Human Cytomegalovirus Stimulates Monocyte-to-Macrophage Differentiation via the Temporal Regulation of Caspase 3

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Monocytes are primary targets for human cytomegalovirus (HCMV) infection and are proposed to be responsible for hematogenous dissemination of the virus. Biologically, monocytes have a short life span of 48 h in the circulation, a period of time during which monocytes must make a cell fate decision on whether to undergo apoptosis or differentiate into a macrophage. We have previously shown that HCMV infection stimulates monocyte-to-macrophage differentiation; however, the mechanism(s) by which HCMV-infected monocytes simultaneously navigate the 48-h “viability gate” and undergo macrophagic differentiation has remained elusive. Studies have demonstrated that the level of caspase 3 and 8 activities in monocytes may mediate the delicate balance between apoptosis and macrophage colony-stimulating factor (M-CSF)-induced myeloid differentiation. Here, we show that HCMV infection, unlike M-CSF treatment, does not induce caspase 8 activity to promote myeloid differentiation. However, HCMV infection does induce a temporal activation of caspase 3, with only a low level of active caspase 3 being observed after the 48-h viability checkpoint. Consistent with the role of a time-dependent activation of caspase 3 in promoting myeloid differentiation, the inhibition of caspase 3 blocked HCMV-induced monocyte-to-macrophage differentiation. Temporal transcriptome and functional analyses identified heat shock protein 27 (HSP27) and Mcl-1, two known regulators of caspase 3 activation, as being upregulated prior to the 48-h viability gate following HCMV infection. Using small interfering RNAs (siRNAs), we demonstrate that HCMV targets the rapid induction of HSP27 and Mcl-1, which cooperatively function to precisely control caspase 3 activity in order to allow for HCMV-infected monocytes to successfully traverse the 48-h cell fate decision checkpoint and commence macrophage maturation. Overall, this study highlights a unique regulatory mechanism employed by HCMV to tightly modulate the caspase 3 activity needed to promote myeloid differentiation, a key process in the viral dissemination and persistence strategy.

Human cytomegalovirus (HCMV), a betaherpesvirus, is endemic throughout the world, with seropositivity reaching 50 to 80% among urban populations in the United States (14). HCMV infection is generally asymptomatic in immunocompetent individuals, although HCMV is now believed to be a primary viral candidate in the etiology of several diseases, including atherosclerosis, inflammatory bowel disease, and glioblastoma multiforme (9, 33, 42). In immunocompromised individuals such as neonates, AIDS patients, and transplant recipients, HCMV infection can lead to multiorgan disease resulting in significant morbidity and mortality (18, 27, 41). The organ disease associated with HCMV infection is a direct consequence of the systemic viral spread to and infection of multiple organ sites that occur during either asymptomatic or symptomatic infections; this process is necessary for the establishment of viral persistence within the infected host (1, 29, 48).

HCMV infection is characterized by a monocyte-associated viremia prior to the onset of viral pathogenesis, suggesting that HCMV may utilize these blood sentinels as vehicles to mediate hematogenous dissemination of the virus to various organ sites (26, 44). In support, monocytes are the primary cell type infected in the blood during acute HCMV infection (44) and are the predominant infiltrating cell type found in infected organs (2, 32). However, although monocytes are “at the right place, at the right time,” these cells have a short life span of approximately 2 days (47) and are not permissive for viral replication (19, 35, 37). To resolve this biological quandary, we have recently shown that HCMV infection stimulates differentiation of short-lived, viral-replication-nonpermissive monocytes into long-lived, viral-repli-

cation-permissive macrophages, making HCMV, to our knowledge, the only identified viral pathogen that can directly induce the monocyte-to-macrophage differentiation process (4, 37). Global transcriptome and functional analyses demonstrated a unique polarization of the differentiating HCMV-infected monocytes toward an M1 proinflammatory phenotype expressing select M2 anti-inflammatory characteristics (3). The unique nature of the HCMV induction of monocyte differentiation indicates that the virus may have evolved a distinct mechanism to modulate the myeloid differentiation process. For the virus, a highly evolved and controlled monocyte-to-macrophage differentiation process would likely allow for an appropriate M1 polarization, which promotes maturation into long-lived macrophages, while concurrently limiting the antiviral activities associated with infection and differentiation.

The rapid initiation of monocyte-to-macrophage differentiation programming following HCMV infection happens prior to viral gene expression, which occurs at 2 to 3 weeks postinfection,

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indicating the involvement of receptor-ligand interactions in the differentiation process (4, 37). Indeed, monocytes challenged with UV-inactivated HCMV or treated with purified glycoprotein B exhibited similar functional changes in a temporal time frame consistent with that of replication-competent virus (37, 49). We recently showed that, in accord with a direct role of viral binding in monocyte activation, activation of the cellular epidermal growth factor receptor (EGFR) and integrins ($\beta 1$ and $\beta 3$) during viral entry was required for many of the unique functional changes seen following HCMV infection (8, 30). We further demonstrated that the subsequent and simultaneous activation of NF- κ B and phosphatidylinositol 3-kinase [PI(3)K] signaling cascades following receptor engagement is responsible for the atypical M1/M2 polarization of monocytes (4). However, the downstream machinery that is regulated by this distinctive HCMV-induced signalosome and that directly modulates the virus-specific myeloid differentiation reprogramming remains unclear.

The short life span of blood monocytes forces these immune surveillance cells to make a cell fate decision on whether to undergo apoptosis or differentiate into long-lived macrophages; thus, in order for monocyte differentiation to progress, they must survive to and through a 48-h viability checkpoint (7, 16, 47). Two distinct viability strategies occur prior to and after this 48-h checkpoint, where different Bcl-2 family members appear to act as temporal “viability gates” along the myelopoiesis differentiation continuum (7, 17, 23, 31). We have previously demonstrated that HCMV inhibits the early activation of caspases via the upregulation of Mcl-1, a member of the Bcl-2 protein family, during the initial stages of infection, allowing for HCMV-infected monocytes to effectively navigate the 48-h viability checkpoint (7). Although caspases are known executioners of apoptosis (10), caspases 3 and 8 also exhibit nonapoptotic functions, including the controlled regulation of myeloid differentiation (12, 20, 40). Inhibition of caspase 3 and 8 activities impedes monocyte differentiation into macrophages following macrophage colony-stimulating factor (M-CSF) stimulation (25, 40). Monocytes undergoing macrophagic differentiation induced by M-CSF treatment do not display caspase 3 and 8 activity until 72 h posttreatment, indicating that the controlled temporal release of caspase activity after the 48-h viability gate is critical to the progression of the monocyte-to-macrophage differentiation process (20). Because our data document that HCMV-infected monocytes successfully traverse the 48-h cell fate decision checkpoint and differentiate into macrophages (7), we have now focused on determining whether caspases are central cogs in the differentiation machinery modulated by HCMV in order to orchestrate the survival and differentiation of monocytes following infection.

