Human Cytomegalovirus Modulates Monocyte-Mediated Innate Immune Responses during Short-Term Experimental Latency *In Vitro*

Vanessa M. Noriega,^{a,b} Kester K. Haye,^{a,b} Thomas A. Kraus,^{a,c} Shanna R. Kowalsky,^d Yongchao Ge,^e Thomas M. Moran,^{a,b} Domenico Tortorella^{a,b}

Department of Microbiology,^a Global Health and Emerging Pathogens Institute,^b Department of Obstetrics, Gynecology and Reproductive Science,^c Department of Pediatrics, Division of Pediatric Infectious Diseases,^d and Department of Neurology,^e Icahn School of Medicine at Mount Sinai, New York, New York, USA

ABSTRACT

The ability of human cytomegalovirus (HCMV) to establish lifelong persistence and reactivate from latency is critical to its success as a pathogen. Here we describe a short-term *in vitro* model representing the events surrounding HCMV latency and reactivation in circulating peripheral blood monocytes that was developed in order to study the immunological consequence of latent virus carriage. Infection of human CD14⁺ monocytes by HCMV resulted in the immediate establishment of latency, as evidenced by the absence of particular lytic gene expression, the transcription of latency-associated mRNAs, and the maintenance of viral genomes. Latent HCMV induced cellular differentiation to a macrophage lineage, causing production of selective proinflammatory cytokines and myeloid-cell chemoattractants that most likely play a role in virus dissemination in the host. Analysis of global cellular gene expression revealed activation of innate immune responses and the modulation of protein and lipid synthesis to accommodate latent HCMV infection. Remarkably, monocytes harboring latent virus exhibited selective responses to secondary stimuli known to induce an antiviral state. Furthermore, when challenged with type I and II interferon, latently infected cells demonstrated a blockade of signaling at the level of STAT1 phosphorylation. The data demonstrate that HCMV reprograms specific cellular pathways in monocytes, most notably innate immune responses, which may play a role in the establishment of, maintenance of, and reactivation from latency. The modulation of innate immune responses is likely a viral evasion strategy contributing to viral dissemination and pathogenesis in the host.

IMPORTANCE

HCMV has the ability to establish a lifelong infection within the host, a phenomenon termed latency. We have established a short-term model system in human peripheral blood monocytes to study the immunological relevance of latent virus carriage. Infection of CD14⁺ monocytes by HCMV results in the generation of latency-specific transcripts, maintenance of viral genomes, and the capacity to reenter the lytic cycle. During short-term latency in monocytes the virus initiates a program of differentiation to inflammatory macrophages that coincides with the modulation of cytokine secretion and specific cellular processes. HCMV-infected monocytes are hindered in their capacity to exert normal immunoprotective mechanisms. Additionally, latent virus disrupts type I and II interferon signaling at the level of STAT1 phosphorylation. This *in vitro* model system can significantly contribute to our understanding of the molecular and inflammatory factors that initiate HCMV reactivation in the host and allow the development of strategies to eradicate virus persistence.

Human cytomegalovirus (HCMV) is a ubiquitous human pathogen with seroprevalence rates of 50 to 90% by adulthood (1). Infection of the immunocompetent host is restricted by cell-mediated immunity, leading to establishment of lifelong latent infection. The advent of AIDS and the development of the field of organ and tissue transplantation has resulted in the resurgence of HCMV-mediated disease (2, 3). While infection of the immunocompetent host is restricted by a robust immune response, patients with inadequate immune function succumb to multiorgan dysfunction, vascular disease, and graft rejection. The threat from HCMV in solid organ or hematopoietic allografts is exacerbated by the additional risk of virus reactivation from latency (4). HCMV latency is defined as the persistence of viral genomes concurrent with a limited but distinct viral gene transcriptional profile. True latency is associated with the absence of detectable production of infectious progeny. Additionally, cells carrying latent viral genomes have the capability to reenter the infection cycle under specific stimuli (5). Cytomegalovirus latency is restricted to myeloid cells, and establishment of dormancy is proposed to occur through the action of viral tegument proteins as

well as epigenetic modifications of the viral genome (6, 7). Despite increased research into this area of HCMV biology, much remains to be understood about the molecular and immune factors that are involved in the establishment of latency and how viral and cellular mechanisms orchestrate persistence. Therefore, recapitulating *in vitro* the cellular microenvironment that leads to latency

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Address correspondence to Domenico Tortorella, Domenico.Tortorella@mssm.edu.

V.M.N. and K.K.H. contributed equally to this work.

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and reactivation will be inherent to our understanding of HCMV pathogenesis.

Early clinical studies analyzing blood from healthy seropositive carriers demonstrated that CD34⁺ bone marrow-derived progenitors could harbor HCMV genomes *in vivo* (8), while CD14⁺ monocytes were the cell type within the peripheral blood compartment that carried and maintained HCMV DNA until terminal differentiation in the periphery (9). These early studies of natural latency in the host laid the groundwork for the development of *in vitro* experimental infection models that could allow further investigation into this phase of the virus life cycle. Experimental models of latency to date have focused on CD34⁺ hematopoietic stem cell (HSC) populations and myeloid-cell precursors, such as granulocyte-macrophage progenitors (GMPs) and CD14⁺ monocytes (10–12). These *ex vivo* models of latency and reactivation have recapitulated many key observations made from natural latent infection of the host, including differentiation-dependent reactivation of the virus and the repressive chromatin structure of the major immediate early promoter (MIEP) (7). Here we report a robust model system of short-term experimental HCMV latency and reactivation utilizing CD14⁺ peripheral blood monocytes, a persistent viral reservoir *in vivo* (13). The use of monocytes as a model system allows the isolation of large numbers of cells from the peripheral blood and therefore circumvents the possible low infectivity for bone marrow-derived cells. Employing this short-term experimental model system, we demonstrated the as-yet-uncharacterized immunological consequence of latent virus carriage. Our findings reveal that latent HCMV preferentially accelerates cellular differentiation of peripheral blood monocytes toward inflammatory macrophages, most likely to promote dissemination in the host. Importantly, latent HCMV activates and controls aspects of innate antiviral immunity in monocytes. This experimental system can provide an efficacious molecular setting for studies focused on immune control during HCMV latency and reactivation in the host.

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MATERIALS AND METHODS

Viruses and cells. Human CD14⁺ monocytes were isolated as previously described (14). In brief, peripheral blood mononuclear cells were isolated by Ficoll density gradient centrifugation (Histopaque; Sigma-Aldrich) from buffy coats of healthy human donors (New York Blood Center). CD14⁺ cells were immunomagnetically purified using anti-human CD14 antibody-labeled magnetic beads and iron-based MiniMACS LS columns (Miltenyi Biotec). Monocytes were maintained in RPMI containing 10% fetal bovine serum (FBS), 100 U/ml penicillin, 100 µg/ml streptomycin, and 500 U/ml human granulocyte-macrophage colony-stimulating factor (GM-CSF) (PeproTech) at 37°C in a humidified atmosphere (95% air–5% CO₂) in 50-ml Falcon tubes. Cell surface phenotyping and visual morphology confirmed these cells as monocytes prior to their use. Monocyte cultures and infections were performed in nonadherent tissue culture vessels and cells were agitated daily to prevent settling in culture. MRC5 human lung fibroblasts were maintained in Dulbecco's modified Eagle medium (DMEM) with 8% fetal bovine serum (FBS), 1 mM HEPES, 100 U/ml penicillin, and 100 µg/ml streptomycin. HCMV TB40/E was a gift from Christian Sinzger (Institute of Virology, University Medical Center Ulm) (15). Virus was propagated on fibroblasts and purified by density gradient centrifugation. Infectious virus yield was assayed by 50% tissue culture infectious dose (TCID₅₀) assay. Monocytes were infected with TB40/E at a multiplicity of infection (MOI) of 3 PFU per cell in RPMI with

1% FBS for 1 h at 37°C with shaking every 15 min and then washed twice with 1 × PBS before culturing. For UV inactivation experiments, the virus inoculum was exposed to UV irradiation at 10 cm from a germicidal lamp (UVP multiple-ray 8-W UV lamp [60 Hz]; Fisher) for 10 min.

