The Mitochondrial Translocator Protein, TSPO, Inhibits HIV-1 Envelope Glycoprotein Biosynthesis via the Endoplasmic Reticulum-Associated Protein Degradation Pathway

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ABSTRACT

The HIV-1 Env glycoprotein is folded in the endoplasmic reticulum (ER), which is necessary for viral entry and replication. Currently, it is still unclear how this process is regulated. The glycoprotein folding in the ER is controlled by the ER-associated protein degradation (ERAD) pathway, which specifically targets misfolded proteins for degradation. Previously, we reported that HIV-1 replication is restricted in the human CD4⁺ T cell line CEM.NKR (NKR). To understand this mechanism, we first analyzed cellular protein expression in NKR cells and discovered that levels of the mitochondrial translocator protein TSPO were upregulated by ~64-fold. Notably, when NKR cells were treated with TSPO antagonist PK-11195, Ro5-4864, or diazepam, HIV restriction was completely disrupted, and TSPO knockdown by short hairpin RNAs (shRNAs) achieved a similar effect. We next analyzed viral protein expression, and, interestingly, we discovered that Env expression was specifically inhibited. Both TSPO knockdown and treatment with TSPO antagonist could restore Env expression in NKR cells. We further discovered that Env proteins were rapidly degraded and that kifunensine, an ERAD pathway inhibitor, could restore Env expression and viral replication, indicating that Env proteins were misfolded and degraded through the ERAD pathway in NKR cells. We also knocked out the TSPO gene in 293T cells using CRISPR/Cas9 (clustered, regularly interspaced, short palindromic repeat [CRISPR]/CRISPR-associated-9) technology and found that TSPO could similarly inhibit Env expression in these cells. Taken together, these results demonstrate that TSPO inhibits Env protein expression through the ERAD pathway and suggest that mitochondria play an important role in regulating the Env folding process.

IMPORTANCE

The HIV-1 Env glycoprotein is absolutely required for viral infection, and an understanding of its expression pathway in infected cells will identify new targets for antiretroviral therapies. Env proteins are folded in the ER and secreted through the classical secretory pathway. The Env folding process involves extensive cross-linking of 10 Cys residues by disulfide bond formation and heavy N-glycosylation on ~30 Asn residues. Currently, it is still unclear how this process is regulated. Here, we studied this mechanism in the HIV nonpermissive human CD4⁺ T cell line CEM.NKR. We found that Env proteins were rapidly degraded through a cellular pathway that specifically targets misfolded proteins, resulting in inhibition of Env expression. Importantly, we have identified a mitochondrial translocator protein, TSPO, which could trigger this degradation by interfering with the Env folding process. Further characterization of TSPO antiviral activity will reveal a novel antiretroviral mechanism that targets the Env protein.

The HIV-1 envelope (Env) glycoprotein is an indispensable viral protein that is absolutely required for viral entry. Like host cell surface and secretory proteins, Env proteins are produced through the classical secretory pathway. They are translated as a 160-kDa type I integral membrane glycoprotein precursor (gp160) on the rough endoplasmic reticulum (ER) and imported into the ER lumen for proper folding and modification. Mature gp160 proteins are exported to the *cis*-Golgi compartment, where they are cleaved by cellular proteases to produce surface gp120 and transmembrane gp41 proteins, which are delivered to the plasma membrane for virion incorporation (1).

A major procedure involved in gp160 maturation is the ER oxidative protein folding process. The gp160 protein has 20 highly conserved Cys residues, which are cross-linked into 10 disulfide bonds so that the protein can be folded into natural conformation (2). Disulfide bond formation stabilizes proteins and facilitates protein folding, which is controlled by ATP, calcium, and the redox state. The ER has the highest Ca²⁺ concentration inside the

cell (3), and depletion of Ca²⁺ in the ER was shown to stall oxidative protein folding (4, 5). In addition, unlike the cytoplasm and mitochondria, which have a reduced redox state, the ER has a rather oxidizing microenvironment, which is essential for disulfide bond formation (6, 7). The ER has many folding enzymes and molecular chaperones to assist this oxidative folding process. In addition, gp160 maturation also requires glycosylation, which is another posttranslational modification that enhances protein folding by improving protein thermal stability. Notably, the ER

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protein folding process is tightly controlled by the ER-associated protein degradation (ERAD) pathway, which retro-transport misfolded proteins to the cytosol and degrades them through the ubiquitin-proteasome system (8).

The translocator protein TSPO, also known as the peripheral-type benzodiazepine receptor (PBR), was originally identified as a high-affinity diazepam-binding protein in peripheral tissues (9). It is widely expressed *in vivo*, particularly in tissues where steroids are synthesized. Inside cells, TSPO is mainly located on the outer mitochondrial membrane, particularly on the outer and inner membrane contact sites (10). Its best-known function is described in steroid biosynthesis (11). TSPO has a C-terminal cholesterol interaction sequence and imports cholesterol from the outer to the inner mitochondrial membrane (12). TSPO is overexpressed in breast cancer patients, and, in particular, its overexpression correlates with advancing stages of the malignancy (13–15). TSPO has a number of pharmacological ligands, including antagonists PK11195, Ro5-4864, and diazepam and agonist FGIN-1-27. Treatment with PK11195 was found to decrease breast cell proliferation, invasion, and migration.