Here, we report that, in contrast to that resulting from M-CSF treatment, HCMV-induced monocyte-to-macrophage differentiation specifically utilizes caspase 3, but not caspase 8, activity. Pro-caspase 3 undergoes two proteolytic cleavage steps necessary for the formation of fully active caspase 3, and examination of caspase 3 cleavage following HCMV infection revealed time-dependent activation, where fully active caspase 3 was not observed until after the 48-h viability gate. We further demonstrated that, while M-CSF inhibits the initial cleavage of pro-caspase 3, HCMV inhibited all cleavage steps prior to the 48-h checkpoint, indicating that the mechanism by which HCMV temporally regulates caspase 3 is distinct from that of M-CSF. Global transcriptome and functional analyses identified Mcl-1 and heat shock protein

27 (HSP27) as being key regulators in the time-dependent activation of caspase 3 by HCMV. Taken together, these results provide evidence that HCMV has evolved a unique mechanism to hijack specific components of the monocyte-to-macrophage differentiation machinery in order to drive differentiation of infected monocytes into the precise cell type that can promote viral dissemination and lifelong persistence within the infected host.

MATERIALS AND METHODS

Virus preparation and infection. HCMV (Towne/E strain; passages 35 to 45) was cultured as previously described in human embryonic lung (HEL) fibroblasts (49). Virus was purified on a 0.5 M sucrose cushion, resuspended in RPMI 1640 medium (Cellgro; Mediatech, Herndon, VA), and used to infect monocytes at a multiplicity of infection (MOI) of 5 for each experiment (37, 38, 49). We found that ~100% of monocytes were infected with Towne/E when an MOI of 5 was used. Mock infection was performed by adding an equivalent volume of RPMI 1640 medium (Cellgro) to monocytes.

Human peripheral blood monocyte isolation and differentiation. University Institutional Review Board and Health Insurance Portability and Accountability Act guidelines were followed for all experimental protocols. Blood was drawn by venipuncture and centrifuged through a Ficoll Histopaque 1077 gradient (Sigma, St. Louis, MO). Mononuclear cells were collected and washed with saline (37, 38, 49). Monocytes were then isolated by centrifugation through a Percoll (Pharmacia, Piscataway, NJ) gradient (50). More than 95% of isolated peripheral blood mononuclear cells were monocytes as determined by CD14-positive staining (data not shown). Cells were washed and suspended in RPMI 1640 (Cellgro) supplemented with 1% human AB serum (Sigma; all experiments were performed in 1% human serum, unless otherwise stated). Macrophage differentiation was induced by infection with Towne/E (MOI, 5) or treatment with 100 ng/ml M-CSF (BD Biosciences, San Jose, CA) and visualized with a Nikon Eclipse TE300 inverted microscope equipped with a Photometrics CoolSNAP_{fx} camera. The average size (arbitrary units) of 50 monocytes for each experimental arm was determined from the captured images using ImageJ software. Results are plotted as means $\pm$ standard deviations (SD). Student's *t* tests were performed, and a *P* value of <0.05 was used as the measure of statistical significance between samples.

Western blot analysis. Monocytes (2×10^6) were harvested in radio-immunoprecipitation assay (RIPA) buffer. Equal amounts of total protein from each sample were added to each well, separated by SDS-polyacrylamide gel electrophoresis, and transferred to polyvinylidene difluoride membranes (Bio-Rad, Hercules, CA). Blots were incubated with an anti-caspase 3 antibody (Calbiochem, Darmstadt, Germany), an anti-caspase 8 antibody (Santa Cruz Biotechnology, Santa Cruz, CA), an anti-HSP27 antibody (Santa Cruz Biotechnology), an anti-Mcl-1 antibody (Santa Cruz Biotechnology), or an anti-actin antibody (Santa Cruz Biotechnology). Blots were then incubated with a horseradish peroxidase (HRP)-conjugated secondary antibody (Santa Cruz Biotechnology) and detected using the ECL Plus protocol (Amersham Pharmacia Biotech, Piscataway, NJ). Results are representative of 3 independent experiments using samples from different donors.

Immunofluorescence. Monocytes plated on fibronectin-coated glass coverslips were infected with Towne/E. Following infection, cells were fixed with 2% paraformaldehyde (Fisher Scientific, Pittsburgh, PA), permeabilized with 0.2% Triton X-100 (Fisher Scientific), and incubated in blocking solution (phosphate-buffered saline [PBS] plus 5% bovine serum albumin [BSA] [Fisher Scientific]), 5% goat serum [Zymed, San Francisco, CA], and human FcR block [Miltenyi Biotec, Auburn, CA]). Indirect immunofluorescence to detect HSP27 localization was performed by incubation with a primary anti-HSP27 antibody (Santa Cruz Biotechnology), followed by incubation with a secondary Alexa Fluor 488-conjugated antibody (Molecular Probes, Carlsbad, CA). The coverslips were mounted in Slow Fade Gold (Molecular Probes), and images were captured using a TCS S5P confocal microscope (Leica Microsystems,

Buffalo Grove, IL). Results are representative of 3 independent experiments using samples from different donors.

Transient transfection and RNA silencing. Monocytes (3×10^6) were resuspended in 100 μ l of room temperature nucleofection solution (human monocyte Nucleofector kit; Amaxa Biosystems, Cologne, Germany) containing 3 μ g Mcl-1 small interfering RNA (siRNA) (Invitrogen, Carlsbad, CA), 3 μ g HSP27 siRNA (Sigma), and 3 μ g control siRNA (Invitrogen) and transfected with an Amaxa Nucleofector electroporator using program Y-001. Following transfection, cells were incubated in RPMI 1640 (Cellgro) supplemented with 1% human AB serum at 37°C (Sigma).

Flow cytometry. Annexin V staining was performed on live monocytes. Cells were incubated in blocking solution (PBS plus 5% BSA [Fisher Scientific], 5% goat serum [Zymed], and human FcR block [Miltenyi Biotec]), followed by staining with anti-CD14-allophycocyanin (APC)-Cy7 (BD Biosciences). Fluorescein isothiocyanate (FITC)-annexin V apoptosis detection kit 1 (BD Biosciences) was used to stain for annexin V and phosphatidylinositol (PI). After antibody staining, the cells were analyzed by flow cytometry using a FACSCalibur system (BD Biosciences). Our gating strategy on forward scatter (FSC)/side scatter (SSC) was set to include cells in the early stages of apoptosis (decreased FSC and increased SSC compared to viable cells) and cells in the late stages of apoptosis (decreased FSC and SSC compared to viable cells).

