# Experimental human cytomegalovirus latency in CD14$^+$ monocytes

**Danna Hargett and Thomas E. Shenk**[1]

Department of Molecular Biology, Princeton University, Princeton, NJ 08544-1014

Contributed by Thomas E. Shenk, September 29, 2010 (sent for review August 17, 2010)

**CD14$^+$ monocytes are a reservoir for latent human cytomegalovirus, and virus replication is reactivated during their differentiation to macrophages or dendritic cells. It has not been clear whether the virus can establish latency upon direct infection of monocytes or whether it must first become quiescent in a progenitor cell that subsequently differentiates to generate a monocyte. We report that infection of primary human monocytes with a clinical strain of human cytomegalovirus exhibits the hallmarks of latency. We established conditions for culturing monocytes that prevent differentiation for at least 25 d, as evidenced by cell surface marker expression. Infection of these monocytes with the FIX clinical strain resulted in transient accumulation of many viral lytic RNAs and sustained expression of four previously described latency-associated transcripts. The amount of viral DNA remained constant after infection, and cell surface and total HLA-DR proteins were substantially reduced on a continuing basis after infection. When treated with cytokine mixtures that stimulate differentiation to a macrophage or dendritic cell phenotype, infected monocytes reactivated virus replication and produced infectious progeny. Treatment of infected monocytes with IL-6 alone also was sufficient for reactivation, and the particles produced after exposure to this cytokine were about fivefold more infectious than virions produced by other treatments. We propose that in vivo microenvironments influence not only the efficiency of reactivation but also the infectivity of the virions produced from latently infected monocytes.**

herpesvirus | myeloid biology | cell culture | antigen presentation | immunology

Human cytomegalovirus (HCMV) is a dangerous opportunistic pathogen (1) that replicates in many cell types but enters latency in others, allowing persistence of the viral genome without production of progeny. Viral DNA and a small subset of viral RNAs have been found in naturally infected CD34$^+$ hematopoietic stem cells (HSCs) and CD33$^+$ progenitor cells (2). Experimental infections of CD34$^+$ and CD33$^+$ cells also display the hallmarks of latency. HCMV DNA and a subset of viral transcripts are present in these cells after infection in culture, and the virus can be reactivated to produce progeny if the cells differentiate (2). The choice of HCMV strain can influence the outcome of infections (3). Two clinical isolates entered and exited latency, whereas the AD169 laboratory strain failed to become latent in CD34$^+$ cells. AD169 lacks the UL138 gene, which is important for entry into latency (3, 4).

Like HSCs, naturally infected CD14$^+$ monocytes harbor HCMV DNA (5–7), and viral replication is activated by differentiation (8, 9). Monocytes from peripheral blood are nonpermissive for HCMV replication (10), but when differentiated to a macrophage phenotype, they support the production of infectious progeny (11, 12). Thus, natural and experimental infections argue that differentiation from monocyte to macrophage or dendritic cell marks a divide between latency and active replication.

Although monocytes harbor latent virus, it has not been clear that direct infection of a monocyte can lead to latency. Latent virus in monocytes could result from prior infection of myeloid progenitors that subsequently differentiate to monocytes (13). Furthermore, infection with the HCMV Towne E strain promotes the differentiation of monocytes (14). Because different responses to infection have been observed for a variety of HCMV strains in monocytes, we revisited the question by developing a system for culturing peripheral blood monocytes in a nondifferentiating state. The FIX clinical isolate of HCMV can enter latency and reactivate from latency in these cells.

## Results

**Maintenance of CD14$^+$ Monocytes Without Detectable Differentiation.** We isolated monocytes from peripheral blood mononuclear cells (PBMCs) by using microbeads with CD14-specific antibody. The isolated cells were maintained in culture plates treated to block cell adherence in medium containing a mixture of cytokines previously used to maintain latently infected myeloid cells (15). The cell surface phenotype was assessed for preparations from three donors at 24 h postisolation (Fig. 1*A*) and found to contain 96.8% ± 0.6% CD14$^+$ cells, with a consistently small variance in the level of CD14 expression. To test for changes caused by selection through the CD14 receptor, we evaluated cell surface marker expression before and after the microbead isolation (Fig. S1). We observed no significant induction of macrophage or dendritic cell markers, and monocyte markers remained unchanged, arguing that the isolation procedure did not induce differentiation.

To monitor the stability of the monocyte phenotype, we analyzed cell surface markers at various times postisolation (Fig. 1*B*). Multiple monocyte markers (CD11b, CD13, and CD14) were present, with little change in levels during the 25-d period in culture. HLA-DR expression remained low, a characteristic of monocytes as opposed to macrophages and dendritic cells, and markers of dendritic cells (CD80 and CD83) and granulocytes (CD15) were not detected. After 24 d in culture, monocytes were transferred to standard culture plates to allow adherence and were maintained in medium containing macrophage colony stimulating factor (M-CSF) plus IL-3 or granulocyte macrophage colony stimulating factor (GM-CSF) plus IL-4, cytokine mixtures that direct differentiation to macrophages or dendritic cells, respectively (16, 17). Six days later, the cells expressed markers (Fig. 1*C*) and morphology (Fig. 1*D*) of macrophages and dendritic cells, showing that our culture conditions do not prevent differentiation after extended time in culture.

