Human Cytomegalovirus Promotes Survival of Infected Monocytes via a Distinct Temporal Regulation of Cellular Bcl-2 Family Proteins

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ABSTRACT

Monocytes play a key role in the hematogenous dissemination of human cytomegalovirus (HCMV) to target organ systems. To infect monocytes and reprogram them to deliver infectious virus, HCMV must overcome biological obstacles, including the short life span of monocytes and their antiviral proapoptotic response to infection. We have shown that virally induced upregulation of cellular Mcl-1 promotes early survival of HCMV-infected monocytes, allowing cells to overcome an early apoptotic checkpoint at around 48 h postinfection (hpi). Here, we demonstrate an HCMV-dependent shift from Mcl-1 as the primary anti-apoptotic player to the related protein, Bcl-2, later during infection. Bcl-2 was upregulated in HCMV-infected monocytes beginning at 48 hpi. Treatment with the Bcl-2 antagonist ABT-199 only reduced the prosurvival effects of HCMV in target monocytes beginning at 48 hpi, suggesting that Mcl-1 controls survival prior to 48 hpi, while Bcl-2 promotes survival after 48 hpi. Although Bcl-2 was upregulated following viral binding/signaling through cellular integrins (compared to Mcl-1, which is upregulated through binding/activation of epidermal growth factor receptor [EGFR]), it functioned similarly to Mcl-1, adopting the early role of Mcl-1 in preventing caspase-3 cleavage/activation. This distinct, HCMV-induced shift from Mcl-1 to Bcl-2 occurs in response to a cellular upregulation of proapoptotic Bax, as small interfering RNA (siRNA)-mediated knockdown of Bax reduced the upregulation of Bcl-2 in infected monocytes and rescued the cells from the apoptotic effects of Bcl-2 inhibition. Our data demonstrate a distinct survival strategy whereby HCMV induces a biphasic regulation of cellular Bcl-2 proteins to promote host cell survival, leading to viral dissemination and the establishment of persistent HCMV infection.

IMPORTANCE

Hematogenous dissemination of HCMV via infected monocytes is a crucial component of the viral survival strategy and is required for the establishment of persistent infection and for viral spread to additional hosts. Our system of infected primary human blood monocytes provides us with an opportunity to answer specific questions about viral spread and persistence in *in vivo*-relevant myeloid cells that cannot be addressed with the more traditionally used replication-permissive cells. Our goal in examining the mechanisms whereby HCMV reprograms infected monocytes to promote viral dissemination is to uncover new targets for therapeutic intervention that would disrupt key viral survival and persistence strategies. Because of this important role in maintaining survival of HCMV-infected monocytes, our new data on the role of Bcl-2 regulation during viral infection represents a promising molecular target for mitigating viral spread and persistence.

Human cytomegalovirus (HCMV) is a ubiquitous host-restricted betaherpesvirus that infects 60 to 90% of the population and persists for the lifetime of the infected individual (1). HCMV infection results in a wide range of pathogenic outcomes dependent upon the age and immune status of the host (2). Infection of immunocompetent hosts is usually asymptomatic or only mildly symptomatic (3, 4); however, it can cause infectious mononucleosis, is a risk factor for the development of cardiovascular disease (5–9), and has been linked to certain types of cancers in otherwise healthy individuals (10–15). In contrast, HCMV infection leads to significant morbidity and mortality in the immunocompromised. HCMV is an important opportunistic pathogen in AIDS patients (16–20), is a leading infectious cause of complications in transplant recipients (21–28), and causes severe neurological disease in congenitally infected neonates (29–34).

