-1 and STAT-1PY. These results demonstrate that cystatin B interferes with the STAT-1 signaling and IFN-β-antiviral responses perpetuating HIV in macrophage reservoirs.

Keywords HIV · Cystatin B · STAT-1 · Macrophages · IFN-β

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Introduction

HIV can infect macrophages without inducing cell death, evading the immune system. The eradication of this virus macrophage reservoir represents an important research challenge. One potential approach identified to diminish HIV replication in macrophage reservoirs is the inhibition of cystatin B (Luciano-Montalvo et al. 2008; Luciano-Montalvo and Meléndez 2009; Rivera et al. 2014). Cystatin B is a cysteine protease inhibitor expressed in most cell types and tissues, however additional functions have been described (Luciano-Montalvo et al. 2008; Aboud et al. 2014; Romas et al. 2014). Of interest, is the finding that cystatin B has been found in low levels in placental macrophages (a less permissive cell for HIV replication), in contrast to high levels found in monocyte-derived macrophages (MDM), which replicates HIV at higher levels (Luciano-Montalvo et al. 2008).

HIV infection in macrophages are known to activate the Signal Transducer and Activator of Transcription-1 (STAT-1) (Magnani et al. 2003), which is a member of a protein family that responds to growth factors and cytokines, such as interferon. When cytokines bind to specific membrane receptors, it induces a signal that stimulates the activation of receptor-associated Janus kinase (JAK). This activated JAK transfers a phosphoryl group to specific residues in the STAT protein. Then, the phosphorylated dimerized STAT enters the nucleus where it can activate transcription of several genes, including interferon-induced genes. This phosphorylated STAT in the nucleus binds specific gene sequences and induces an antiviral response.

Our group has previously reported that cystatin B induces HIV replication in MDM (Luciano-Montalvo et al. 2008) and

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interacts with STAT-1 (Luciano-Montalvo and Meléndez 2009). Furthermore, we have reported that cystatin B inhibits the interferon β, (IFN-β) response, STAT-1PY, and its nuclear translocation in Vero cells (Rivera-Rivera et al. 2012). In order to further elucidate the mechanism of cystatin B induction of HIV replication, we determined the regulation of STAT-1 signaling circuits in cystatin B deficient and IFN-β HIV-infected human macrophages.

Materials and methods

Monocyte isolation and differentiation into macrophages

Peripheral blood mononuclear cells (PBMC) obtained from healthy donors and isolated by Ficoll gradient were seeded to Lab-Tek Chamber Slides. The adherent monocytes were differentiated for 7 days into monocyte-derived macrophages (MDM) using RPMI-1640 medium supplemented with 10 % human serum, 20 % fetal bovine serum (MDM media).

Small interfering RNA treatment against cystatin B

To inhibit protein expression of cystatin B, small interference RNA (siRNA) (Acell smart pool, Dharmacon, Sigma, MO) was used, while non-targeting siRNA was used as control. The MDM were either incubated with cystatin B siRNA or non-targeting siRNA for 72 h as per manufacturer's instructions.

IFN-β treatment or HIV-1 infection of MDM

For the IFN-β treatment, cultured MDM treated with siRNA were washed with serum-free medium and then, they were either treated with 500u/mL IFN-β (PBL Assay Science, NJ) for 24 h, or left untreated as negative controls.

For HIV-1 infection, cultured MDM treated with siRNA were washed 3× with serum-free media, inoculated with 0.1MOI of HIV-1_{ADA} and cultured overnight. HIV-1 containing supernatants was removed and replaced with MDM media. The HIV infection was monitored for 12 days and supernatants collected at 3, 6, and 12 days post-infection to determine HIVp24 antigen by ELISA, as per the manufacturer's instructions (Express BioTech, Maryland, USA).