**Nondifferentiating Monocytes Can Be Infected with an HCMV Clinical Isolate.** Monocytes were infected with a GFP-expressing derivative of the FIX clinical isolate (3) (Fig. 2*A*). At a multiplicity of 1 pfu/cell, FIX expressed GFP in 35% of monocytes, and the portion of infected cells increased to 97% at a multiplicity of 3. The AD169 laboratory strain (3) entered monocytes much less efficiently than FIX. At a multiplicity of 20, AD169 expressed

Author contributions: D.H. and T.E.S. designed research; D.H. performed research; D.H. and T.E.S. analyzed data; and D.H. and T.E.S. wrote the paper.

The authors declare no conflict of interest.

[1]To whom correspondence should be addressed. E-mail: tshenk@princeton.edu.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1014509107/-/DCSupplemental.

MICROBIOLOGY

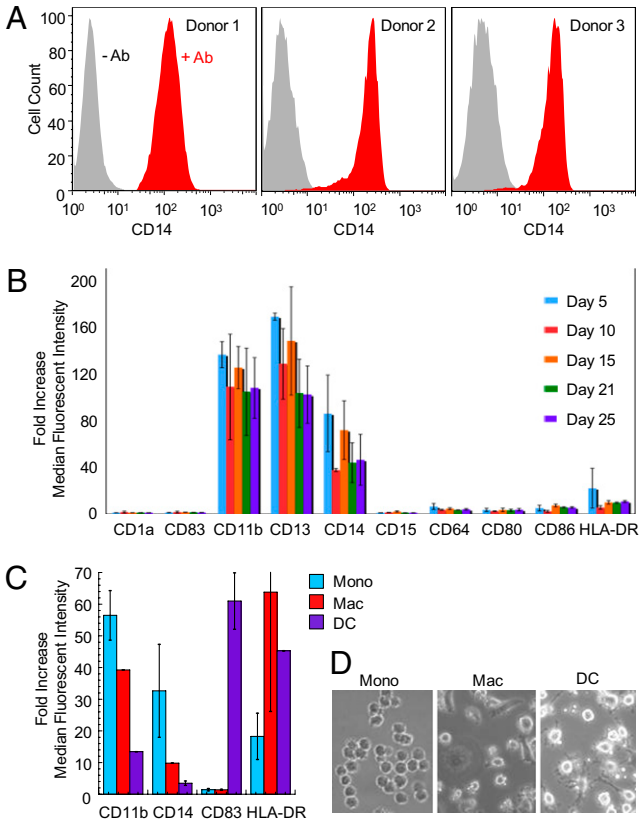


**Fig. 1.** Monocyte culture. (*A*) Reproducible isolation of CD14$^{+}$ populations. Monocytes from three donors were prepared, maintained in culture for 24 h, and then analyzed for CD14 expression by flow cytometry. Samples receiving no antibody (gray) were compared with samples treated with fluorescently conjugated anti-CD14 antibody (red). (*B*) Stable expression of cell surface markers over an extended period in culture. Cells from two donors were monitored by flow cytometry with phycoerythrin (PE)-CY7–conjugated anti-CD14 and PE-conjugated antibodies to various marker proteins. Fold increases in median fluorescent intensity relative to isotype controls were calculated for cells from the population of CD14$^{high}$ backgated cells. The mean plus SE are presented. (*C*) Induction of macrophage and dendritic cell markers in response to cytokine treatment. After 24 d in culture, monocytes (Mono; blue bars) were transferred to standard tissue culture plates in medium containing either M-CSF plus IL-3 (Mac; red bars) or GM-CSF plus IL-4 (DC; purple bars); 6 d later, cell surface markers were analyzed by flow cytometry, and the mean fold increase in median fluorescent intensity (±SE) relative to isotype controls for cells from two donors is displayed. (*D*) Morphology of undifferentiated vs. differentiated monocytes. Cells were visualized by phase microscopy.

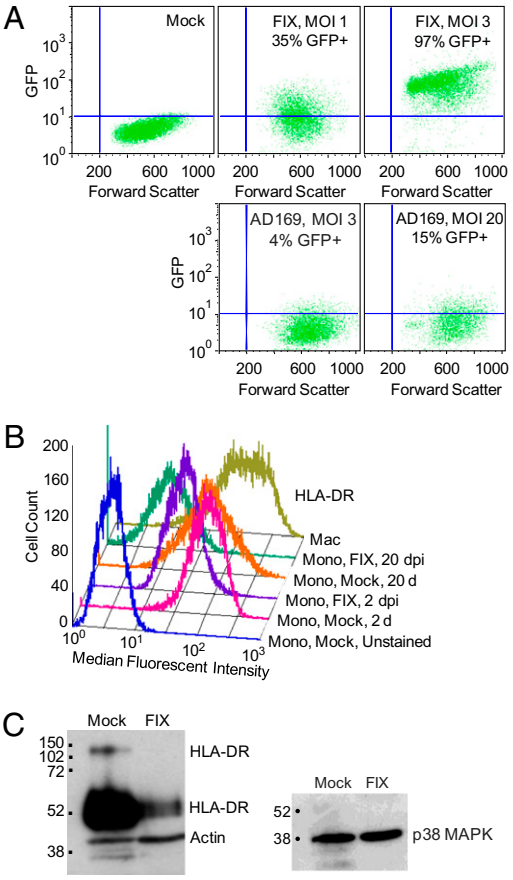


**Fig. 2.** Infection of monocytes. (*A*) Cultured monocytes are efficiently infected with the FIX strain of HCMV. After 1 d in culture, monocytes were mock-infected or infected with FIX- or AD169-expressing GFP at the indicated multiplicities of infection (MOI). Two days later, cells were analyzed by flow cytometry for GFP expression. (*B*) HLA-DR surface expression is reduced after infection of monocytes. Cells were mock-infected or infected with a FIX derivative expressing an IE2–GFP fusion protein and assayed for HLA-DR by flow cytometry at 2 or 20 d post infection (dpi). Cells differentiated to a macrophage (Mac) phenotype were also analyzed as a control. (*C*) Reduced total cell HLA-DR after infection of monocytes. Cells were mock-infected or infected with FIX and assayed for HLA-DR 5 d later by Western blot. Intact αβ heterodimers and monomers are labeled. The blot was probed for β-actin and p38 MAPK as controls.