HCMV pathogenesis and disease result from viral spread to multiple organ sites following primary HCMV infection, a process which appears to be a critical step in the viral persistence strategy, as it allows for the establishment of lifelong persistence within the host, as well as for viral shedding and spread to additional hosts (1,

35, 36). Monocytes are the primary blood-borne targets for HCMV infection and are thought to be centrally involved in the hematogenous dissemination of the virus to target organ systems (37–41). We propose that HCMV reprograms the biology of infected monocytes, creating the ideal cell type to serve as “Trojan horses” to carry HCMV to target host organ sites and then to

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promote persistence within those sites as an infected macrophage (42). In support, we have reported that HCMV-infected monocytes show a significantly enhanced motility compared to the motility of unstimulated monocytes and of those treated with lipopolysaccharide (LPS) and phorbol myristate acetate (PMA) (36, 42–45). These virus-mediated changes in monocyte motility lead to increased transendothelial migration, a process that biologically would allow HCMV-infected monocytes to exit the peripheral blood and enter host organ tissues, where shedding to additional hosts can occur. In addition, we have identified that HCMV-infected monocytes undergo a distinct monocyte-to-macrophage differentiation program with a biased polarization toward an M1-like macrophage (42, 46–48). Monocyte-to-macrophage differentiation is ultimately required for viral persistence because monocytes are not initially permissive for viral gene expression and replication, unlike their differentiated counterparts, macrophages, which support the full viral life cycle (42). We propose that HCMV takes advantage of the inherent plasticity of the monocyte/macrophage system in order to create a cell type that balances effective hematogenous dissemination (monocytes) with replication and persistence (tissue macrophages). Finally, we have described a prosurvival phenotype in HCMV-infected monocytes whereby cells remain viable for months following infection, contrary to their naturally limited biological life span (48, 49). Enhanced survival of HCMV-infected monocytes is essential to the viral persistence strategy, as monocytes are naturally short-lived cells that are programmed to undergo apoptosis within 48 to 72 h of entering the circulation (50, 51), a problematic biological scenario for HCMV with its slow DNA replication cycle of days to weeks *in vivo* (1).

It is documented in replication-permissive cell types that HCMV employs viral antiapoptotic proteins to maintain the viability of infected cells (52–54). In monocytes, however, we have shown that viral antiapoptotic transcripts are not expressed during at least the first 72 h of infection (49). In fact, we consistently see no *de novo* viral gene expression in our system until approximately 2 weeks postinfection, when monocyte-to-macrophage differentiation is complete (42, 48). Taken together, these data suggest that viral antiapoptotic proteins are unlikely to participate in the long-term survival of infected monocytes/macrophages prior to the initiation of viral gene programs around 2 weeks postinfection. In the absence of viral gene expression, HCMV appears to have evolved an alternative mechanism for controlling cellular survival of infected monocytes. We have previously described a mechanism by which HCMV promotes monocyte survival via signaling events resulting from viral binding to the epidermal growth factor receptor (EGFR) on the surface of monocytes (49). In fact, many of the HCMV-induced changes we previously observed in infected monocytes result from signaling through a combination of two signaling axes: EGFR and cellular integrins (36, 45). We have shown that viral binding to EGFR (55) and both $\beta 1$ and $\beta 3$ integrins (56, 57), via glycoprotein B (gB) and the gH/gL/UL128-131 complex, respectively, as well as downstream signaling events are required for HCMV entry into monocytes. In addition, we have demonstrated that signaling through these receptors is responsible for the increase in infected monocyte motility (43, 55–57) and allows HCMV to govern the apoptotic signalosome in infected monocytes (49).