Monocyte-to-macrophage differentiation staining was done on monocytes fixed in 2% paraformaldehyde at room temperature for 10 min. Cells were incubated in blocking solution (PBS plus 5% BSA [Fisher Scientific], 5% goat serum [Zymed], and human FcR block [Miltenyi Biotec]), followed by staining with anti-CD71-APC (BD Biosciences) and anti-CD14-APC-Cy7 (BD Biosciences) in blocking solution. Labeled monocytes were then analyzed by flow cytometry using a FACSCalibur system (BD Biosciences).

Microarray data accession number. The GEO accession number for the Affymetrix microarray data is GSE11408.

RESULTS

Caspase activity is required for the induction of monocyte-to-macrophage differentiation following HCMV infection. We have previously shown that HCMV infection is able to initiate the monocyte-to-macrophage differentiation process, which is critical to the viral dissemination strategy since, unlike monocytes, macrophages are permissive for viral replication (4, 37). Because several studies have provided evidence that caspases are essential to M-CSF-induced differentiation of monocytes (12, 20, 40), we investigated if caspase activity is also essential in mediating HCMV-induced macrophagic differentiation. Primary peripheral blood monocytes were infected with HCMV in the presence or absence of a general caspase inhibitor z-VAD-fmk and examined for morphological changes associated with differentiation 3 days postinfection. In accord with our previous studies (4, 37), HCMV-infected monocytes exhibited morphological differentiation as demonstrated by increases in granularity, cell spreading, and size (Fig. 1A), as well as increased expression of macrophage cell surface markers CD71 (transferrin receptor) and CD163 (haptoglobin-hemoglobin scavenger receptor) (see Fig. S1 in the supplemental material). Inhibition of caspase activity with z-VAD-fmk treatment abrogated HCMV-induced cellular differentiation of infected monocytes, which was confirmed by quantitative analysis of cell size (Fig. 1B) and by flow cytometric analysis examining the frequency of CD71⁺ monocytes (Fig. 1C). These results show that HCMV targets caspase activity in order to directly regulate the myeloid differentiation process.

HCMV targets caspase 3, but not caspase 8, activity to induce macrophagic differentiation. Caspases 3 and 8 have been shown

FIG 1 HCMV induces monocyte-to-macrophage differentiation in a caspase-dependent manner. (A, B, C) Peripheral blood monocytes were given treatment with or without general caspase inhibitor z-VAD-fmk for 1 h. Following treatment, cells were mock infected or HCMV infected (MOI, 5) for 72 h (A and B) or 96 h (C). (A) Images representative of 3 independent experiments from different blood donors. (B) Monocyte surface area was determined with Scion Image software using photomicrographs at 72 hpi. (C) At 96 hpi, monocytes were stained with APC-CD71 and analyzed by flow cytometry. Asterisks indicate significant differences ($P < 0.05$) between the indicated control samples and the infected sample.

to regulate monocyte-to-macrophage differentiation following treatment with M-CSF (25, 40). Consequently, we next focused on the contributions of caspases 3 and 8 to the HCMV-induced differentiation process. During M-CSF-induced differentiation, the activation of caspase 8 after the 48-h viability gate is central for the progression of differentiation (20) (see Fig. S2 in the supplemental material). However, in contrast to M-CSF treatment, HCMV in-

FIG 2 Caspase 8 is not involved in the HCMV-induced myeloid differentiation process. (A) Peripheral blood monocytes were mock infected or HCMV infected (MOI, 5) for 1 day (D1), 2 days (D2), or 3 days (D3), after which whole-cell lysates were harvested. Pro-caspase 8 (55 kDa) and cleaved caspase 8 (18 kDa) were detected by immunoblotting. Membranes were re probed for β -actin as a loading control. (B, C) Monocytes were given treatment with or without the caspase 8-specific inhibitor z-IETD-fmk for 1 h. Cells were then mock infected or HCMV infected (MOI, 5) for 72 h, after which photomicrographs were taken (B) and monocyte surface area was determined with Scion Image software using photomicrographs at 72 hpi (C; asterisks indicate significant differences ($P < 0.05$) from results of control treated monocytes). (A, B, C) Data are representative of 3 independent experiments with samples from different blood donors.

fection appears to inhibit the proteolytic cleavage of pro-caspase 8 (55-kDa form) into the active cleaved form of caspase 8 (18-kDa form) for the first 3 days postinfection (Fig. 2A; see Fig. S2 in the supplemental material), suggesting only a limited role for caspase 8 in mediating HCMV-induced differentiation, at least at the early stages of the process. Indeed, treatment with z-IETD-fmk, a caspase 8-specific inhibitor, prior to infection with HCMV had no effect on the morphological differentiation of infected monocytes (Fig. 2B and C). We confirmed that z-IETD-fmk treatment

blocked M-CSF-induced differentiation (see Fig. S3 in the supplemental material), indicating that the induction of monocyte-to-macrophage differentiation by HCMV does not require caspase 8 and that different molecular players control different differentiation events. It should be pointed out that, over the course of multiple experiments with different donors, inhibition of caspase 8 activation following HCMV infection was not 100% at day 3 (D3) for each donor (Fig. 2A); however, treatment with z-IETD-fmk did not affect HCMV-induced myeloid differentiation in any of the experiments. Thus, our data suggest that caspase 8 activity must reach a certain threshold level to mediate monocyte-to-macrophage maturation and that the minimal levels of caspase 8 activity in HCMV-infected monocytes are not sufficient to induce differentiation.