GFP in only 14% of cells. These results are consistent with earlier work showing that AD169 enters many cell types inefficiently because of a mutation in its UL131 ORF (18).

We assessed whether HCMV induced the differentiation of monocytes by monitoring HLA-DR surface expression, which increases on differentiation to either a macrophage or dendritic cell phenotype (19, 20). Although differentiation was observed by others when a different HCMV strain and adherent culture conditions were used (21), infection with the FIX clinical isolate did not induce monocyte differentiation, as assessed by HLA-DR cell surface fluorescence (Fig. 2*B*). In fact, infection reduced the cell surface level of HLA-DR in the cell population by a factor of about 3 on day 2 and a factor of 6 at 20 d post infection (dpi). A similar reduction in HLA-DR was observed after infection of granulocyte–macrophage progenitor cells with a different clinical isolate (22). We also tested levels of HLA-DR by Western blot (Fig. 2*C*). At 5 dpi, HLA-DR was substantially reduced in infected compared with mock-infected monocytes. To control for the possibility that the down-regulation of HLA-DR resulted from a general reduction in host protein synthesis, we assayed for levels of p38 MAPK, which has a half life in monocytic cells of about 24 h (23) and is not induced by infection (24). p38 levels were unchanged, ruling out a global effect. Consistent with the failure of HLA-DR to be elevated, the morphology of the infected monocytes did not change (Fig. S2). These results argue that infection with FIX does not induce differentiation of monocytes but rather, down-regulates HLA-DR expression.

**Transient Expression of Lytic Transcripts with Prolonged Expression of Latency-Associated RNAs in Monocytes.** A subset of lytic HCMV RNAs is transiently expressed after infection of CD34$^{+}$ HSCs (25) or granulocyte–macrophage progenitor cells (26). We infected monocytes with FIX and monitored the accumulation of the UL122 and UL123 immediate-early RNAs, whose immediate early 2 and 1 (IE2 and IE1) protein products are essential for lytic replication (1). Reverse transcription-coupled quantitative PCR (RT-qPCR) revealed that both RNAs are present at 1 d, their levels were increased at 2 d, and then, they decreased over time

until at 10 dpi, the signal was indistinguishable from that for mock-infected cells (Fig. 3 *Upper Left*). During lytic infection, UL123 RNA typically accumulates in much higher levels than the UL122 transcript (27), but this did not seem to be the case in FIX-infected monocytes. Early and late RNAs were also monitored in monocytes infected with the clinical strain (Fig. 3 *Lower*). Both classes of RNA were present at 1 dpi and then steadily declined before becoming indistinguishable from mock-infected cells by 10 dpi. In contrast to the clinical isolate, the AD169 laboratory strain expressed viral RNAs throughout the 10-d time course (Fig. 3 *Upper Right*), reminiscent of the earlier observation that AD169 does not extinguish production of its RNAs after infection of $CD34^+$ HSCs (3). Although our analysis does not discriminate RNA fragments from functional mRNAs, we can nevertheless conclude that both the viral strain and the monocyte host environment influence the pattern of HCMV RNA accumulation.

Viral DNA was maintained but failed to accumulate after infection of monocytes with FIX (Fig. 4*A*). About 0.5 genomes/cell were detected, somewhat below the range of 2–13 genomes/cell reported for naturally infected mononuclear cells in G-CSF–mobilized blood (28).

Four HCMV latency-associated transcripts have been described. UL138 RNA is expressed in $CD14^+$ and $CD34^+$ cells from HCMV seropositive individuals, and a UL138-deficient FIX derivative failed to enter latency after infection of $CD34^+$ cells (3). Latency unique nuclear antigen (LUNA) RNA is coded opposite to the UL81-82 coding region, and it accumulates during latency in monocytes and bone marrow cells (29). Bone marrow cells from infected donors express latency-associated cmvIL-10 (LAcmvIL-10) RNA (30), which encodes a variant of the HCMV IL-10 (vIL-10), and infected THP1 monocyte-like cells express RNA encoding the US28 chemokine receptor (31). All four RNAs were detected on days 1–10 after infection of monocytes (Fig. 4*B*). Two amplification products were evident for LAcmvIL-10 RNA on days 7 and 10, which likely correspond to unspliced and spliced transcripts (30). Importantly, UL123 RNA was reduced at day 7 and no longer evident on day 10. We conclude that the FIX virus enters a quiescent state with continued expression of a limited set of RNAs after infection of cultured monocytes.

