



Research article

VISTA facilitates phagocytic clearance of HIV infected CEM-SS T cells

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ARTICLE INFO

Keywords:

Human immunodeficiency virus (HIV)
 V domain immunoglobulin suppressor of T cell activation (VISTA)
 Phagocytosis
 T cells
 Apoptosis

ABSTRACT

Phagocytosis is a critical component of the innate immune response to viral infection, resulting in the clearance of infected cells while minimizing the exposure of uninfected cells. On the other hand, phagocytosis of HIV-infected T cells may cause phagocytes, such as macrophages and dendritic cells, to be infected, thus leading to HIV cell-to-cell transmission. V domain immunoglobulin suppressor of T cell activation (VISTA, gene *Vsir*, aliases *Gi24*, *Dies-1*, *PD-1H*, and *DD1 α*) has been identified as an immune checkpoint molecule that possesses dual activities when expressed on APCs and T cells. Our study found that VISTA might play a significant role during the immune response to HIV infection via apoptosis upregulation and subsequent phagocytosis of infected CEM-SS T cells. HIV-induced apoptosis and monocytic cell engulfment were tested utilizing CEM-SS T cells as target cells and the monocytic cell line THP-1 as phagocytic cells. Cells were infected with a GFP-labeled HIV strain, NL4-3. HIV-infected CEM-SS T cells displayed greater apoptotic activity (approximately 18.0%) than mock-infected controls. Additionally, phagocytosis of HIV-infected CEM-SS T cells was increased approximately 4-fold. Expression of VISTA on infected CEM-SS T cells was detected in 16.7% of cells, which correlated with the increased phagocytosis observed. When an antagonistic antibody against VISTA was used, the number of phagocytosed cells was reduced by a factor of 2, which was replicated utilizing human stem cell-derived dendritic cells. Phagocytosis was also confirmed by the upregulation of IL-1 β expression, which was 5-fold higher in infected cells than in control cells. We also found that VISTA overexpression on both phagocytes and HIV-infected CEM-SS T cells facilitated phagocytosis. Our study suggests that VISTA may act as a direct ligand in the phagocytosis of HIV-infected T cells.

1. Introduction

Monocytes, macrophages and dendritic cells play critical roles in defending against infection by microbial pathogens, including viruses, by inducing host cellular and humoral immune responses to fight against microbes. Phagocytosis is one component of innate cellular responses. Phagocytes can either directly bind pathogen-associated molecular pattern molecules (PAMPs) located on the surface of pathogens with cellular receptors to phagocytize the pathogens [1] or indirectly phagocytize pathogen-infected cells [2, 3]. Phagocytes also initiate the innate immune response by secreting inflammatory cytokines/chemokines during pathogen-induced phagocytosis [4, 5].

In the course of HIV infection, myeloid cells, especially long-lived macrophages, have been identified as an important reservoir [6, 7, 8], and they also contribute to HIV-induced chronic inflammation [6, 9, 10]. This myeloid cell-based HIV reservoir is of importance because these cells can act as antigen-presenting cells (APCs), thus transmitting the virus to

CD4⁺ T cells, the predominantly targeted cell type for most HIV strains. Macrophages have been reported to spread HIV by cell-to-cell transmission at the virological synapse [11, 12], and one of the mechanisms of HIV infection of macrophages is selective phagocytosis of HIV-infected CD4⁺ T cells by macrophages [13]. However, the mechanisms by which these HIV-infected cells are recognized by phagocytes and the consequent signaling involved in the phagocytic clearance of HIV-infected CD4⁺ T cells have not yet been identified.

VISTA was recently identified as a new immune checkpoint molecule [14, 15, 16, 17, 18, 19], and evidence has shown that it acts as an inhibitory ligand in T cell activation but plays a stimulatory role when expressed on APCs [20]. We previously found that HIV-infected patient monocytes had significantly elevated levels of VISTA expression and that this overexpression was highly correlated with inflammatory responses [21]. In a subsequent study, we performed a gene array analysis and found that VISTA-overexpressing monocytes showed a proinflammatory profile, and further analysis revealed that pathways related to

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phagocytosis were highly likely to be involved [22]. Therefore, we postulated that VISTA may play a role in the phagocytic clearance of HIV-infected CD4⁺ T cells.

In this study, we investigated whether VISTA expression is correlated with phagocytosis of HIV-infected T cells. We also studied whether HIV-induced cell apoptosis is related to VISTA expression and whether this induced apoptosis is a possible mechanism underlying the phagocytic clearance of HIV-infected T cells. Finally, we examined whether VISTA plays a role in the regulation of cytokine secretion during phagocytosis. We showed that VISTA expression in APCs facilitated the phagocytic clearance of HIV-infected CEM-SS T cells and that VISTA overexpression on APCs upregulated IL-1 β secretion. We speculated that VISTA might act as a phagocytosis ligand expressed on both APCs and HIV-infected cells, thus enabling the phagocytic clearance of HIV-infected CEM-SS T cells.

2. Materials and methods

2.1. Cells, viruses, antibodies, and chemicals

Fluorophore-labeled anti-CD11b, anti-CD11c, and anti-VISTA monoclonal antibodies and corresponding isotype controls for flow cytometry were obtained from BioLegend. Carboxyfluorescein diacetate succinimidyl ester (CFSE), pHrodo and VMQC were purchased from Invitrogen.

The cell lines 293T, THP-1, and CEM-SS were purchased from ATCC, and the HIV strains NL4-3 and NL4-3 GFP were obtained from the NIH AIDS Reagent program. Cells were thawed and cultured in the laboratory. Cells were expanded, and cells with 95% viability were used for experiments.

2.2. Generation and characterization of apoptotic CEM-SS T cells

One million CEM-SS T cells were harvested and incubated with camptothecin (CPT) at a concentration of 1 μ M overnight. The apoptotic status was defined as annexin V-positive, propidium iodide-negative staining by flow cytometry. For phagocytosis assays, cells were harvested and stained with pHrodo or CFSE for half an hour at room temperature. The cells were then washed twice with 10% FBS medium.

For HIV-induced cell apoptosis, CEM-SS T cells were infected with HIV (NL4-3) at an MOI of 0.01 and incubated at 37 °C and 5% CO₂ for 6 days. Apoptotic cells were characterized by annexin V and PI staining. For phagocytosis assays, cells were stained with pHrodo or CFSE as described above.

