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Cellular and Viral Control over the Initial Events of Human Cytomegalovirus Experimental Latency in CD34$^+$ Cells$^\nabla$

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Human cytomegalovirus (HCMV) persists for the life of its host by establishing a latent infection. The identification of viral and cellular determinants of latency is the first step toward developing antiviral treatments that target and might clear or control the reservoir of latent virus. HCMV latency is established in CD34$^+$ cells when expression of viral immediate early (IE) proteins that initiate lytic infection is silenced. Viral IE gene expression during lytic infection is controlled by a cellular intrinsic immune defense mediated by promyelocytic leukemia nuclear body (PML-NB) proteins such as Daxx and histone deacetylases (HDACs). This defense is inactivated at the start of lytic infection by the HCMV virion tegument protein pp71, which upon viral entry traffics to the nucleus and induces Daxx degradation. Here we show that a similar defense is present, active, and not neutralized during experimental latency in CD34$^+$ cells infected *in vitro* because tegument-delivered pp71 remains in the cytoplasm. Artificial inactivation of this defense by HDAC inhibition or Daxx knockdown rescues viral IE gene expression upon infection of CD34$^+$ cells with a laboratory-adapted viral strain but not with clinical strains. Interestingly, coinfection of CD34$^+$ cells with clinical viral strains blocked the ability of an HDAC inhibitor to activate IE1 and early protein expression during infection with a laboratory-adapted strain. This suggests that in addition to the intrinsic defense, HCMV clinical strains contribute an HDAC-independent, *trans*-acting dominant means of control over viral gene expression during the early stages of experimental HCMV latency modeled *in vitro* in CD34$^+$ cells.

Human cytomegalovirus (HCMV) is a betaherpesvirus that infects the majority of the population, causing disease in patients with inadequate immune function (48). There is no effective vaccine against HCMV, and treatments for productive, lytic viral replication exist but are often ineffective because of toxicity and the development of resistant viruses. Furthermore, the establishment of a lifelong latent infection for which there is no therapy means that curing an HCMV-infected patient is currently unachievable. Thus, all aspects of HCMV replication, but particularly the establishment, maintenance, and reactivation of latent infections, are under intense study for the identification of proteins and processes that can be novel targets of antiviral treatments.

HCMV latency has been observed and studied in various cell populations that represent incompletely differentiated cells within the myeloid lineage (18, 34, 35, 44, 70, 76). Recently, CD34$^+$ hematopoietic progenitor cells infected either *in vivo* (true latency) or *in vitro* (experimental latency) have emerged as the most commonly examined cell population for studies of HCMV latency (4, 5, 13, 14, 55, 57–59). Differentiation of CD34$^+$ cells either prior to or after HCMV infection precludes latency and activates (or reactivates) the expression of viral lytic-phase genes, leading to a productive infection (57, 58, 70, 76). Thus, HCMV gene expression and the outcome of infection (latent or lytic) are referred to as differentiation dependent (67).

Lytic, persistent, and latent HCMV infections are studied *in vitro* by using two different classes of viral strains. One, exemplified by strain AD169, represents viruses that have been propagated for many years *in vitro* in fibroblasts. These are termed laboratory strains. The second class, called clinical strains, represents viruses that were isolated more recently from infected individuals and have undergone limited passage *in vitro*. Clinical strains retain tropism for endothelial and epithelial cells that is lost in laboratory viral strains. This extended tropism is mediated by viral proteins that allow clinical-strain virions to enter a broader range of cell types than laboratory-strain virions (68). The genes that encode these tropism extenders are found near or within a region of the genome termed the ULb′ locus that is present in low-passage clinical strains of HCMV but is absent in high-passage laboratory-adapted strains (3).

Clinical strains of HCMV are clearly able to establish, maintain, and reactivate from experimental latency modeled in CD34$^+$ cells (13, 14, 57, 58). Two studies that have analyzed the ability of the laboratory-adapted AD169 strain to latently infect CD34$^+$ cells have yielded antithetical results. In one report, HCMV AD169 was found to establish experimental latency in CD34$^+$ cells, maintain latency for at least 11 days, and fully reactivate a productive infection (5). The other study observed detectable levels of productive replication during the carriage of HCMV AD169 in CD34$^+$ cell culture for 14 days (13). Whether these disparate results extend from biological differences between the cells and viruses utilized or from technical components of the individual assays that were employed remains an area of research and debate.

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Mechanisms governing the establishment, maintenance, and reactivation of HCMV latency are poorly understood. It is thought that in order for HCMV to establish latency, expression of the viral immediate early (IE) genes that initiate lytic infection (IE1 and IE2) must be silenced (48, 66). The viral major IE promoter (MIEP) controls the expression of IE1 and IE2, which are produced from differentially spliced transcripts (48). At the start of a lytic infection, expression from the MIEP is initially subject to transcriptional silencing by a cellular intrinsic immune defense mediated by the promyelocytic leukemia nuclear body (PML-NB) proteins ATRX, Daxx, and PML (2, 42, 56, 62, 72, 73, 79). PML-NB proteins associate with infecting viral genomes (28), and together with histone deacetylases (HDACs), they silence IE gene expression by establishing a repressive chromatin structure at the MIEP (7, 16, 79). IE gene expression initiates in part through the action of pp71 (39), a tegument protein found in HCMV virions (32) that traffics to PML-NBs in the nucleus, displaces ATRX from PML-NBs (42), and induces the SUMOylation (23) and ubiquitin-independent degradation (24) of Daxx. The subsequent expression of IE1 and IE2 then neutralizes the repressive effects of the PML and HDAC proteins (53, 54, 72, 73). Thus, pp71 orchestrates the stepwise inactivation of the PML-NB-mediated intrinsic defense meant to silence viral IE gene expression, facilitating the start of the HCMV lytic replication cycle (32, 63). Interestingly, despite encoding multiple viral proteins that either directly or indirectly impair HDAC function, wild-type HCMV IE gene expression and productive viral replication are still enhanced by small-molecule HDAC inhibitors in multiple cell types *in vitro* (37, 45–47, 62, 79).

This PML-NB-mediated intrinsic defense also acts to silence IE gene expression during quiescent infection of incompletely differentiated NT2 and THP-1 cells (61). Similar to true or experimental latency in $CD34^+$ cells, these cell systems display differentiation-dependent viral gene expression and productive, lytic replication. HCMV IE genes are silenced upon infection of undifferentiated NT2 and THP-1 cells but are expressed upon infection of cultures of these cells that were previously induced to differentiate (12, 43, 51, 77). However, unlike for $CD34^+$ cells, the subsequent differentiation of previously infected NT2 or THP-1 cells rarely results in lytic reactivation (43, 81), although stimulation of the cyclic AMP signaling pathway can lead to IE gene expression in previously infected NT2 cells (33, 82). Thus, in order to distinguish them from true or experimental viral latency, infections in these cells are termed quiescent or latent-like. The PML-NB-mediated intrinsic defense is not inactivated at the start of quiescent HCMV infections, because tegument-delivered pp71 is sequestered in the cytoplasm of these cells (61), permitting the maintenance of high levels of Daxx, which contributes to the repression of viral IE gene expression.