**Reactivation of HCMV from Monocytes.** To test for reactivation of FIX, infected monocytes were maintained in culture for 10–14 d to allow for loss of lytic viral RNA expression. Then, to induce differentiation, monocytes were transferred to plates with a normal cell-binding surface, and the medium was supplemented with cytokines supporting differentiation to macrophages (M-CSF + IL-3) or dendritic cells (GM-CSF + IL-4). IL-6 was also tested, because antibody to IL-6 inhibits reactivation of another herpesvirus (32) and IL-6 modulates the differentiation of monocytes (19, 33). In an initial experiment, monocytes from two donors with quiescent FIX genomes were transferred to monolayers of fibroblasts, subjected to the cytokine treatments, and assayed for the induction of fluorescent foci 11 d later (Fig. 5*A*). No infectivity was detected in the lysate of monocytes from donor 1, one infectious unit was found in the lysate from donor 2, and culture medium harvested before the induction of reactivation contained no detectable virus. All three stimuli induced the production of infectious virus, and IL-6 was about 2.5-fold more effective at inducing foci than the other treatments. Next, the experiment was repeated without adding fibroblasts. Infected monocytes from four donors were allowed to adhere to the plastic surface of culture plates and analyzed 11 d later for differentiation. Each treatment induced the production of virus (Fig. 5*B Left*). IL-6 again generated the largest amount of infectivity, and no virus was detected in cells or media collected before reactivation. Viral DNA accumulation was monitored by qPCR, and the three treatments each caused an ~250-fold increase in the level of intracellular DNA relative to the amount in uninduced monocytes (Fig. 5*B Right*). This experiment used a derivative of FIX virus expressing a GFP-tagged IE2 protein. Expression of the fusion protein was observed by fluorescence microscopy in fibroblasts but not in monocytes undergoing reactivation, suggesting that the viral protein accumulates to a lower level during reactivation than in normal lytic replication. To evaluate whether the viral DNA detected during reactivation was encapsidated in a virion, we subjected reactivation supernatants to DNase I treatment either before or after virion disruption. Extracellular DNA from all three reactivation stimuli was protected from degradation before disruption (Fig. S3), indicating that each reactivation stimulus

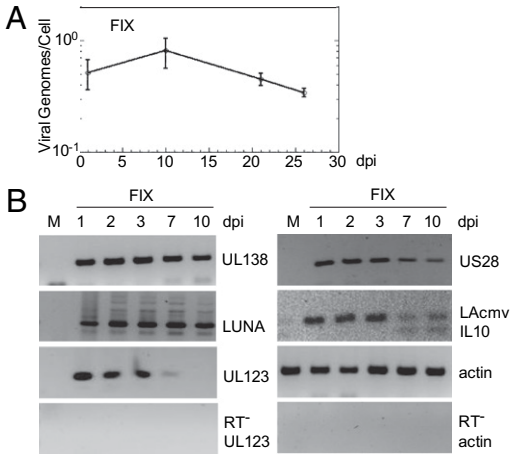


**Fig. 4.** Genome maintenance and expression of latency-associated RNAs in monocytes. (*A*) Maintenance of the FIX genome. DNA was isolated from monocytes at various times after infection, and viral DNA was quantified by qPCR. Samples were run in quadruplicate, and viral genomes per cell were calculated by dividing the number of genomes (normalized to actin) by the cell number. ANOVA was performed to test for variation in DNA content over time and yielded a *P* value of 0.44, supporting the interpretation that the means do not differ. (*B*) Latency-associated RNAs are present in FIX-infected monocytes throughout a 10-d time course. Total RNA was isolated from monocytes and assayed by RT-PCR (40 cycles) followed by gel electrophoresis of the products. Control assays were performed without reverse transcriptase ($RT^-$) to monitor for DNA contamination. Negative images are displayed to better visualize DNA bands.

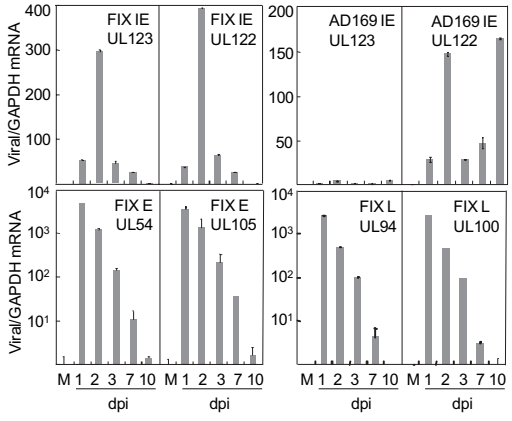


**Fig. 3.** Transient expression of HCMV lytic RNAs in monocytes. One day after isolation, monocytes were mock-infected (M) or infected with FIX or AD169, and RNA was prepared on various days postinfection. Representative immediate-early (IE), early (E), and late (L) viral RNAs were quantified by using RT-qPCR. Samples were assayed in quadruplicate; standard curves were generated using purified viral DNA with a β-actin insertion, and quantities of viral RNAs were normalized to cellular GAPDH RNA levels.