To ensure its spread and survival in infected monocytes, HCMV must subvert the innate biological programming of a

monocyte to undergo apoptosis within 48 to 72 h of entering the circulation (49, 50) and thwart any antiviral cellular response triggered by infection (58, 59). Our recent work identifies an *in vitro* viability checkpoint that exists at around 48 h following isolation of primary human monocytes (49), consistent with the *in vivo* life span of these cells (50). This 48-h viability checkpoint constitutes an initial survival hurdle that infected monocytes must overcome, and it appears that two distinct survival strategies are required for HCMV-infected monocytes on either side of this viability checkpoint. We have previously reported that HCMV, in the absence of *de novo* viral gene expression, regulates the expression of a cellular antiapoptotic protein, Mcl-1, to navigate the initial cell survival checkpoint up to 48 h postinfection (hpi) (49). Because Mcl-1 levels in HCMV-infected monocytes decline to those in mock-infected monocytes by 72 hpi, Mcl-1 cannot be responsible for long-term survival of HCMV-infected monocytes once the initial 48-h viability gate has been successfully negotiated. Nonetheless, we observe continued survival beyond the 48-h checkpoint in HCMV-infected monocytes compared to their mock-infected counterparts (48). It is this question that we have begun to address, and our new results are presented in this article.

We describe a distinct HCMV-driven regulation of cellular Bcl-2 proteins that constitutes a multiphasic survival strategy designed to favor infected-cell survival throughout the course of infection. We show that between 48 and 72 hpi, HCMV-infected monocytes undergo a functional shift from Mcl-1 to Bcl-2 as the primary antiapoptotic player responsible for promoting cell survival. This Mcl-1-to-Bcl-2 shift is in contrast to the regulation and functionality of these same proteins in normal homeostatic monocytes that are manipulated *ex vivo* to survive beyond their natural life span by virtue of survival signals resulting from cellular adhesion. Data from our previous survival studies (49) suggest that Mcl-1 is required for early survival of infected monocytes through the 48-h viability checkpoint. Here we show that a later shift to Bcl-2 extends infected cell survival beyond this initial checkpoint. HCMV-induced regulation of Mcl-1 and Bcl-2 is mediated via distinct signaling events (EGFR-dependent upregulation of Mcl-1 [49] and integrin-dependent upregulation of Bcl-2 [present study]) following viral binding to the two cellular receptors, which we have previously shown must signal cooperatively to trigger viral entry (55–57). The shift from Mcl-1 to Bcl-2 occurs in response to an upregulation of proapoptotic Bax, a protein which appears to mediate the antiviral proapoptotic response to HCMV infection and can be bound and inactivated by Bcl-2 but not Mcl-1. Overall, this study suggests that HCMV has evolved to utilize a select expression program in monocytes to regulate cellular antiapoptotic proteins in answer to the apoptotic challenges associated with HCMV infection of monocytes.

MATERIALS AND METHODS

Virus preparation. HCMV (Towne/E strain [passages 38 to 41], BADwt, and BAD rUL131) was cultured in human embryonic lung fibroblasts and then purified through a 0.5 M sucrose gradient (42–44, 47–49, 55–57, 60–66). Virus was resuspended in RPMI 1640 medium (BioWhittaker, Lonza, Walkersville, MD) and used to infect cells at a multiplicity of infection (MOI) of 5, which yields an ~99% infection rate in primary human monocytes (42–44, 47–49, 55–57, 62, 63). Mock infection of monocytes was achieved using equivalent volumes of virus-free RPMI 1640 medium. BADwt (lacking the gH/gL/UL128-131 pentameric complex) and BAD rUL131 (containing a repaired UL131A mutation, such that the gH/gL/UL128-131 pentameric complex is restored) are AD169-derived

bacterial artificial chromosome (BAC) viruses. Both BAC-based viruses were previously constructed and characterized (67).

Human peripheral blood monocyte isolation. Primary human monocytes were collected from healthy donors by venipuncture as previously described (47–49, 55–57, 62, 63). Briefly, 240 ml of blood was drawn from each donor and centrifuged through a Ficoll Histopaque 1077 gradient (Sigma-Aldrich Co., St. Louis, MO) at $200 \times g$ for 30 min at room temperature. Leukocytes were collected and then subjected to multiple 10-min washes in room temperature normal saline (B. Braun Medical Inc., Irvine, CA) at $150 \times g$ to remove platelets. Cells were then layered over a 45% and 52.5% isosmotic Percoll (GE Healthcare Life Sciences, Pittsburgh, PA) gradient and centrifuged at $400 \times g$ for 30 min at room temperature, yielding >95% pure, unactivated monocytes with >95% survival by trypan blue exclusion. Isolated monocytes were then washed twice with saline and resuspended in RPMI 1640 medium (BioWhittaker) supplemented with 1% human serum (Sigma-Aldrich). This procedure has been approved by the Institutional Review Board for the Protection of Human Research Subjects and follows all guidelines outlined by the Health Insurance Portability and Accountability Act.