The PML-NB-mediated defense that silences HCMV IE gene expression acts by facilitating the establishment of a repressive chromatin structure at the MIEP. Unacetylated histone H4 and heterochromatin protein-1 (HP-1) associate with the MIEP in lytically infected fibroblasts prior to pp71 function (79), as well as in quiescently infected NT2 and THP-1 cells (25, 51). Interestingly, indistinguishable markings of repressed chromatin were also found at the MIEP in $CD34^+$ cells with infections either initiated by *in vitro* infection (experimental latency) or established naturally *in vivo* (true latency) within infected individuals (57, 58). This suggests that a conserved cellular process, perhaps the PML-NB-mediated intrinsic defense, may function in assembling repressive chromatin at the MIEP of infecting HCMV genomes regardless of the cell type or mode of infection (*in vivo* versus *in vitro*).

Here we show that the PML-NB defense contributes to the silencing of viral IE gene expression that is observed when HCMV infects $CD34^+$ cells. However, the PML-NB defense is apparently not the only means by which viral IE gene expression is repressed when experimental latency is established in $CD34^+$ cells. We also found, using coinfection experiments in $CD34^+$ cells, that clinical HCMV strains exert a *trans*-dominant silencing of viral IE gene expression even when the PML-NB intrinsic defense is artificially inactivated with an HDAC inhibitor. Our data suggest that HCMV utilizes both cellular and viral mechanisms to silence viral IE gene expression during the initial stages of experimental latency in $CD34^+$ cells infected *in vitro* and that the functional virus-specific mechanism is encoded only by low-passage clinical strains of HCMV.

MATERIALS AND METHODS

Cells and viruses. Normal human dermal fibroblasts (NHDFs), TERT-immortalized human fibroblasts (TERT-HFs), human foreskin fibroblasts (HFFs), THP-1 cells, and differentiated THP-1 macrophage were cultured as previously described (61). Human $CD34^+$ hematopoietic progenitor cells derived from cord blood (Lonza) were maintained in HPGM medium (Lonza) supplemented with recombinant human stem cell factor (SCF) (25 ng/ml), recombinant human thrombopoietin (TPO) (50 ng/ml), and recombinant human Fms-related tyrosine kinase 3 ligand (Flt3) (50 ng/ml), all from PeproTech Inc. $CD34^+$ cells were maintained in culture for no more than 10 days (three passages). The percentages of cells from two separate and representative lots that remained $CD34^+$ at different periods of maintenance with these culture conditions were determined by flow cytometry by using a phycoerythrin (PE)-conjugated anti-$CD34^+$ antibody essentially as described previously (14). The differentiation status of these cells was maintained well for 7 days (99.5%, 99.1%, and 80.2% $CD34^+$ cells on days 1, 4, and 7, respectively) but declined after longer periods of cell culture (34.7% $CD34^+$ cells on day 10). Thus, HCMV IE gene expression experiments were conducted with cells that had been cultured 7 days or less. $CD34^+$ cells were differentiated in HPGM medium as previously described (57). Briefly, $CD34^+$ cells were grown for seven days in HPGM medium supplemented with transforming growth factor beta (TGF-β) (0.5 ng/ml), Flt3 (100 ng/ml), SCF (20 ng/ml), tumor necrosis factor alpha (TNF-α) (50 units/ml), and recombinant human granulocyte-macrophage colony-stimulating factor (GM-CSF) (100 ng/ml; Fitzgerald). After seven days, lipopolysaccharide (LPS) (50 ng/ml) was added for an additional three days. Viruses used were AD169 and green fluorescent protein (GFP)-expressing FIX and TB40/E strains. Cells were infected in minimal volume for 60 min, followed by the addition of medium to normal culture volumes. Note that the titers used represent "fibroblast infectious units" and do not necessarily represent the efficiency with which the virus entered or infected other cell types, most notably $CD34^+$ cells. Multiplicities of infection (MOIs) were selected in an attempt to equalize the potentials to synthesize IE genes in the different cell types. Transduction with recombinant adenoviruses has been described previously (61).

Inhibitors, antibodies, and Western blots. Valproic acid (VPA) (1 mM; Sigma) dissolved in water was added 18 h before infection (HFFs) (37), 3 h before infection (THP-1- and THP-1-derived macrophage cells) (61), or at the time of infection ($CD34^+$) with HCMV. The following antibodies were from commercial sources: anti-Daxx (D7810) and antitubulin (DM 1A) (both from Sigma), anti-PML (H-238 and sc-966; Santa Cruz), anti-Sp100 (AB1380; Chemicon), anti-UL44 (CA006-100; Virusys), and anti-CD34 (555822; BD Pharmingen). Antibodies against pp71 (IE-233), IE1 (1B12), and pp28 (CMV157) and secondary antibodies have been previously described (61, 62). For Western blot analysis, either equivalent numbers of cells lysed in radioimmunoprecipitation assay (RIPA) buffer with protease inhibitors (see Fig. 6D) or equivalent protein

concentrations from cell lysates prepared in RIPA buffer with protease inhibitors (all other Western analyses) were analyzed as previously described (62).

Indirect immunofluorescence. Mock- or HCMV-infected THP-1 or $CD34^+$ cells were collected by low-speed centrifugation, treated with trypsin (0.5 mg/ml) for 5 min at 37°C, again collected by low-speed centrifugation, and resuspended in cold phosphate-buffered saline (PBS). Cells were allowed to attach to water-washed coverslips as previously described (61). Additionally, $CD34^+$ cells were differentiated on coverslips prior to infection. Cells were fixed with 1 to 4% paraformaldehyde in PBS and processed as described previously (61). Images were visualized and photographed using a Zeiss Axiovert 200 M deconvolution fluorescence microscope with a 60× objective.

siRNA, shRNA, and transfections. $CD34^+$ cells were transfected using Amaxa Biosystems technology with small interfering RNA (siRNA) (1.25 μg/1.0×10^6 cells) or with expression plasmids (1 μg/5.0×10^5 cells, pSG5pp71 or pSG5pp71Did2-3) by using the human CD34 cell Nucleofector kit (VPA-1003; Amaxa Biosystems), following the manufacturer's protocol. Previously described synthetic, annealed, 21-base oligonucleotides (siRNA) were purchased from Dharmacon (61, 62). Cells transfected with siRNA were cultured for 48 h and then infected with HCMV AD169 (MOI of 1) for 24 additional hours. Cells transfected with pp71 expression plasmids were cultured for 36 h prior to collection for indirect immunofluorescence. The generation of TERT-immortalized HFs and THP-1 cells constitutively expressing a short hairpin RNA (shRNA) directed at Daxx was achieved using a retrovirus encoding the hDx2 sequence as previously described (61).

RT-PCR. Total RNA was isolated from $CD34^+$ cells infected with HCMV at an MOI of 1 for 24 h after treatment with VPA or siRNA as described above by using the RNeasy minikit (catalog no. 74104; Qiagen). Isolated RNA was quantified, and equivalent amounts were treated with RNase-free DNase (M6101; Promega) following the manufacturer's protocol. Equivalent quantities of RNA were subsequently used in a reverse transcription (RT)-PCR (35 cycles), using the SuperScript III one-step RT-PCR system (catalog no. 12574-026; Invitrogen). Primer pairs included IE1 sense (5′-CGTCCTTGACACGATGGAGT) and antisense (5′-ATCTGTTTGACGAGTTCTGCC) primers (that span the intron between exon 2 and exon 3) and primers for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as previously described (31). PCR products were separated on 1.5% agarose gels, and bands were quantified using Quantity-One software (Bio-Rad).