TSPO interacts with an outer mitochondrial membrane protein, the voltage-dependent anion channel (VDAC) protein (16), which is considered to be a component of the mitochondrial permeability transition pore (mPTP) complex. The mPTP complex is hypothesized to consist of VDAC, the inner membrane protein adenine nucleotide translocase (ANT), and the mitochondrial matrix protein cyclophilin D (CypD) (17). This complex controls mitochondrial membrane integrity by maintaining a proton gradient across the inner mitochondrial membrane, with negative charges inside, which is referred as the mitochondrial transmembrane potential ($\Delta\psi_m$). $\Delta\psi_m$ allows carrier proteins to exchange small molecules between the mitochondrial matrix and cytoplasm for energy production, which is required for the integrity of the mitochondrial membrane (18). High levels of intracellular reactive oxygen species (ROS) and Ca^{2+} overload can disrupt mPTP specificity and allow uncontrolled entry of small solutes into the mitochondrial matrix driven by electrochemical forces, resulting in immediate dissipation of $\Delta\psi_m$ and osmotic swelling of the mitochondrial matrix. Osmotic swelling of the mitochondrial matrix and a consequent rupture of the outer membrane result in release of apoptogenic proteins into the cytoplasm and initiation of apoptosis (17). Trivalent arsenic (As^{III}) directly inhibits mitochondrial thioredoxin reductase activity and elevates intracellular ROS levels (19), which results in opening of the mPTP and apoptosis (20). Conversely, Bcl-2, a human proto-oncogene, increases mitochondrial membrane integrity by stabilizing the mPTP complex and inhibits apoptosis (20, 21). Notably, PK11195, Ro5-4864, and diazepam could also enhance apoptosis by targeting the mPTP complex (21–23). These observations suggested that TSPO contributes to mitochondrial membrane integrity and protects cells from apoptosis.

Previously, we reported that HIV-1 replication is strongly restricted in the human CD4^+ T cell line CEM.NKR (NKR) (24–26). NKR cells were originally isolated from the human T lymphoblastoid cell line CEM based on its resistance to natural killer (NK) cell-mediated lysis (27). How these NKR cells became NK cell resistant is still unknown. We now report that NKR cells overexpress TSPO, resulting in inhibition of Env expression. We found that Env proteins were rapidly degraded in NKR cells and that inhibition of the ERAD pathway could effectively restore Env ex-

pression and HIV-1 replication. These results demonstrate that Env proteins are likely misfolded in NKR cells, resulting in Env degradation via the ERAD pathway, and suggest that mitochondria could influence the Env folding process in the ER.

MATERIALS AND METHODS

Chemicals. Resazurin, As_2O_3 , PK11195, Ro5-4864, diazepam, FGIN-1-27, kifunensine (KIF), brefeldin A, thapsigargin, and tunicamycin were purchased from Sigma.

Cells. Human T cell lines CEM.NKR (NKR), CEM-T4 (T4), and CEM-SS (SS) were obtained from the NIH AIDS Research and Reference Reagent Program. 293T cells and the original CEM cells (CCRF-CEM) were purchased from ATCC. The CEM.NKR subclones N1-NP, N2-NP, N5-P, and N8-SP were described before (26). All T cell lines were cultured in RPMI 1640 medium with 10% fetal bovine serum (HyClone). 293T cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% bovine calf serum (HyClone).

Plasmids. The pNL- ΔVpr vector was described previously (25). Two pGIPZ lentiviral vectors expressing miR-TSPO1 (V2LHS_112183) and miR-TSPO2 (V3LHS_331646) and a pSM2C retroviral vector expressing a control microRNA (miRNA; HMR4867) were purchased from Open Biosystems. The lentiviral packaging vector $\Delta 8.9$ containing the HIV-1 *gag*, *pol*, and *rev* genes was obtained from the laboratory of D. Trono. The retroviral packaging vector pCap expressing murine leukemia virus (MLV) *gag* and *pol* genes was obtained from the laboratory of P. Cannon. A human TSPO expression vector was obtained from the laboratory of K. Gallo, Michigan State University (MSU). The TSPO gene was then subcloned into the pcDNA3.1/V5-His-TOPO vector by a TOPO-cloning strategy (Invitrogen). The pcDNA3.3-TOPO vector expressing human codon-optimized Cas9 was obtained from the G. M. Church laboratory through Addgene (28). To express the TSPO guide RNA as shown in Fig. 7A, a 455-bp gBlock that contained the U6 promoter, 19-bp target, guide RNA scaffold, and termination signal sequences was ordered from Integrated DNA Technologies (IDT) and cloned into the pGEM-T Easy vector (Promega) after PCR amplification, according to the protocol of Mali et al. (28).

Measurement of cell growth and apoptosis. To measure cell growth under As_2O_3 treatment (Fig. 1A), cells were cultured in 96-well plates at a density of 2×10^6 cells/ml and treated with $1 \mu\text{M}$ As_2O_3 for various periods. Viable cells were counted by resazurin dye staining at each time point. Resazurin is a nonfluorescent blue dye, which can be reduced to the pink and highly red fluorescent resorufin in metabolically active cells (29). To count cells at each time point, $80 \mu\text{M}$ resazurin was added to the culture, and cultures were incubated for 4 h. After that, samples were measured spectrophotometrically at the 570-nm wavelength to detect the increase in pink absorbance. For each cell line, a standard curve indicating the number of viable cells at the corresponding absorbance was created. Relative cell viability was then calculated according to a standard curve. Levels of apoptotic induction by As_2O_3 , shown in Fig. 1B, were measured by a Mitochondrial Membrane Potential Detection Kit (Stratagene). Cells were cultured in 24-well plates at a density of 1×10^6 cells/ml and treated with $1 \mu\text{M}$ As_2O_3 for 12 h. After that, cells were incubated in 500 μl of $0.1 \times$ JC-1 reagent solution (Stratagene) for 30 min. After a washing step, cells were resuspended in 500 μl of $1 \times$ assay buffer, and cell fluorescence was measured on a flow cytometer using the FL1 and FL2 channels.