Immunofluorescence studies

Uninfected macrophages, fixed after 24 h of IFN-β stimulation, and HIV-infected macrophages at 12 days post-infection, were permeabilized with 0.5 % Triton-x 100 in 5 % BSA, incubated overnight with mouse anti-human cystatin B antibody (Sigma, MO) and rabbit anti-human unphosphorylated STAT-1 (USTAT-1) [C-terminal, Abcam, MA] primary antibodies and for 1 h with ALEXA-conjugated secondary anti-mouse or anti-rabbit antibodies (Invitrogen). Blue fluorescent DAPI was used as nuclear stain.

Cells were analyzed for immunofluorescence using a confocal microscopy. Images were obtained on a Zeiss Axiovert 200 M confocal microscope with a LSM 510 or on a Nikon Eclipse E400 fluorescence microscope with a SPOT Insight QE camera and SPOT 5.1 software.

Protein-protein interactions and protein phosphorylation using proximity ligation assay

The MDM were cultured in eight well permanox chamber slides and treated with IFN-β or inoculated with HIV-1_{ADA}, were then fixed with 4 % paraformaldehyde in PBS, permeabilized with 0.5 % Triton-x 100 in 5 % BSA, and used for proximity ligation assay (PLA) as per manufacturer's instructions (Sigma-Aldrich, MO). The PLA enables the visualization of protein interactions or phosphorylation in cell samples.

The PLA employs two different kinds of antibodies against the proteins that are in close proximity followed by a pair of oligonucleotide-labeled secondary antibodies (anti-rabbit PLA plus probe, anti-mouse PLA minus probe). A signal visualized as an individual fluorescent spot is generated only when the two PLA probes are bound in close proximity. The STAT-1 tyrosine phosphorylation (STAT-1PY) was detected using primary antibody against STAT-1 (rabbit anti-human STAT-1, Abcam, MA) and mouse anti-human primary antibody directed against tyrosine phosphorylation (Abcam, MA). Images were obtained on a Nikon Eclipse E400 fluorescence microscope with a SPOT Insight QE camera and SPOT 5.1, ×40 objective.

Statistical analysis and quantification

An unpaired two-tailed Student's *t* test was used to compare HIV p24 levels after treatment with cystatin B siRNA compared with controls lacking siRNA. One-tailed Student's *t* test was used to compare cystatin B siRNA treatment with no-targeting siRNA control. PLA signals from three different fields were quantified by Imaris® and analyzed using unpaired two-tailed Student's *t* test by comparing the mean of spots/nucleus. Error bars are ± SEM.

Results

Effect of cystatin B modulation in HIV replication in human MDM

Following silencing of cystatin B, HIV infection decreased significantly ($p < 0.05$) at 12dpi, as demonstrated

by decreased HIVp24 antigen levels in culture supernatants by ELISA. Cell viability was not affected by siRNA treatment, and non-targeting siRNA showed similar results as controls lacking siRNA. These results confirmed studies previously reported by our group (Luciano-Montalvo et al. 2008).

Effect of cystatin B modulation in USTAT-1 activation in IFN-β treated human MDM

Although the percent of cystatin B silencing was only 9–10 %, all the cells that were silenced showed the following results.

In the presence of cystatin B, USTAT-1 was retained in the cytoplasm before (Fig. 1, panels 1–3) and after IFN-β treatment (Fig. 1, panels 4–6). However, in the absence of cystatin B, USTAT-1 is located within the nucleus of IFN-β treated MDM (Fig. 1, panels 10–12). Unstimulated MDM treated with cystatin B siRNA (Fig. 1, panels 7–9) and IFN-β treated MDM treated with non-targeting siRNA (Fig. 1, panels 13–15) served as controls, showing USTAT-1 retention in the cytoplasm in both settings. Lower power images for Fig. 1 were included as supplementary Fig. S1.