Antibodies and immunoblot analysis. Cells lysis and immunoblot analysis were performed as previously described (16). Monoclonal antibodies against HCMV immediate early 1 (IE1) protein (P63-27) and phosphoprotein 65 (pp65) were obtained from William Britt (University of Alabama, Birmingham). Polyclonal anti-US2 antibody was generated as described previously (16). Anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was purchased from Upstate/Millipore. Antibodies to STAT1, phosphorylated STAT1, STAT2, and phosphorylated STAT2 were purchased from Cell Signaling.

DNA isolation and PCR. Cells were resuspended in solution containing 400 mM NaCl, 10 mM Tris (pH 7.4), 10 mM EDTA, 20% SDS, and 10 mg/ml proteinase K and incubated overnight at 37°C. Samples were extracted with phenol-chloroform and incubated with 10 mg/ml RNase A. DNA was precipitated with 100% ethanol and resuspended in 10 mM Tris (pH 8). Samples were used in a PCR with primers to viral IE1 (UL123) or β-actin. The primers used were as follows: IE1 Forward, 5'-GCCTTCCC TAAGACCACCAA-3'; IE1 Reverse, 5'-ATTTTCTGGGCATAAGCCAT AATC-3'; β-actin Forward, 5'-CATTGCCGACGGATGCA-3'; β-actin Reverse, 5'-GCCGATCCACACGGAGTACT-3'.

Quantitative PCR. To calculate viral genome number, quantitative PCR (qPCR) was performed by SYBR green assay using a Roche LightCycler 480 II. The concentration of viral DNA (UL123) at each time point was normalized to that of the β-actin gene. A standard curve to quantify genome copy number was generated using serial dilutions of the AD169 genome maintained within a bacterial artificial chromosome (BAC).

RNA isolation, reverse transcription, and PCR. RNA was isolated using the Absolutely RNA miniprep kit (Stratagene) according to the manufacturer's protocol. cDNA was prepared using the Transcriptor first-strand cDNA synthesis kit (Roche) [reverse transcription with oligo(dT) primers, 60 min at 50°C, inactivation at 85°C for 5 min] and used in a PCR with primers to latency-specific transcripts (cDNA amplification performed with 2-min extensions at 72°C for 35 cycles). The primers used were as follows: US28 Forward, 5'-TTTGGTGGATCTTTGCCGTG-3'; US28 Reverse, 5'-ACGAAAGCACCAGCATGAG-3'; UL138 Forward, 5'-TGCGCATGTTTTGAGCTAC-3'; UL138 Reverse, 5'-ACGGGTTT CAACAGATCGAC-3'; pp65 Forward, 5'-CCGACAACGAAATCCACA AT-3'; pp65 Reverse, 5'-TTCTGACCCTGAACCGTAGC-3'; RNA2.7 Forward, 5'-AAGATTACCGTCCTTACGAG-3'; RNA2.7 Reverse, 5'-GT GTCTACTACTCTGTGTG-3'; RL8A Forward, 5'-TGCCGTACGTGA TGCCTCA-3'; RL8A Reverse, 5'-AAAACAGCGGACAGTCCCACGCT G-3'; US3 Forward, 5'-ATGAAGCCGGTGTGTTGGTGCTC-3'; US3 Reverse, 5'-TTAAATAAATCGCAGACGGGC-3'.

Viral reactivation. Equal numbers of mock-infected and TB40/E-infected monocytes (see above) were placed in culture with monolayers of fibroblasts or human umbilical vein endothelial cells (HUVEC) (ATCC). Monolayers were monitored daily for cytopathic effect (CPE). Immunofluorescence was performed as previously described (16). Cell images were documented using an Olympus 1X70 microscope and analyzed using Q Capture Pro software (Media Cybernetics). Images were generated using Adobe Photoshop 7.0 (Adobe Systems, Inc.). At 100% CPE, cells were harvested and subjected to immunoblot analysis.

Flow cytometry. Flow cytometry was performed as previously described (16) with fluorophore-conjugated antibodies specific for CD14, CD33, CD163, CD169, major histocompatibility complex (MHC) class II, and CD1a (Beckman Coulter; BD-Pharmingen). Mean fluorescent intensity (MFI) was calculated from two or three independent experiments.

Multiplex ELISA. Cell supernatants were harvested and measured as part of a multiplex enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's protocol (Upstate/Millipore). Plates were read in a Luminex plate reader (Millipore), and data were analyzed using soft-

ware from Applied Cytometry Systems. Samples from three independent experiments were assayed in triplicate.

Microarray analysis. Total RNA isolated from mock-infected or TB40/E-infected monocytes was utilized for Illumina BeadArray analysis. Samples were processed by the Mount Sinai Genomics Core Facility (Institute for Genetics and Genomic Sciences) using the human HT-12 v4 expression BeadChip and scanned using the Illumina HiScanSQ system. The microarray data were processed by quantile normalization (17) using the Bioconductor lumi package (18). The normalized microarray data were transformed by the log₂ function and analyzed by using the linear model in the Bioconductor limma package (19). By comparing virus infection with the mock control, we found 348 upregulated genes and 221 downregulated genes that consistently showed at least 2-fold changes and a multiple-testing-adjusted *P* value of less than 0.05 for all time points postinfection, where the *P* values have been adjusted by false discovery rates. Hierarchical clustering was performed on these Euclidean distances with the agglomeration method “ward” (20, 21), resulting in a dendrogram that places samples with smaller Euclidean distances in neighboring positions. While the constraints imposed by the dendrogram were maintained, the samples were further reordered according to the average of the log₂-transformed gene expression measurements. After the samples and the genes were reordered, a heat map was generated for the microarray expression matrix. Heat map analysis was performed in the statistics programming language R (open source software at <http://www.r-project.org>) version 2.15.1 (22). The enriched pathways of the two gene lists were explored using the NIAID/NIH Database for Annotation, Visualization, and Integrated Discovery (DAVID) (23).