Microarray analysis. Total cellular RNAs were extracted from CEM-SS, CEM-T4, and CEM.NKR cells using TRIzol reagent (Invitrogen). Single-stranded antisense cDNAs were then synthesized using Ovation RNA Amplification System V2 (NuGEN Technologies). This kit utilizes the Ribo-SPIA (single primer isothermal amplification) technology that uses a DNA/RNA chimeric primer to produce large amounts of cDNA from a small quantity of total RNA. Amplified cDNAs were fragmented and biotinylated using an FL-Ovation cDNA Biotin Module V2 kit (NuGEN Technologies). The labeled antisense cDNAs were hybridized to the Human Genome U133 Plus 2.0 Array (Affymetrix), washed, and stained

using an Affymetrix Hybridization Wash Stain Kit. GeneChip probe arrays for gene expression were analyzed using the GeneChip Operating Software (GCOS) at the MSU Research Technology Support Facility.

Viral infection of human T cell lines. HIV-1 virions were produced from 293T cells by transfection of various proviral constructs using a standard calcium phosphate transfection protocol. Typically, 20 μ g of HIV-1 proviral DNA was used for each transfection in a 100-mm culture dish with 40% confluence of cells, and viruses were quantitated by p24^{Gag} enzyme-linked immunosorbent assay (ELISA). To infect T cells, viruses equivalent to 50 ng of p24^{Gag} were inoculated into 2×10^5 cells and incubated at 37°C for 3 h. After removal of the inoculum, followed by three extensive washes, cells were cultured in 24-well plates for 16 days in the presence or absence of various concentrations of As₂O₃ or TSPO ligand. Culture supernatants were then collected every 2 days for measurement of p24^{Gag} by ELISA, followed by supplementation with fresh medium.

Detection of HIV-1 protein expression in virions produced from T cell lines. A total of 2×10^7 cells were infected with vesicular stomatitis virus G protein (VSV-G) pseudotyped HIV-1 equivalent to 500 ng of p24^{Gag} by spinoculation. In particular, cells were placed in a 48-well plate with the virus and centrifuged at $1,200 \times g$ at 25°C for 2 h. Cells were then washed three times and cultured in the presence of 0.25 μ M As₂O₃ or 50 μ M PK11195 or without any treatment for 48 h. After that, supernatants were collected, and virions were pelleted down by a Sorvall S100-AT6 rotor in a Discovery m150se micro-ultracentrifuge at 4°C and 70,000 rpm for 1 h. Virions were then lysed by 1% Triton X-100. Cells were lysed by a lysis buffer (25 mM Tris-HCl, pH 7.8, 2 mM dithiothreitol, 2 mM 1,2-diaminocyclohexane-*N,N,N',N'*-tetraacetic acid, 10% glycerol, 1% Triton X-100). Both cell and viral lysate were analyzed by Western blotting with specific antibodies.

Establishment of TSPO knocked down NKR-KD1 and NKR-KD2 cell lines. Recombinant lentiviruses expressing TSPO short hairpin RNAs (shRNAs) were produced from 293T cells cultured in a 100-mm dish after transfection of 10 μ g of pGIPZ, 10 μ g of Δ 8.9, and 1 μ g VSV-G expression vectors using the calcium phosphate transfection method. Viruses were collected from the supernatants after 48 h and used to infect NKR cells. Infected cells were cultured in the presence of 1 μ g/ml puromycin. Two stable knockdown (KD) cell lines were finally created: NKR-KD1 cells expressing miR-TSPO1 and NKR-KD2 cells expressing miR-TSPO2. An NKR cell line expressing a control miRNA (NKR-Ctrl) was established by a different strategy. Recombinant retroviruses expressing a control miRNA were produced from 293T cells after transfection of 10 μ g of pSM2C, 10 μ g of pCgp, and 1 μ g of VSV-G expression vectors. Viruses were collected to infect NKR cells in the presence of 1 μ g/ml puromycin, and a stable cell line was finally created.

Pulse-chase analysis. HIV-1-infected T cells were pulse labeled for 60 min with 250 μ Ci of EasyTag Express ³⁵S protein labeling mixture (PerkinElmer Life Sciences). The initial pulse-labeled (time zero [$t = 0$]) samples were harvested. The remaining radiolabeled samples were incubated with normal Dulbecco's modified Eagle's medium plus 10% fetal bovine serum and harvested at various time points. The harvested cell pellets were lysed in radioimmunoprecipitation assay buffer for 15 min on ice and clarified at $14,000 \times g$ at 4°C for 10 min. Samples were incubated with anti-gp120 antibodies, and Env proteins were immunoprecipitated by protein G-conjugated beads (Roche Applied Science). After being washed, the samples were analyzed by SDS-PAGE. The gels were scanned by a Typhoon 9200 instrument, and the images were quantified by ImageQuant TL software (Amersham Biosciences).