Effect of cystatin B modulation in USTAT-1 activation in HIV-infected human MDM

For these series of experiments, the percent of cystatin B silencing in MDM was 12–15 % and all the cells that were silenced showed similar results. Uninfected MDM expressing cystatin B showed cytoplasmic localization of USTAT-1 (Fig. 2, panels 1–3). After cystatin B gene silencing, USTAT-1 was retained in the cytoplasm of uninfected MDM (Fig. 2, panels 4–6) as well as in HIV-infected MDM treated with non-targeting siRNA control (Fig. 2, panels 7–9). However, USTAT-1 was located within the nucleus in HIV-infected MDM treated with cystatin B siRNA (Fig. 2, panels 10–12). Lower power images for Fig. 2 were included as supplementary Fig. S2.

Effect of cystatin B modulation in STAT-1PY in IFN-β treated human MDM

In situ PLA and primary antibody specifically directed against tyrosine-phosphorylated STAT-1 were used to confirm the localization of STAT-1PY. Both IFN-β treated and untreated MDM showed STAT1-PY to be localized in the cytoplasm in the presence of cystatin B (Fig. 3a). However, after

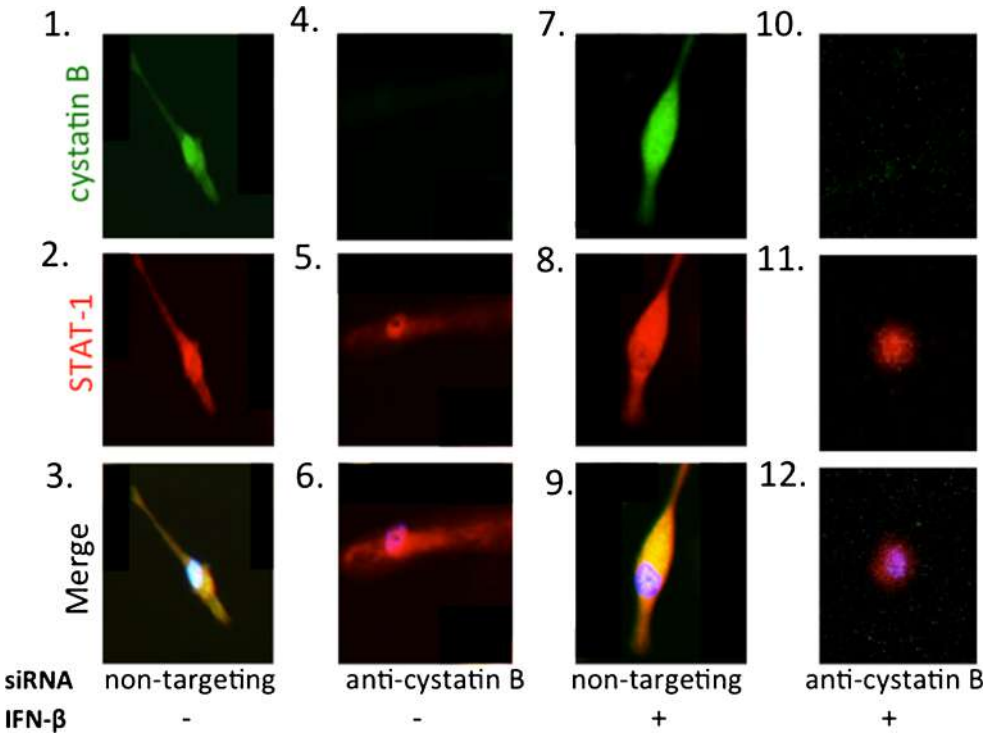


Fig. 1 Cystatin B inhibits the nuclear translocation of USTAT-1 in IFN-β-treated MDM. Uninfected MDM induced by IFN-β were assayed by immunofluorescence for cystatin B (*green*) and USTAT-1 (*red*). Blue fluorescent DAPI was used as nuclear stain. MDM expressing cystatin B before (**1–3**) and after IFN-β treatment (**7–9**), showed a cytoplasmic localization of STAT-1. In absence of cystatin B, USTAT-1 was retained in the cytoplasm of untreated MDM (**4–6**), but USTAT-1 was completely translocated to the nucleus in IFN-β treated MDM after silencing of cystatin B (**10–12**). Non-targeting siRNA was used as a control for the cystatin B silencing procedure (**1–3** and **7–9**). Confocal images were obtained on a Zeiss confocal microscope Axiovert 200 M with a LSM 510, ×40. The results are representative of experiments performed using three different donors