2.3. Preparation of human HSC-derived DCs

CD34⁺ cells were isolated from cord blood using an EasySep human CD34-positive selection kit (STEMCELL Technologies, Canada). Cells were expanded by adding chemokine cocktails (CC100, STEMCELL Technologies, Canada) for three days, and the cells were then supplemented with recombinant human IL4 (50 ng/ml) and GM-CSF (50 ng/ml) cytokines and incubated at 37 °C and 5% CO₂ for 5 additional days. Cell differentiation was characterized by staining for human CD11c.

2.4. Preparation of a VISTA-overexpressing lentivirus

To construct a transfer plasmid, the DNA sequence encoding the VISTA gene was cloned into the pLVX lentiviral vector (Genentech, CA). To enhance VISTA expression, the endogenous CMV promoter in pLVX was replaced with a CASI promoter [23]. To make a VISTA-overexpressing lentivirus, 20 μ g of pLVX-VISTA transfer plasmid was cotransfected into 1×10^7 293 T cells with two additional plasmids, Vpr (15 μ g) and VSVG (9 μ g), using a PEI transfection protocol we described previously [24, 25]. After 48–72 h of transfection, the supernatants were harvested and ultracentrifuged to concentrate the lentivirus.

2.5. Overexpression of PD1H on THP1 and CEM-SS T cells

THP-1 or CEM-SS T cells were transduced with the VISTA-overexpressing lentivirus using the spin transduction method described previously [24]. Briefly, 5×10^5 cells were seeded in standard RPMI 1640 medium supplemented with 5 μ g/ml polybrene, and then the lentivirus was added to the cells at an MOI of 20. The cell plates were centrifuged at 300 g for 2 h, followed by two additional incubations at 37 °C and 5% CO₂ for 2 h. Then, the medium was replaced with RPMI medium supplemented with 10% inactivated FBS (ThermoFisher, CA) and 1% PenStrep (100 U/ml penicillin and 100 μ g/ml streptomycin). The cells were incubated for 48 h, and VISTA expression was monitored by staining the cells with an anti-VISTA mAb.

2.6. Phagocytosis assay

For the CPT-induced apoptotic cell phagocytosis assay, 3×10^5 THP-1 cells were incubated with 100 ng/ml PMA to activate the cells. The cells were then seeded in 12-well plates. The next day, the supernatant was removed, and fresh RPMI medium containing 10% FBS and 1% PenStrep was added. CPT-induced CEM-SS T cells were labeled with pHrodo according to the manufacturer's instructions.

For flow cytometry analysis of phagocytosis, 3×10^5 THP-1 cells were stained with CMTPX (Invitrogen). For target cells, uninfected or HIV-infected CEM-SS T cells were stained with 1 mg/ml pHrodo (Invitrogen) in 150 μ l of PBS buffer for 30 min. Then, the cells were extensively washed with cold PBS and mixed with the THP-1 cells at an effector:target ratio of 1:5. The plate was incubated at 37 °C and 5% CO₂ for 4–12 h. For fluorescence microscopy analysis, the plate was extensively washed with PBS three times to remove nonadherent cells. The plate was then examined under a fluorescence microscope (Zeiss Axio Observer) for pHrodo and CFSE using bright field, Texas Red or GFP filter sets. The phagocytic index was equal to the number of ingested cells (number of THP-1 cells/100).

For the flow cytometry assay, cells were scratched and harvested from the plate directly. Cells were gated by FSC and SSC, and single- or double-positive cells were described as positive engulfment cells. The data were analyzed using FlowJo software.

2.7. Cytokine bead array assay

Inflammatory cytokine concentrations were measured in the supernatants of cultures using the BD Cytometric Bead Array (CBA) according to the manufacturer's instructions. The data were analyzed with FCAP Array software. The concentrations of the cytokines were calculated according to a standard curve.

2.8. Statistical analysis

All experiments were repeated twice to ensure the repeatability. Statistical analyses were performed using GraphPad Prism version 7. Data were compared between groups using unpaired Student's t tests. Statistical significance was defined as *P \leq 0.05, **P \leq 0.01, and ***P \leq 0.001.

3. Results

3.1. HIV infection causes increased phagocytosis of CEM-SS T cells by monocyte-derived THP-1 cells

To investigate phagocytosis, we first used an apoptosis model in which a T cell line, CEM-SS, was induced to undergo apoptosis using the apoptosis inducer camptothecin, while a monocytic cell line, THP-1, was used as the phagocytes. Phagocytosis will occur once activated THP-1 cells are mixed with apoptotic CEM-SS T cells. To detect phagocytosis, we labeled CEM-SS T cells with the dye pHrodo. Due to the unique pH

sensitivity characteristics of this dye, when apoptotic CEM-SS T cells are phagocytosed by phagocytes (entered an acidic environment), the dye pHrodo emitted red fluorescence, while the dye did not fluoresce in free CEM-SS T cells. After 36 h of induction with PMA, THP-1 cells highly expressed the marker CD11c, indicating that the monocytic THP-1 cells were differentiated into macrophage/dendritic cell-like cells, as shown in Supplementary Fig. 1A. We then incubated the activated THP-1 cells with normal or apoptotic cells, and the results showed that after 24 h of incubation, the number of engulfed CEM-SS T cells in the apoptosis group was significantly greater than that in the control group without apoptosis induction (Supplementary Fig. 1B).

Next, we investigated whether HIV infection of CEM-SS T cells causes cell apoptosis and increased CEM-SS T cell phagocytosis. To this end, we first infected CEM-SS T cells with a laboratory-generated HIV strain, NL4-3, and then stained the CEM-SS T cells with fluorophore-labeled anti-Annexin V and anti-HIV P24 antibodies after 12 h and 36 h of HIV infection. The results showed that 13.3% of the HIV-infected cells were undergoing apoptosis (showing HIV P24 and Annexin V double positive) after 36 h of infection (Figure 1A, the gating strategy is shown in Supplementary Fig. 2). However, CEM-SS T cells without HIV infection or the infected cells sampled at 12 h showed only a background level of apoptosis, which is caused by intrinsic programmed cell death (Figure 1A). We then incubated the above-described HIV-infected CEM-