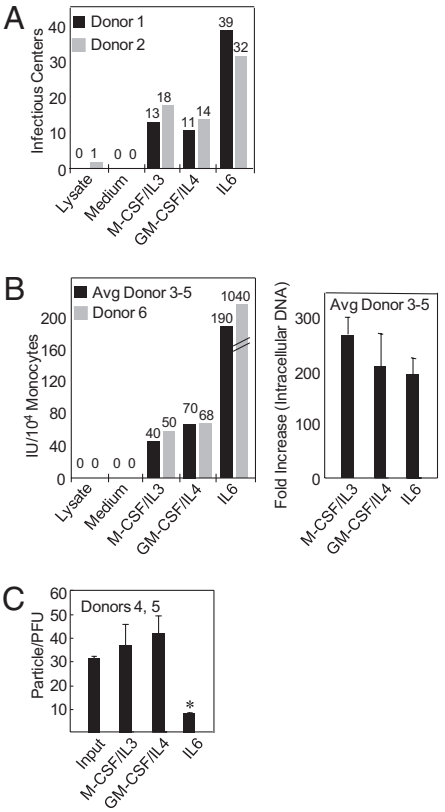


**Fig. 5.** Reactivation of FIX by differentiation of monocytes. Ten days before initiating a reactivation assay, CD14$^+$ monocytes were infected with FIX virus (donors 1, 2, and 6) or a derivative of FIX expressing an IE2-GFP fusion protein (donors 3–5). (*A*) Production of virus by coculture of monocytes with fibroblasts in medium supplemented with cytokines. After 11 d, infectious centers containing more than or equal to five infected cells were counted. As controls, lysates of infected monocytes and medium from infected cultures, prepared at the start of the reactivation experiment, were added to fibroblasts. (*B Left*) Production of virus by transferring monocytes to standard cell culture plates in medium supplemented with cytokines. After 11 d, supernatants were harvested and assayed for infectious units (IUs; i.e., the number of IE1-positive fibroblasts generated at 24 h postinfection). Monocyte lysates and medium were assayed as controls. (*Right*) Amplification of viral DNA on differentiation of monocytes. Total cell DNA was prepared (donors 3–5), quantified by qPCR, and reported as the fold increase over the amount of viral DNA in infected monocytes immediately before reactivation. (*C*) IL-6–mediated reactivation generates virions with enhanced infectivity. Particle to PFU ratios were calculated by dividing the amount of DNA in DNase before samples (Fig. S3) by the number of infectious units for donors 4 and 5. The asterisk indicates a $P$ value $< 0.0001$ compared with the input virus (produced in fibroblasts) and IL-6–induced virus (produced in differentiating monocytes) by the Student $t$ test.

induced the release of virions into the supernatant and not merely free viral genomes as a consequence of cell death.

We also calculated a particle to plaque-forming units ratio for each reactivation condition (Fig. 5*C*). IL-6 treatment resulted in a fivefold increase in the infectivity of virus particles compared with virus produced in fibroblasts or monocytes after treatment with the other cytokines.

In sum, monocytes from six donors harboring quiescent HCMV genomes produced infectious virus when stimulated to differentiate. The rates of reactivation were similar to those observed for CD34$^+$ HSCs infected with a clinical isolate of HCMV (25).

## Discussion

Other herpesviruses reactivate from latency after the ectopic expression of one or more immediate-early transactivating proteins (34–37). In contrast, expression of the immediate-early IE1 and IE2 proteins in myeloid cells carrying latent HCMV failed to stimulate the production of virus (38). Thus, understanding the elements of the cellular environment that support latency and reactivation is crucial to our understanding of HCMV pathogenesis.

Numerous conditions have been tested for culturing CD14$^+$ monocytes. Suspension culture in Teflon vessels did not prevent monocyte differentiation (39), and therefore, we combined culture on a nonadherent surface with a cytokine-rich environment similar to that was used previously to support the culture of bone marrow cells (15). Analysis of cell surface markers showed that this method maintained populations of monocytes without evidence of differentiation for 25 d (Fig. 1*B*), and the cells were able to respond to differentiation signals after 24 d in culture (Fig. 1 *C* and *D*). These monocytes were efficiently infected with the FIX clinical isolate of HCMV (Fig. 2*A*), and infection did not induce differentiation as judged by morphology (Fig. S2) or HLA-DR levels (Fig. 2 *B* and *C*). Viral RNAs required for lytic replication were transiently expressed after infection with FIX (Fig. 3), as observed for CD34$^+$ HSCs and CD33$^+$ progenitor cells (25, 26), and four latency-associated RNAs were detected on day 10 after infection (Fig. 4*B*)—a time at which other lytic transcripts were extinguished. Viral genomes were maintained in the infected cultures (Fig. 4*A*), and the production of infectious virus was reactivated by subjecting cultures to differentiation-inducing signals (Fig. 5). Thus, infection of cultured monocytes elicited the hallmarks of latency.

The normal HCMV temporal cascade of RNA accumulation did not occur in monocytes after infection with FIX, because the early and late transcript levels peaked earlier than immediate-early transcripts (Fig. 3). Furthermore, late RNAs accumulated without viral genome replication, which normally precedes high-level late mRNA expression. A portion of the late RNAs may be delivered to cells as constituents of virions. Alternatively, the myeloid cell environment could allow IE1/IE2–independent-early and late RNA accumulation. The availability of late gene products immediately after infection might not serve a purpose, but they could protect cells from toxic effects of immediate-early products.

Our experimental monocyte infections highlight two possibly interrelated immunosuppressive elements of HCMV latency. First, HLA-DR cell surface levels are down-regulated in infected monocytes (Fig. 2 *B* and *C*), probably inhibiting viral antigen presentation; second, RNA coding for the latency-associated form of the viral IL-10 homolog, LAcmvIL-10 (30), is expressed in FIX-infected monocytes (Fig. 4*B*). If LAcmvIL-10 RNA is functional and the cytokine is expressed, it may help to suppress differentiation and down-regulate HLA-DR, because the cell surface antigen was not down-regulated in granulocyte–monocyte progenitor cells infected with mutants lacking LAcmvIL-10 and mutant-infected myeloid cells induced T cell proliferation to a greater extent than cells infected with wild-type virus (40). If vIL-10–mediated down-regulation of HLA-DR impacts T helper cell differentiation, this could suppress anti-HCMV antibody production and the development of a robust HCMV-specific cytotoxic T cell response, because CD4$^+$ cells maintain HCMV-specific CD8$^+$ populations and contribute importantly to HCMV disease in solid organ transplant patients (41).