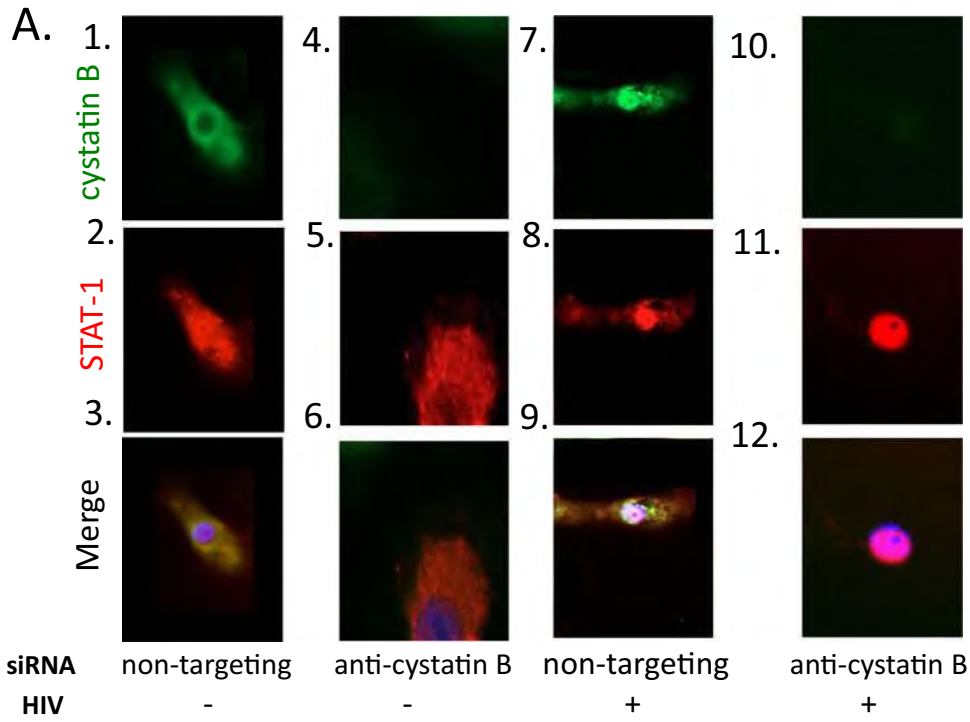


Fig. 2 Cystatin B inhibits the nuclear translocation of USTAT-1 in HIV-infected MDM. Nuclear translocation of STAT-1 was observed in HIV-1 infected MDM treated with cystatin B siRNA. At 12 DPI, HIV-infected MDM were assayed by immunofluorescence for cystatin B (*green*) and USTAT-1 (*red*). Blue fluorescent DAPI was used as nuclear stain. Non-targeting siRNA was used as siRNA control. Cystatin B was silenced in MDM cultures using siRNA. Uninfected MDM expressing cystatin B showed a predominantly cytoplasmic localization of USTAT-1 (**1**–**3**). After cystatin B gene silencing, STAT-1 was retained in the cytoplasm of uninfected MDM (**4**–**6**) as well as in HIV-infected MDM treated with non-targeting siRNA (**7**–**9**). At 12 DPI, USTAT-1 was totally translocated to the nucleus in HIV-infected MDM after cystatin B gene silencing (**10**–**12**). Images were obtained on a Nikon Eclipse E400 fluorescence microscope with a SPOT Insight QE camera and SPOT 5.1, ×40. These results are representative of two independent experiments performed using two different donors