In contrast to its effect on caspase 8 activation, HCMV infection of monocytes induced a time-dependent change in the activation state of caspase 3 (Fig. 3A). Pro-caspase 3 (32 kDa) is proteolytically cleaved into a 20-kDa intermediary protease with partial activity, which then undergoes a second autocatalytic cleavage event to generate the fully active 17-kDa protease (15). Freshly isolated monocytes abundantly expressed the 32-kDa pro-caspase 3 and the 20-kDa intermediary caspase but did not express the fully active 17-kDa caspase 3 (Fig. 3A). At D1 and D2 after mock infection, the 20-kDa caspase 3 was readily cleaved into the 17-kDa protease in mock-infected monocytes, which is likely due to the rapid decline in Mcl-1 protein expression (7), and thus cleavage acts as a molecular clock (controlling the 48-h viability gate) to ensure the short life span of these cells. In HCMV-infected monocytes, we observed the accumulation of the 32-kDa pro-caspase 3 and the 20-kDa intermediary caspase 3 and the absence of the 17-kDa caspase 3 (prior to 72 hours postinfection [hpi]). These results indicate that HCMV can inhibit both caspase 3 cleavage steps, ensuring the minimal formation of fully active caspase 3 prior to the 48-h viability gate. M-CSF treatment also prevented the formation of the 17-kDa caspase 3 prior to the monocyte cell fate decision checkpoint; however, there was only a single complete blockade in the first cleavage step of pro-caspase 3, suggesting a different mechanism of caspase 3 regulation following M-CSF treatment (Fig. 3B). After the 48-h viability gate, HCMV-infected and M-CSF-treated monocytes expressed the 17-kDa caspase 3 (Fig. 3A and B). To assess the role of this temporal activation of caspase 3 in the viral induction of monocyte-to-macrophage differentiation, we first utilized caspase 3-specific inhibitor z-DEVD-fmk, which inhibits the formation of fully active caspase 3 (see Fig. S4 in the supplemental material) without affecting HCMV binding or entry into monocytes (data not shown). The presence of z-DEVD-fmk abrogated the morphological differentiation of HCMV-infected and M-CSF-treated monocytes, confirming that the full activation of caspase 3 after the 48-h viability checkpoint is necessary for the induction of macrophage differentiation following HCMV infection and M-CSF treatment (Fig. 3C, D; see Fig. S5 in the supplemental material). Taken together, these data indicate that HCMV regulates caspase 3 activity to mediate myeloid differentiation and that the regulatory mechanisms of caspase 3 activation during HCMV-induced differentiation are distinct from that of M-CSF-induced differentiation.

HCMV induces the transient upregulation and the long-term cytoplasmic expression of HSP27. The ability of HCMV to rapidly block the secondary cleavage of the 20-kDa intermediary caspase 3 into the 17-kDa fully active caspase 3 prior to the 48-h

FIG 3 HCMV temporally modulates caspase 3 activation in order to regulate macrophagic differentiation following infection. (A, B) Peripheral blood monocytes were mock infected, HCMV infected (MOI, 5), or M-CSF treated for 1 day (D1), 2 days (D2), or 3 days (D3), after which whole-cell lysates were harvested. Pro-caspase 3 (32 kDa) and cleaved caspase 3 (20 kDa and 17 kDa) were detected by immunoblotting. (C) Monocytes were given treatment with or without the caspase 3-specific inhibitor z-DEVD-fmk for 1 h. Following treatment, cells were mock infected or HCMV infected (MOI, 5) for 72 h and photomicrographs were taken. (D) Monocyte surface area was determined with Scion Image software using photomicrographs at 72 hpi. The asterisk indicates a significant difference ($P < 0.05$) from results for the various control treated monocytes. (A, B, C, D) Data are representative of 3 independent experiments with samples from different blood donors.

FIG 4 HCMV specifically targets the upregulation of HSP27 in monocytes during the early stages of infection. (A) Monocytes were mock infected or HCMV infected (MOI, 5) for 24 h. Following infection, Affymetrix microarray analysis was performed to determine fold changes in gene expression (8). The asterisk indicates a significant difference from results for mock-infected, untreated monocytes. (B) Monocytes were mock infected or HCMV infected (MOI, 5) for 1 day (D1), 2 days (D2), or 3 days (D3), after which whole-cell lysates were harvested. Total HSP27 was detected by immunoblotting. Membranes were reprobed for β -actin as a loading control. Data are representative of 3 independent experiments with samples from different blood donors.

viability gate suggests that the viral regulation of caspase 3 is a key control mechanism in the HCMV-mediated induction of myeloid differentiation. To begin to decipher how HCMV prevents the cleavage of the 20-kDa caspase 3, we examined our previous transcriptome analysis for the upregulation of transcripts known to encode proteins that inhibit the autoproteolytic activation of caspase 3 at 24 hpi (8). Members of the inhibitor of apoptosis (IAP) family, including cIAP1, cIAP2, XIAP, and survivin, have been shown to inhibit apoptosis through direct interactions with the active sites of caspases, such that conversion into fully active proteases is abrogated (11, 34). We did not detect the presence of XIAP and survivin in infected monocytes, nor did we detect any change in cIAP1 and cIAP2 mRNA levels following infection, suggesting that IAPs have at best a limited role in the early blockage of the cleavage of the 20-kDa caspase 3 into the 17-kDa caspase 3 (Fig. 4A). Members of the heat shock proteins (HSPs) are also key regulators of cell death and survival (21), and, similar to the IAPs, HSP27 has been demonstrated to directly bind to caspase 3 to prevent autoproteolytic processing into the fully active 17-kDa isoform (46). Our transcriptome analysis showed a 5-fold increase in HSP27 gene transcript levels at 24 hpi (Fig. 4A), which was confirmed at the protein level by Western blot analysis (Fig. 4B). The upregulation of HSP27 expression was transient, peaking at 24 hpi and returning to mock-infection levels by 72 hpi (Fig. 4B). Furthermore, using confocal microscopy we showed that HCMV infection prevented the nuclear localization of HSP27 for at least 3 days postinfection (Fig. 5), a biological event that is necessary for HSP27 to mediate its function and inhibit the autoproteolytic cleavage of caspase 3 (46). These results indicate that HCMV has a two-pronged approach to regulate HSP27 function: (i) to induce

FIG 5 HCMV infection inhibits nuclear localization of HSP27. Monocytes were mock infected or HCMV infected (MOI, 5) for 1 day (D1), 2 days (D2), or 3 days (D3). Following infection, cells were fixed and immunofluorescence staining was performed to examine HSP27 cellular localization. Blue expression represents nuclei stained by DAPI (4',6-diamidino-2-phenylindole), and green expression represents HSP27 staining/localization. Results are representative of 3 independent experiments with samples from different donors.

the rapid upregulation of HSP27 protein expression during the early stages of infection and (ii) to facilitate the long-term cytoplasmic expression of HSP27.

HCMV targets the upregulation of HSP27 and Mcl-1 to cooperatively regulate caspase 3 activity during the early stages of monocyte infection. Our previous studies have demonstrated that Mcl-1 acts as a rapidly inducible, short-term effector of cell viability targeted by HCMV to allow infected monocytes to survive past the 48-h viability gate (7). Unlike HSP27, which blocks the second autoproteolytic cleavage of caspase 3, Mcl-1 has been

FIG 6 HCMV-induced Mcl-1 and HSP27 work in concert to rapidly prevent the two-step activation cleavage of caspase 3 following infection. Monocytes were transfected using nucleofection (nucleofected) with control siRNA, Mcl-1 siRNA, HSP27 siRNA, or Mcl-1 and HSP27 siRNAs. Immediately following transfection, cells were mock infected or HCMV infected (MOI, 5) for 48 h. Mcl-1 and HSP27 (A) or pro-caspase 3 (32-kDa) and cleaved caspase 3 (20-kDa and 17-kDa) (B) were then detected by immunoblotting. Panels A and B show results from the same experiment. Membranes were reprobbed for β -actin as a loading control. Results are representative of 3 independent experiments using samples from different donors.