Q-PCR. DNA and RNA were isolated from 750,000 recombinant adenovirus (rAD)-transduced cells by using the DNeasy tissue kit (catalog no. 69504; Qiagen) and RNeasy minikit (catalog no. 74104; Qiagen), respectively, according to the manufacturer's instructions. RNA was treated with RNase-free DNase as described above. Subsequently, 1 μg of DNase-treated RNA was used for a first-strand cDNA synthesis procedure using the SuperScript III first-strand synthesis system (18080-051; Invitrogen) with provided oligo(dT) primers according to the manufacturer's specifications. Reactions without reverse transcriptase controlled for contaminating DNA (data not shown). DNA and cDNA were used in a quantitative real-time PCR (Q-PCR) to determine the relative activity of the MIEP assayed by expression of pp65 transcripts using previously described primer/probe sets (61) or that of the EF1α promoter expressing pp71. Primer pairs for pp71 were as follows: forward, 5′-CGTTCATTTGGAACACC GAC, and reverse, 5′-CTCTTCCTCTTCTTCCTCCTCTTC. The pp71 probe was 6-carboxyfluorescein (6FAM)–TCTCACACCCGGTAAACCGGACAAA–6-carboxytetramethylrhodamine (TAMRA) (Applied Biosystems). Expression levels of cellular β-actin quantitated with a primer pair and probe set previously described (61) were analyzed as a control and varied no more than 3-fold in all samples examined. Unknown sample values were determined based on a standard curve of known copy numbers of pp65, beta-actin (61), and pp71.

RESULTS

The PML-NB intrinsic defense is functional against low-passage clinical isolates of HCMV. Evidence supporting the presence of an intrinsic immune defense that blocks HCMV IE gene expression at the start of lytic and quiescent infections has been obtained in experiments utilizing high-passage laboratory-adapted viral strains such as AD169 (2, 56, 61, 62, 72, 73). If the PML-NB defense were to control the establishment of true HCMV latency *in vivo*, it would also have to be active against circulating viruses that are more similar to low-passage clinical viral strains than to AD169. We suspected that clinical isolates such as FIX (19) and TB40/E (69) would also be subject to the PML-NB-mediated intrinsic defense because the MIEPs (from −1140 to +970 [the ATG codon], where the major transcription initiation site is +1) of low-passage clinical viral strains are 98% identical to that of AD169 (data not shown). Our experiments proved this to be the case. Inhibition of HDAC activity by valproic acid (VPA) increased IE1 expression at the start of a low-multiplicity-of-infection (MOI) lytic infection of fibroblasts with laboratory (AD169) and clinical (FIX and TB40/E) viral strains (Fig. 1A). Likewise, telomerase-immortalized fibroblasts (TERT-HFs) constitutively expressing a short hairpin RNA directed at Daxx displayed higher levels of IE1 than the parental TERT-HFs when infected at a low MOI with AD169, FIX, or TB40/E (Fig. 1B). These results show that HDACs and Daxx negatively regulate the HCMV MIEP of clinical strains at the start of lytic infections in fibroblasts. Note that infection of fibroblasts with equal multiplicities of AD169 or clinical viral strains resulted in comparable steady-state levels of IE1 at 6 h postinfection (hpi) both in the absence and in the presence of the HDAC inhibitor (Fig. 1A) even though the particle-to-PFU ratios of clinical and laboratory strains are often found to differ dramatically.

Using equivalent approaches, we made the similar observation that Daxx and HDACs also silence IE1 expression from infecting FIX and TB40/E viral genomes during quiescent infections of THP-1 cells. Specifically, treatment of THP-1 cells with VPA (Fig. 1C) or infection of Daxx-knockdown THP-1 cells (Fig. 1D) partially rescued IE gene expression for both AD169 and clinical viral strains. VPA treatment did not permit transit of tegument-delivered pp71 to the nucleus of undifferentiated THP-1 cells (data not shown). It was retained in the cytoplasm, as we previously demonstrated (61).

Interestingly, IE1 expression was more robust in AD169-infected THP-1 cells in the presence of VPA (Fig. 1C) or in infected THP-1-shDaxx cells (Fig. 1D) than in cells infected with FIX or TB40/E clinical strains at the same multiplicity. This effect was not observed during lytic infection of fibroblasts (Fig. 1A and B). Such a difference may result from the different particle-to-PFU ratios of the viral stocks, because the PML-NB intrinsic defense is the sole or dominant repressive mechanism found in differentiated cells, or perhaps because of a clinical-strain-specific restriction to HCMV infection prior to IE gene expression in undifferentiated cells. Regardless of the mechanism, this observation may explain the different conclusions drawn about the contribution of Daxx to IE gene silencing during quiescent infection of NT2 cells between two independent studies, one of which used AD169 (61) while the other used the clinical strain Toledo (17).

An analysis of IE gene expression after infection of THP-1 cells previously differentiated into macrophage cells yielded results very similar to those observed in fibroblasts. Infections at identical MOIs resulted in similar levels of IE gene expression (Fig. 1E), which were enhanced by both VPA treatment (Fig. 1E) and Daxx knockdown (Fig. 1F). In total, these experiments show that the FIX and TB40/E clinical viral strains express more IE1 when the PML-NB-mediated intrinsic defense is inactivated in both differentiated (Fig. 1A, B, E, and F) and undifferentiated (Fig. 1C and D) cells. Thus, we conclude that the PML-NB intrinsic defense that silences the MIEP is

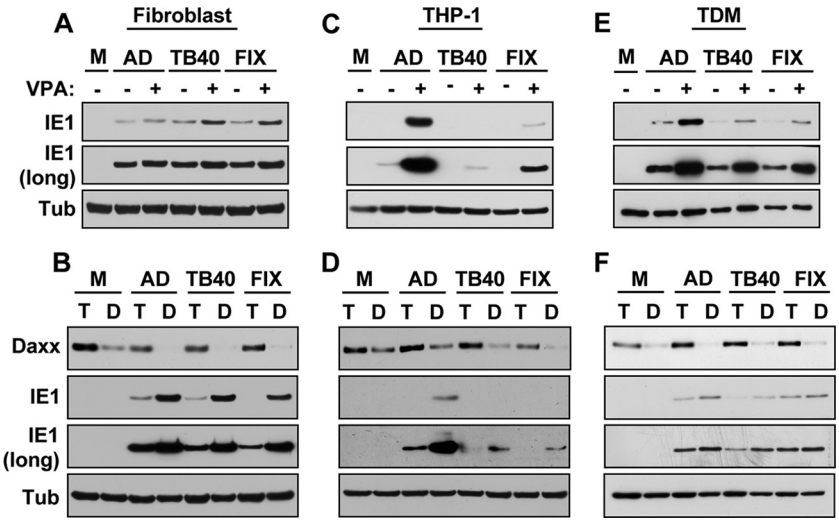

FIG. 1. HCMV clinical isolates are susceptible to Daxx-mediated MIEP silencing. (Panel A) HFFs were mock infected (M) or treated without (−) or with (+) VPA ~18 h prior to infection with AD169 (AD), TB40/E (TB40), or FIX at a multiplicity of infection (MOI) of 0.05. Lysates were harvested 6 h postinfection (hpi) and analyzed by Western blotting. (Panel B) TERT-HFs (T) or those constitutively expressing an shRNA to Daxx (D) were infected with AD169, TB40/E, or FIX at an MOI of 0.5. Lysates were harvested at 6 hpi and analyzed by Western blotting. (Panel C) THP-1 monocytes were mock infected or infected with AD169, TB40/E, or FIX in the absence (−) or presence (+) of VPA at an MOI of 1 for 12 h. Lysates were analyzed by Western blotting. (Panel D) THP-1 cells (T) or those expressing shRNA to Daxx (D) were infected with AD169, TB40/E, or FIX at an MOI of 1 for 24 h and analyzed by Western blotting. (Panel E) THP-1-derived macrophage (TDM) were mock infected or treated without (−) or with (+) VPA for 3 h prior to infection with AD169, TB40/E, or FIX at an MOI of 0.5. Lysates were harvested 12 hpi and analyzed by Western blotting. (Panel F) THP-1-derived macrophage (T) or those expressing shRNA to Daxx (D) were infected with AD169, TB40/E, or FIX at an MOI of 0.5 for 12 h and analyzed by Western blotting. For all experiments, tubulin (Tub) was used as a loading control. Short and long exposures are shown for IE1.

active against low-passage clinical strains of HCMV during both lytic and quiescent infections.