RESULTS

HCMV establishes short-term experimental latency in CD14⁺ monocytes. Latent viral genomes are detected in CD14⁺ monocytes following natural infection (24). Given their role in latency *in vivo*, the ease of their isolation and culture, and their abundance in peripheral blood, CD14⁺ monocytes represent an excellent model system to study HCMV latency and reactivation within the circulating myeloid lineage. To that end, we analyzed the short-term outcome of infection of CD14⁺ peripheral blood monocytes. A shorter time course of infection was chosen to mimic the life span of monocytes in circulation as well as to improve upon the extended culture of monocytes in previous latency models (12, 25). Additionally, this time frame would coincide with the established role of the infected monocyte in HCMV dissemination (26, 27). Cells were either mock infected or infected with HCMV TB40/E and monitored over a six-day time course for deposition of viral genomes, viral gene expression, and RNA transcription (Fig. 1). To begin, total DNA isolated from mock-infected or HCMV-infected monocytes and HCMV-infected fibroblasts (positive control) was subjected to nucleotide analysis for the UL123 (IE1) gene (Fig. 1A). IE1-specific amplicons were identified exclusively from HCMV-infected CD14⁺ monocytes (Fig. 1A, lanes 2, 4, and 6) and not mock-infected samples (Fig. 1A, lanes 1, 3, and 5). When qPCR was utilized, approximately 4 viral genomes per cell were detected (see Materials and Methods). Interestingly, this number did not increase over the time course, suggesting that monocytes maintained, but did not replicate, the viral genome. The results confirm that HCMV is able to initiate entry into and infection of CD14⁺ monocytes.

HCMV latency involves a repression of viral proteins associated with lytic replication in cells harboring latent genomes (5). To determine whether short-term infection of monocytes initiated production of viral proteins associated with the lytic life cycle, expression of the immediate early transactivator IE1 and the ma-

jor tegument protein pp65 was examined (Fig. 1B). A similar experiment was performed in fibroblasts to demonstrate protein expression during productive infection (Fig. 1C). TB40/E-infected CD14⁺ monocytes did not express IE1 proteins over the six-day time course (Fig. 1B, lanes 1 to 6). Virion-associated pp65 was observed at day 1 postinfection (Fig. 1B, lane 8), demonstrating entry of the virus and deposition of tegument proteins. However, newly synthesized pp65 did not accumulate during infection (Fig. 1B, lanes 9 to 12). In contrast, the same proteins associated with lytic infection were expressed kinetically in infected fibroblasts (Fig. 1C, lanes 2, 4, and 6 [IE1] and lanes 8, 10, and 12 [pp65]). The data demonstrate that infection of CD14⁺ monocytes by HCMV failed to initiate expression of particular viral genes associated with lytic infection.

Analysis of CD34⁺ HSCs and CD14⁺ monocytes from HCMV-seropositive carriers have identified viral RNAs associated with latency (5). A number of cytomegalovirus latency-associated transcripts (CLTs) have been identified and characterized: latency unique nuclear antigen (LUNA), the viral interleukin 10 homologue (vIL-10), UL138 transcripts, the chemokine receptor US28 (28–31), and, more recently, additional genes, including RNA2.7, RNA4.9, and UL95 (32). To determine if TB40/E-infected monocytes express known CLTs along with other viral transcripts, RNA was isolated over a six-day time course and subjected to reverse transcription-PCR (RT-PCR) with primers to various HCMV open reading frames (Fig. 1D). Consistent with previous CD14⁺-cell-based infection systems (33, 34), TB40/E-infected monocytes expressed two well-described CLTs, UL138 (Fig. 1D, lanes 1 to 8) and US28 (Fig. 1D, lanes 9 to 16), with sustained expression of each CLT throughout the time course. Contrary to previous latency systems, where a period of dysregulated gene expression occurs (10, 12, 32, 35), HCMV-infected monocytes failed to express the well-characterized IE1- and pp65-specific RNAs associated with lytic infection (Fig. 1D, lanes 33 to 40 and 41 to 48). Because IE1 (UL123)-specific and pp65 (UL83)-specific transcripts have been shown to be expressed at significantly high levels during productive infection (36), their absence suggests the establishment of experimental latency during the six-day time course of infection. Further analysis of TB40/E-infected monocytes was performed, as recent work has demonstrated HCMV to have a broader transcriptional profile during latency (32). Analysis of TB40/E-infected monocytes during short-term experimental latency identified the expression of RNA2.7 (Fig. 1D, lanes 17 to 24), a long noncoding RNA identified during long-term (18 days) infection of monocytes (32), and the noncoding transcripts RNA1.2 and RNA4.9 (data not shown). These findings are consistent with RNA sequencing data from TB40/E-infected monocytes (V. M. Noriega and D. Tortorella, unpublished data). Interestingly, the RL8A and RL9A transcripts, which are located downstream of RNA1.2, were not detected in TB40/E-infected monocytes (Fig. 1D, lanes 49 to 56; also data not shown), supporting the model of selective transcription of viral genes during latency. Strikingly, analysis of some well-characterized viral immune evasion genes (16) revealed that US3 (Fig. 1D, lanes 25 to 32), US2, UL111A (vIL-10), and UL32 (data not shown) were expressed exclusively by TB40/E-infected monocytes, while these latently infected monocytes tested negative for US11 (data not shown). The selective expression of the viral immune evasion genes may play a role in limiting T cell activation. The data validate the paradigm that HCMV latency is associated with a particular viral transcriptional

FIG 1 HCMV TB40/E establishes latency in CD14⁺ peripheral blood monocytes. (A) CD14⁺ monocytes that had been mock infected (M) or infected with HCMV TB40/E (V) were harvested at the indicated times postinfection and subjected to DNA isolation. Samples were used as a template for PCR amplification of HCMV UL123 (IE1) (lanes 1 to 6) and cellular β -actin (lanes 8 to 13) genes. DNA from TB40/E-infected fibroblasts was used as a positive control (MRC5; lanes 7 and 14). IE1 and β -actin-specific DNA fragments and relative DNA standards are indicated. Total cell lysates from CD14⁺ monocytes (B) and mock-infected (M) or TB40/E-infected (V) MRC5 fibroblasts (C) were subjected to SDS-PAGE and immunoblot analysis. (D) CD14⁺ monocytes that had been mock infected (M) or TB40/E infected (V) were harvested at the indicated times postinfection and subjected to RNA isolation. Samples were then reverse transcribed and used as the template in PCR with primers specific to the indicated viral genes. A sample lacking RNA template was included as an RT control [(-)RNA]. RNA isolated from TB40/E-infected fibroblasts was included as a positive control (MRC5). UL138, US28, RNA2.7, US3, IE1, pp65, RL8A, β -actin, and relative DNA standards are indicated. (E to G) CD14⁺ monocytes that had been mock infected (M) or TB40/E infected (V) were harvested at 1, 3, and 6 days postinfection and cocultured with MRC5 fibroblasts at a 1:1 ratio. Cell monolayers were allowed to reach 100% CPE (mean = 7 days) and then subjected to immunofluorescence microscopy (E) using antibody specific to IE1 antigen (P63-27) or harvested for immunoblot analysis (F). A similar reactivation experiment was performed with HUVEC (G) as the indicator cells.

profile. Furthermore, the data confirm that HCMV establishes experimental latency in CD14⁺ monocytes and demonstrate that a shorter time course of latent infection can be utilized to determine the impact of quiescent virus on cellular immune responses.