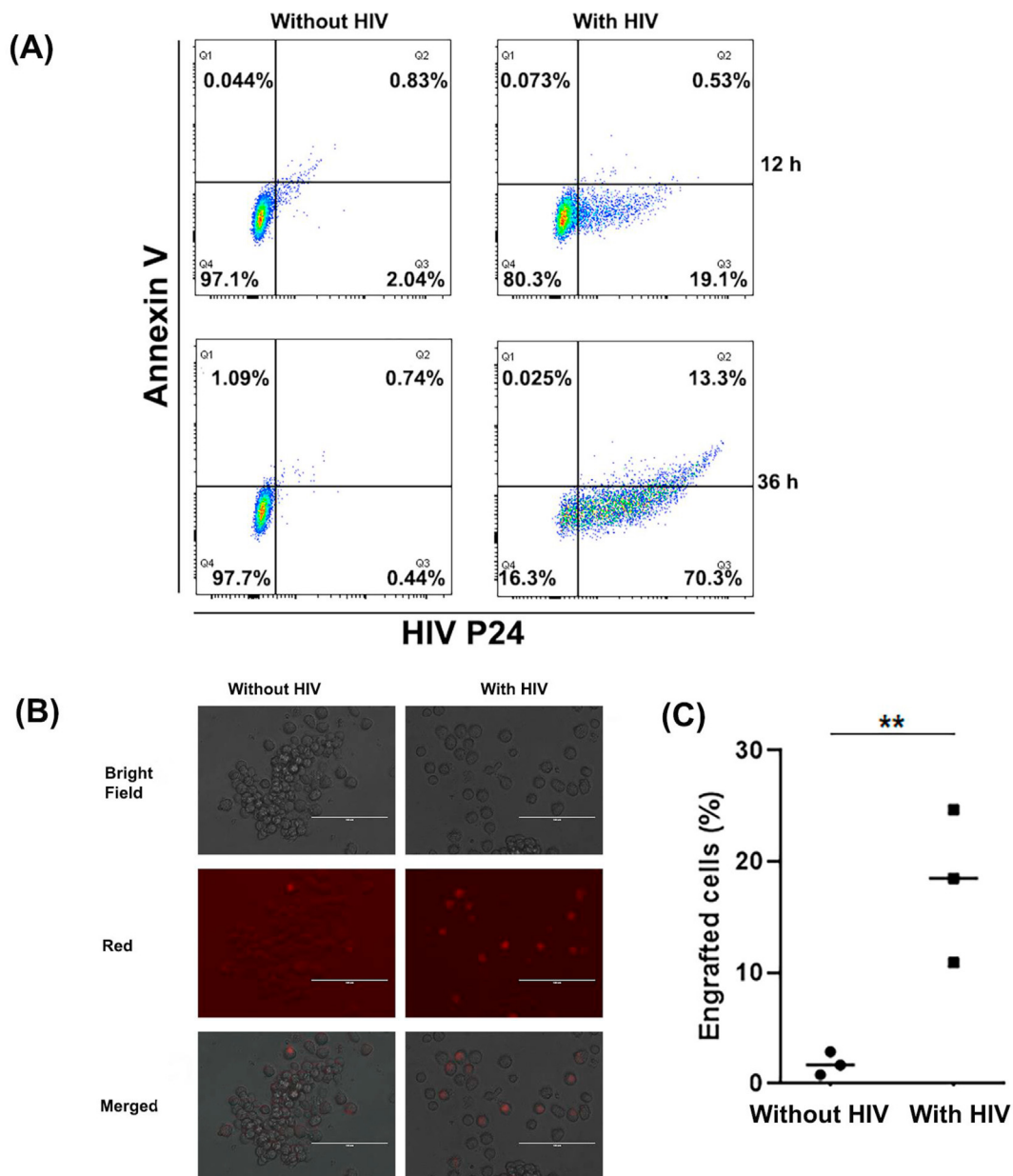


Figure 1. HIV infection causes CEM-SS T cell apoptosis and phagocytosis. (A) CEM-SS T cells were infected with HIV NL4-3, and the cells were sampled on 12h, 36h, stained with FITC-labeled anti-Annexin V and PE-labeled HIV P24 mAbs, and analyzed by FACS. Uninfected CEM-SS T cells were used as a negative control. The gating strategy is shown in supplementary Fig.1. (B) Representative figures showing that HIV-infected or uninfected CEM-SS T cells were stained with pHrodo and then mixed with PMA-activated THP-1 cells, and after 24 h, phagocytosis was observed by fluorescence microscopy. Bright field (left) and RFP channel fluorescence microscopy showed PMA-activated THP-1 cells phagocytosed HIV-infected and uninfected CEM-SS T cells. The merged channel was used to clearly show the phagocytic events (showing red). (C) Engulfed cells were counted (five random fields were counted in each well), and the histogram shows the percentages of engulfed cells in the groups of cells with and without HIV infection. The experiments were repeated twice in triplicate, and the statistical analysis are based on the averages of the three independent experiments.

SS T cells with PMA-activated THP-1 cells and detected phagocytosis after 24 h of incubation. Compared with that of uninfected CEM-SS T cell phagocytosis, the rate of HIV-infected CEM-SS T cell phagocytosis was significantly higher (an average phagocytosis rate of 1.5% without HIV infection vs. 19% with HIV infection) (Figure 1B and C).

We further developed a method to characterize the phagocytosis of HIV-infected CEM-SS T cells using flow cytometry. For this purpose, we used a modified GFP-expressing NL4-3 strain to infect CEM-SS T cells for 6 days and the dye CMPTX (APC channel in flow cytometry) to stain activated THP-1 cells and then mixed the cell lines together. After 24 h of incubation, we checked fluorescence in the FITC and APC channels. We found that the phagocytosed cells showed a unique “sticky” population, which was distinct from the populations of CEM-SS T cells and THP-1 cells (Figure 2). Compared with control cells (Figure 2B), HIV-infected CEM-SS T cells showed an ~4-fold higher rate of phagocytosis (3.2% vs 13.2%) (Figure 2B and C).

3.2. HIV-infected CEM-SS T cells express high levels of VISTA, and VISTA levels are highly correlated with phagocytosis

We evaluated VISTA expression in HIV (NL4-3 GFP)-infected CEM-SS T cells after 36 h of infection and found that the VISTA expression level increased to 13% of the infected cell culture (Figure 3A). After we gated the HIV-infected population by GFP expression, we found that the elevated VISTA expression was solely contributed by the HIV-infected cells (Figure 3B). We then tested whether VISTA plays a role in phagocytosis. For this purpose, we used a VISTA-specific monoclonal antibody (mAb) to block VISTA expressed on the surface of HIV-infected CEM-SS T cells for 2 h, mixed activated THP-1 cells with the HIV-infected CEM-SS T cells, and detected phagocytosis after 5 h of incubation. The results showed that the THP-1 cells had a background phagocytosis rate of ~2.9% (Figure 3C and D), while HIV-infected CEM-SS T cells induced an average phagocytosis rate of 11.0% (Figure 3E). Interestingly, once we applied the anti-VISTA mAb to block VISTA expression, the phagocytosis rate dropped to ~6.9% (Figure 3F), suggesting that VISTA plays a direct role during phagocytosis.