CD34$^+$ cells contain PML-NB proteins and structures. The susceptibility of clinical strains to the PML-NB-mediated intrinsic defense that silences HCMV IE gene expression (Fig. 1) and the indistinguishable chromatin structure found at the MIEP during early lytic, quiescent, and latent infections (25, 51, 57, 58, 79) led us to hypothesize that viral latency in CD34$^+$ cells is controlled, in part, by the PML-NB intrinsic defense. Western blots indicated that Daxx, PML, and Sp100, all PML-NB constituents, were expressed in CD34$^+$ cells (Fig. 2A). Multiple experiments consistently showed an increase in Daxx levels in CD34$^+$ cells compared to fibroblasts. Conversely, PML levels were reduced in CD34$^+$ cells, and the spectra of isoforms likely resulting from both alternative splicing and differential SUMOylation (30) present in the two cell

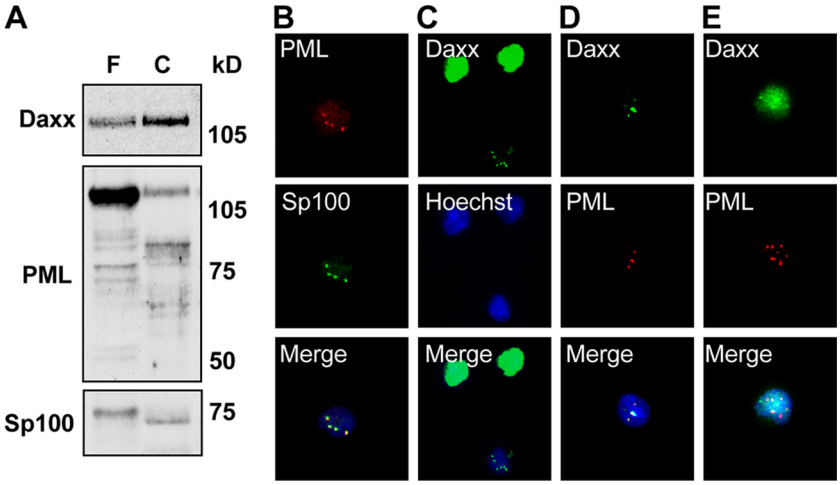

FIG. 2. CD34$^+$ cells contain PML-NBs. (Panel A) Fifty micrograms of protein lysate from NHDFs (F) or CD34$^+$ cells (C) were analyzed by Western blotting. Approximate protein sizes in kDa are shown to the right. (Panel B) CD34$^+$ cells were immobilized on coverslips and stained for PML and Sp100 (red and green, respectively). Nuclei were counterstained with Hoechst stain (blue). (Panel C) CD34$^+$ cells stained for Daxx (green). (Panel D) CD34$^+$ cells stained for Daxx (green) and PML (red) for which Daxx is punctate. (Panel E) CD34$^+$ cells stained for Daxx (green) and PML (red) for which Daxx is diffuse. The magnification for all images in this figure is ×60.

types were different. However, all isoforms contain the RBCC/Trim motif that is required for the antiviral effects of PML (60). Sp100 levels were similar in the two cell types, but like for PML, differentially modified proteins and/or different protein isoforms were expressed. Similar to the case for PML, multiple Sp100 isoforms have been found to display antiviral activities (52).

Indirect immunofluorescent staining showed that PML and Sp100 were found to colocalize in punctate domains (Fig. 2B) in all cells examined. Interestingly, Daxx localization varied in different $CD34^+$ cells (Fig. 2C), where it was found either in punctate domains that colocalized with PML (Fig. 2D) or dispersed throughout the nucleus, even though PML remained punctate (Fig. 2E). $CD34^+$ cells contain less PML and more Daxx than fibroblasts (Fig. 2A). Because Daxx is recruited to PML-NBs specifically by the PML protein (27), perhaps Daxx binding sites on PML become saturated in some $CD34^+$ cells, causing the extra Daxx to disperse throughout the nucleus. Alternatively, because cells at multiple stages of hematopoietic differentiation express CD34 (78), differing Daxx distributions may be due to the heterogeneous nature of $CD34^+$ cell populations.

We counted the number of cells containing punctate or diffuse Daxx prior to or 24 h after HCMV AD169 infection (MOI of 3). We found that 81% ± 2% of mock-infected cells showed diffuse Daxx, whereas 68% ± 6% of HCMV-infected cells (identified by staining for tegument-delivered pp71) contained diffuse Daxx (punctate Daxx was observed in the remainder of the cells). Although small, this difference is statistically significant ($P = 0.002$). The biological significance of this observation (if any) is presently unclear, and this experiment is unable to discern between HCMV preferentially entering $CD34^+$ cells with punctate Daxx and the virus somehow modulating PML-NB structure after viral entry.

Daxx proteins found outside the context of PML-NBs appear to be stronger transcriptional repressors than PML-NB-localized Daxx (38). Thus, the majority of $CD34^+$ cells (those with diffuse Daxx) may provide an inherently more repressive environment for the viral MIEP (which is silenced by Daxx) than fibroblasts do (see below). From these observations, we conclude that $CD34^+$ cells express PML, Sp100, and Daxx isoforms with potential antiviral activities (10) and have the capacity to assemble PML-NBs. Thus, the PML-NB-mediated intrinsic defense against HCMV could conceivably be active in $CD34^+$ cells.

The HCMV MIEP is an inefficient promoter in $CD34^+$ cells. The experiments described above show that PML-NB proteins that institute an intrinsic defense against HCMV by silencing the viral MIEP are present in $CD34^+$ cells, and their steady-state levels and localizations indicate that $CD34^+$ cells might be more restrictive to MIEP function than fibroblasts. To test this, we determined the activities, on a per-DNA-copy basis, of the MIEP and the cellular EF1α promoter in a series of cell types used to study HCMV infection. The EF1α promoter was selected because it is often used to express proteins in undifferentiated cells (6, 41, 61). In these experiments, we delivered the MIEP to cells by recombinant adenoviral (rAD) transduction. Serotype 5 adenoviruses enter $CD34^+$ cells with an efficiency similar to that of HCMV, and in $CD34^+$ cells successfully entered, they replicate with efficiencies approaching 100% (64), indicating that there are no substantial postentry blocks (e.g., inability to deliver genomes to the nucleus) to gene expression from adenoviral genomes in $CD34^+$ cells. For these reasons, and because adenoviral genomes (26), like those of HCMV (28), associate with PML-NBs in infected cells, we chose adenoviral transduction (as opposed to retroviral transduction or plasmid transfection) for these experiments. Internally controlled quantitative (reverse transcriptase or straight) real-time PCR (Q-PCR) was used to determine the relative amounts of mRNA compared to DNA copies of the genes expressed from the MIEP or EF1α promoter in each cell type examined.