Myeloid-cell differentiation is crucial for reactivation in both experimental and natural latency (7, 13). Reactivation is likely mediated by the differentiation-dependent regulation of lytic genes essential for replication. Supernatants harvested from mock-infected and TB40/E-infected monocytes throughout the time course were noninfectious when titers were determined on permissive fibroblasts (data not shown), indicating that TB40/E-infected monocytes do not produce infectious progeny. Additionally, treatment of infected monocytes with IL-6, shown to induce reactivation in previous latency models (12, 34), did not initiate productive infection in our short-term latency system (Noriega and Tortorella, unpublished). While UL138 has been proposed to mediate reactivation by sensitizing cells to tumor necrosis factor alpha (TNF- α) (37), treatment with TNF- α alone did not induce reactivation in monocytes (Noriega and Tortorella, unpublished). Reactivation from latency *in vivo* most likely occurs due to undefined stimuli provided by extracellular factors and neighboring cells. Coculture of HCMV-infected HSCs with fibroblasts can induce reactivation from experimental latency in myeloid progenitors (10, 38). To stimulate reactivation of latently infected monocytes, cells were harvested during a six-day time course and cocultured with uninfected MRC5 monolayers. Cells were monitored for cytopathic effect (CPE), and coculture lysates were analyzed for expression of viral proteins (Fig. 1F). CPE was observed solely from fibroblasts cocultured with TB40/E-infected monocytes (see Fig. S1 in the supplemental material). Viral lytic genes of each transcriptional class were observed from TB40/E-infected monocyte/fibroblast cocultures (Fig. 1F, lanes 1 to 6, 7 to 12, and 13 to 18). Interestingly, TB40/E-infected monocytes cultured in Transwell plates with indicator fibroblasts did not result in CPE or transfer of virus (data not shown), suggesting that cell-cell contact may be necessary for reactivation from short-term latency. To confirm that fibroblasts were productively infected, viral IE1 expression was validated by fluorescence microscopy (Fig. 1E). Similarly, lytic gene expression was observed when latently infected monocytes were cocultured with endothelial cells (Fig. 1G, lanes 1 to 6 and 7 to 12). Supernatants taken from TB40/E-infected monocytes 24 h postinfection did not result in infection of fibroblasts (data not shown), excluding the possibility that remaining infectious particles were responsible for fibroblast infection in the coculture reactivation system. The data demonstrate that, in the presence of specific stimuli, monocytes can reactivate and disseminate HCMV following short-term latent infection. Taken together, the results strongly verify that CD14⁺ peripheral blood monocytes represent an excellent system to define the immune or inflammatory response during the establishment and maintenance of HCMV latency.

HCMV promotes the differentiation of CD14⁺ monocytes to a macrophage lineage during short-term latency. Myeloid progenitors undergo a differentiation program involving changes in surface markers during their maturation and trafficking from the bone marrow to the periphery (39). To determine if HCMV infection alters the physiology of short-term latently infected monocytes, the myeloid-progenitor marker CD33 and the classical monocytic marker CD14 were assessed (Fig. 2A). While both samples expressed comparable levels of CD33 on day 1 postinfection,

TB40/E-infected monocytes downregulated CD33 expression by days 3 and 6 (Fig. 2A, left). CD33 expression is downregulated with development of the myeloid lineage, resulting in low-level expression on peripheral granulocytes and tissue macrophages (40). This suggests that latently infected monocytes commit to a specific myeloid lineage. Remarkably, when CD14 expression was assessed, TB40/E-infected monocytes rapidly upregulated this marker compared to mock-infected cells (Fig. 2A, right). Monocyte-to-macrophage differentiation during inflammation can upregulate CD14 surface expression (41). The results suggest that latently infected monocytes commit early to a macrophage phenotype. Remarkably, similar results were found in a CD14⁺-cell-based system for dissemination (42, 43), validating our short-term model of latency.

To further define the surface composition of latently infected monocytes, a selection of macrophage surface markers were assessed: CD163, a member of the macrophage group B scavenger receptor cysteine-rich (SRCR) superfamily (44); major histocompatibility complex (MHC) class II molecules (45); and CD169 (SIGLEC-1), a macrophage sialoadhesion molecule (46). The most pronounced effect was observed with CD169 (Fig. 2B, center). CD169 surface expression was upregulated on latently infected monocytes in comparison to mock-infected samples throughout the time course (Fig. 2B, center). Interestingly, CD169 gene expression was identified as being upregulated in a GMP model of latency (47), demonstrating that physiological changes observed during long-term latency (2 weeks) are reflected in our CD14⁺-cell-based short-term model system. Furthermore, CD163 levels were consistently higher on TB40/E-infected monocytes than mock-infected samples (Fig. 2B, left). MHC class II molecules were increased on HCMV-infected monocytes on days 1 and 6 postinfection (Fig. 2B, right). An increase in HLA-DR protein levels was also found during long-term infection (2 weeks) of CD14⁺ monocytes by HCMV (48). Interestingly, infection with UV-irradiated TB40/E also caused upregulation of CD163 and CD169 (Fig. 2C), excluding a role for *de novo* viral latency transcripts as the trigger for upregulation of these surface molecules. This suggests that virus binding or tegument proteins may facilitate establishment of latency. A role for tegument protein pp71 in the initial events of HCMV latency has been proposed in a CD34⁺ experimental system (6). The results make it evident that skewing of monocyte-to-macrophage progression occurs during short-term HCMV latency.

Monocytes are a common precursor of macrophages and bone marrow-derived dendritic cells (mDCs) (49). To determine if latent HCMV manipulates differentiation of monocytes toward mDCs during short-term latency, cells were placed under DC culturing conditions immediately following infection. Cells were assessed 6 days postinfection for monocyte/macrophage (CD14) and mDC (CD1a) surface markers (Fig. 2D). Mock-infected monocytes downregulated surface CD14 and upregulated expression of CD1a, evidence of mDC differentiation (Fig. 2D, left). Remarkably, TB40/E-infected monocytes maintained expression of CD14 and failed to upregulate CD1a (Fig. 2D, right). HCMV infection inhibits DC differentiation in order to avoid host immune recognition (50), which may explain this result seen in TB40/E-infected monocytes. Alternately, the virus may preferentially inhibit differentiation to DCs to ensure that infected CD14⁺ monocytes enter tissue to become macrophages and reactivate virus, thus promoting dissemination in the host. Taken together,

FIG 2 Latent HCMV alters monocyte cell lineage commitment. CD14⁺ monocytes that had been mock infected or TB40/E infected were harvested at 1, 3, and 6 days postinfection and subjected to flow cytometry analysis using fluorophore-conjugated antibodies to CD33 and CD14 (A) or CD163, CD169, and MHC class II polypeptides (B). Data compiled from three independent experiments are presented as the change (fold) in normalized mean fluorescence intensity (MFI) relative to that of day 1 mock-infected cells. All error bars show standard deviations (SD). (C) CD14⁺ monocytes infected with TB40/E or UV-irradiated TB40/E (TB40/E^{UV}) were harvested 1 day postinfection and subjected to flow cytometry analysis using fluorophore-conjugated antibodies to CD14, CD163, and CD169. Gates represent the isotype control for each sample. (D) CD14⁺ monocytes that had been mock infected or TB40/E infected for 1 h at 37°C were placed into culture medium supplemented with 1,000 U/ml human IL-4 and 500 U/ml human GM-CSF for 6 days. Samples were subjected to flow cytometry analysis for CD14 and CD1a using the respective antibodies. Gates represent the isotype control for each sample.

the data demonstrate that short-term HCMV latent infection preferentially reprograms monocytes toward a macrophage lineage and limits their differentiation into DCs.