3.3. VISTA blockade reduced HSC-derived DC engulfment of HIV-infected cells

We further tested whether primary CD34 + hematopoietic stem cell (HSC)-derived phagocytes can also engulf HIV-infected cells and whether

the anti-VISTA mAb can reduce the phagocytosis by these cells. To this end, we isolated human CD34 + HSCs from cord blood and then differentiated the HSCs into DCs by supplementation with recombinant human IL-4 and GM-CSF for 5 days. After mixing HIV-infected CEM-SS T cells treated with or without anti-VISTA mAb blockade with the HSC-derived DCs, we examined DC engulfment of the HIV-infected CEM-SS T cells after 24 h. The results showed that before the incubation, the DC differentiation rate of the HSCs was ~50% (Figure 4B), and HIV-positive CEM-SS T cells composed ~98.8% of the target cell population (Figure 4C). After mixing the two cell types together and incubating them for 24 h, the engulfment results showed a higher engulfment background level (CEM-SS T cells without HIV infection), an engulfment rate of 18.2% (Figure 4D), due to intrinsic apoptosis in the CEM-SS T cells. However, HIV infection more than doubled the phagocytosis rate to 41.4% (Figure 4E). As expected, after blockade of VISTA, the engulfment level dropped to the background level (21.3%). These results indicated that HSC-derived DCs could also efficiently phagocytose HIV-infected CEM-SS T cells and that VISTA expression facilitated DC engulfment of HIV-infected CEM-SS T cells.

3.4. Apoptotic CEM-SS T cells expressed high levels of VISTA during HIV infection

As phagocytosis of apoptotic cells is an intrinsic homeostatic mechanism for maintaining normal homeostasis [26, 27], we wondered whether VISTA can mediate phagocytosis of HIV-infected CEM-SS T cells via the apoptotic pathway. We stained HIV-infected CEM-SS T cells with fluorophore-labeled antibodies against HIV-P24, VISTA, and Annexin V, and then we gated the populations to determine whether the HIV-infected CEM-SS T cell population undergoing apoptosis had a higher level of VISTA expression. As shown in Figure 5B, 37.8% of the CEM-SS T cells were infected with HIV, while only 6.8% of the CEM-SS T cells were undergoing apoptosis. The apoptotic CEM-SS T cells, either noninfected or HIV infected (red and blue curves shown in Figure 5C), showed a higher level of VISTA expression than the nonapoptotic CEM-SS T cells, and the apoptotic HIV-infected T cells had the highest level of VISTA expression. However, the nonapoptotic CEM-SS T cells, either noninfected or HIV infected, demonstrated only a background level of VISTA expression. These results suggested that HIV infection caused apoptosis and VISTA expression, followed by recognition of the apoptotic cells by phagocytes and initiation of the process of phagocytosis.

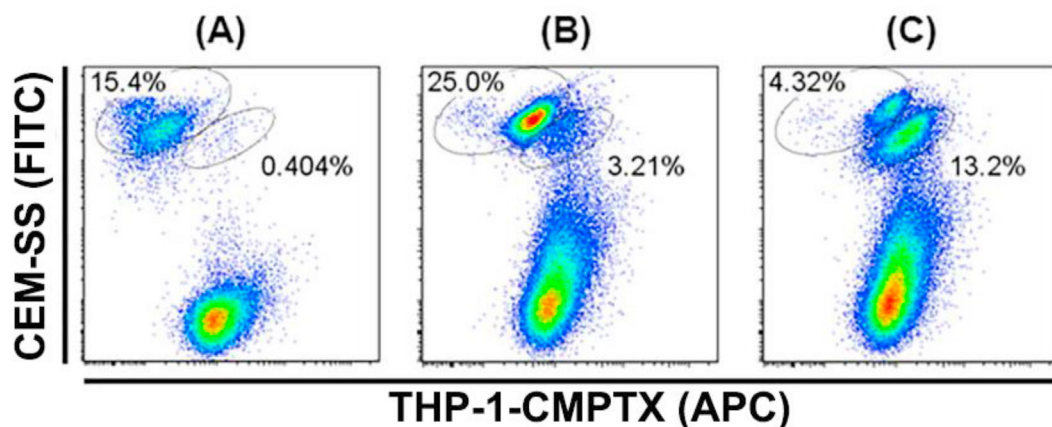


Figure 2. HIV infection causes increased phagocytosis of CEM-SS T cells. CEM-SS T cells were infected with GFP-expressing HIV NL4-3 for 6 days. To evaluate phagocytosis, PMA-activated, CMPTX-stained THP-1 cells were mixed with NL4-3-infected CEM-SS T cells, and after 24 h, phagocytosis was assessed by FACS analysis. (A) CMPTX-labeled THP-1 cells mixed with CFSE-labeled CEM-SS T cells, immediately checked by FACS (negative control); (B) CMPTX-labeled THP-1 cells mixed with CFSE-labeled CEM-SS T cells, and after 24 h, checked by FACS; and (C) CMPTX-labeled THP-1 cells mixed with HIV NL4-3-infected CFSE-labeled CEM-SS T cells, and after 24 h, checked by FACS.