When infected monocytes were moved from a substrate that discourages attachment to a substrate that supports cell attachment and the medium was supplemented with cytokines that stimulate differentiation (Fig. 1 *C* and *D*), HCMV DNA replication was induced, and infectious progeny accumulated (Fig. 5). We have not yet tested whether the change in the adherence properties of the surface and cytokine mixtures contributes to reactivation. However, we suspect that both are important, because, as discussed above, both variables have been shown to influence the

differentiation state of monocytes. M-CSF plus IL-3 or GM-CSF plus IL-4, which induce differentiation to a macrophage or dendritic cell phenotype, were equally efficient in reactivating the production of virus. IL-6 also induced reactivation. It induces M-CSF receptors on monocytes, allowing them to consume their autocrine M-CSF and differentiate to a macrophage phenotype (19, 33). Furthermore, IL-6 can activate nuclear factor for IL-6 (NF-IL-6) in monocytic cells (42, 43); NF–IL-6 is a member of the CCAAT/enhancer binding protein (C/EBP) family of transcription factors (44). The HCMV major immediate-early promoter contains a C/EBP binding site (45), and therefore, IL-6 might simultaneously initiate monocyte differentiation and help to induce expression of the IE1 and IE2 proteins.

Virions produced during IL-6–mediated reactivation were more infectious for fibroblasts than virus produced after treatment with other cytokines (Fig. 5*C*). Perhaps the microenvironment in which a reactivation event occurs influences the infectivity of the virus produced and has a marked effect on whether the reactivation leads to HCMV spread with active disease.

In sum, we have validated a monocyte model for HCMV latency and reactivation. This system has potential advantages relative to earlier models: (*i*) monocytes are readily available and can be cultured for an extended period without detectable differentiation, (*ii*) monocytes are efficiently infected by a clinical HCMV isolate, and (*iii*) reactivation can be induced using defined mixtures of cytokines.

## Materials and Methods

**Cells and Viruses.** Human MRC-5 fibroblasts were cultured in DMEM supplemented with 10% FBS. PBMCs were isolated from buffy coats (New Jersey Blood Center) by centrifugation in Ficoll–Paque gradients (Pharmacia-Amersham). $CD14^+$ monocytes were purified from PBMCs using CD14 microbeads (Miltenyi Biotec) according to the manufacturer's protocol. After isolation, cells were resuspended in monocyte suspension medium (Iscove DMEM: 20% heat-inactivated FBS, 25 mM Hepes, 50 ng/mL M-CSF, 50 ng/mL stem cell factor [SCF], 50 ng/mL G-CSF, 50 ng/mL GM-CSF, 50 ng/mL IL-3; cytokines from R&D Systems) at a density of $10^6$ cells/mL on low cell-binding plates (Nunc HydroCell). Medium was replaced every 3 d. To induce differentiation, monocytes were cultured on standard culture plasticware in Iscove DMEM containing 20% heat-inactivated FBS with 100 ng/mL GM-CSF and 25 ng/mL IL-4 to generate dendritic cells or 100 ng/mL M-CSF and 100 ng/mL IL-3 to produce macrophages.

The BAC-derived AD169 and FIX strains were engineered to express GFP from an SV40 promoter, producing BAD*in*GFP and FX*in*GFP (3). The variant expressing IE2 with a C-terminal GFP fusion partner, FX*in*UL122GFP, was also generated from the FIX BAC clone. Virus stocks were propagated in MRC-5 cells, and virus was partially purified by centrifugation through a 20% sorbitol cushion before being resuspended in DMEM with 10% FBS and stored at −80 °C. Virus titers were determined on MRC-5 cells. Unless otherwise stated, infections were at a multiplicity of 3 pfu/cell.

**Assays for Reactivation from Latency.** Monocytes were differentiated by adherence to plastic in medium supplemented with cytokines at 10 and 14 dpi. The release of virus from monocytes cocultured with MRC-5 cells was quantified by counting infectious centers (≥5 adjacent GFP+ cells). The yield of virus produced by monocytes in the absence of fibroblasts was assayed by plating virus onto fibroblasts and quantifying IE1-expressing cells 96 h later.

**Protein and Nucleic Acid Analysis.** For analysis of cell surface proteins, cells in PBS containing 0.5% BSA were reacted with phycoerythrin (PE)-conjugated antibodies to HLA-DR, CD1a, CD1b, CD1d, CD11a, CD11b, CD11c, CD13, CD14, CD15, CD16, CD64, CD80, CD86, and CD209 or allophycocyanin (APC)-conjugated CD83. For double-labeling experiments, PE-Cy7–conjugated CD14 antibody was used. All antibodies were from BD Biosciences, except anti-CD1b, which was from Miltenyi Biotec. Analyses were performed using a FACSSort flow cytometer (BD Biosciences Immunocytometry Systems), and data analysis, median fluorescent intensity calculations, and graphs were generated using FLOWJO flow cytometry software (Tree Star). HLA-DR was also monitored by Western blot assay with monoclonal antibody B8.12.2 (Beckman Coulter) and a secondary goat anti-mouse antibody (Jackson Laboratory). Western blots for p38 MAPK used a polyclonal antibody (Cell Signaling) and secondary goat anti-rabbit antibody (Jackson Laboratory). Protein bands reacting with antibody were detected by chemiluminescence (GE Healthcare).