HCMV-infected CD14⁺ monocytes produce an inflammatory immune response during short-term latency. Productive HCMV infection induces genes involved in innate immune activation and inflammation (51). This induction occurs in the absence of virus replication and is triggered by recognition of glycoprotein B (gB) and viral double-strand DNA (dsDNA) (52, 53). HCMV infection of total PBMCs suggests that Toll-like receptor 2 (TLR2) recognizes virion components, triggering inflammatory

cytokine secretion (54). Interestingly, treatment of monocytes with a TLR2 ligand, LTA-SA, induced upregulation of CD14 and CD169 (see Fig. S2 in the supplemental material), a result observed following TB40/E infection (Fig. 2) but not infection with an RNA virus (NDV) or lipopolysaccharide (LPS) treatment (see Fig. S2 in the supplemental material). While activation of innate responses seems detrimental, increasing evidence suggests that inflammation may expedite HCMV replication and dissemination (55). Indeed, inflammatory cytokines facilitate mononuclear cell recruitment and migration into tissues (56), providing a pathway for dissemination. Therefore, is there an inflammatory secretome

FIG 3 HCMV alters the cytokine/chemokine profile of latently infected monocytes. Supernatants from CD14⁺ monocytes that had been mock-infected or TB40/E-infected were harvested at 1, 3, and 6 days postinfection and subjected to multiplex ELISA. Data are presented as changes in proinflammatory cytokines (A) and leukocyte chemoattractants (B and C). Supernatants from three independent experiments were sampled in triplicate. Error bars show SD. A similar experiment was performed where supernatants from CD14⁺ monocytes mock-infected or infected with TB40/E or UV-inactivated TB40/E (TB40/E^{UV}) were harvested at the indicated times postinfection and subjected to multiplex ELISA. Data are presented as changes in proinflammatory cytokines (D) and leukocyte chemoattractants (E). Supernatants from two independent experiments were sampled in triplicate. Error bars show SD.

associated with short-term HCMV latency in monocytes? To address this, supernatants from mock-infected or HCMV-infected cells were analyzed by multiplex ELISA (Fig. 3A to C; also, see Table S1 in the supplemental material). TB40/E-infected monocytes demonstrated selective secretion of the proinflammatory cytokines CXCL10, TNF- α , and IL-6 and minimal secretion of alpha interferon (IFN- α) (Fig. 3A). These findings expand our knowledge of the monocyte transcriptional profile following exposure to HCMV. Following binding and entry of HCMV (4 h postinfection), the virus stimulates a distinct proinflammatory transcriptome with polarization toward an M1 monocyte/macrophage (42, 57). Our results show that this proinflammatory environment is maintained throughout short-term latency and as long as 6 days postinfection. It was quite unexpected that latent virus could thrive in this inflammatory milieu, since many of these cytokines have the capacity to promote cellular immune responses. However, increasing evidence now links HCMV infection with the progression of inflammatory diseases, suggesting that the virus benefits from this microenvironment (58). In addition, latent HCMV caused marked secretion of cellular growth factors, including VEGF, G-CSF, and GM-CSF (see Table S1 in the supplemental material). In the host, these growth factors may be co-opted by the virus to communicate to neighboring cells or to promote cellular differentiation or proliferation. VEGF may be upregulated during

infection of kidney fibroblasts by US28 (59), a transcript present in our latency system. Interestingly, latently infected monocytes differentially secreted chemokines involved in leukocyte recruitment. CCL13 and CCL24 secretion was downregulated during short-term latency, perhaps providing an advantage to virus persistence due to their ability to recruit T lymphocytes (60, 61). In contrast, CCL2, CCL7, and CCL8 secretion was increased, possibly to attract additional monocytes to sites of latent infection. Interestingly, an increase in CCL2 and CCL8 secretion was observed in a CD34⁺-cell-based latency system, which led to an increase in CD4⁺ T-cell recruitment but also inhibited cytokine secretion and cytotoxicity by these responding cells (62). The data suggest that during latency, HCMV modulates cytokine/chemokine secretion for biased recruitment of immune cells to propagate latency in the host.

The detection of inflammatory cytokine secretion suggests that components of the HCMV virion and/or transcription of viral CLTs contribute to activation of immune responses. Upon recognition of pathogen-derived nucleic acids, hematopoietic cells, including macrophages and DCs, can produce type I IFN and inflammatory cytokines (63). To examine the role of viral transcription in the inflammatory response during short-term latency, the cytokine/chemokine profile of monocytes infected with TB40/E or UV-inactivated TB40/E was determined (Fig. 3D and

FIG 4 Genome-wide expression profiling of latently infected monocytes demonstrates activation of innate immune responses. RNA harvested at 1, 3, and 6 days postinfection from CD14⁺ monocytes that had been either mock infected or TB40/E infected was used for whole-genome profiling by Illumina BeadArray analysis. Microarray data were processed by quantile normalization and transformed by the $\log_2$ function prior to generation of a heat map expression matrix. Gene expression profiles were generated for 348 upregulated genes (A) and 221 downregulated genes (B). Samples were then further analyzed using the NIAID/NIH DAVID bioinformatics database. Results of pathway analysis for the top 31 upregulated genes and top 14 downregulated genes are shown in [Tables 1 and 2](#).

E). Though IFN- α was secreted at comparable levels after infection with TB40/E and UV-inactivated TB40/E, secretion of CXCL10 and TNF- α was drastically reduced with UV-inactivated virus ([Fig. 3D](#)), suggesting that the viral genome or a latency transcript potentiates proinflammatory cytokine secretion. Though CCL2 ([Fig. 3E](#)) and CCL3 (see Table S2 in the supplemental material) show comparable secretion with both virus samples, CCL8 ([Fig. 3E](#)) and CCL7 (see Table S2 in the supplemental material), which are both involved in recruitment of monocytes, were drastically reduced with UV-inactivated TB40/E. HCMV virion components may regulate secretion of CCL13 ([Fig. 3E](#)), CCL1, and CCL24 (see Table S2 in the supplemental material), as both live virus and UV-irradiated virus demonstrated limited secretion of these chemokines. The data demonstrate that the inflammatory response of short-term latently infected CD14⁺ monocytes may be differentially regulated by components of the HCMV virion or viral transcripts to possibly regulate latency and dissemination ([32](#)).

Latent HCMV triggers global modulation of gene expression in monocytes. Cytokine/chemokine secretion of CD14⁺ monocytes is dramatically altered by latent HCMV ([Fig. 3](#)). Are additional cellular processes modulated by the virus during experimental short-term latency? To address this question, whole-genome expression profiling was performed ([Fig. 4](#)). RNA transcripts prepared from mock-infected or TB40/E-infected monocytes were hybridized to array platforms representing >47,000 gene probe sets. Latent HCMV caused modulation of cellular transcription in monocytes, resulting in a myriad of upregulated ([Fig. 4A](#)) and downregulated ([Fig. 4B](#)) genes. Gene expression data were further analyzed to identify cellular pathways most highly impacted by HCMV latency ([Fig. 4](#); [Tables 1 and 2](#)). In accordance with the multiplex ELISA data, transcripts involved in the inflammatory response were highly upregulated by virus infection over the three-day time course ([Fig. 4A](#)). Interestingly, genes governing host antiviral responses and immune defense were also upregulated ([Fig. 4A](#)). Latent HCMV infection caused