Establishment of TSPO knockout 293T cells using CRISPR/Cas9 technology. 293T cells grown in a 100-mm dish were transfected with 10 μ g of Cas9 (clustered, regularly interspaced, short palindromic repeat [CRISPR]-associated-9) expression vector, 10 μ g of TSPO guide RNA expression vector, and 1 μ g of pEGFP-N1 (where EGFP is enhanced green fluorescent protein) (Clontech) vector using the calcium phosphate transfection method. Cells were trypsinized 4 days after transfection and resuspended in DMEM. Single cells were isolated into 96-well plates using a BD Influx sorter gated on GFP fluorescence. Clonal cell populations were expanded, and whole-cell extracts were analyzed by Western blotting. In addition, genomic DNA samples were extracted from 1×10^5 cells by lysing and digesting cells in 25 μ l of buffer (10 mM Tris-HCl, pH 8.0, 50 mM NaCl, 5 mM EDTA, 0.1% SDS, 60 μ g/ml proteinase K) and incubation at 55°C for 1 h. Following another incubation at 98°C for 10 min, samples were diluted at a 1:10 ratio with water, and 2 μ l was used to amplify the 88-bp fragment encompassing the gene inactivation locus in 20 μ l of PCR buffer using TSPO-ko-S and TSPO-ko-A as a primer pair. The sequence of these two primers is shown in Fig. 7A. The PCR fragments were analyzed in a 10% polyacrylamide gel using 1 $\times$ Tris-borate-EDTA (TBE) as a buffer after ethidium bromide staining.

Western blotting. A goat anti-human TSPO antibody was purchased from Novus. Goat anti-actin antibody was purchased from Santa Cruz. Mouse monoclonal anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody was from Meridian Life Science. Antibodies against VDAC, ATPase, and CypD used in the experiments shown in Fig. 2B were purchased from MitoSciences. HIV-1 viral proteins were detected by antibodies from the NIH AIDS Research and Reference Reagent Program, and their catalogue numbers are 1513 (Gag), 526 (gp41), 521 (gp120), and 969 (Vpu). Horseradish peroxidase (HRP)-conjugated anti-human, -rabbit, -goat, or -mouse immunoglobulin G secondary antibodies were purchased from Pierce. Detection of the HRP-conjugated antibody was performed using an enhanced chemiluminescence detection kit (Amersham Bioscience).

RESULTS

Nonpermissive NKR cells are more resistant to apoptosis than the permissive cells. During studies of HIV restriction in NKR cells, we isolated, by limiting dilution, several NKR subclones which exhibited different levels of HIV resistance. The N5-P clone is highly permissive, the N8-SP is semipermissive, and the N1-NP and N2-NP clones are nonpermissive (permissiveness is indicated in clone designations as P, SP, and NP, respectively) for HIV-1 infection (26). We have reported that As₂O₃ could effectively disrupt HIV-1 restriction in NKR cells (25). As₂O₃ is highly toxic to cells due to its ability to induce apoptosis. Interestingly, when NKR cells were treated with As₂O₃, it was found that NKR, N1-NP, N2-NP, and N8-SP cells showed much higher viability than N5-P and the original CEM cells (Fig. 1A). This result suggested that the nonpermissive or semipermissive NKR clones could be more resistant to As₂O₃-induced apoptosis than the permissive clones.

To verify this suggestion, we directly measured the apoptotic cell population by flow cytometry after staining As₂O₃-treated cells with the mitochondrial dye JC-1. In healthy cells, JC-1 normally stays in mitochondria due to the negatively charged environment. However, when the $\Delta\psi_m$ dissipates, JC-1 diffuses into

FIG 1 NKR cell resistance to apoptosis induction. (A) Resistance to cell death. Indicated cells were treated with 1 μ M As₂O₃, and cell viability was detected by conversion of resazurin into resorufin (see Materials and Methods). The viability of untreated cells (control, Ctrl) was considered as 100% and that of treated cells was calculated and presented as relative values. (B) Resistance to apoptosis. Indicated cells were treated with 1 μ M As₂O₃, and cells were stained with JC-1. Cell fluorescence was then measured on a flow cytometer using the FL1 and FL2 channels. Each of these experiments was repeated at least twice, and consistent results were obtained.

FIG 2 High levels of TSPO expression in NKR cells. (A) Microarray determination of mitochondrial protein expression. mRNA expression profiles in CEM.NKR (NKR), CEM-SS (SS), and CEM-T4 (T4) cells were analyzed by Human Genome U133 Plus 2.0 Array (Affymetrix). After that, a total of 246 mitochondrial proteins were selected, and their levels of expression were compared as relative ratios, as indicated. (B) TSPO protein expression in CEM-derived cells. The indicated cell lysate was prepared from a total of 1×10^7 cells, and one-sixth of the sample was analyzed by Western blotting using the indicated antibodies. (C) To compare the difference between TSPO expression levels in N2-NP and N5-P cells, the lysate from N2-NP cells was serially diluted and then compared to that of an undiluted N5-P cell-derived sample by Western blotting. Each of these experiments was repeated at least twice, and consistent results were obtained.

the cytoplasm and changes its color from red to green, indicating that cells are under apoptosis. It was found that more cells were shifted from red to green in CEM and N5-P than in N1-NP, N2-NP, N8-SP, and NKR cells after As_2O_3 treatment (Fig. 1B). Thus, the nonpermissive cells are more resistant to apoptosis than the permissive cells, and there is a correlation between HIV resistance and apoptosis resistance among CEM and NKR cells and NKR clones.

Nonpermissive NKR cells express much more TSPO than permissive cells. To understand why NKR cells were resistant to the mitochondrion-targeted apoptosis induction, we compared mitochondrial protein expression profiles in NKR, CEM-SS (SS), and CEM-T4 (T4) cells by microarray analysis. SS and T4 cells are two other CEM-derived cell lines, which are highly permissive for HIV-1 infection. We selected a total of 246 well-known mitochondrial proteins, and tried to identify proteins that are enriched in NKR but not in SS and T4 cells. It was found that the mRNA levels of ATPase and TSPO were upregulated by 2- to 4-fold in NKR cells (Fig. 2A). We then compared their protein expression levels by Western blotting. It was found that, indeed, TSPO expression was significantly upregulated in NKR cells compared to levels in CEM cells although ATPase expression was similar (Fig. 2B). Interestingly, N1-NP, N2-NP, and N8-SP cells also expressed very high levels of TSPO, whereas TSPO expression levels in N5-P cells were very low. We also compared VDAC, CypD, and ATPase expression levels in these cells, and no differences were observed (Fig. 2B). We further quantitated the difference in TSPO expression levels in N2-NP and N5-P cells by Western blotting and found that N2-NP cells expressed ~64-fold more TSPO protein than N5-P cells (Fig. 2C). Thus, the nonpermissive and semipermissive NKR cells expressed much more TSPO than the permissive cells, which correlated with their apoptosis resistance. Importantly, we have found a correlation between TSPO expression and HIV-1 resistance in these NKR cells.