reported to block cytochrome *c* release from the mitochondria, which in turn results in the inhibition of the initial cleavage of pro-caspase 3 (45). Because protein expression of both HSP27 (Fig. 4B) and Mcl-1 (7) is transiently upregulated during the early stages of infection, we proposed that HCMV utilizes HSP27 and Mcl-1 to cooperatively block the activation of caspase 3 prior to the 48-h cell fate decision checkpoint in infected monocytes. To investigate the role of HSP27 and Mcl-1 in regulating the early survival of HCMV-infected monocytes, we utilized a siRNA knockdown approach that achieved a $\geq 90\%$ knockdown of both proteins in mock-infected and HCMV-infected monocytes 48 hpi (Fig. 6A). Knockdown of HSP27, Mcl-1, or both in mock-infected monocytes resulted in the cleavage of pro-caspase 3 and the formation of the fully active 17-kDa caspase 3; the loss of Mcl-1 appears to trigger a more robust induction of caspase 3 activation (Fig. 6A). Little or none of the intermediate 20-kDa form of caspase 3 was observed. Consistent with the different levels of caspase 3 activation following knockdown of HSP27 and Mcl-1, Mcl-1-deficient monocytes exhibited a higher frequency of apoptosis than HSP27-deficient cells (see Fig. S6 in the supplemental material). In addition, cells deficient in both HSP27 and Mcl-1 displayed no difference in the rates of cell death compared to monocytes transfected with Mcl-1 siRNA alone, suggesting that Mcl-1 is the major player regulating “normal” monocyte viability (in the absence of infection). In contrast to results for mock-infected monocytes, knockdown of HSP27 did not induce the formation of the 17-kDa caspase 3 in HCMV-infected monocytes

(Fig. 6B). Furthermore, although knockdown of Mcl-1 resulted in the cleavage of the 32-kDa pro-caspase 3 in infected cells, we observed only a limited cleavage of the 20-kDa intermediary caspase 3 into the fully active 17-kDa caspase 3. Only infected monocytes deficient for both HSP27 and Mcl-1 exhibited a robust activation of caspase 3, indicating that in infected cells, but not in mock-infected cells, HSP27 and Mcl-1 work in concert to regulate caspase 3 cleavage/activation.

We next examined the apoptotic rates the HCMV-infected monocytes following transfection with siRNAs and found that HSP27 knockdown did not affect the levels of apoptosis, while Mcl-1 knockdown and HSP27/Mcl-1 knockdown induced 1.8-fold and 2.4-fold increases in apoptosis, respectively (Fig. 7). In contrast, uninfected Mcl-1- and HSP27-deficient monocytes exhibited levels of apoptosis similar to those of uninfected Mcl-1-deficient monocytes (see Fig. S6 in the supplemental material), indicating that HSP27 enhances the ability of Mcl-1 to inhibit cell death only in HCMV-infected cells. These data imply that HCMV may induce HSP27 to function as an additional safeguard to ensure that any pro-caspase 3 cleaved during infection would be prevented from reaching an active caspase 3 state. It should also be pointed out that a low-level induction of apoptosis in mock-transfected/HCMV-infected monocytes, compared to that in mock-transfected/mock-infected cells, was observed (Fig. 7A and B). This slight induction of cell death may be a result of an overactivation of monocytes from the double stimuli (nucleofection and HCMV infection), which is known to induce apoptosis of myeloid cells (22). In accord, HCMV infection lowers the rate of apoptosis in nontransfected cells (7), and transfection of mock-infected cells did not affect the rate of apoptosis (see Fig. S6 in the supplemental material). We also found that knockdown of Mcl-1 and HSP27 consistently resulted in a 2- to 3-fold increase in apoptosis of HCMV-infected monocytes, which, in contrast to that of mock-infected cells, did not increase over time (data not shown). Since Bcl-2 and heat shock protein family members are also known to directly mediate differentiation of several cell types (17, 21), we examined whether Mcl-1 and HSP27 affect the differentiation of HCMV-infected cells not undergoing apoptosis following Mcl-1 and HSP27 depletion. Indeed, we found that reduced levels of Mcl-1 and HSP27 inhibited myeloid differentiation of HCMV-infected monocytes, as determined by the decrease in CD71 surface expression (Fig. 7G). Together, these data suggest that Mcl-1 and HSP27 play a dual role in directly regulating apoptosis and differentiation through the “fine-tuning” of caspase 3 activity.

DISCUSSION

The ability of HCMV to directly initiate monocyte-to-macrophage differentiation is critical for its dissemination and persistence strategy, since, unlike monocytes, macrophages are permissive for viral replication (13, 19, 37). We have previously shown that HCMV drives monocyte differentiation toward a proinflammatory M1 phenotype (3), which we argue provides infected monocytes with the necessary cellular and molecular tools to mediate viral spread by promoting motility and transendothelial migration into surrounding organ tissue (6, 37–39). An in depth analysis of the differentiation outcome of HCMV-infected monocytes revealed an atypical M1 polarization that exhibited decreased expression of certain antiviral M1 cytokines and increased levels of anti-inflammatory M2 cytokines (3). This selective modulation of M1- and M2-associated factors suggests that HCMV

FIG 7 HCMV-induced Mcl-1 and HSP27 cooperatively function to inhibit apoptosis and drive myeloid differentiation during the early stages of infection. Monocytes were nucleofected with no siRNA (A, B), control siRNA (C), Mcl-1 siRNA (D), HSP27 siRNA (E), or Mcl-1 and HSP27 siRNAs (F). Immediately following transfection, cells were mock infected (A) or HCMV infected (B, C, D, E, F) (MOI, 5) for 48 h. Following infection, cells were stained with FITC-annexin V and PI to detect apoptotic cells and flow cytometric analysis was performed. (G) Monocytes were nucleofected with no siRNA (red), control siRNA (blue), or Mcl-1 and HSP27 siRNAs (green). Cells were then infected with HCMV (MOI, 5). At 48 hpi, infected monocytes were stained with APC-CD71 and analyzed by flow cytometry. (A to G) Results are representative of 3 independent experiments from different donors.

has evolved a strategy to stimulate unique monocyte differentiation programming in order to balance viral spread with immune evasion. Indeed, we have demonstrated that the initiation of a distinct viral ligand/cellular receptor-mediated signalsome during viral entry is responsible for the unique characteristics associated

with macrophagic differentiation following HCMV infection (4, 5, 8, 30). In the current study, we expand our understanding of the molecular mechanisms by which HCMV induces a distinctive monocyte-to-macrophage differentiation process by examining how HCMV modulates the cellular differentiation machinery and balances this modulation with that of the survival machinery.