MTT viability assay. MTT [3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide] cell viability assays were performed according to the manufacturer's suggested protocol (Roche Diagnostics, Mannheim, Germany). Briefly, primary monocytes were isolated, infected, and plated in triplicate (100,000 cells per well; 100- μ l final volume per well) in 96-well microtiter plates. Cells were then incubated at 37°C to allow attachment before undergoing MTT analysis. At each indicated time point, 10 μ l of MTT labeling solution (Roche) was added to the appropriate wells. Cells were then incubated at 37°C for an additional 4 h before the addition of 100 μ l of MTT solubilization solution (Roche). Following an additional 20 h of incubation at 37°C, absorbance for each well was read at 590 nm (colorimetric indicator of tetrazole-formazan reduction) and at 690 nm (cellular debris subtracted as background) using a Costar plate reader.

ABT-737 and ABT-199 inhibitors. Isolated monocytes were mock infected or HCMV infected under nonadherent conditions at 37°C for 1 h and then plated in 96-well plates in RPMI 1640 medium supplemented with 1% human serum. Plated cells were allowed to adhere and then treated with dimethyl sulfoxide (DMSO) solvent control or various concentrations of ABT-737 (Selleckchem, Houston, TX) or ABT-199 (Selleckchem). ABT-737/ABT-199 treatment at 5 hpi was then used as the 0-h time point for the assays. MTT cleavage was performed at the adjusted 0-, 24-, 48-, 72-, and 96-h time points to assess cell viability. Paired Student's *t* tests were performed using IBM SPSS Statistics software 20.0 (IBM) to identify statistically significant differences in survival rates between drug-treated and DMSO-treated control groups.

Z-DEVD-FMK and Z-VAD-FMK inhibitors. Isolated monocytes were mock infected or HCMV infected under nonadherent conditions at 37°C for 1 h and then plated on 96-well plates in RPMI 1640 medium supplemented with 1% human serum. Plated cells were allowed to adhere and then were treated with control DMSO, ABT-199, Z-DEVD-FMK caspase-3 inhibitor (Santa Cruz Biotechnology, Santa Cruz, CA), and/or Z-VAD-FMK pan-caspase inhibitor (Santa Cruz Biotechnology). Drug treatment at 5 hpi was used as the adjusted 0-h time point. MTT cleavage was performed to assess cell viability at 0 and 72 h. IBM SPSS Statistics software 20.0 (IBM) was used to identify statistical significance (by paired Student's *t* test) between survival rates in drug-treated and DMSO-treated control groups.

Bax-specific siRNA. Isolated monocytes were mock infected, HCMV infected, or LPS treated under nonadherent conditions for 1 h at 37°C. At 1 h, cells were nucleofected with a nontargeting control small interfering RNA (siRNA; Integrated DNA Technologies, Coralville, IA) or with a Bax-specific siRNA (Integrated DNA Technologies) and then plated onto 96-well plates in RPMI 1640 medium supplemented with 10% human serum. Plated cells were allowed to adhere for 4 h. After 4 h (the adjusted 0-h time point), control DMSO or ABT-199 was added to each well. An

MTT assay was then performed at 0, 24, 48, 72, and 96 h to assess cell viability.