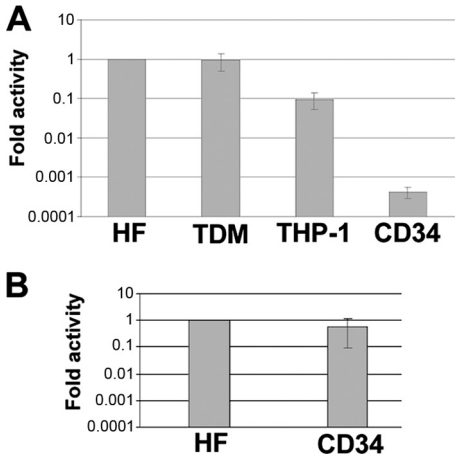

FIG. 3. The HCMV MIEP is less active in $CD34^+$ cells than in other cell types. (Panel A) NHDF (HF), THP-1-derived macrophage (TDM), THP-1, and $CD34^+$ cells were transduced with a recombinant adenovirus (rAD) expression system driven by the HCMV MIEP and harvested 24 h posttransduction. The relative quantity of expressed mRNA compared to input rAD genomic DNA was determined by Q-PCR (see Materials and Methods). Fold activity of the MIEP was compared to levels found in NHDFs, which were empirically set at 1. Averages and standard errors of the means for five independent experiments are shown. (Panel B) NHDF and $CD34^+$ cells were transduced with an rAD expression system driven by the EF1α promoter and analyzed as described for panel A. Results from three independent experiments are shown, with the averages and standard errors of the means indicated.

We found that the MIEP is over 100-fold less active in $CD34^+$ cells than in THP-1 cells and over 1,000-fold less active in $CD34^+$ cells than in THP-1-derived macrophage and in fibroblasts (which showed equivalent MIEP activities) (Fig. 3A). However, the EF1α promoter had similar activity, on a per-molecule basis, in $CD34^+$ cells and fibroblasts (Fig. 3B). These experiments indicate that the HCMV MIEP is inherently less active in $CD34^+$ cells than in other cell types routinely used to study HCMV infection. As mentioned above, the poor activity of the MIEP in $CD34^+$ cells may be due in part to higher Daxx and lower PML levels in $CD34^+$ cells (Fig. 2A), because Daxx is known to repress the MIEP (56, 62, 79) and PML is known to inhibit Daxx-mediated transcriptional repression (38). Alternatively or in addition, other cellular repressors (40, 42, 72, 80) or the proposed lack of activating transcription factors (17, 67) may also contribute to the poor function of the HCMV MIEP in $CD34^+$ cells.

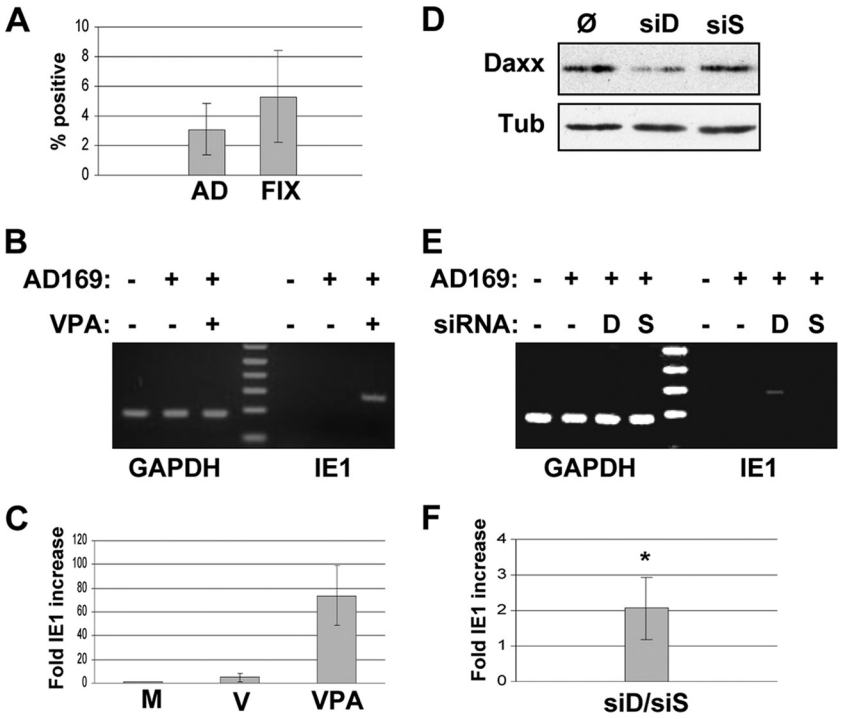

FIG. 4. Daxx and histone deacetylase activity control HCMV AD169 IE gene expression during infection of $CD34^+$ cells. (Panel A) $CD34^+$ cells were infected with AD169 (AD) or FIX at an MOI of 5 or 3, respectively. After 24 h, cells were harvested, treated with trypsin, immobilized on coverslips, and stained for pp71. The percentages of cells staining positive for pp71 are shown. Standard deviations are indicated with bars. (Panel B) $CD34^+$ cells were mock treated (−) or treated with VPA (+) and either mock infected (−) or infected with AD169 (+) at an MOI of 1 as indicated. Cells were harvested 24 hpi, and total RNA was extracted and analyzed by RT-PCR for expression of IE1. Cellular GAPDH served as an internal control. (Panel C) Fold IE1 activation (normalized to GAPDH levels) for samples shown in panel B is shown. M, mock; V, AD169; VPA, AD169 + VPA. Bars indicate standard deviations. (Panel D) $CD34^+$ cells were transfected without siRNA (Ø) or transfected with siRNA directed at Daxx (siD) or Skp-1 (siS). Forty-eight hours later, cells were infected with AD169 at an MOI of 1 for 24 h. Cells were harvested, and a fraction was used for Western blot analysis. Tubulin (Tub) was used as a normalization control. For the experiment shown, the Daxx level in the Daxx-knockdown cells was 42% of the untreated cells. In the Skp-1-knockdown cells, the Daxx level was 96% of the untreated cells. (Panel E) RNA was isolated from the remaining cells (treated as described for panel D above) and used in an RT-PCR assay for IE1 and GAPDH. Mock-infected (−) or AD169-infected (+) cells and cells transfected without siRNA (−), with siRNA directed at Daxx (D), or with siRNA directed at Skp-1 (S) were analyzed. (Panel F) Results from five independent experiments as outlined (panels D and E) were analyzed, and fold activation of IE1 in cells transfected with siRNA to Daxx over those transfected with siRNA to Skp-1 (siD/siS) is shown with the standard deviation (*, P value $<$ 0.05).