enhanced expression of interferon-stimulated genes (ISGs), including Mx1, IRF7, and STAT1 ([Table 1](#)). This suggests that latent infection of monocytes triggers aspects of innate immune sensing. TB40/E-infection caused upregulation of inflammatory and chemotaxis factors, including CCL8, CXCL10, and CCL7 ([Table 1](#)). A more comprehensive list of the genes upregulated more than 2-fold (see [Tables S4 and S5](#) in the supplemental material) further demonstrates that innate immune factors, antigen presentation molecules, protein degradation factors, and apoptotic regulators were upregulated in the TB40/E-infected monocytes. The array data were quite consistent with the cytokine profile in that genes upregulated in the microarray analysis, such as CCL7 and CCL8, were also increased in the multiplex ELISA ([Fig. 3](#); also, see [Tables S1 and S2](#) in the supplemental material). Other genes, such as CCL1 and CCL17, did not change in either the microarray or multiplex ELISA, while CCL24 decreased in both types of analysis. Strikingly, CCL13, IL-15, and TRAIL were observed to increase only in the microarray analysis, suggesting that these genes may be regulated at the translational or posttranslational level (see [Tables S1 and S4](#) in the supplemental material). Remarkably, latent HCMV caused downregulation of genes involved in translation initiation (EIF3L), elongation (EEF1 β 2), and protein biosynthesis (RPL5 and RPL21) ([Table 2](#)). This may be a means to counteract the upregulation of the aforementioned antiviral mRNAs or perhaps a method to decrease the general metabolism of infected cells. Additionally, lipid biosynthesis genes (ALDH1A2 and LPL) were also downregulated ([Table 2](#)). This is in stark contrast to lytic infection, during which the virus induces lipogenesis ([64](#)). Analysis of all genes downregulated more than 2-fold (see [Tables S4 and S5](#)) revealed that a majority of genes could be categorized into protein translation or lipid metabolism factors. Collectively, the analysis of mRNA and protein secretion of virus-infected monocytes supports the paradigm that latent HCMV infection correlates with a unique innate immune signature and this response may be key to maintaining viral latency and preventing lytic viral replication.

TABLE 1 Examples of upregulated cellular genes during HCMV short-term latency

Biological process	Symbol	Gene annotation	Fold increase	Function
Antiviral defense	MX1	Myxovirus (influenza virus) resistance 1, interferon-inducible protein p78 (mouse), mRNA	55	GTPase
	IFIT1	Interferon-induced protein with tetratricopeptide repeats 1, transcript variant 2, mRNA	39	RNA binding
	IFIT3	Interferon-induced protein with tetratricopeptide repeats 3, mRNA	34	RNA binding
	ISG15	Interferon-stimulated gene, 15 kDa, mRNA	32	Ubiquitin-like modifier
	ISG20	Interferon-stimulated exonuclease gene, 20 kDa, mRNA	28	RNase
	OAS2	2'-5'-oligoadenylate synthetase 2, 69/71 kDa, transcript variant 1, mRNA	27	RNA synthetase
	IFIT2	Interferon-induced protein with tetratricopeptide repeats 2, mRNA	25	RNA binding
	MX2	Myxovirus (influenza virus) resistance 2 (mouse), mRNA	24	GTPase
	RSAD2	Radical S-adenosyl methionine domain containing 2, Cig5, viperin mRNA	22	Lipid synthesis
	OASL	2'-5'-oligoadenylate synthetase-like, transcript variant 2, mRNA	16	RNA synthetase
	IRF7	Interferon regulatory factor 7, transcript variant b, mRNA	15	Transcription factor
	STAT1	Signal transducer and activator of transcription 1, 91 kDa, transcript variant alpha, mRNA	14	Transcription factor
	OAS3	2'-5'-oligoadenylate synthetase 3, 100 kDa, mRNA	13	RNA synthetase
	OAS1	2',5'-oligoadenylate synthetase 1, 40/46 kDa, transcript variant 2, mRNA	10	RNA synthetase
	IFI35	Interferon-induced protein 35, mRNA	9	Transcription factor
	IFIH1	Interferon induced with helicase C domain 1, MDA5 mRNA	7	RNA helicase
Host immune response	INDO	Indoleamine-pyrrole 2,3 dioxygenase, mRNA	30	Amino acid degradation
	CFB	Complement factor B, mRNA	22	Protease
	SERPING1	Serpin peptidase inhibitor, clade G (C1 inhibitor), member 1, transcript variant 2, mRNA	18	Protease inhibitor
	SP110	SP110 nuclear body protein, transcript variant b, mRNA	6	Transcription factor
	PSMB9	Proteasome (prosome, macropain) subunit, beta type, 9 (large multifunctional peptidase 2), transcript variant 1, mRNA	5	Protease
	NCF1C	Neutrophil cytosolic factor 1C pseudogene, non-coding RNA	4	ROI production
	TAP2	Transporter 2, ATP-binding cassette, sub-family B (MDR/TAP), transcript variant 2, mRNA	3	Peptide transporter
Inflammatory response	CCL8	Chemokine (C-C motif) ligand 8, mRNA	111	Leukocyte trafficking
	CXCL10	Chemokine (C-X-C motif) ligand 10, mRNA	41	Leukocyte trafficking
	CCL7	Chemokine (C-C motif) ligand 7, mRNA	9	Leukocyte trafficking
	CCL13	Chemokine (C-C motif) ligand 13, mRNA	9	Leukocyte trafficking
	CCL19	Chemokine (C-C motif) ligand 19, mRNA	9	Leukocyte trafficking
	TNFSF10	Tumor necrosis factor (ligand) superfamily, member 10, TRAIL mRNA	9	Apoptosis
	CCL5	Chemokine (C-C motif) ligand 5, mRNA	6	Leukocyte trafficking
	IL18BP	Interleukin 18-binding protein, transcript variant A, mRNA	4	Immunosuppression
	CD48	CD48 molecule, mRNA	3	Lymphocyte activation

Cig5, cytomegalovirus-inducible gene 5; MDA5, melanoma differentiation-associated protein 5; PKR, protein kinase, RNA associated; ROI, radical oxygen intermediate; TRAIL, TNF-related apoptosis-inducing ligand; viperin, virus inhibitory protein, endoplasmic reticulum associated, interferon inducible.

Latent HCMV infection alters the ability of monocytes to respond to innate immune triggers. Trafficking of monocytes from the peripheral blood to sites of infection is critical for control and clearance of pathogens. Following recruitment, monocytes function to promote immune defense by inducing production of type I interferon and proinflammatory cytokines (65). To determine if latently infected monocytes can mount responses against known triggers of innate immunity, cells that had been either mock infected or TB40/E infected were challenged with Newcastle disease virus (NDV), LPS, or IFN- β (Fig. 5). Supernatants were collected following treatment and analyzed by multiplex ELISA. The proinflammatory cytokine IL-1 α was marginally secreted by mock-

infected and TB40/E-infected monocytes incubated with NDV and IFN- β yet was highly upregulated by uninfected cells in response to LPS (Fig. 5), while this was not true for virus-infected cells. The cytokines CXCL10 and CCL8 were produced to significant levels by mock-infected monocytes in response to NDV, IFN- β , and LPS, yet TB40/E-infected cells failed to produce both CXCL10 and CCL8 after secondary challenge (Fig. 5). While the chemokine CX3CL1 was secreted at low levels by both mock-infected and TB40/E-infected monocytes, only secondary challenge with LPS caused increased production of CX3CL1 in virus-infected cells (Fig. 5), whereas mock-infected cells secreted CX3CL1 in response to all treatments. Interestingly, a trend of