Several studies have shown that, despite being executioners of apoptosis (10), caspases 3 and 8 also play necessary roles in mediating myeloid differentiation (25, 40). The inhibition of either caspase using pharmacological inhibitors blocked M-CSF-induced macrophagic differentiation. In contrast, our studies determined that only caspase 3, but not caspase 8, is required for HCMV-driven macrophage maturation, suggesting that caspase 3 is a dominant player in the virus-induced differentiation process. The reasons for this disparity in the requirement for caspase 8 during M-CSF- and HCMV-induced differentiation remain unclear, but perhaps caspase 8 activity is detrimental to the viral life cycle. Unlike M-CSF, HCMV is a proapoptotic insult to the cell; thus, infection can result in the release of antiviral proapoptotic cytokines (3, 49). Because caspase 8 mediates the initiation of the extrinsic apoptosis pathway (10), via the activation of death receptors at the cell surface, the release of proapoptotic cytokines could induce an overactivation of caspase 8 leading to the induction of cell death of the infected monocyte. In the face of proapoptotic signaling from death receptors, the shutdown of caspase 8 activity by HCMV likely enhances infected-monocyte survival. On the other hand, caspase 3 is an effector caspase activated by either the extrinsic pathway (via caspase 8) or the intrinsic pathway (via mitochondrial depolarization) (10) and is tightly regulated by members of the Bcl-2 family of proteins (45). We have previously shown that HCMV controls expression of the antiapoptotic members of the Bcl-2 family (7). Thus, HCMV may have evolved a strategy to selectively mediate myeloid differentiation through the specific control of a single caspase (caspase 3) that can be fine-tuned by Bcl-2 proteins, a process not possible with caspase 8.

Our data indicate that, aside from utilizing antiapoptotic Bcl-2 proteins to block the initial cleavage of pro-caspase 3, HCMV possesses an additional regulatory mechanism to control caspase 3 activation by inhibiting the second autoproteolytic cleavage step. Certain IAPs have been shown to inhibit apoptosis via direct binding with caspase 3 to prevent autocatalytic maturation (11) and are upregulated upon M-CSF-induced myeloid differentiation (51), yet transcriptome analysis indicates that HCMV does not upregulate this family of proteins. IAPs are multifunctional proteins and have been shown to be involved in the initiation of innate antiviral signaling (24); the lack of detectable upregulation of IAPs following HCMV infection may imply a potentially deleterious effect on the virus. Similar to IAPs, HSP27 also directly interacts with caspase 3 to inhibit cleavage (46) but has not been shown to directly possess antiviral activity. In fact, several viruses, including adenovirus (43), respiratory syncytial virus (36), and herpes simplex virus 1 (HSV-1) (28), target HSP27 activity to enhance viral replication directly through many of its nonapoptotic functions; however, further studies are needed to determine if HSP27 plays a direct role in promoting HCMV replication during myeloid differentiation. Nonetheless, our data demonstrate that HCMV targets the caspase 3 regulatory function of HSP27 during the early stages of infection to promote survival of the infected monocyte through the 48-h viability gate.

The rapid and transient upregulation of HSP27 protein expres-

sion following HCMV infection of monocytes is different from the kinetics observed following M-CSF treatment, where a significant increase in HSP27 levels is not observed until 3 days posttreatment and where levels continue to increase thereafter (46). We suggest two possibilities for this kinetic difference in expression: (i) in contrast to M-CSF, HCMV does not effectively block the cleavage of pro-caspase 3 and (ii) unlike M-CSF, which only needs to inhibit the intrinsic biological programming of monocytes to activate the caspase cascade upon entry into the circulation, HCMV must also subvert the proapoptotic signaling initiated during the antiviral response. Thus, HCMV may induce the rapid upregulation of HSP27 expression to ensure that the intermediary caspase 3 is not cleaved into the fully active form. Together with our previous data demonstrating the central role of Mcl-1 in regulating the molecular clock of infected monocytes (7), our data here indicate that Mcl-1 and HSP27 work in concert to regulate caspase 3 activity prior to the 48-h viability gate.

The cooperative effort of Mcl-1 and HSP27 to block caspase 3 activation during the first 48 h following HCMV infection suggests that monocytes become hypersensitive to the proapoptotic effects of caspase 3. In support, knockdown of Mcl-1 in HCMV-infected monocytes, which resulted in low basal levels of fully active caspase 3 similar to that seen in mock-infected cells, significantly increased the frequency of apoptosis, indicating that the cellular apoptotic microenvironment of monocytes is dramatically altered following infection. Our previous global transcriptome studies showed the upregulation of several proapoptotic proteins following HCMV infection, suggesting an increase in the proapoptotic cellular state (3, 5, 7), which is likely part of the antiviral response. However, our studies have also shown that HCMV stimulates the upregulation of a vast array of cellular antiapoptotic proteins to rapidly counteract the progression of the antiviral proapoptotic events (7). These data suggest that a disturbance in the HCMV-induced antiapoptotic “brake” could tip the delicate balance between survival and death signals within the HCMV-induced differentiating monocyte toward apoptosis.

Our study also showed that Mcl-1 and HSP27 not only function to allow for the survival of HCMV-induced differentiating monocytes but also directly promote the progression of monocyte-to-macrophage differentiation following HCMV infection. Despite >90% knockdown of Mcl-1 and HSP27 protein expression, 30 to 70% (donor dependent) of HCMV-infected monocytes remained viable, and these percentages, unlike the percentages of viable mock-infected cells, did not decrease with time. Studies have demonstrated that members belonging to the Bcl-2 and heat shock protein families can directly stimulate the differentiation of several cell types (17, 21). In accord, we found that Mcl-1 and HSP27 depletion blocked HCMV-induced differentiation. It remains unclear what determines whether Mcl-1 and HSP27 regulate apoptosis or differentiation, but the precise level of caspase 3 activity may be a deciding factor. Perhaps, a minimal threshold level of caspase 3 is required to facilitate myeloid differentiation and exceeding this threshold drives the differentiating monocyte into a quiescent state, where, if caspase 3 activity continues to increase, the induction of apoptosis is then initiated. Regardless of the mechanism, our data indicate that Mcl-1 and HSP27 play an active role in stimulating monocyte-to-macrophage differentiation following HCMV infection.