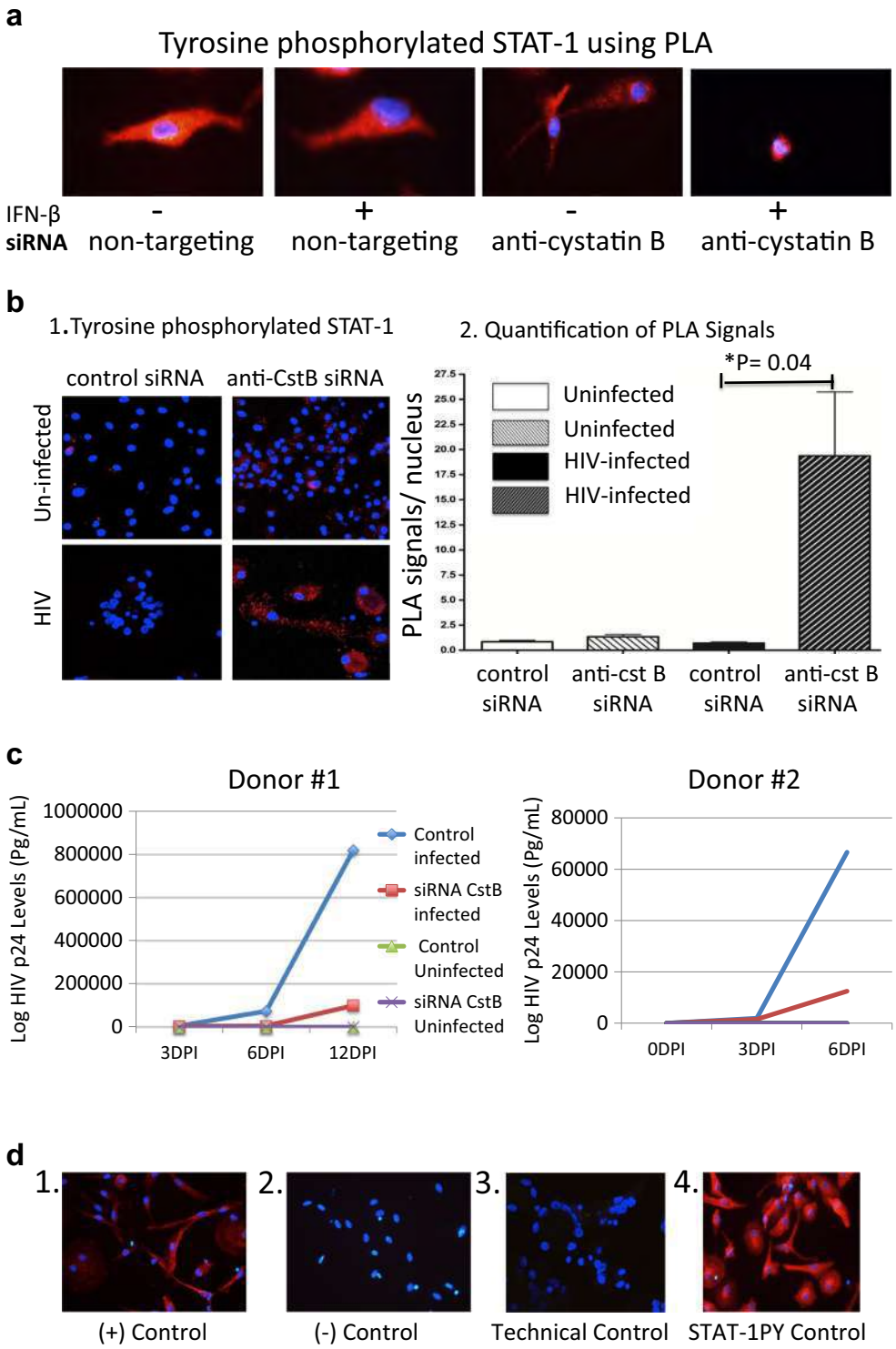
silencing cystatin B and treatment with IFN-β, MDM showed STAT-1PY located within the nucleus (Fig. 3a).