TABLE 2 Examples of downregulated cellular genes during HCMV short-term latency

Biological process	Symbol	Gene annotation	Fold decrease	Function
Lipid biosynthesis	CH25H	Cholesterol 25-hydroxylase, mRNA	5	Hydrolase
	PPARG	Peroxisome proliferator-activated receptor gamma, transcript variant 2, mRNA	5	Transcription factor
	LPL	Lipoprotein lipase, mRNA	4	Lipase
	LTA4H	Leukotriene A4 hydrolase, mRNA	4	Peptidase
Cellular metabolism	ALDH1A2	Aldehyde dehydrogenase 1 family, member A2, transcript variant 3, mRNA	10	Dehydrogenase
	TACSTD2	Tumor-associated calcium signal transducer 2, mRNA	9	Cell surface receptor
	CST6	Cystatin E/M, mRNA	7	Cysteine protease
	CCND2	Cyclin D2, mRNA	5	Kinase complex
	BCAT1	Branched chain aminotransferase 1, cytosolic, mRNA	4	Transaminase
Protein synthesis	RPL21	Ribosomal protein L21, mRNA	3	60S ribosome subunit
	TTC3	Tetratricopeptide repeat domain 3, transcript variant 1, mRNA	3	Ubiquitin ligase
	EEF1β2	Eukaryotic translation elongation factor 1 beta 2, transcript variant 1, mRNA	2	Guanine exchange factor
	EIF3L	Eukaryotic translation initiation factor 3, subunit L, mRNA	2	Translation preinitiation complex
	RPL5	Ribosomal protein L5, mRNA	2	60S ribosome subunit

protein secretion by TB40/E-infected monocytes in response to LPS challenge alone was found for several cytokines, including IL-10 and CCL4 (Fig. 5) as well as IL-6, CXCL1, CCL3, CCL4, and TNF-α (see Table S3 in the supplemental material). For each of the aforementioned cytokines, latently infected cells appeared to respond robustly to LPS treatment but not IFN-β or NDV challenge (Fig. 5). The results demonstrate that latently infected monocytes respond selectively to triggers of innate immunity. This implies that latent HCMV alters the cellular function of the monocyte to benefit carriage of the virus and inhibit premature reactivation of the virus and/or immune detection. Most importantly, TB40/E-infected monocytes failed to upregulate IFN-α (see Table S3 in the supplemental material) in response to second-

ary challenge, suggesting that latent virus alters the ability of the cell to exert immunoprotective responses.

Latent HCMV modulates interferon signaling in CD14⁺ monocytes. Infection of monocytes by HCMV presents a significant biological challenge to the virus. Monocytes are major contributors to the innate antiviral immune response, functioning in phagocytosis, antigen presentation, and cytokine production (65). In response to pathogens, monocytes can become activated and differentiate to macrophages or dendritic cells, which are both potent stimulators of T-cell-mediated immunity and the activation of which would be unfavorable to the virus. Infection of monocytes by HCMV induces expression of cellular transcripts associated with antiviral responses (Table 1), yet latently infected monocytes secrete minimal amounts of IFN-α (Fig. 3A) and respond aberrantly to secondary challenge with type I IFN or virus infection (Fig. 5). This suggests that during short-term latency, HCMV can manipulate antiviral signaling for its benefit. The ability of HCMV to counteract the interferon response during productive infection has been well documented (66). Our results indicate that latent virus also has the ability to modulate type I IFN activity. To determine what level of IFN signaling is targeted by latent HCMV, monocytes that had been either mock infected or TB40/E infected were treated at day 3 postinfection with IFN-β and harvested for analysis (Fig. 6). Both mock-infected and TB40/E-infected monocytes expressed comparable levels of the cell surface type I IFN receptor (IFNAR1/2) following IFN-β treatment (data not shown). Therefore, we turned our attention to the classical Janus kinase/STAT (Jak/STAT) signaling pathway, which is activated downstream of the IFN receptor. JAK1 protein expression and phosphorylation were unaffected by latent HCMV infection (data not shown). However, when STAT1 phosphorylation was assessed following IFN-β treatment, TB40/E-infected monocytes demonstrated reduced phosphorylation of STAT1 in comparison to mock-infected monocytes (Fig. 6A, lanes 1 to 4). Surprisingly, total STAT1 levels remained comparable between mock-infected and TB40/E-infected monocytes (Fig. 6A, lanes 5 to 8), despite the fact that TB40/E infection caused upregulation of mRNA for STAT1 (Table 1). This suggests that, in addition to

FIG 5 Latent HCMV alters monocyte responses to activators of innate immunity. CD14⁺ monocytes that had been either mock infected or TB40/E infected were cultured for 5 days and then either left untreated (Mock) or subjected to secondary challenge with Newcastle disease virus (NDV; MOI = 1), IFN-β (100 U/ml), or LPS (100 ng/ml) in fresh medium. The following day, supernatants were collected and subjected to multiplex ELISA.

FIG 6 Latent HCMV restricts interferon signaling at the level of STAT1 phosphorylation. (A) CD14⁺ monocytes that had been mock infected or TB40/E infected were treated at day 3 postinfection with 1,000 U/ml of IFN- β for 30 min and then harvested for immunoblot analysis. P-STAT1 and P-STAT2, phosphorylated STAT1 and STAT2. (B) CD14⁺ monocytes that had been mock infected or TB40/E infected were treated at day 3 postinfection with 1,000 U/ml of IFN- γ for 30 min and then harvested for immunoblot analysis. (C) Levels of phosphorylated STAT1 versus total STAT1 were quantified by densitometry for results in both panels A and B.

decreasing its phosphorylation, HCMV may exert translational control of STAT1 message, perhaps through downregulation of protein biosynthesis pathways (Table 2). When quantified, virus-infected monocytes demonstrated a 2-fold decrease in STAT1 phosphorylation in comparison to mock-infected cells (Fig. 6C). STAT2 phosphorylation was unaffected in latently infected monocytes (Fig. 6A, lanes 9 to 12), demonstrating the specificity of the blockade in type I IFN signaling during short-term latency. A similar 2-fold inhibition of STAT1 phosphorylation was observed when TB40/E-infected monocytes were challenged with type II interferon (IFN- γ) (Fig. 6B, lanes 1 to 4, and 6C), implying a selective inhibition of this signaling molecule. This broad inhibition of STAT1 phosphorylation may represent an immune evasion mechanism employed by HCMV during latent infection as a means to antagonize innate immunity. Taken together, the results establish the modulation of both type I and II interferon signaling during short-term experimental latency in CD14⁺ monocytes.

DISCUSSION

Latency permits HCMV to persist indefinitely within the host, a strategy that contributes to its success as a human pathogen. Here we describe a robust short-term *in vitro* latency system utilizing human peripheral blood monocytes to examine the immunological circumstances surrounding HCMV latency in the host. In this model system, the virus enters cells, and traditional lytic genes are immediately silenced (Fig. 1B and D). During latency, viral ge-

nomes were maintained (Fig. 1A), and transcripts associated with latency were observed (Fig. 1D). This is quite revolutionary considering that prior experimental latency models utilized systems in which transient IE gene expression is observed (12, 67) or long-term culture of infected cells is required to achieve latency (12, 38). The immediacy of latency *in vitro* correlated with numerous physiological changes to the infected monocyte, including the selective expression of inflammatory factors and modulation of innate immune responses. Latent virus induced the upregulation of cellular and immune factors consistent with monocyte differentiation and migration (Fig. 2 to 4). Furthermore, HCMV could be reactivated upon monocyte coculture with permissive fibroblasts and endothelial cells (Fig. 1E to G). Collectively, the data demonstrate that our system represents an authentic short-term setting for studying the immunological events of viral latency *in vitro*.