Complete disruption of HIV-1 restriction in nonpermissive NKR cells by TSPO antagonists. In order to understand how TSPO regulates HIV-1 replication, we treated the nonpermissive N2-NP cells with a number of different TSPO pharmacological ligands, which included TSPO antagonists PK11195, Ro5-4864, and diazepam and TSPO agonist FGIN-1-27. First, we treated cells with increasing amounts of PK11195 and Ro5-4864 (4 μ M, 8 μ M, 16 μ M, 32 μ M, or 64 μ M) and measured HIV-1 replication. It was found that both PK11195 and Ro5-4864 increased HIV-1 replication in a dose-dependent manner, and treatment with 16 μ M PK11195 or 32 μ M Ro5-4864 increased viral replication by more than 100-fold (Fig. 3A). This result suggested that PK11195 and Ro5-4864 could completely disrupt HIV-1 restriction in these cells. We further tested the activity of diazepam and FGIN-1-27 in N2-NP and N5-P cells. It was found that, like PK11195 and Ro5-4864, diazepam could also fully rescue HIV-1 replication in N2-NP cells, and FGIN-1-27 was completely inactive (Fig. 3B, left panel). In contrast, all of these compounds inhibited HIV-1 replication in N5-P cells although the levels of inhibition varied (Fig. 3B, right panel). Thus, TSPO antagonists could effectively disrupt HIV-1 restriction in the nonpermissive NKR cells.

As noted earlier, TSPO transports cholesterol into mitochondria via a direct interaction (12). We wondered whether cholesterol could also stimulate HIV-1 replication in NKR cells by interacting with TSPO. NKR, N2-NP, N5-P, and SS cells were treated with regular cholesterol (Chol) or cholesterol mixed with methyl- β -cyclodextrin to make it more water soluble (β -CD Chol), and viral replication was determined. It was found that both Chol and β -CD Chol could increase HIV-1 replication in NKR and N2-NP cells but not in N5-P and SS cells although the effect of Chol and β -CD Chol was less than that of As_2O_3 (Fig. 3C). Thus, we concluded that cholesterol could partially disrupt HIV-1 restriction in NKR cells.

FIG 3 Disruption of HIV-1 restriction in NKR cells by TSPO ligands. (A) Effects of PK11195 and Ro5-4864 on HIV-1 restriction. N2-NP cells were infected with HIV-1 (pNL4-3) and treated with increasing amounts of PK11195 or Ro5-4864. Viral replication was determined by measuring the p24^{Gag} in the culture supernatant using ELISAs. (B) Effects of other TSPO ligands on HIV-1 restriction. N2-NP and N5-P cells were treated with 50 μ M FGIN-1-27, 65 μ M PK11195, 50 μ M Ro5-4864, or 5 μ M diazepam during HIV-1 infection, and viral replication was determined. The concentration of these compounds used in this experiment was the highest that did not affect cell viability (data now shown). (C) Effects of cholesterol on HIV-1 restriction. NKR, N2-NP, N5-P, and SS cells were treated with 0.2 μ M As₂O₃, 50 μ M cholesterol (Chol), or 25 μ M water-soluble cholesterol (β -CD Chol) or remained untreated during HIV-1 infection, and viral replication was determined. Each of these experiments was repeated at least twice, and consistent results were obtained.

As₂O₃ and PK11195 restore Env biogenesis in NKR cells. To understand how HIV-1 replication is restricted in NKR cells, we infected NKR, N2-NP, N8-SP, and N5-P cells with HIV-1 in the presence or absence of As₂O₃ or PK11195 treatment and analyzed

viral protein expression. As shown in Fig. 4A, in the absence of any treatment, HIV-1 replicated poorly in NKR and N2-NP cells and replicated productively in N5-P and N8-SP cells. Notably, when virions were purified from the supernatant of these infected cell

FIG 4 (A) HIV-1 replication in the original NKR, nonpermissive subclone N2-NP, semipermissive subclone N8-SP, and permissive subclone N5-P cells. A total of 2×10^5 cells were incubated with equal amounts of HIV-1 (pNL4-3), and viral replication was determined every other day by p24^{Gag} ELISA. (B and C) As₂O₃ and PK11195 increase Env expression. Indicated cells were spinoculated with VSV-G pseudotyped HIV-1 and cultured in the presence or absence of 0.25 μ M As₂O₃ or 50 μ M PK11195. Virions were purified from culture supernatants by ultracentrifugation. Viral protein expression in virions (B) and virus-producing cells (C) was detected by Western blotting as indicated. Each of these experiments was repeated at least twice, and consistent results were obtained.