Affymetrix gene microarray and analysis. Reanalysis of Affymetrix gene data from our previous studies was used to assemble a transcriptional profile of antiapoptotic Bcl-2 family genes at 4 (62), 24 (55), and 48 (43) hpi. Affymetrix Microarray Suite version 5.0 was used to compile data from each of the replicates. One-way analysis of variance (ANOVA) tests were performed on the mock-infected versus HCMV-infected replicates to calculate *P* values. A *P* value of ≤ 0.05 was used as the criterion for statistical significance; this correlates with a ≥ 1.5 -fold change in regulation between mock-infected and HCMV-infected groups and is marked with an asterisk in Fig. 1B. The GEO accession numbers for these data are GSE11408 (62), GSE17948 (55), and GSE19772 (43). For a complete description of the experimental procedures used, see our previous publications (43, 55, 62).

Semiquantitative RT-PCR. Isolated monocytes were treated and infected as detailed in individual experimental descriptions. RNA was harvested using RNA STAT-60 (Tel-Test, Inc., Friendswood, TX) and made into cDNA. PCR was performed with primers specific for Bcl-2 (sense, 5'-GCATGCGCCTCTGTTGATTTCT-3'; antisense, 5'-AGGCATGTGACTTCACTTGTGGC-3'), Bax (sense, 5'-GCTGTTGGGCTGGATC CAAG-3'; antisense, 5'-TCAGCCCATCTTCTCCAGA-3'), and 18S rRNA (sense, 5'-CGAGCCGCCTGGATACC-3'; antisense, 5'-CAGTTC CGAAAACCAACAAATAG-3') as an internal control.

Western blot protein analyses. Isolated monocytes were treated and infected per individual experimental design. Total whole-cell lysates were harvested with lysis buffer (50 mM Tris-HCl at pH 7.5 [Fisher Scientific, Fair Lawn, NJ], 5 mM EDTA [Bio-Rad Laboratories, Hercules, CA], 100 mM sodium chloride [Fisher Scientific], 1% Triton X-100 [Fisher Scientific], 0.1% sodium dodecyl sulfate [MP Biomedicals, Inc., Solon, OH], and 10% glycerol [MP Biomedicals, Inc.]) supplemented with $1\times$ protease inhibitor (Sigma-Aldrich), $1\times$ phosphatase inhibitor I (Sigma-Aldrich), and $1\times$ phosphatase inhibitor III (Sigma-Aldrich) for 30 min on ice. Lysates were then centrifuged at $16,000 \times g$ for 5 min at 4°C. Supernatants were collected and stored at -20°C until analyzed. Protein concentrations of the harvested samples were determined by the Bradford protein assay (Bio-Rad). Samples were then denatured by boiling with Laemmli's solution (2 parts Laemmli's SDS-sample buffer [Boston Bio-products, Boston, MA] to 1 part β -mercaptoethanol [Fisher Scientific]) for 5 min. Equivalent amounts of total cellular protein from each sample were separated by SDS-PAGE, transferred to polyvinylidene difluoride (PVDF) membranes, and then immunoblotted. Blots were blocked in 5% bovine serum albumin (BSA)-Tris-buffered saline (TBS)-Tween 20 solution and then probed with an anti-Bcl-2 antibody (Ab) (Santa Cruz Biotechnology), an anti-caspase-3 Ab (Calbiochem, San Diego, CA), an anti-Bax Ab (Santa Cruz Biotechnology), or an anti-actin Ab (Santa Cruz Biotechnology) as a loading control. Blots were rinsed in TBS-Tween 20 and then probed with horseradish peroxidase (HRP)-conjugated secondary antibodies (GE Healthcare Life Sciences) and developed using Super-Signal West Pico chemiluminescent substrate (Fisher Scientific) according to the manufacturer's protocol. Densitometric analysis was performed using Quantity One Basic software (Bio-Rad) to quantify changes in protein expression.