The data support a paradigm for HCMV latency highlighting the role of the peripheral blood monocyte in carriage and dissemination of virus as well as in manipulation of host immune responses during latency (Fig. 7). Primary HCMV infection most likely initiates with lytic replication in the mucosal epithelium. We propose that infection spreads to circulating monocytes, where HCMV can establish latent infection. Latently infected monocytes upregulate macrophage surface markers and secrete inflammatory cytokines and monocyte chemoattractants. This in turn may allow the dissemination of virus throughout the host and into tissue where the HCMV-infected monocytes would reactivate

FIG 7 Model of HCMV latency, reactivation, and dissemination. Following localized replication, HCMV encounters circulating CD14⁺ monocytes in the periphery of the host. Upon binding and entry of the virus, tegument proteins most likely aid in remodeling the cell for latent carriage of the virus. During this time, the DNA genome is maintained with limited transcription of viral genes. Latently infected monocytes undergo a differentiation process involving upregulation of cell surface macrophage markers and secretion of inflammatory cytokines and monocyte chemoattractants. This is concomitant with the activation and modulation of monocyte-mediated innate immune responses by latent virus. These short-lived monocytes enter tissue and receive the correct signal for reactivation of latent virus, thus becoming productively infected macrophages that can then disseminate virus to monocytes responding to inflammatory cues, potentiating latency in the host.

from latency. Viral reactivation probably requires multiple signals received from neighboring cells to ensure that HCMV replication occurs in a cell type appropriate for viral propagation. Our reactivation data do not exclude the possibility that viral genomes are directly transferred to uninfected cells, thereby providing a strategy to disseminate virus in the absence of robust viral replication and assembly. This suggests a “safe haven” for the virus within the monocyte until a specific set of stimuli trigger the transport of the viral genome. Interestingly, our experimental latency model would provide an excellent system to examine this very point.

Cytomegalovirus latency in monocytes correlates with selective expression of cellular and innate immune factors. Notably, latently infected monocytes begin a differentiation process toward the macrophage lineage (Fig. 2) likely initiated by recognition of virions by TLR2 (see Fig. S2 in the supplemental material). Latency drives the differentiation of monocytes toward macrophages, but this was insufficient for complete reactivation, as the lytic gene pp65, a major component of the mature virion, was not observed (Fig. 1B). Tegument proteins may aid in remodeling monocytes for latency, as infection with UV-irradiated TB40/E also upregulated macrophage surface markers and induced unique inflammatory responses (Fig. 2C and 3). Interestingly, both TLR2 antagonism and UV-treated TB40/E caused increased cell death (data not shown), while TB40/E-infected monocytes remained viable throughout the time course, supporting data in-

dicating that HCMV may modulate prosurvival pathways of traditionally short-lived monocytes (68). The downregulation of cellular processes, including protein and lipid biosynthesis (Table 2), further supports the paradigm that HCMV modulates the physiology of monocytes during short-term latency. Latent HCMV may perhaps alter processes involved in protein translation as a means to inhibit expression of viral lytic genes. The downregulation of particular genes suggests that associated pathways are key processes to establish and maintain HCMV latency. Interestingly, a number of genes that were upregulated by TB40/E infection of monocytes did not result in a concomitant increase of protein levels. Although infection of monocytes caused an increase in mRNA for STAT1, the levels of total STAT1 protein remained the same between mock-infected and TB40/E-infected cells (Fig. 6A and B). A similar result was found for CCL13, IL-15, and TNFSF10 (TRAIL) (Table 1), with virus infection causing an increase in mRNA which did not translate to an increase in secretion of these proteins (Fig. 3C; also, see Table S1 in the supplemental material). Secretion of these proteins may be detrimental to viral latency; therefore, the virus selectively inhibits these factors through modulation of transcription and/or translation. The identification of viral and cellular genes, including those encoding inflammatory factors, which govern the establishment of latency and the switch to lytic replication will provide major insight into the cellular basis of HCMV latency and reactivation.

Quiescent viral genomes or latency-associated transcripts trigger the release of inflammatory factors from infected monocytes during short-term experimental latency (Fig. 3). Whether infected monocytes recognize pathogen-associated patterns, inducing inflammation, or latency transcripts hijack cellular cytokine/chemokine secretion pathways remains to be elucidated. Regardless, the virus thrives despite, or perhaps because of, these early immune responses (Fig. 3; Table 1). Thus, what is the significance of activating an innate immune response during latency? A plausible model is the recruitment of circulating monocytes to potentiate latency in the host. In fact, viral dissemination may utilize specialized CX3CR1^{hi} monocytes that are attracted to sites of infection (69). Alternatively, secreted inflammatory factors may be beneficial to the establishment of latency by creating an intracellular antiviral state, thus limiting lytic replication. Additionally, inflammatory cytokine secretion may contribute to the resistance of latently infected cells to selective extracellular stimuli (Fig. 5) and aid the virus in evading elimination by the immune system. Remarkably, the proinflammatory milieu alone could not induce reactivation or lytic gene expression (Fig. 1B), implying that multiple signals likely trigger reactivation. Interestingly, pretreatment of cells with IFN- β did not inhibit infection of monocytes or establishment of latency, as viral genomes could be detected 1 day postinfection following overnight IFN- β treatment (see Fig. S3 in the supplemental material). Exogenous application of IL-6, TNF- α , IFN- β , or the immunosuppressive compound estrogen or prednisone could not reactivate latent virus in monocytes (Noriega and Tortorella, unpublished). The natural course of lytic HCMV infection triggers cellular immune responses that the virus is able to circumvent (70). The cellular processes governing induction of both innate and inflammatory immune responses most likely represent additional pathways co-opted by HCMV during infection of monocytes to establish persistence, although this remains to be analyzed in other latency systems.

Understanding the molecular dynamics by which HCMV establishes, maintains, and reactivates from latency will enable the development of regimens to target *in vivo* reservoirs of virus. Here we report the establishment and characterization of experimental short-term latency in CD14⁺ monocytes. Our system possesses advantages over prior latency models, including the availability of monocytes and the speed with which the virus enters latency. The large numbers of monocytes that can be harvested and infected, coupled with high infectivity rates (~70%) (Fig. 2C), can allow global analysis of HCMV latency through use of current technologies such as deep sequencing. Of paramount importance are the immunological findings that latent HCMV remodels monocytes to generate macrophages that can reactivate virus following cues from neighboring cells, that latently infected monocytes promote inflammation, leading to myeloid-cell recruitment and enhancement of latency, that latency is associated with a global modulation of cellular processes, including innate immune sensing, and that latent HCMV can inhibit aspects of interferon signaling and modulate the cellular antiviral state. This model system represents a significant advancement in the field of herpesvirus latency and an excellent tool to examine aspects of immune control during HCMV latency and reactivation.

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