FIG 5 TSPO knockdown increases HIV-1 replication. (A) Comparison of TSPO expression in TSPO knockdown (KD) and control cell lines by Western blotting. These cell lines were created by stable transduction of two different TSPO-specific shRNAs or a control (Ctrl) shRNA, as described in Materials and Methods. (B) Indicated cells were infected with wild-type (WT) or Δ Vpr HIV-1, and viral replication was determined by p24^{Gag} ELISA. (C) Comparison of Env expression levels in TSPO knockdown and control cell lines. Indicated cells were infected with HIV-1, and the Env and Gag expression were determined by Western blotting.

cultures, it was found that virions from NKR and N2-NP cells contained much less gp41 and gp120 than those from N5-P and N8-SP cells (Fig. 4B, lanes 1 to 4). We then determined the levels of viral protein expression in virus-producing cells. Consistently, it was found that the steady-state levels of Env gp41 and gp160 proteins were substantially reduced in NKR and N2-NP cells, and levels of the gp41 protein in N8-SP cells were also slightly reduced compared to the level in N5-P cells (Fig. 4C, lanes 1 to 4). In sharp contrast, Vpu and Gag (p24^{Gag} and p55^{Gag}) proteins were expressed at comparable levels in all of these cells (Fig. 4C). Importantly, when NKR and N2-NP cells were treated with PK11195 or As₂O₃, the levels of Env protein expression in these infected cells as well as in the virions they produced were all significantly increased, and the expression of Vpu and Gag proteins was not affected (Fig. 4B and C, lanes 5 to 10). Thus, there is a specific inhibition of Env expression in NKR and N2-NP cells that blocks HIV-1 replication, and this inhibition can be overcome by treatment with either PK11195 or As₂O₃.

TSPO knockdown in NKR cells rescues Env expression and HIV-1 replication. Because TSPO expression correlated with HIV-1 resistance in NKR cells and because TSPO antagonists could disrupt HIV-1 restriction in NKR cells, we wondered whether the high level of TSPO expression was responsible for HIV-1 restriction. To answer this question, we knocked down TSPO expression by short hairpin RNAs (shRNAs) and determined HIV-1 replication. The parental NKR cells were transduced with a lentiviral vector that expressed two different TSPO-specific shRNAs, and two stable knockdown (KD) cell lines (NKR-KD1 and NKR-KD-2) were finally established by puromycin selection. A control cell line was similarly established by stable transduction of a control shRNA (NKR-Ctrl). We first compared TSPO expression levels in these

cell lines. It was found that TSPO expression was significantly reduced in both NKR-KD1 and NKR-KD2 cells compared to levels in N2-NP and NKR-Ctrl cells (Fig. 5A). We then determined HIV-1 replication in these cells. NKR-KD1, NKR-KD2, and NKR-Ctrl cells were infected with wild-type (WT) HIV-1, and viral replication levels were compared. Recently, we reported that HIV-1 replication in NKR cells is Vpr dependent (25). Thus, a Vpr-deficient (Δ Vpr) virus was also included in this experiment as a control. It was found that WT HIV-1 replication was significantly increased in NKR-KD1 and NKR-KD2 cells relative to the level in NKR-Ctrl cells, whereas the Δ Vpr virus still replicated very poorly in all of these cells (Fig. 5B). Thus, TSPO could inhibit HIV-1 replication in NKR cells, but this inhibition is different from what we reported previously (25). We further compared Env expression levels in the WT virus-infected cells and found that the NKR-KD1 and NKR-KD2 cells expressed much higher levels of gp41 protein than the NKR-Ctrl cells (Fig. 5C). Thus, TSPO knockdown could effectively increase HIV-1 replication by recovering Env expression in NKR cells.

Env proteins are rapidly degraded through the ERAD pathway. Env proteins are translated from the same viral mRNA templates where Vpu protein is translated (30). Because Vpu expression was not inhibited in NKR cells (Fig. 4C), we hypothesized that Env expression is inhibited at the protein level. We then compared the Env half-lives in N2-NP and N5-P cells by a pulse-chase experiment. It was found that in N5-P cells, the gp120 protein half-life was $\sim$ 16 h, which was reduced to $\sim$ 4 h in N2-NP cells (Fig. 6A). Thus, Env proteins were rapidly degraded in N2-NP cells, indicating that they are highly unstable in these cells.

To explore this Env degradation mechanism, we determined whether the ERAD pathway was responsible for this HIV-1 inhi-

FIG 6 Env proteins are rapidly degraded through the ERAD pathway. (A) Detection of rapid Env degradation. N5-P and N2-NP cells were infected with HIV-1 (pNL4-3) for 24 h. Cells were pulse labeled with [³⁵S]Met-Cys for 1 h and then incubated for the indicated length of time. Env proteins were immunoprecipitated with an anti-gp120 antibody and analyzed by autoradiography, followed by quantification using the Image-Quant TL program. (B) Kifunensine (KIF) rescues HIV-1 replication in N2-NP cells. HIV-infected N2-NP and N5-P cells were treated with increasing amounts of KIF, and viral replication was determined by p24^{Gag} ELISA. (C) KIF rescues Env expression in NKR and N2-NP cells. Indicated cells were spinoculated with VSV-G pseudotyped HIV-1 and cultured in the absence or presence of 40 μ M KIF for 48 h. Cells were lysed, and Env and Gag expression levels were determined by Western blotting. (D) Activation of the ERAD pathway inhibits Env expression. N5-P and T4 cells were treated with brefeldin A (10 μ g/ml), tunicamycin (5 μ g/ml), or thapsigargin (5 μ g/ml) for 48 h during HIV-1 infection or remain untreated, and viral protein expression was determined by Western blotting.