The MIEP is repressed by the PML-NB defense in HCMV-infected $CD34^+$ cells. We next asked if the HDAC and Daxx components of the PML-NB defense silence IE gene expression upon HCMV infection of $CD34^+$ cells. We employed RT-PCR as a direct assay for MIEP function in $CD34^+$ cells for multiple reasons, including the low infection efficiency of these cells reported previously (13, 34) and assayed here by tegument delivery of pp71 (Fig. 4A), the poor activity of the MIEP in these cells (Fig. 3A), the potential for microRNA-mediated silencing of the IE1 transcript (15, 49), and most importantly, to keep our experimental conditions consistent with the previous RT-PCR examinations of viral gene expression during latency (17, 44, 58, 76). In all experiments, mRNA levels for the glyceraldehyde phosphate dehydrogenase (GAPDH) housekeeping gene were used for normalization. We found a significant enhancement of IE1 mRNA production (~15-fold over virus alone) in $CD34^+$ cells treated with the HDAC inhibitor VPA after infection with HCMV AD169 (Fig. 4B and C). We also found that siRNA-mediated knockdown of Daxx in $CD34^+$ cells (Fig. 4D) permitted a reproducible and statistically significant ($P = 0.025$) increase in AD169 IE1 expression compared to transfection of $CD34^+$ cells with siRNA to a control protein Skp-1 (Fig. 4E and F). Although the magnitude of Daxx knockdown and the activation of IE gene expression are modest, five separate experiments using two different lots of $CD34^+$ cells gave comparable results. Thus, HDACs and the PML-NB protein Daxx, which are part of an intrinsic immune defense against HCMV (63, 74), contribute to the silencing of viral IE gene expression when $CD34^+$ cells are infected *in vitro* with a laboratory-adapted strain of HCMV. This finding is in agreement with previous data demonstrating a repressed chromatin structure at the MIEP during latency (57, 58) that is indistinguishable from that assembled by the PML-NB defense prior to lytic infection (79).

Our demonstration that the PML-NB defense contributes to silencing of the MIEP in HCMV-infected $CD34^+$ cells led us to ask why it is not neutralized by tegument-delivered pp71, as it is when lytic infection initiates (62). Identical to the cases for NT2 and THP-1 cells (61), we found tegument-delivered pp71 exclusively in the cytoplasm of $CD34^+$ cells infected with either AD169 or FIX (Fig. 5A). In contrast, we found that prior

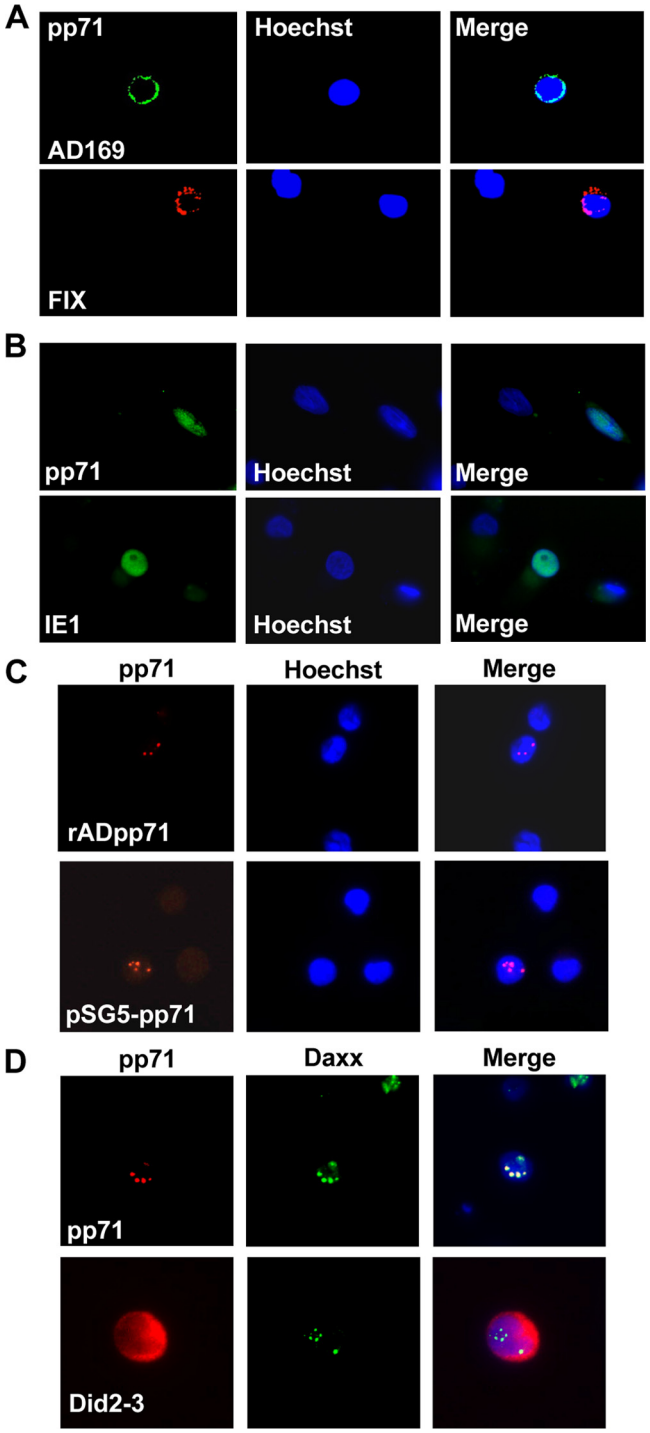

FIG. 5. Differentiation-dependent localization of tegument-delivered pp71 and IE1 expression during HCMV infection of CD34$^+$ cells. (Panel A) CD34$^+$ cells were infected with AD169 or FIX at an MOI of 5 or 3, respectively. After 24 h, cells were harvested, treated with trypsin, immobilized on coverslips, and stained with an antibody to pp71 (AD169, green; FIX, red). (Panel B) CD34$^+$ cells were differentiated into dendritic cells and infected with AD169 at an MOI of 5 for 24 h. Adherent cells on coverslips were analyzed for the presence of pp71 or IE1 (both green). (Panel C) CD34$^+$ cells were transduced with a recombinant adenovirus that expresses pp71 (rADpp71) at 1,000 particles per cell for 24 h or transfected with a pp71 expression plasmid (pSG5-pp71) for 36 h and analyzed for pp71 expression (red). (Panel D) CD34$^+$ cells were transfected with expression plasmids for wild-type pp71 (pp71) or a pp71 mutant unable to bind Daxx (Did2-3) for 24 h and stained with antibodies to pp71 (red) and Daxx (green). In all panels, nuclei were counterstained with Hoechst stain (blue). The image magnification was ×60.

differentiation of CD34$^+$ cells into dendritic cells and subsequent infection with AD169 permitted both the nuclear localization of tegument-delivered pp71 and IE1 expression (Fig. 5B). Identical to the cases for other cell types we have examined (61), pp71 that is newly expressed by either adenoviral transduction or plasmid transfection efficiently localizes to the nuclei of CD34$^+$ cells (Fig. 5C). In CD34$^+$ cells in which Daxx had a punctate nuclear localization, newly expressed wild-type pp71 also appeared as punctate dots that colocalized with Daxx (Fig. 5D). pp71 was found throughout the nucleus in cells with a diffuse pattern of Daxx staining (data not shown). The Did2-3 pp71 mutant, unable to bind Daxx (20), showed a diffuse staining pattern (Fig. 5D) both in the nucleus and in the cytoplasm. Thus, while tegument-delivered pp71 is trapped in the cytoplasm, *de novo*-expressed wild-type pp71, presumably by binding to Daxx, is recruited to PML-NBs in CD34$^+$ cells, as expected.

In addition to the PML-NB defense, clinical strains of HCMV employ a dominant means to silence IE gene expression during latency. Our data indicate that artificial inactivation of the PML-NB intrinsic defense allows for the expression of viral IE genes upon infection of CD34$^+$ cells with the high-passage laboratory-adapted HCMV strain AD169. We next asked if this also held true for the FIX and TB40/E clinical strains of HCMV. In contrast to our results with AD169, we found that addition of VPA to CD34$^+$ cells prior to infection with either FIX (Fig. 6A and B) or TB40/E (Fig. 6C) failed to induce IE1 gene expression. This result is unlikely to be due to differing infection efficiencies of the two viral strains, because the numbers of CD34$^+$ cells that received tegument proteins upon HCMV infection were similar for representative laboratory and clinical virus strains (Fig. 4A). Thus, we conclude that clinical strains, in addition to the PML-NB defense, experience an additional postentry, HDAC-independent block to IE gene expression in CD34$^+$ cells compared to a laboratory-adapted viral strain infecting these cells.