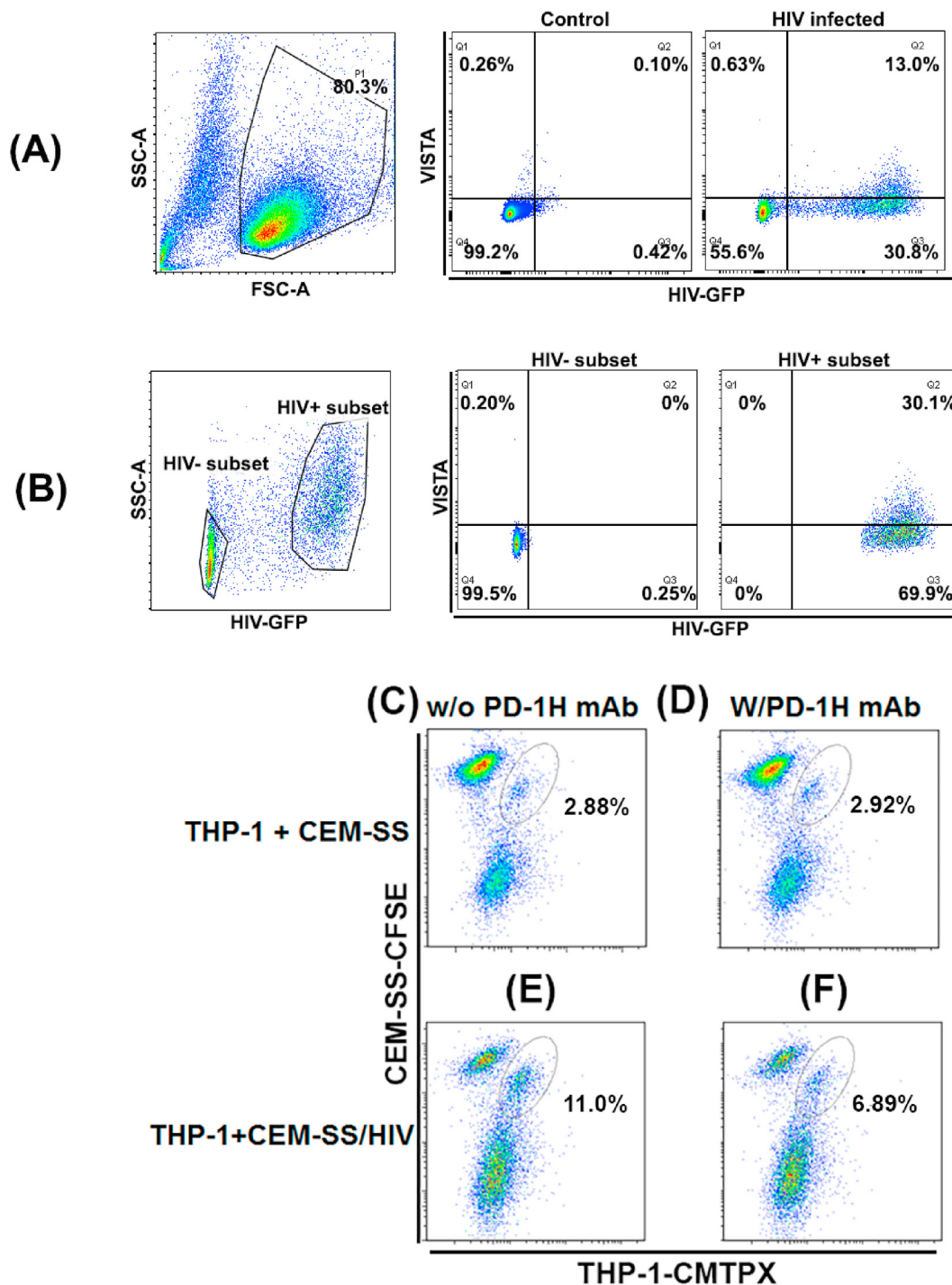


Figure 3. HIV-infected CEM-SS T cells express high levels of VISTA, and VISTA levels are highly correlated with phagocytosis. (A) VISTA expression level of HIV-infected CEM-SS T cells, detected by FACS after 48 h of infection. VISTA Uninfected CEM-SS T cells was used as control. Left panel shows the gating strategy. (B) 36 H post-infected cells were sampled for FACS analysis. The left panel shows the gating strategy. The HIV-positive and HIV negative cell populations were separately gated for checking VISTA expression. (C) and (D) CMPTX-labeled THP-1 cells mixed with uninfected cells stained with CFSE and treated without (C) or with an anti-VISTA mAb (D). After 5 h, phagocytosis was detected by FACS analysis. (E) and (F) CMPTX-labeled THP-1 cells mixed with HIV-infected cells stained with CFSE and treated without (C) or with the anti-VISTA mAb (D). After 5 h, phagocytosis was detected by FACS analysis.

3.5. VISTA expression on both phagocytes and HIV-infected T cells facilitates phagocytosis

The above experiments reveal that VISTA facilitates phagocytosis when VISTA is expressed on CEM-SS T cells and suggest that VISTA may play a role in apoptosis by triggering phagocytosis. We then wondered whether VISTA triggers the apoptotic pathway and thus initiates

phagocytosis or whether apoptosis triggers VISTA expression, with VISTA acting as a ligand to initiate phagocytosis. To distinguish these possibilities, we overexpressed VISTA on both phagocytes and CEM-SS T cells using either a GFP-expressing lentivirus or a VISTA-overexpressing lentivirus. VISTA expression after lentiviral transduction is shown in Figure 6A. We then mixed both cells and detected phagocytosis after 5 h. As shown in Figure 6B and C, when either THP-1 cells or CEM-SS T cells