Our work here begins to decipher how HCMV concurrently allows infected monocytes to navigate the 48-h viability check-

FIG 8 A potential model depicting the mechanism by which HCMV temporally regulates caspase 3 activation in order to direct monocyte-to-macrophage differentiation. Peripheral blood monocytes are biologically programmed to undergo cell death within 48 h of entering the circulation from the bone marrow. Upon HCMV infection, antiapoptotic regulators Mcl-1 and HSP27 are rapidly upregulated to prevent the formation of fully activated caspase 3 prior to the 48-h viability gate. Once HCMV-infected monocytes have proceeded through the 48-h viability checkpoint, Mcl-1 and HSP27 return to basal levels. HCMV is able to continue to partially inhibit the first cleavage step of pro-caspase 3 by an unknown mechanism but is unable to inhibit the second autocatalytic cleavage step of caspase 3, resulting in low-level activation. We argue that the presence of low levels of fully activated caspase 3 after the 48-h viability gate is necessary for mediating HCMV-induced monocyte-to-macrophage differentiation. Overall, HCMV appears to have evolved a unique regulatory mechanism to temporally control caspase 3 activation in order to ensure the simultaneous induction of monocyte-to-macrophage survival and differentiation, necessary steps for the progression of viral dissemination and the establishment of lifelong viral persistence within the infected host.

point and to undergo monocyte-to-macrophage differentiation. Specifically, we demonstrated that HCMV temporally regulates caspase 3 activation such that fully active caspase 3 is not observed until after 48 hpi. Although caspase 3 is regulated in a similar time-dependent manner during M-CSF-induced differentiation, our data indicated that the mechanism of regulation by HCMV is distinct from that observed with known macrophage differentiation agents. In HCMV-infected monocytes, prior to the 48-h viability gate, the coordinated action of HSP27 and Mcl-1 prevents the sequential activation cleavage steps for caspase 3, ensuring that the presence of fully activated caspase 3 is minimized until differentiating monocytes have survived to and through the cell fate decision checkpoint (see model presented in Fig. 8). After the 48-h viability gate, HCMV blocks the first cleavage event for pro-caspase 3 by an unknown means; however, because the blockage is not 100%, the low numbers of pro-caspase 3 proteins that do undergo the first cleavage step will become fully activated. This resulting low-level activation appears to be required for HCMV-induced macrophagic differentiation. Overall, the study begins to highlight the complex molecular interplay between survival and differentiation that a pathogen may have to manipulate. Specifically, our data showing that inhibition of caspase 3 after 48 hpi leads to a complete inhibition of monocyte-to-macrophage differentiation but allows for survival of the HCMV-infected monocyte, while the overactivation of caspase 3 leads to cell death, demonstrate the complex nature of the caspase 3 control mechanism utilized by HCMV in order to negotiate the delicate line between myeloid apoptosis and maturation. By elucidating the precise mechanism of how HCMV uniquely modulates the monocyte dif-

ferentiation machinery to promote viral dissemination and persistence, we may provide insight into novel cellular therapeutic targets against HCMV-infected monocytes, where replication does not occur until weeks postinfection.

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REFERENCES

1. Bissinger AL, Sinzger C, Kaiserling E, Jahn G. 2002. Human cytomegalovirus as a direct pathogen: correlation of multiorgan involvement and cell distribution with clinical and pathological findings in a case of congenital inclusion disease. *J. Med. Virol.* 67:200–206.
2. Booss J, Dann PR, Griffith BP, Kim JH. 1989. Host defense response to cytomegalovirus in the central nervous system. Predominance of the monocyte. *Am. J. Pathol.* 134:71–78.
3. Chan G, Bivins-Smith ER, Smith MS, Yurochko AD. 2008. Transcriptome analysis reveals human cytomegalovirus reprograms monocyte differentiation toward an M1 macrophage. *J. Immunol.* 181:698–711.
4. Chan G, Bivins-Smith ER, Smith MS, Yurochko AD. 2009. NF- κ B and phosphatidylinositol 3-kinase activity mediates the HCMV-induced atypical M1/M2 polarization of monocytes. *Virus Res.* 144:329–333.
5. Chan G, Bivins-Smith ER, Smith MS, Yurochko AD. 2008. Transcriptome analysis of NF- κ B- and phosphatidylinositol 3-kinase-regulated genes in human cytomegalovirus-infected monocytes. *J. Virol.* 82:1040–1046.
6. Chan G, Hemmings DG, Yurochko AD, Guilbert LJ. 2002. Human cytomegalovirus-caused damage to placental trophoblasts mediated by immediate-early gene-induced tumor necrosis factor- α . *Am. J. Pathol.* 161:1371–1381.
7. Chan G, et al. 2010. PI(3)K-dependent upregulation of Mcl-1 by human cytomegalovirus is mediated by epidermal growth factor receptor and inhibits apoptosis in short-lived monocytes. *J. Immunol.* 184:3213–3222.
8. Chan G, Nogalski MT, Yurochko AD. 2009. Activation of EGFR on monocytes is required for human cytomegalovirus entry and mediates cellular motility. *Proc. Natl. Acad. Sci. U. S. A.* 106:22369–22374.
9. Cobbs CS, et al. 2002. Human cytomegalovirus infection and expression in human malignant glioma. *Cancer Res.* 62:3347–3350.
10. Cohen GM. 1997. Caspases: the executioners of apoptosis. *Biochem. J.* 326(Pt 1):1–16.
11. Deveraux QL, et al. 1998. IAPs block apoptotic events induced by caspase-8 and cytochrome c by direct inhibition of distinct caspases. *EMBO J.* 17:2215–2223.
12. Droin N, et al. 2008. A role for caspases in the differentiation of erythroid cells and macrophages. *Biochimie* 90:416–422.
13. Fish KN, Depto AS, Moses AV, Britt W, Nelson JA. 1995. Growth kinetics of human cytomegalovirus are altered in monocyte-derived macrophages. *J. Virol.* 69:3737–3743.
14. Fowler KB, Pass RF. 1991. Sexually transmitted diseases in mothers of neonates with congenital cytomegalovirus infection. *J. Infect. Dis.* 164:259–264.
15. Han Z, Hendrickson EA, Bremner TA, Wyche JH. 1997. A sequential two-step mechanism for the production of the mature p17:p12 form of caspase-3 in vitro. *J. Biol. Chem.* 272:13432–13436.
16. Hashimoto SI, Komuro I, Yamada M, Akagawa KS. 2001. IL-10 inhibits granulocyte-macrophage colony-stimulating factor-dependent human monocyte survival at the early stage of the culture and inhibits the generation of macrophages. *J. Immunol.* 167:3619–3625.
17. Haughn L, Hawley RG, Morrison DK, von Boehmer H, Hockenbery DM. 2003. BCL-2 and BCL-XL restrict lineage choice during hematopoietic differentiation. *J. Biol. Chem.* 278:25158–25165.
18. Ho M. 1977. Virus infections after transplantation in man. Brief review. *Arch. Virol.* 55:1–24.
19. Ibanez CE, Schrier R, Ghazal P, Wiley C, Nelson JA. 1991. Human cytomegalovirus productively infects primary differentiated macrophages. *J. Virol.* 65:6581–6588.
20. Jacquelin A, et al. 2009. Colony-stimulating factor-1-induced oscillations