We then asked if this additional restriction to IE gene expression was observed in mixed coinfections in which CD34$^+$ cells were infected with equal multiplicities of the laboratory-adapted AD169 virus and a clinical strain of HCMV. We found that in CD34$^+$ cultures coinfected with heterogeneous viral mixtures, the VPA-induced activation of IE1 gene expression from the AD169 genome was not observed (Fig. 6A, B, and C). Thus, the additional restriction to IE gene expression observed upon HCMV infection of CD34$^+$ cells by clinical viral strains acts in *trans* and in a dominant fashion, as it was able to restrict the ability of VPA to permit IE gene expression from coinfecting AD169 genomes.

These experiments indicate that in VPA-treated CD34$^+$ cells infected with laboratory (AD169) but not clinical (FIX or TB40/E) strains of HCMV, latency is not established, but rather lytic infection is initiated because the viral IE genes are expressed. We confirmed this by detecting the expression of

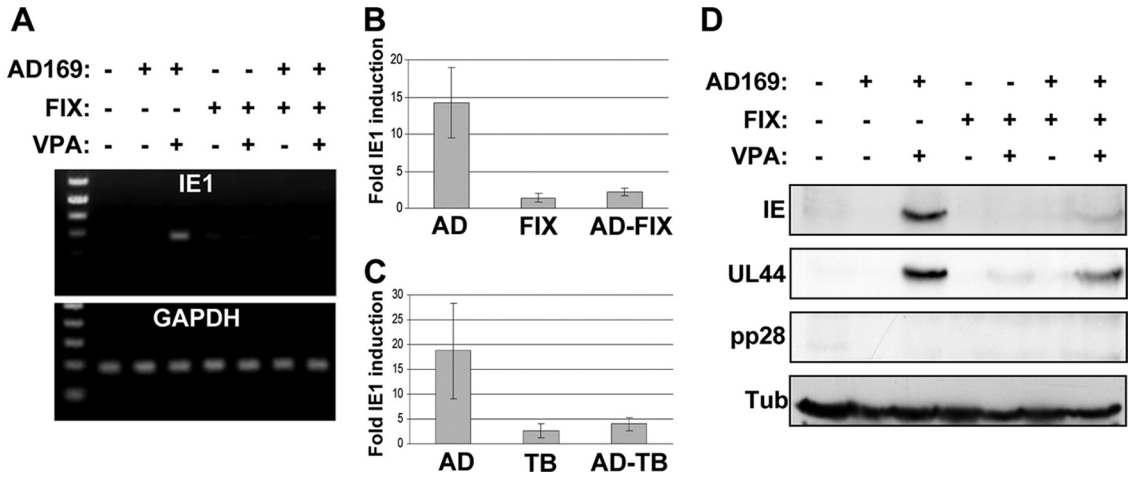

FIG. 6. HCMV clinical isolates institute a dominant block to IE gene expression. (Panel A) $CD34^+$ cells were mock infected (−), infected (+) with AD169 (MOI of 1) or FIX (MOI of 1), or coinfected with both (each at an MOI of 1 added to the cells simultaneously [total MOI of 2]) in the absence (−) or presence (+) of VPA. Cells were harvested 24 hpi, and isolated RNA was used in an RT-PCR for IE1 and GAPDH expression. (Panel B) Fold activation of IE1 during infection in the presence of VPA compared to virus alone was determined for experiments described for panel A. (Panel C) Experiments were conducted as outlined above by using AD169 (AD; MOI of 1), clinical isolate TB40/E (TB; MOI of 1), or both (AD-TB; each at an MOI of 1, added to the cells simultaneously [total MOI of 2]). Fold IE1 activation with virus plus VPA over virus alone is shown. For panels B and C, results from four (B) or three (C) independent experiments with standard errors of the means shown. (Panel D) $CD34^+$ cells left untreated (−) or treated at the time of infection with 1 mM VPA (+) as indicated were infected with AD169 (MOI of 1) or FIX (MOI of 1) or coinfected with a mixture of AD169 and FIX (each at an MOI of 1 for a total MOI of 2) for 24 h, after which the cells were washed free of virus and VPA and cultured for an additional 6 days. Equal cell numbers were lysed in RIPA buffer, and their protein contents were analyzed by Western blotting with the indicated antibodies. Tubulin was used as a loading control. Note that titers are based on fibroblast infection and do not represent the efficiency with which HCMV enters $CD34^+$ cells (Fig. 4A).

IE1 at the protein level, as well as the expression of the UL44 protein (encoded by an early gene), by Western blot analyses in VPA-treated $CD34^+$ cells infected with AD169, whereas IE1 and UL44 expression levels were substantially lower in those infected with FIX or coinfected with AD169 and FIX (Fig. 6D). However, this initiated lytic infection that progresses at least to the early phase appears to be abortive, because we were unable to detect expression of the pp28 protein encoded by the true late gene UL99 (9). This result is identical to what we observed in NT2 cells (a model for HCMV quiescence), in which IE1 and UL44 protein expression but not pp28 protein expression was observed upon infection of Daxx-knockdown cells (61).

In total, our data demonstrated that HCMV experimental latency in $CD34^+$ cells infected *in vitro* is established in part by a cellular intrinsic immune defense mediated by Daxx and HDACs because tegument-delivered pp71 fails to enter the nucleus and inactive this defense by degrading Daxx. Furthermore, we demonstrated that an additional mechanism, revealed only by the comparison of laboratory and clinical viral strains, acts in an HDAC-independent, *trans*-dominant manner to silence the MIEP during experimental latency in $CD34^+$ cells infected *in vitro* with clinical strains of HCMV.

DISCUSSION

Latency enables HCMV to persist for the life of the infected host by avoiding both immune clearance and the action of currently available antiviral drugs. Elucidating viral and cellular factors that contribute to the establishment, maintenance, or reactivation of latent infections may enable development of a treatment regimen that targets the latent reservoir of virus. Candidates for viral functions that may control HCMV latency, such as astUL81-82/LUNA (1, 59), miR-UL112-1 (15, 49), UL138 (13, 55), and UL111A/LAcmvIL-10 (5), are emerging. Work presented here adds both pp71 and an unidentified, clinical-strain-specific function to this growing list. All of these could represent viable viral targets for a therapy designed to eliminate or control latently infected cells.

However, evolution rates for herpesviruses exceed those of their hosts (8), and thus, viruses resistant to any individual antiviral treatment targeting a virally encoded function will eventually develop. Thus, we have focused on identifying cellular determinants of HCMV latency, with the expectation that it is less likely that viruses will develop resistance to an antiviral treatment focused on a cellular factor. The work shown here demonstrates that a cellular intrinsic immune defense contributes to the establishment of HCMV latency in $CD34^+$ cells. We observed a substantial (15-fold) increase in IE1 gene expression when HDACs were inhibited with VPA in $CD34^+$ cells infected with the high-passage laboratory-adapted AD169 strain of HCMV (Fig. 4C and 6B and C). Furthermore, we quantitated a modest but statistically significant increase in IE1 gene expression when Daxx levels were reduced in $CD34^+$ cells prior to infection with AD169 (Fig. 4F) despite our inability to achieve high-level knockdown of Daxx in these cells (Fig. 4D). As HCMV has multiple ways to inactivate HDACs (53, 54, 62), and as small-molecule HDAC inhibitors increase IE gene expression even in wild-type-virus-infected cells (37, 45–47, 62, 79), our finding that VPA is a better inducer of IE gene expression than is a partial Daxx knockdown is not surprising. We should note that the IE gene expression observed in these experiments should not be confused with reactivation of truly

latent virus but more likely represents the failure of this laboratory-adapted virus to properly establish latency in the absence of the PML-NB-mediated intrinsic defense.