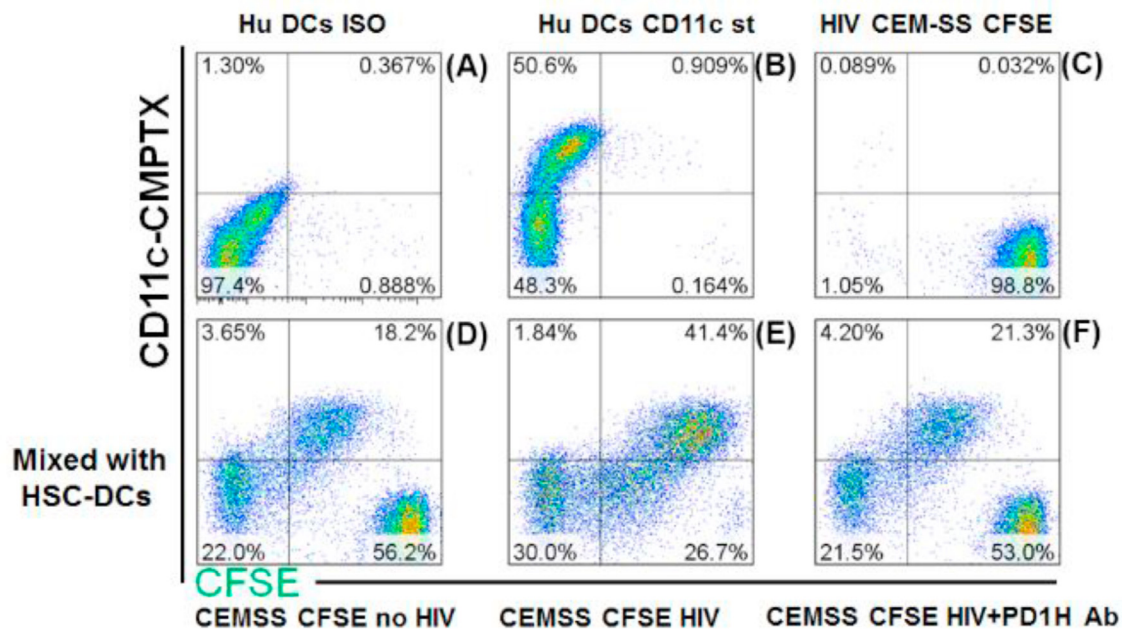


Figure 4. VISTA blockade reduced HSC-derived DC engulfment of HIV-infected cells. CEM-SS T cells were infected with HIV NL4-3 for 6 days and treated with or without an anti-VISTA mAb for 2 h. Then, the cells were labeled with CFSE and mixed with HSC-derived DCs, and phagocytosis was detected by FACS after 24 h of incubation. APC-labeled human CD11c was used to detect DCs, and the FITC channel was used to detect CFSE-labeled CEM-SS T cells. (A) DCs stained with an isotype antibody; (B) DCs stained with an APC-conjugated anti-human CD11c antibody; (C) CEM-SS T cells stained with CFSE; (D) CEM-SS T cells mixed with DCs for 24 h; (E) HIV-infected CEM-SS T cells mixed with DCs for 24 h; and (F) anti-VISTA mAb-treated HIV-infected CEM-SS T cells mixed with DCs for 24 h.

overexpressed VISTA, phagocytosis increased significantly compared with when corresponding cells did not express VISTA, and there was no significant difference in the expression of VISTA between THP-1 and CEM-SS T cells. As expected, when both cell lines overexpressed VISTA, phagocytosis reached an even higher level. These results show that VISTA expressed on either phagocytic or apoptotic cells facilitates phagocytosis, suggesting that VISTA may act as a phagocytosis ligand, leading to the recognition of apoptotic cells by phagocytes.

3.6. IL-1 β expression is upregulated during phagocytosis of HIV-infected CEM-SS T cells, and VISTA overexpression in CEM-SS T cells upregulates IL-1 β secretion

Previous studies have shown that phagocytosis by macrophages induces inflammasome activation and release of the proinflammatory

cytokine IL-1 β [28, 29]. We then examined whether this phenomenon also occurs during phagocytosis of HIV-infected cells and whether VISTA plays a role in the regulation of IL-1 β secretion. To explore these questions, we mixed THP-1 cells and HIV-infected or uninfected CEM-SS T cells. After 24 h of incubation, we confirmed that phagocytosis occurred (data not shown). Next, we collected the supernatants from the mixed cell cultures and quantified the IL-1 β concentration by ELISA. As shown in Figure 7A, the supernatant from the HIV-infected CEM-SS T mixed cell culture had a significantly higher level of IL-1 β than the supernatant from the mixed cell culture without HIV infection. We then examined whether VISTA overexpression in CEM-SS T cells can regulate IL-1 β secretion and how this regulation occurs. Therefore, we transduced HIV-infected CEM-SS T cells with a VISTA-expressing lentiviral vector, mixed the cells with THP-1 cells, and checked cytokine secretion by a bead array assay. As shown in Figure 7B, after VISTA overexpression, the secretion of the proinflammatory cytokines IL-1 β was upregulated.

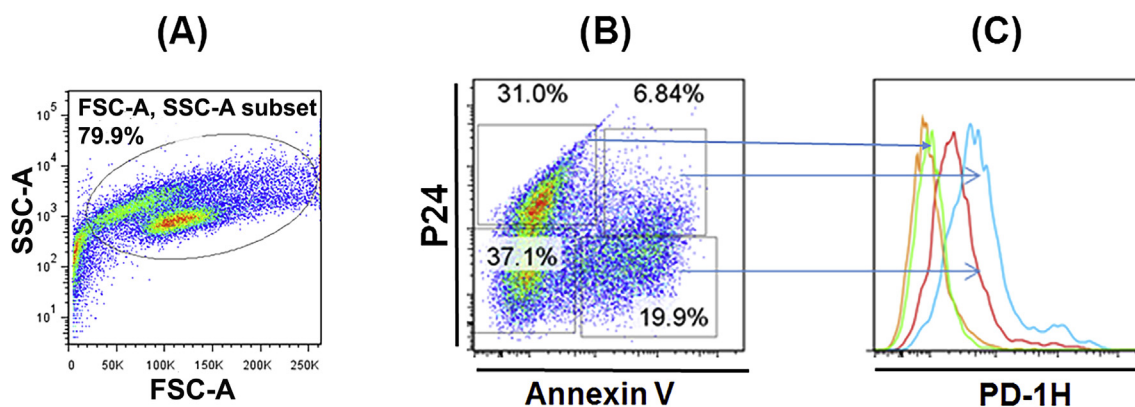


Figure 5. Apoptotic CEM-SS T cells expressed high levels of VISTA during HIV infection. HIV-infected CEM-SS T cells (6 days of infection) were stained with a PE-labeled anti-HIV P24 mAb, FITC-labeled Annexin V, and APC-labeled anti-VISTA antibody and analyzed by FACS. (A) Gating strategy; (B) gated populations showing Annexin V and P24 staining positivity or negativity; and (C) VISTA expression level for each population in (B).

4. Discussion

In this study, we showed that HIV infection of CEM-SS T cells increased phagocytosis and that VISTA expression was correlated with phagocytosis. We further demonstrated that blocking VISTA with a mAb reduced phagocytosis of HIV-infected CEM-SS T cells and that VISTA was associated with the apoptotic pathway in a manner related to facilitating phagocytosis. In addition, we found that VISTA expressed on both phagocytes and HIV-infected CEM-SS T cells facilitated phagocytosis,

suggesting that VISTA might serve as a ligand that directly mediates phagocytosis.