Intracellular viral DNA and DNA from cell supernatants were assayed (46) by qPCR using primers for the HCMV UL123 ORF and β-actin (Table S1). Viral RNA was assayed (3) by qRT-PCR using primers for viral RNAs, GAPDH, or β-actin (Table S1).

**ACKNOWLEDGMENTS.** We thank Christine DeCoste (Princeton University) for advice for FACS experiments. This work was supported by grants from the National Institutes of Health (CA85786 and AI 087672). D.H. received fellowship support from the Leukemia and Lymphoma Society.

1. Mocarski ES, Shenk T, Pass RF (2007) Cytomegaloviruses. *Fields Virology*, eds Knipe DM, Howley PM (Wolters Kluwer Health/Lippincott Williams & Wilkins, Philadelphia), 5th Ed, pp 2701–2772.
2. Sinclair J (2008) Human cytomegalovirus: Latency and reactivation in the myeloid lineage. *J Clin Virol* 41:180–185.
3. Goodrum F, Reeves M, Sinclair J, High K, Shenk T (2007) Human cytomegalovirus sequences expressed in latently infected individuals promote a latent infection in vitro. *Blood* 110:937–945.
4. Petrucelli A, Rak M, Grainger L, Goodrum F (2009) Characterization of a novel Golgi apparatus-localized latency determinant encoded by human cytomegalovirus. *J Virol* 83:5615–5629.
5. Schrier RD, Nelson JA, Oldstone MB (1985) Detection of human cytomegalovirus in peripheral blood lymphocytes in a natural infection. *Science* 230:1048–1051.
6. Stanier P, et al. (1989) Persistence of cytomegalovirus in mononuclear cells in peripheral blood from blood donors. *BMJ* 299:897–898.
7. Taylor-Wiedeman J, Sissons JG, Borysiewicz LK, Sinclair JH (1991) Monocytes are a major site of persistence of human cytomegalovirus in peripheral blood mononuclear cells. *J Gen Virol* 72:2059–2064.
8. Söderberg-Nauclér C, Fish KN, Nelson JA (1997) Reactivation of latent human cytomegalovirus by allogeneic stimulation of blood cells from healthy donors. *Cell* 91: 119–126.
9. Reeves MB, MacAry PA, Lehner PJ, Sissons JG, Sinclair JH (2005) Latency, chromatin remodeling, and reactivation of human cytomegalovirus in the dendritic cells of healthy carriers. *Proc Natl Acad Sci USA* 102:4140–4145.
10. Rice GP, Schrier RD, Oldstone MB (1984) Cytomegalovirus infects human lymphocytes and monocytes: Virus expression is restricted to immediate-early gene products. *Proc Natl Acad Sci USA* 81:6134–6138.
11. Ibanez CE, Schrier R, Ghazal P, Wiley C, Nelson JA (1991) Human cytomegalovirus productively infects primary differentiated macrophages. *J Virol* 65:6581–6588.
12. Lathey JL, Spector SA (1991) Unrestricted replication of human cytomegalovirus in hydrocortisone-treated macrophages. *J Virol* 65:6371–6375.
13. Minton EJ, Tysoe C, Sinclair JH, Sissons JG (1994) Human cytomegalovirus infection of the monocyte/macrophage lineage in bone marrow. *J Virol* 68:4017–4021.
14. Smith MS, Bentz GL, Alexander JS, Yurochko AD (2004) Human cytomegalovirus induces monocyte differentiation and migration as a strategy for dissemination and persistence. *J Virol* 78:4444–4453.
15. Zhuravskaya T, et al. (1997) Spread of human cytomegalovirus (HCMV) after infection of human hematopoietic progenitor cells: Model of HCMV latency. *Blood* 90: 2482–2491.
16. Sallusto F, Lanzavecchia A (1994) Efficient presentation of soluble antigen by cultured human dendritic cells is maintained by granulocyte/macrophage colony-stimulating factor plus interleukin 4 and downregulated by tumor necrosis factor alpha. *J Exp Med* 179:1109–1118.
17. Young DA, Lowe LD, Clark SC (1990) Comparison of the effects of IL-3, granulocyte-macrophage colony-stimulating factor, and macrophage colony-stimulating factor in supporting monocyte differentiation in culture. Analysis of macrophage antibody-dependent cellular cytotoxicity. *J Immunol* 145:607–615.
18. Wang D, Shenk T (2005) Human cytomegalovirus UL131 open reading frame is required for epithelial cell tropism. *J Virol* 79:10330–10338.
19. Chomarat P, Banchereau J, Davoust J, Palucka AK (2000) IL-6 switches the differentiation of monocytes from dendritic cells to macrophages. *Nat Immunol* 1: 510–514.
20. Freudenthal PS, Steinman RM (1990) The distinct surface of human blood dendritic cells, as observed after an improved isolation method. *Proc Natl Acad Sci USA* 87: 7698–7702.
21. Chan G, Bivins-Smith ER, Smith MS, Smith PM, Yurochko AD (2008) Transcriptome analysis reveals human cytomegalovirus reprograms monocyte differentiation toward an M1 macrophage. *J Immunol* 181:698–711.
22. Slobedman B, Mocarski ES, Arvin AM, Mellins ED, Abendroth A (2002) Latent cytomegalovirus down-regulates major histocompatibility complex class II expression on myeloid progenitors. *Blood* 100:2867–2873.
23. Shiratsuchi H, Basson MD (2005) Activation of p38 MAPKalpha by extracellular pressure mediates the stimulation of macrophage phagocytosis by pressure. *Am J Physiol Cell Physiol* 288:C1083–C1093.
24. Johnson RA, Huong SM, Huang ES (2000) Activation of the mitogen-activated protein kinase p38 by human cytomegalovirus infection through two distinct pathways: A novel mechanism for activation of p38. *J Virol* 74:1158–1167.