Taken together, the immunofluorescence and PLA studies demonstrate that cystatin B retains USTAT-1 and STAT-1PY in the cytoplasm independent of the presence or absence of IFN-β. Results of experimental controls of Fig. 3 were included as supplementary figure (Fig. S3). PLA was also used to confirm cystatin B silencing, as absence of cystatin B/STAT-1 interaction is expected in the absence of cystatin B. Cystatin B/STAT-1 interaction is observed in both IFN-β treated and untreated MDM, but this interaction was not detected after silencing cystatin B with siRNA, (Fig. S3a). As expected, cystatin B/STAT-1 interaction decreased after cystatin B silencing of both uninfected and HIV-infected MDM (12DPI). These results demonstrated that cystatin B was silenced (Fig. S3a). Non-targeting siRNA was used as control for siRNA treatment (Fig. S3b).

Effect of cystatin B modulation in STAT-1PY in HIV-infected human MDM

In situ PLA was used to determine the STAT1PY levels and localization in HIV-infected MDM treated with cystatin B

Fig. 3 The role of cystatin B in STAT-1 activation in MDM is mediated by STAT-1PY. The effect of cystatin B modulation in STAT-1PY were examined in induced MDM. **a** Cystatin B inhibits the STAT-1PY nuclear translocation in IFN-B-induced MDM. Uninfected MDM induced by IFN-β were assayed with PLA in situ. Non-targeting siRNA and unstimulated MDM were used as controls. Both, unstimulated and IFN-β treated MDM showed a cytoplasmic localization of STAT1-PY. In IFN-β-treated MDM, STAT-1PY is translocated to the nucleus after silencing of cystatin B. **b** Cystatin B inhibits STAT-1PY in HIV-infected MDM. (*1*) In situ PLA was used to determine the STAT1PY in HIV-infected MDM treated with cystatin B siRNA. siRNA treatment against cystatin B increased the STAT-1 tyrosine phosphorylation in HIV-infected MDM. (*2*) An unpaired two-tailed Student's *t* test was used to compare the PLA signals of tyrosine-phosphorylated-STAT1 in uninfected and HIV-infected MDM treated with or without anti-cystatin B siRNA. **c** HIVp24 antigen levels from MDM supernatants of two donors at 3, 6, and 12 days post-infection shows a productive infection. **d** Experimental controls included the following: (*1*) biological positive control consisting of uninfected MDM stained for cystatin B/cathepsin B interacting proteins, (*2*) biological negative control consisting of HIV-infected MDM that lost cystatin B/cathepsin B interaction, (*3*) a technical control included the absence of the primary antibody, and (*4*) STAT-1PY in IFN-β-treated MDM. Images were obtained on a Nikon Eclipse E400 fluorescence microscope with a SPOT Insight QE camera and SPOT 5.1, ×40. These results are representative of two independent experiments using two different donors



siRNA. Cystatin B silencing increased the STAT-1PY signal in HIV-infected MDM at 12DPI that was retained in the cytoplasm (Fig. 3b panels 1–2). This prominent STAT-1PY signal was not observed in either uninfected or HIV-infected MDM, in the presence of cystatin B (Fig. 3b panels 1–2) or with non-targeting siRNA (Fig. S3b).

Discussion

This study has disclosed a previously unknown function for cystatin B, a cysteine protease inhibitor of cathepsin B, which is the inhibition of the nuclear transportation of both USTAT-1 and STAT-1PY in IFN-β-treated MDM. In the presence of

cystatin B, USTAT-1 is retained in the cytoplasm before and after IFN-β treatment. However, in the absence of cystatin B, all the USTAT-1 in the IFN-β treated MDM remain in the nucleus. Furthermore, PLA studies of IFN-β-treated MDM silenced for cystatin B detected STAT-1PY only in the nucleus. These findings demonstrate that cystatin B inhibits the USTAT-1 and STAT-1PY nuclear transportation in IFN-β treated MDM.