Monocytes, macrophages and DCs act as scavengers to eliminate pathogen-infected cells by phagocytosis, but research on the phagocytosis of HIV-infected T cells has been underrepresented. Unlike most pathogens, HIV can infect macrophages and DCs and replicate in these cells [30, 31, 32, 33]; thus, the phagocytosis of HIV-infected T cells represents a cell-to-cell transmission mechanism of HIV infection in these cells. More importantly, these antigen-presenting cells can present HIV to

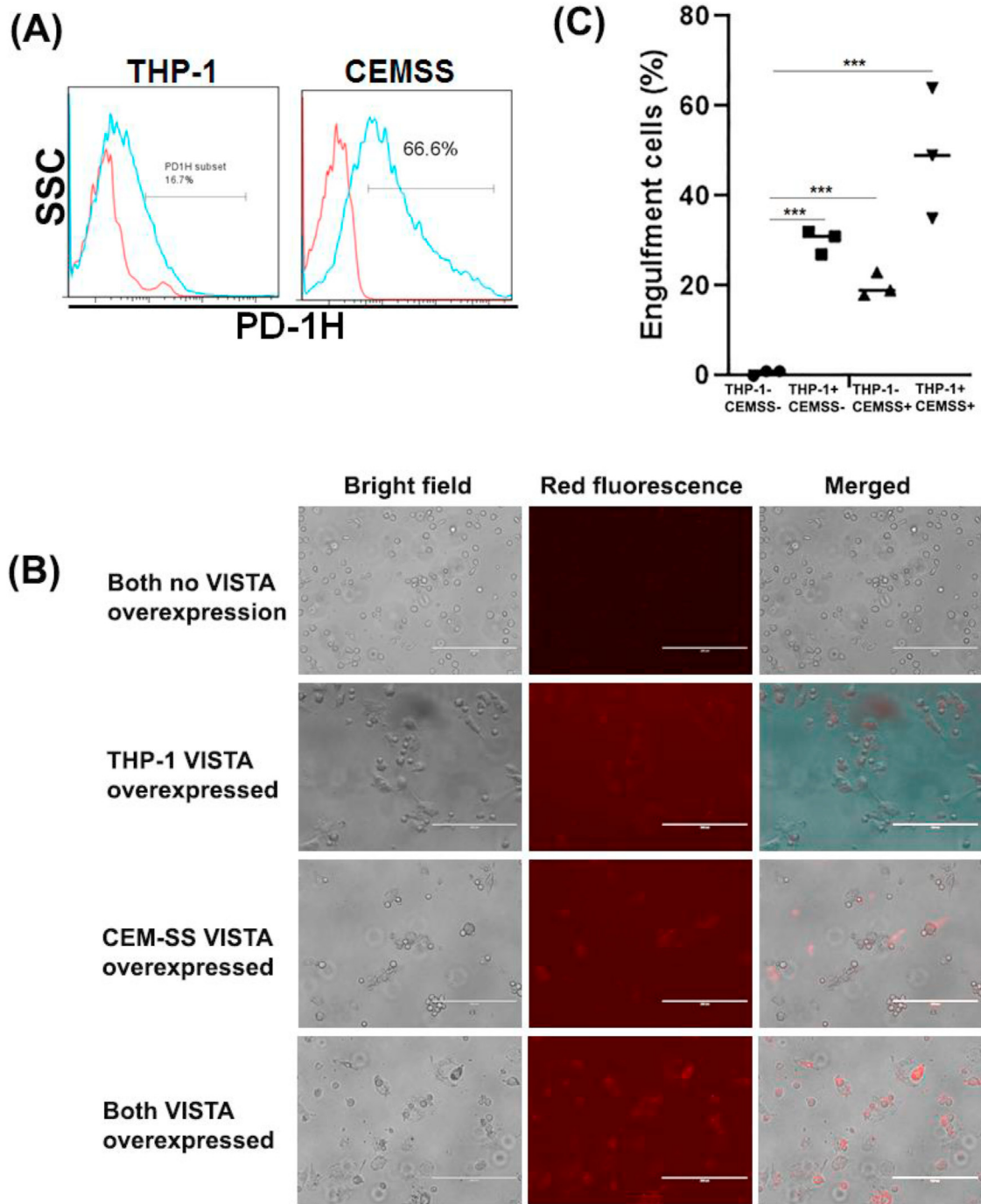


Figure 6. VISTA expression on both phagocytes and HIV-infected CEM-SS T cells facilitates phagocytosis. (A) THP-1 (left) and CEM-SS T cells (right) were transduced with GFP (red curve) and VISTA-overexpressing (blue curve) lentiviruses, and the VISTA expression level was determined by staining the cells with an APC-labeled anti-VISTA mAb. (B) THP-1 cells (without or with VISTA overexpression) and pHrodo-stained CEM-SS T cells (without or with VISTA overexpression) were mixed together, and the phagocytosis rate was determined by fluorescence microscopy. (C) The histogram shows the phagocytosis rates for the different groups in (B).

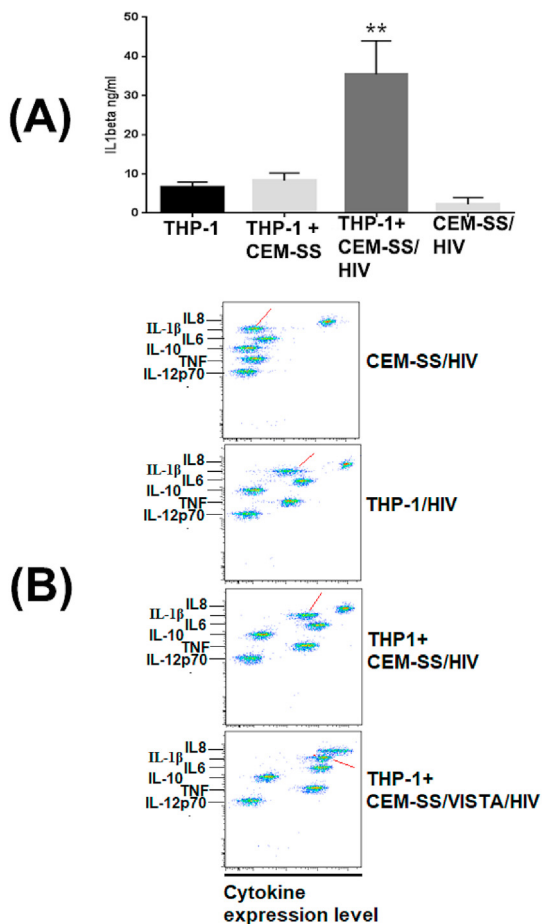


Figure 7. IL-1 β expression is upregulated during phagocytosis of HIV-infected CEM-SS T cells, and VISTA overexpression on CEM-SS T cells upregulates IL-1 β secretion. (A) THP-1 cells were mixed with HIV-infected or uninfected CEM-SS T cells. After 24 h of incubation, the supernatants were collected and evaluated with an IL-1 β ELISA to assess the expression level. The supernatants from only THP-1 cells or HIV-infected CEM-SS T cells were used as controls. (B) The inflammatory bead assay showed the IL-1 β secretion changes during the phagocytosis of HIV-infected CEM-SS T cells by VISTA-overexpressing THP-1 cells.