If the establishment phase of experimental HCMV latency in $CD34^+$ cells *in vitro* is operationally defined as the delivery of functional viral genomes to the nucleus without the subsequent expression of the IE genes, then our data show that cellular HDAC and Daxx proteins, components of the PML-NB intrinsic defense against the virus, are required for the proper establishment of HCMV latency in this model system. Thus, accumulating data (45–47, 51, 61, 63, 74, 79), in addition to work presented here, indicate that PML-NB proteins and/or HDACs silence viral gene expression and suppress productive viral replication in multiple cell types (fibroblast, epithelial, endothelial, NT2, T2RA, THP-1, THP-1-derived macrophage, and $CD34^+$ cells) as well as during multiple types of infections (lytic, quiescent, and latent).

The PML-NB intrinsic defense is not inactivated in $CD34^+$ cells upon HCMV infection (as it is in fibroblasts), because tegument-delivered pp71 fails to reach the nucleus (Fig. 5A). The molecular mechanism controlling pp71 subcellular localization is unknown, but recent transient ectopic expression data suggest that it is modulated by phosphorylation (65). However, we have been unable to control the subcellular localization of tegument-delivered pp71 with kinase or phosphatase inhibitors in studies that are similar to one we have previously performed with success (22). Defining the mechanisms that control pp71 partitioning to the nucleus or cytoplasm is an active area of research in our laboratory, because artificial control of pp71 subcellular localization could represent an antiviral intervention point for both lytic and latent HCMV infections.

While it seems to play an important, early role during the establishment of latency for HCMV and perhaps for other herpesviruses as well (11), the PML-NB-mediated defense is not the only mechanism that controls MIEP function and thus HCMV latency in $CD34^+$ cells. For example, we show here the existence of at least one viral measure that silences IE gene expression during the establishment of latency by clinical viral strains (Fig. 6). We speculate that HCMV likely uses multiple, perhaps overlapping and partially redundant mechanisms to restrict viral IE gene expression during the establishment of latency. These would include permitting the intrinsic defense to silence IE gene expression (Fig. 4), establishing latency in a cell type with a fortuitous mixture of cellular transcriptional repressors and activators (Fig. 3) (17, 21, 40, 67, 71, 80), expressing viral microRNAs to potentially regulate viral gene translation (15, 49), and expressing a *trans*-acting, dominant, HDAC-independent means to silence viral IE gene expression (Fig. 6). Multiple silencing systems might provide fail-safe mechanisms ensuring sufficient IE gene suppression even if one or more of them is inactivated or bypassed as well as allow for the fine-tuning of IE gene expression during reactivation.

Although the PML-NB intrinsic defense appears to control, in part, experimental latency in $CD34^+$ cells infected *in vitro*, it may be insufficient to establish or maintain the latent state under some (13) but not other (5) experimental conditions. Interestingly, our results suggest that, in addition to the PML-NB intrinsic defense, clinical strains impart an HDAC-independent *trans*-dominant restriction to viral IE1 gene expression during experimental latency in $CD34^+$ cells *in vitro* (Fig. 6). We have not yet identified the clinical-strain-specific function that mediates this repression. While the UL138 gene is required for the maintenance of latency under some (13) but not other (5) experimental conditions, previous work indicates that the protein it encodes does not regulate the viral MIEP (55). Therefore, pUL138 is unlikely to be responsible for the prolatency viral function we observed here. Other candidates for clinical-isolate-specific viral factors contributing to IE gene silencing during latency include functions encoded by the limited set of viral transcripts that are expressed during latency (1, 4, 13, 14, 29, 36, 55), as well as additional genes encoded within the ULb′ region of the genome that is absent in laboratory-adapted virus strains (3, 50).

The low infection efficiency of HCMV in $CD34^+$ cells implies that if this unidentified *trans*-acting dominant restriction acts in an intrinsic (cell-autonomous) manner, then HCMV may preferentially enter only a subset of $CD34^+$ cells. If this is not the case, then perhaps this newly identified dominant restriction could act through a secreted factor in an extrinsic (non-cell-autonomous) manner. Clearly, additional work to determine which viral gene(s) functions in concert with HDACs and Daxx to restrict MIEP activity during HCMV latency is required. Importantly, our finding that IE gene expression during $CD34^+$ cell infection with AD169 is controlled in part by the PML-NB-mediated defense but not the additional clinical-strain-specific restriction allows AD169 to be used to study the role of PML-NB proteins during latency. Furthermore, it allows the use of AD169 for gain-of-function studies to identify and characterize other impediments to IE gene expression during HCMV latency in $CD34^+$ cells characteristic only of clinical viral strains.

The lytic infection cycle that is initiated in VPA-treated $CD34^+$ cells infected with AD169 progresses past the immediate early stage and into the early phase, as evidenced by the expression of the UL44 protein (Fig. 6D). However, we were unable to detect the expression of the pp28 protein encoded by the true late gene UL99. We interpret these experiments to indicate that the lytic infection initiated in these undifferentiated cells is abortive (nonproductive). This result mirrors our previous data in our report of an abortive infection in undifferentiated NT2 cells with reduced levels of Daxx (61) and is consistent with the numerous reports indicating that HCMV fails to productively replicate in undifferentiated cells (12, 18, 34, 35, 44, 75, 77). The significant changes cells undergo upon differentiation appear to dissolve multiple impediments to productive HCMV replication, with the restriction of viral IE gene expression being only one of many blocking points (albeit an early and prominent one).

We hypothesize that the inefficiency with which undifferentiated cells support productive viral replication represents a biologically relevant pressure against HCMV that may have selected for viruses that can effectively suppress IE gene expression upon entry into undifferentiated cells. Efficient IE gene silencing would prevent the initiation of an abortive lytic infection in undifferentiated cells until after differentiation, when the cell becomes fully permissive for a productive infection. Preventing the initiation of an abortive infection by silencing viral IE gene expression may be additionally important to help protect the reservoir of latently infected cells from

potent cellular T-cell responses to viral IE antigens until which time differentiation renders them competent to produce infectious progeny virions.

Our working model for the establishment of HCMV latency in $CD34^+$ cells posits that at least three circumstances conspire to silence viral IE gene expression promptly upon viral entry: the low inherent activity of the MIEP in these cells (Fig. 3A), the PML-NB intrinsic defense (Fig. 4), and an unidentified viral function characteristic only of low-passage clinical strains of HCMV (Fig. 6). We suggest that HCMV latency may have developed in part as a response to a cellular intrinsic antiviral defense but that the efficient establishment and/or maintenance of the latent state has been facilitated by the evolution of at least one and likely multiple viral functions. Eschewing the immediate inactivation of this defense (of which the virus is clearly capable) in favor of establishing a latent reservoir of virus appears to allow HCMV recurrent opportunities for lytic amplification and virion dissemination throughout the lifetime of the infected host via the reactivation of latent infections.

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