- in phosphatidylinositol-3 kinase/AKT are required for caspase activation in monocytes undergoing differentiation into macrophages. *Blood* 114: 3633–3641.
21. Lanneau D, de Thonel A, Maurel S, Didelot C, Garrido C. 2007. Apoptosis versus cell differentiation: role of heat shock proteins HSP90, HSP70 and HSP27. *Prion* 1:53–60.
 22. Liu B, et al. 2001. Molecular consequences of activated microglia in the brain: overactivation induces apoptosis. *J. Neurochem.* 77:182–189.
 23. Liu H, et al. 2006. Regulation of Mcl-1 expression in rheumatoid arthritis synovial macrophages. *Arthritis Rheum.* 54:3174–3181.
 24. Lopez J, Meier P. 2010. To fight or die—inhibitor of apoptosis proteins at the crossroad of innate immunity and death. *Curr. Opin. Cell Biol.* 22: 872–881.
 25. Maelfait J, Beyaert R. 2008. Non-apoptotic functions of caspase-8. *Biochem. Pharmacol.* 76:1365–1373.
 26. Manez R, et al. 1996. Time to detection of cytomegalovirus (CMV) DNA in blood leukocytes is a predictor for the development of CMV disease in CMV-seronegative recipients of allografts from CMV-seropositive donors following liver transplantation. *J. Infect. Dis.* 173:1072–1076.
 27. Masur H, Whitcup SM, Cartwright C, Polis M, Nussenblatt R. 1996. Advances in the management of AIDS-related cytomegalovirus retinitis. *Ann. Intern. Med.* 125:126–136.
 28. Mathew SS, Della Selva MP, Burch AD. 2009. Modification and reorganization of the cytoprotective cellular chaperone Hsp27 during herpes simplex virus type 1 infection. *J. Virol.* 83:9304–9312.
 29. Mocarski ES, Shenk T, Pass RF. 2007. Cytomegaloviruses, p 2701–2772. *In* Knipe DM, Howley PM (ed), *Fields virology*, vol 2. Lippincott Williams & Wilkins, Philadelphia, PA.
 30. Nogalski MT, Chan G, Stevenson EV, Gray S, Yurochko AD. 2011. Human cytomegalovirus-regulated paxillin in monocytes links cellular pathogenic motility to the process of viral entry. *J. Virol.* 85:1360–1369.
 31. Opferman JT, et al. 2005. Obligate role of anti-apoptotic Mcl-1 in the survival of hematopoietic stem cells. *Science* 307:1101–1104.
 32. Pulliam L. 1991. Cytomegalovirus preferentially infects a monocyte derived macrophage/microglial cell in human brain cultures: neuropathology differs between strains. *J. Neuropathol. Exp. Neurol.* 50:432–440.
 33. Rahbar A, et al. 2003. Evidence of active cytomegalovirus infection and increased production of IL-6 in tissue specimens obtained from patients with inflammatory bowel diseases. *Inflamm. Bowel Dis.* 9:154–161.
 34. Salvesen GS, Duckett CS. 2002. IAP proteins: blocking the road to death's door. *Nat. Rev. Mol. Cell Biol.* 3:401–410.
 35. Sinclair J, Sissons P. 1996. Latent and persistent infections of monocytes and macrophages. *Intervirology* 39:293–301.
 36. Singh D, McCann KL, Imani F. 2007. MAPK and heat shock protein 27 activation are associated with respiratory syncytial virus induction of human bronchial epithelial monolayer disruption. *Am. J. Physiol. Lung Cell. Mol. Physiol.* 293:L436–L445.
 37. 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.
 38. Smith MS, Bentz GL, Smith PM, Bivins ER, Yurochko AD. 2004. HCMV activates PI(3)K in monocytes and promotes monocyte motility and transendothelial migration in a PI(3)K-dependent manner. *J. Leukoc. Biol.* 76:65–76.
 39. Smith MS, et al. 2007. Roles of phosphatidylinositol 3-kinase and NF- κ B in human cytomegalovirus-mediated monocyte diapedesis and adhesion: strategy for viral persistence. *J. Virol.* 81:7683–7694.
 40. Sordet O, et al. 2002. Specific involvement of caspases in the differentiation of monocytes into macrophages. *Blood* 100:4446–4453.
 41. Stagno S, et al. 1982. Congenital cytomegalovirus infection: the relative importance of primary and recurrent maternal infection. *N. Engl. J. Med.* 306:945–949.
 42. Streblow DN, Dumortier J, Moses AV, Orloff SL, Nelson JA. 2008. Mechanisms of cytomegalovirus-accelerated vascular disease: induction of paracrine factors that promote angiogenesis and wound healing. *Curr. Top. Microbiol. Immunol.* 325:397–415.
 43. Suomalainen M, Nakano MY, Boucke K, Keller S, Greber UF. 2001. Adenovirus-activated PKA and p38/MAPK pathways boost microtubule-mediated nuclear targeting of virus. *EMBO J.* 20:1310–1319.
 44. Taylor-Wiedeman J, Sissons P, Sinclair J. 1994. Induction of endogenous human cytomegalovirus gene expression after differentiation of monocytes from healthy carriers. *J. Virol.* 68:1597–1604.
 45. van Delft MF, Huang DC. 2006. How the Bcl-2 family of proteins interact to regulate apoptosis. *Cell Res.* 16:203–213.
 46. Voss OH, et al. 2007. Binding of caspase-3 prodomain to heat shock protein 27 regulates monocyte apoptosis by inhibiting caspase-3 proteolytic activation. *J. Biol. Chem.* 282:25088–25099.
 47. Whitelaw DM. 1972. Observations on human monocyte kinetics after pulse labeling. *Cell Tissue Kinet.* 5:311–317.
 48. Yurochko AD. 2008. Human cytomegalovirus modulation of signal transduction. *Curr Top. Microbiol. Immunol.* 325:205–220.
 49. Yurochko AD, Huang ES. 1999. Human cytomegalovirus binding to human monocytes induces immunoregulatory gene expression. *J. Immunol.* 162:4806–4816.
 50. Yurochko AD, Liu DY, Eierman D, Haskill S. 1992. Integrins as a primary signal transduction molecule regulating monocyte immediate-early gene induction. *Proc. Natl. Acad. Sci. U. S. A.* 89:9034–9038.
 51. Zhang J, Li Y, Shen B. 2002. Up-regulation of XIAP by M-CSF is associated with resistance of myeloid leukemia cells to apoptosis. *Leukemia* 16:2163–2165.