bition. First, we treated N2-NP and N5-P cells with kifunensine (KIF), a widely used ERAD inhibitor, and determined HIV-1 replication. It was found that when increasing concentrations of kifunensine (5 μ M, 10 μ M, 20 μ M, or 40 μ M) were used, viral replication was increased in a dose-dependent manner in N2-NP cells (Fig. 6B). In contrast, similar treatments did not apparently affect HIV-1 replication in N5-P cells. Thus, KIF could rescue HIV-1 replication in N2-NP cells. Second, we determined whether the KIF treatment could increase Env expression in NKR and N2-NP cells. NKR and N2-NP cells were infected with HIV-1 in the presence or absence of KIF treatment, and Env expression was compared to that in infected N5-P cells by Western blotting. It was found that KIF significantly increased gp41 expression, but not Gag expression, in both NKR and N2-NP cells, for which the levels were comparable to the level in N5-P cells (Fig. 6C). Thus, KIF could specifically increase Env expression in nonpermissive NKR cells. Finally, we determined whether activation of the ERAD pathway in HIV-permissive cells could inhibit Env expression. N5-P and T4 cells were treated with three different ERAD induc-

ers, brefeldin A, thapsigargin, and tunicamycin, and viral protein expression was determined. Brefeldin A inhibits protein transport from the ER to the Golgi apparatus, depletes Ca²⁺ from the ER, and tunicamycin directly inhibits protein N-glycosylation. It was found that both brefeldin A and tunicamycin strongly blocked HIV-1 Env expression but not Gag expression and that thapsigargin also slightly inhibited Env expression in T4 cells (Fig. 6D). Thus, activation of the ERAD pathway could specifically target the Env protein for degradation. Taken together, these results demonstrated that the ERAD pathway is likely responsible for Env inhibition in nonpermissive NKR cells.

TSPO inhibits Env expression in 293T cells. To understand whether the TSPO inhibitory effect on Env expression applies to other cell systems, we studied TSPO activity in 293T cells. We first tried to knock out the *TSPO* gene using the most recently developed CRISPR/Cas9 (clustered, regularly interspaced, short palindromic repeat [CRISPR]/CRISPR-associated-9) system (28, 31). This system uses a 19-nucleotide (nt) specific RNA to direct the Cas9 nuclease to the genomic target via Watson-Crick base pair-

FIG 7 TSPO inhibits HIV-1 Env expression in 293T cells. (A) Schematic illustrating Cas9 inactivation of the human *TSPO* locus. Numbers indicate the nucleotide positions in the *TSPO* open reading frame. The 19-bp guide RNA target sequence is shown in green, and the protospacer-adjacent motif (PAM) is shown in red. The sense primer TSPO-ko-S and antisense primer TSPO-ko-A sequences that were used to amplify this gene locus are underlined. (B) Analysis of TSPO protein expression in three clones (A2, A3, and A4) isolated from 293T cells transfected with Cas9 and TSPO guide RNA expression vectors by Western blotting. (C) An 88-bp DNA fragment was PCR amplified from the *TSPO* locus of the A3 clone and wild-type (WT) 293T cells using primers TSPO-ko-S and TSPO-ko-A and analyzed by 10% TBE-polyacrylamide gel. M, marker. (D) Wild-type and A3 cells were transfected with the HIV-1 proviral clone pNL4-3, and Gag and Env protein expression levels were analyzed by Western blotting. (E) Indicated amounts of pNL4-3 and TSPO expression vectors were transfected into A3 cells, and TSPO and HIV-1 viral protein expression levels were analyzed by Western blotting.

ing, which allows Cas9 to precisely introduce a DNA double-strand break (DSB) in front of a protospacer-adjacent motif (PAM). This cleavage activates the error-prone nonhomologous end-joining (NHEJ) pathway or the homology-directed repair (HDR) pathway to repair the DSB, which simultaneously introduces small insertions/deletions (indels) at the cleavage site, resulting in disruption of the reading frame and gene expression. We designed a specific guide RNA to target the human *TSPO* locus (Fig. 7A), which was coexpressed with a human codon-optimized *Cas9* gene in 293T cells. After that, we isolated a number of clones by limiting dilution and found one clone, A3, which did not express any TSPO protein (Fig. 7B). We then amplified an 88-bp DNA fragment from the targeted locus and identified a small deletion in the A3 cells (Fig. 7C). These results demonstrated that the *TSPO* gene was successfully knocked out in A3 cells.

Next, we studied HIV-1 viral protein expression in A3 cells. First, we compared Env expression in A3 and wild-type 293T cells. After cells were transfected with the HIV-1 proviral vector pNL4-3, we observed that both A3 and the wild-type cells expressed similar levels of Gag proteins, but the levels of Env expression were increased in the A3 cells (Fig. 7D). Second, we overexpressed the *TSPO* gene in these cells and compared viral gene expression levels. It was found that although TSPO overexpression did not cause inhibition of HIV-1 replication in wild-type cells (data not shown), it specifically decreased Env but not Gag expression in A3 cells (Fig. 7E). Taken together, these results demonstrated that TSPO could also inhibit Env protein expression in 293T cells.

DISCUSSION

In this report, we investigated the mechanism of how HIV-1 replication is restricted in NKR cells. As noted earlier, the mechanism of how NKR cells became NK cell resistant is still unclear (27). It was demonstrated before that these cells express normal levels of

various major histocompatibility complex class I (MHC-I) molecules, which are the NK cell ligands that are required for NK cell engagement to their target cells (27). Thus, NK cell resistance should not occur at the engagement step. After engagement, NK cells secrete granzymes into target cells, which dissipate $\Delta\psi_m$ by opening the mPTP and kill target cells by apoptosis (32). We have found that NKR cells are resistant to apoptosis when treated with As_2O_3 (Fig. 1). Thus, it is highly likely that the NK cell resistance is associated with apoptosis resistance in these cells. In addition, we have found that TSPO is overexpressed in NKR cells, and TSPO expression correlates with apoptosis resistance in NKR cells (Fig. 2). These results suggest that TSPO overexpression should increase mitochondrial membrane integrity by stabilizing the mPTP complex, which is consistent with previous observations made by other groups.