25. Goodrum FD, Jordan CT, High K, Shenk T (2002) Human cytomegalovirus gene expression during infection of primary hematopoietic progenitor cells: A model for latency. *Proc Natl Acad Sci USA* 99:16255–16260.
26. Cheung AK, Abendroth A, Cunningham AL, Slobedman B (2006) Viral gene expression during the establishment of human cytomegalovirus latent infection in myeloid progenitor cells. *Blood* 108:3691–3699.
27. Bresnahan WA, Shenk TE (2000) UL82 virion protein activates expression of immediate early viral genes in human cytomegalovirus-infected cells. *Proc Natl Acad Sci USA* 97:14506–14511.
28. Slobedman B, Mocarski ES (1999) Quantitative analysis of latent human cytomegalovirus. *J Virol* 73:4806–4812.
29. Bego M, Maciejewski J, Khaiboullina S, Pari G, St Jeor S (2005) Characterization of an antisense transcript spanning the UL81-82 locus of human cytomegalovirus. *J Virol* 79: 11022–11034.
30. Jenkins C, Abendroth A, Slobedman B (2004) A novel viral transcript with homology to human interleukin-10 is expressed during latent human cytomegalovirus infection. *J Virol* 78:1440–1447.
31. Beisser PS, Laurent L, Virelizier JL, Michelson S (2001) Human cytomegalovirus chemokine receptor gene US28 is transcribed in latently infected THP-1 monocytes. *J Virol* 75:5949–5957.
32. Kriesel JD, et al. (1997) Anti-interleukin-6 antibodies inhibit herpes simplex virus reactivation. *J Infect Dis* 175:821–827.
33. Mitani H, et al. (2000) Activity of interleukin 6 in the differentiation of monocytes to macrophages and dendritic cells. *Br J Haematol* 109:288–295.
34. Halford WP, Kemp CD, Isler JA, Davido DJ, Schaffer PA (2001) ICP0, ICP4, or VP16 expressed from adenovirus vectors induces reactivation of latent herpes simplex virus type 1 in primary cultures of latently infected trigeminal ganglion cells. *J Virol* 75:6143–6153.
35. Lukac DM, Renne R, Kirshner JR, Ganem D (1998) Reactivation of Kaposi's sarcoma-associated herpesvirus infection from latency by expression of the ORF 50 transactivator, a homolog of the EBV R protein. *Virology* 252:304–312.
36. Sun R, et al. (1998) A viral gene that activates lytic cycle expression of Kaposi's sarcoma-associated herpesvirus. *Proc Natl Acad Sci USA* 95:10866–10871.
37. Countryman J, Miller G (1985) Activation of expression of latent Epstein-Barr herpesvirus after gene transfer with a small cloned subfragment of heterogeneous viral DNA. *Proc Natl Acad Sci USA* 82:4085–4089.
38. Yee LF, Lin PL, Stinski MF (2007) Ectopic expression of HCMV IE72 and IE86 proteins is sufficient to induce early gene expression but not production of infectious virus in undifferentiated promonocytic THP-1 cells. *Virology* 363:174–188.
39. van der Meer JW, et al. (1982) Characteristics of human monocytes cultured in the Teflon culture bag. *Immunology* 47:617–625.
40. Cheung AK, et al. (2009) The role of the human cytomegalovirus UL111A gene in down-regulating CD4+ T-cell recognition of latently infected cells: Implications for virus elimination during latency. *Blood* 114:4128–4137.
41. Waller EC, Day E, Sissons JG, Wills MR (2008) Dynamics of T cell memory in human cytomegalovirus infection. *Med Microbiol Immunol (Berl)* 197:83–96.
42. Natsuka S, et al. (1992) Macrophage differentiation-specific expression of NF-IL6, a transcription factor for interleukin-6. *Blood* 79:460–466.
43. Akira S (1997) IL-6-regulated transcription factors. *Int J Biochem Cell Biol* 29: 1401–1418.
44. Akira S, et al. (1990) A nuclear factor for IL-6 expression (NF-IL6) is a member of a C/EBP family. *EMBO J* 9:1897–1906.
45. Prösch S, Heine AK, Volk HD, Krüger DH (2001) CCAAT/enhancer-binding proteins alpha and beta negatively influence the capacity of tumor necrosis factor alpha to up-regulate the human cytomegalovirus IE1/2 enhancer/promoter by nuclear factor kappaB during monocyte differentiation. *J Biol Chem* 276:40712–40720.
46. Goodrum F, Jordan CT, Terhune SS, High K, Shenk T (2004) Differential outcomes of human cytomegalovirus infection in primitive hematopoietic cell subpopulations. *Blood* 104:687–695.