healthy T cells, thus causing wide spread of the virus. Furthermore, long-lived macrophages can establish a latent infection, and these infected long-lived macrophages can migrate to tissues where they become tissue-resident cells; thus, phagocytosis of HIV-infected T cells by phagocytes can become an important source of the latent HIV reservoir. Therefore, identifying the possible mechanisms underlying the phagocytosis of HIV-infected T cells may provide potential therapeutic targets to intervene in HIV infection and reduce the HIV reservoir.

Consistent with studies by the Sattentau group [13], we found that phagocytic activity increased after HIV infection of CEM-SS T cells. We also found that VISTA was highly expressed on the surface of apoptotic cells, both HIV infected and noninfected, and that VISTA blockade could reduce phagocytosis of HIV-infected CEM-SS T cells, implying that a possible mechanism underlying HIV-infected CEM-SS T cell engulfment is that VISTA molecules are involved in the apoptotic pathway in a manner that regulates the phagocytic process. Coincidentally, during the clearance of influenza virus-infected cells by macrophages, an apoptosis-dependent mechanism of phagocytosis was revealed by two studies [34, 35]. Nevertheless, given that phagocytosis of either healthy or apoptotic HIV-infected cells can occur [13], other mechanisms that may be involved in the phagocytosis of HIV-infected CEM-SS T cells cannot be excluded. Notably, instead of using primary T cells for VISTA

overexpression, we used a T cell line, CEM-SS, as the model for HIV infection and phagocytosis, partly because it is difficult to achieve high lentiviral transduction efficiency and a high VISTA expression level in primary T cells. Moreover, using a high MOI for lentiviral transduction of primary cells, which is necessary to achieve a significant VISTA expression, may lead to substantial cell death and the consequential phagocytosis that will interfere with VISTA-induced phagocytosis. Thus, the results would not reflect the actual situation in the course of HIV infection in humans.

It has been reported that the expression of the tumor suppressor gene p53 can activate VISTA expression and that VISTA then acts as a ligand to induce phagocytosis [36]. In this hypothesis, p53 induction of VISTA expression in apoptotic cells is a critical component for phagocytosis. Indeed, when VISTA was overexpressed in CEM-SS T cells, we found a significantly increased level of phagocytosis. Hence, both studies by our group and the Lee group suggested that VISTA might act as a direct ligand for phagocytosis. Unfortunately however, the only potential binding molecule with VISTA has been identified as IgSF11 (or BT-IgSF) by high throughput screening of a transmembrane protein expression library [37], but there is no any finding reported it as an engulfment receptor, thus the VISTA-induced mechanism of phagocytosis needs to be further investigated. Strikingly, when we overexpressed VISTA in phagocytes, phagocytosis was also increased significantly, suggesting that VISTA may directly enhance the phagocytotic activity in phagocytes, which is distinct from the mechanism of p53-initiated apoptosis-dependent pathway. Given that VISTA may act as receptor or ligand [38], and that VISTA can bind to itself via its extracellular domain [36], we speculate that VISTA overexpressed on the phagocytic cells may act as a phagocytic receptor, and it may directly bind to VISTA (ligand) on the apoptotic T cells, thus triggering the phagocytosis-related pathway.

It is worth noting that our study demonstrated that the level of the proinflammatory cytokine IL-1 β was elevated during phagocytosis of HIV-infected CEM-SS T cells. Generally, apoptosis has been characterized as active programmed cell death that avoids eliciting inflammation [24]. Therefore, beyond the mechanism of apoptosis-induced phagocytosis, there must be another mechanism that is responsible for the secretion of proinflammatory cytokines. To identify the possible mechanism, we stained mixed cell cultures containing phagocytes and HIV-infected CEM-SS T cells with fluorophore-labeled Annexin-V and 7-AAD (staining for dead cells). We found that most of the cells demonstrated characteristics of apoptosis (Annexin-V staining), while 4.5% of the cells showed pyroptosis-like characteristics (7-AAD staining or double staining) (Supplementary Fig. 3), indicating that some of the HIV-infected CEM-SS T cells were engulfed by phagocytes for pyroptosis and that during this process, proinflammatory cytokines were released. However, whether this process of phagocytosis contributes to the chronic inflammation normally seen in HIV-infected patients remains to be further investigated.

Our future studies will focus on identifying the other mechanisms that may be involved in phagocytosis of HIV-infected CEM-SS T cells other than the apoptosis-induced mechanism described here, and several important questions remains to be answered. For instances, if, as we speculated that, VISTA plays a role as phagocytic receptor, is the phagocytosis mechanism similar to the well-identified mechanism of Fc γ receptor or complement receptor-mediated phagocytosis? Whether and how VISTA overexpression impacts the sequential phagocytosis events after receptor-ligand binding, such as actin cross-linking and remodeling, activation of internalization/engulfment, phagosome formation following lysosomal processing, etc.? As we have shown that blockade of VISTA by an anti-VISTA antibody reduces phagocytosis events, then how VISTA blockade will affect other phagocytosis-related protein expression? In addition, whether knockout of VISTA and some key apoptosis genes will affect phagocytosis of HIV-infected T cells, as well as how HIV-infected T cell phagocytosis regulates phagocyte immunotolerance? All of these are exciting problems to be solved in the future.

Declarations

Author contribution statement

Xuequn Xu, Guohua Yi: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Sean Petersen, Cynthia Rodriguez: Performed the experiments.

Funding statement

This work was supported by the following grants: RO1 HL125016, from NIH/NHLBI, UG3 AI150550 and R03 AI120696 from NIH/NIAID to G.Y.

Data availability statement

Data included in article/supp. material/referenced in article.

Competing interest statement

The authors declare no conflict of interest.

Additional information

Supplementary content related to this article has been published online at <https://doi.org/10.1016/j.heliyon.2021.e07496>.

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