Furthermore, cystatin B plays a role in STAT-1 activation of HIV-infected MDM. Our data demonstrated that at 12DPI, USTAT-1 is retained mainly in the cytoplasm of HIV-infected MDM and in uninfected MDM controls. After cystatin B silencing, USTAT-1 moves to the nucleus. In addition, cystatin B also plays a role in STAT-1 tyrosine phosphorylation. Huang and collaborators reported that HIV infection of MDM stimulated STAT-1PY at 3, 5, and 7 DPI, but more prominently at 5 DPI (Huang et al. 2009). However, in this study we found that in the presence of cystatin B, there are no differences in STAT-1PY levels and localization between uninfected and HIV-infected MDM at 12 DPI. The difference in the HIV-induced-STAT-1PY at earlier points of time (1–7 DPI) versus at 12 DPI can be due to differences in cystatin B levels induced by HIV infection (Luciano-Montalvo et al. 2008). Our findings reveal cystatin B inhibiting STAT-1PY in HIV-infected MDM at 12 DPI. There is a possibility that low levels of STAT-1PY in HIV-infected macrophages could potentially interfere with the interferon-induced-antiviral response, facilitate the evasion of host immune surveillance, and allow HIV replication.

We found that USTAT-1 is retained in the nucleus of HIV-infected MDM at 12 DPI, in the absence of cystatin B. By silencing cystatin B in HIV-infected MDM, STAT-1PY was induced and remained in the cytoplasm. In contrast, our data showed nuclear transportation of both USTAT-1 and STAT-1PY in the IFN-β treated uninfected MDM silenced for cystatin B. PLA studies performed during cystatin B silencing of IFN-β treated cells confirmed that STAT-1PY is transported to the nucleus, since the PLA signal of STAT-1PY is shown only in the nucleus. The circuit of STAT-1 signaling and the nuclear transportation of USTAT-1 in unstimulated and IFN-β treated cells have been described (Vinkemeier 2004). In unstimulated cells, STAT-1 is predominantly in the cytoplasm. However, it has been reported that USTAT (USTAT1, USTAT3, USTAT5) are constantly moving between cytoplasm and nucleus (Meyer et al. 2002; Marg et al. 2004) and nuclear localization signals have been identified in STAT1, STAT-3, and STAT-5 DNA binding domains (Melen et al. 2001; Meyer et al. 2002; McBride et al. 2002; Ma et al. 2003). Furthermore, after stimulation with cytokines, such as IFN, one portion of USTAT-1 continues its flow between nucleus and cytoplasm (Cheon and Stark 2009). Other studies reported that STAT-1 dimers do not require to be stimulated by cytokines such as IFN-β to move in and be stored in the nucleus (Meyer et al. 2003). To fully understand the role of IFN-β in USTAT-1 nuclear/cytoplasm movement, additional research is required.

Our findings demonstrated that cystatin B inhibits STAT-1PY and in absence of cystatin B, HIV infection hijacks USTAT-1 to the nucleus of MDM. The exact mechanism to explain how HIV retains proteins within the nucleus has not been described. At present, we do not have enough data to identify possible cellular/viral factors that might be involved in the nucleocytoplasmic trafficking of transcription factors such as STAT-1.