We observed a nice correlation between TSPO expression and Env inhibition in NKR cells. First, we found that Env expression was inhibited in only NKR and N2-NP cells, where TSPO was significantly overexpressed, and it was not inhibited in CEM, N5-P, SS, and T4 cells, where TSPO was expressed at lower levels (Fig. 4C and 5A). Second, when we knocked down TSPO expression in NKR cells, we detected a significant increase in Env expression (Fig. 5B). Third, when NKR and N2-NP cells were treated with TSPO antagonists PK11195, Ro5-4864, and diazepam, we found that the viral restriction as well as Env inhibition was disrupted (Fig. 3A and 4C). Lastly, when *TSPO* was knocked out from 293T cells, Env expression was increased, whereas TSPO overexpression resulted in a dose-dependent inhibition of Env expression (Fig. 7D and E). These results suggest that TSPO overexpression is probably responsible for Env inhibition in NKR cells. We have collected several pieces of evidence to suggest how TSPO could inhibit Env expression. When we determined Env protein stability by pulse-chase experiments, we found that the Env half-life in N2-NP cells was significantly reduced compared to that in

N5-P cells (Fig. 6A), indicating that rapid Env degradation occurred in the nonpermissive NKR cells. In addition, we found that treatment of N2-NP cells with KIF could increase Env expression and HIV-1 replication (Fig. 6B and C), and treatment of N5-P and T4 cells with brefeldin A and tunicamycin could specifically inhibit Env expression. These results demonstrate that Env proteins are degraded through the ERAD pathway.

Because the ERAD pathway selectively targets misfolded glycoproteins for degradation, our results further suggest that Env proteins should be misfolded in NKR cells. Indeed, we have more evidence to support this conclusion. We have reported that As_2O_3 could disrupt HIV-1 restriction in NKR cells (25), and we showed here that it could specifically increase Env expression (Fig. 4C). As noted earlier, As_2O_3 increases intracellular ROS levels, and the Env folding process requires formation of 10 disulfide bonds by cross-linking of 20 Cys residues, which depends on a highly oxidizing redox state in the ER. We therefore speculate that As_2O_3 could promote Env folding by improving the ER oxidative redox state, resulting in an increase in Env expression. Thus, our results also suggest that the ER in the nonpermissive NKR cells may have an unfavorable redox state, which may not support Env folding, resulting in Env misfolding and degradation.

Although it is well known that Env proteins are folded in the ER, the precise mechanism of this process is still unclear. Because TSPO overexpression could influence Env folding, it suggests that mitochondria could play an important role in regulating the ER oxidative protein folding process. In fact, accumulated evidence has suggested a tight association between the ER and mitochondria. As cellular power plants, mitochondria generate most of the ATP, and they are also responsible for production of intracellular ROS. On the other hand, the ER is the main site of lipid synthesis, and it also serves as the principal Ca^{2+} storage site. In order to exchange these metabolites, these two organelles have to communicate with each other by establishing physical interactions (33). Indeed, the ER contains a specialized subcompartment that is called the mitochondrial-associated ER membrane (MAM), which physically connects the ER to mitochondria (34). In *Saccharomyces cerevisiae*, the ER and mitochondria are tethered by a protein complex called the ER-mitochondria encounter structure (ERMES), which includes two integral outer mitochondrial membrane proteins, Mdm10 and Mdm34, one ER integral membrane protein, Mmm1, and a cytosolic protein, Mdm12 (35). In mammalian cells, MAM is supported by a protein complex consisting of the outer mitochondrial membrane protein VDAC, the ER Ca^{2+} channel inositol (1,4,5)-triphosphate receptor (IP3R), growth receptor protein 75 (GRP75), mitofusin-2, and the cargo-sorting protein PACS2 (36). MAM supports essential lipid and Ca^{2+} transfer from the ER to mitochondria, which play an important role in transduction of calcium signaling and maintenance of ER homeostasis. Because the ER oxidative protein folding is dependent on ATP, high levels of Ca^{2+} , and a highly oxidizing redox state, an important role of MAM in this protein folding process has been proposed (34). In fact, it was shown that mitochondrial ROS production could directly control cell surface protein expression (37). In addition, knockdown of PACS-2 expression not only uncouples the ER from mitochondria but also induces a misfolded protein response in the ER, confirming that MAM has a direct impact on ER oxidative protein folding (38).

Because VDAC is found in the protein complex that is essential for MAM formation, we speculate that the mPTP complex plays a

role in ER-mitochondrion communication. Indeed, it was reported that ANT overexpression could reduce ER-mitochondrion Ca^{2+} flux (39, 40), suggesting that further stabilization of the mPTP complex could have a negative impact on ER-mitochondrion communication. Because TSPO interacts with mPTP via VDAC, it is possible that TSPO overexpression in NKR cells could interfere with ER-mitochondrion communication, with the result that the ER cannot maintain an optimized environment to support Env folding. It should be noted that TSPO overexpression in NKR cells does not cause global inhibition of oxidative protein folding because it was shown that MHC-I protein expression is not inhibited in these cells (27). TSPO overexpression might target only viral proteins such as HIV-1 gp160, whose folding process involves extensive disulfide bond cross-linking. Nevertheless, our results indicate that mitochondria play an important role in the ER oxidative protein folding process. Further illustration of this mechanism will explore a novel antiretroviral mechanism by targeting the Env protein.

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