Moreover, the hijacking of USTAT-1 to the nucleus could have several consequences. To begin with, USTAT-1 retained in the nucleus cannot be phosphorylated because phosphorylation takes place in the cytoplasm. Secondly, USTAT-1 in the nucleus can activate signals other than those induced by STAT-1PY, including the induction of new USTAT-1. Cheon and Stark (Cheon and Stark 2009) reported that STAT-1PY and USTAT-1 stimulate the STAT-1 gene to increase the levels of more USTAT-1. Microarray studies have reported at least 20 genes induced by USTAT-1 in BJ fibroblast human line cells (Cheon and Stark 2009). Thirdly, another study proposed that USTAT-1 can act as a transcription factor (Kossow et al. 2012). Additional studies will be necessary to fully understand the role of USTAT-1 in the nucleus of HIV-infected MDM.

During HIV infection of MDM in vitro there is an increased expression of cystatin B, which parallels increased number of round cells. Round MDM has been associated with M1, pro-inflammatory phenotype, and spindle MDM with M2 anti-inflammatory profile (Mantovani et al. 2004; Eligini et al. 2015). Others reported that M1 polarization of macrophages restricts HIV replication during virus entry and post-entry steps (Cassetta et al. 2013; Burdo et al. 2015) while HIV infection can also switch macrophage polarization to M2 (Sang et al. 2015). Since IFNs signaling affect macrophage polarization (Sang et al. 2015), it is possible that cystatin B is playing a role in M1/M2 switch during HIV infection. Therefore, studies of M1 and M2 cytokines and surface CD16 + CD163 + markers together with cystatin B expression in macrophages represent an important area of interest for conducting studies in the future.

Essentially, both cystatin B and HIV infection regulate the STAT-1 signaling circuit in MDM. Cystatin B decreases STAT-1PY and its movement to the nucleus, while HIV infection retains USTAT-1 in the nucleus preventing its exit to the cytoplasm and its eventual phosphorylation. Other STAT signaling deficiencies in macrophages induced by HIV have been reported. For example, HIV-1 infection of human MDM in vitro resulted in the disruption of GM-CSF-induced STAT5A activation (Warby et al. 2003), and neonatal MDM was not induced by IFN because IFN-γ-mediated STAT-1 phosphorylation was significantly decreased (Maródi et al. 2001). Since the STAT protein family responds to interferon to stimulate an anti-proliferative and antiviral response, deficiencies of STAT signaling in HIV-infected macrophages may restrict HIV replication

and allow for the formation of viral reservoirs that escape host immune surveillance.

This study revealed that cystatin B has a crucial role in the inhibition of STAT-1 signaling activation in MDM, suggesting that cystatin B plays an important role in perpetuating HIV inside macrophages. The adverse role of intracellular cystatin B in HIV-infected macrophages contrasts with its suggested protective role in the extracellular rectal, oral, and cervicovaginal mucosa proteome (Burgener et al. 2011; Romas et al. 2014). These studies suggest that cystatin B has a dual role depending on its localization; extracellular cystatin B protects against HIV infection in mucosa (Burgener et al. 2011; Romas et al. 2014) but induces HIV replication inside macrophages reservoirs (Luciano-Montalvo et al. 2008). Thus, inhibiting intracellular cystatin B with siRNA may be a potential therapeutic approach against HIV reservoirs in macrophages. However, a drawback of this study is the finding that not all macrophages can internalize siRNA. As a result, further studies are required to improve silencing of cystatin B and facilitate the delineation of possible therapies targeting HIV-1 reservoir in macrophages.

In addition to its role in inducing HIV replication, cystatin B plays an important role as an inhibitor of cathepsin B secretion but this function is affected in HIV-1 infected MDM. The lack of interaction of cystatin B with cathepsin B in HIV-infected MDM results in increased neurotoxic activity (Rodriguez-Franco et al. 2012; Cantres-Rosario et al. 2013). Therefore, gaining knowledge on the signaling intermediates of cystatin B may eventually provide novel targets for development of inhibitory factors, leading to the eradication of HIV macrophage reservoirs and the reduction of HIV-induced neurotoxicity.

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Compliance with ethical standards

Conflict of Interest LMM has a patent application approved for cystatin B, related to this manuscript. Pat No. 8,143,231.

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