TUNEL staining apoptosis assay. Isolated monocytes were mock infected or HCMV infected and then treated with control DMSO or ABT-199 (Selleckchem) and plated in 96-well cell culture plates. At 24, 48, and 72 h, a TUNEL (terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end labeling) assay was performed as previously described (49). Briefly, cells were fixed with methanol and washed with phosphate-buffered saline (PBS) before the portion of apoptotic cells with DNA fragmentation was identified via the TUNEL reaction. The TUNEL reaction was then terminated using 300 mM sodium chloride and 30 mM sodium citrate. Cells were then washed with PBS, and a 30-min incubation at room temperature in 3% H_2O_2 was performed to neutralize endogenous peroxidase activity. After the cells were washed in PBS, a sec-

ondary streptavidin-conjugated antibody (Streptavidin Biotin System Histostain-SP kit; Zymed, Markham, CA) was added for 1 h, followed by an additional PBS wash. 3-Amino-9-ethylcarbazole substrate (AEC substrate kit; Fisher Scientific) was added to cells for 5 min to yield a dark-brown precipitate in apoptotic cells with nicked DNA. Cells were then washed with PBS, fixed in 4% paraformaldehyde, and visualized by bright-field microscopy, and the numbers of apoptotic cells versus total cells were counted. Statistically significant differences in rates of apoptosis between ABT-199-treated groups and DMSO-treated controls at each time point were determined by performing paired Student's *t* tests using IBM SPSS Statistics software 20.0 (IBM).

RESULTS

Bcl-2 family proteins are required for monocyte survival beyond the 48-h viability gate. HCMV upregulation of the cellular antiapoptotic protein Mcl-1 allows infected monocytes to navigate the initial viability checkpoint at 48 h, securing the short-term survival of these cells (49). We hypothesized that HCMV regulates an additional Mcl-1-related antiapoptotic protein to extend the survival of infected monocytes beyond the 48-h viability gate. To test this initial hypothesis, we treated mock-infected and HCMV-infected monocytes with ABT-737, a small-molecule inhibitor of the antiapoptotic Bcl-2 family proteins: Bcl-2, Bcl-xL, and Bcl-w (68, 69). Mechanistic studies reveal that ABT-737 binds Bcl-2, Bcl-xL, and Bcl-w, preventing their antagonistic effect over the proapoptotic Bcl-2 family proteins, Bax and Bak; however, ABT-737 itself does not directly induce apoptosis (68, 69). Viability of treated cells was assessed at 72 h by an MTT assay. Mock-infected cells show a slight decrease in viability at 72 h (~80% survival) compared to the HCMV-infected cohorts (~90% survival) (Fig. 1A), as documented previously (49), even when plated to induce adhesion-associated survival and differentiation signals. Treatment with ABT-737 reduced the viability of both mock-infected and HCMV-infected monocytes (Fig. 1A), suggesting that Bcl-2 family antiapoptotic proteins, Bcl-2, Bcl-xL, and/or Bcl-w, play a role in extending monocyte survival. Of note, however, higher concentrations of ABT-737 were required to observe cellular death in HCMV-infected monocytes than in their mock-infected counterparts (Fig. 1A). Statistically significant differences in percent cell survival were observed in mock-infected monocytes following treatment with 1 μ M ABT-737; however, a higher dosage of 8 μ M ABT-737 was required for significant cell death in HCMV-infected cells. We extrapolate that a dosage of ~1 μ M versus ~6 μ M ABT-737 is required for a 50% reduction in viability of mock-infected versus HCMV-infected cells, respectively. These data hint at a threshold level of antiapoptotic Bcl-2, Bcl-xL, and/or Bcl-w being required for monocyte survival and suggest that HCMV-infected monocytes express greater levels of one or more of these proteins.

Because ABT-737 functions as an inhibitor of multiple Bcl-2 family antiapoptotic proteins (Bcl-2, Bcl-xL, and Bcl-w) (68, 69), we next sought to determine which of these proteins might be differentially regulated in HCMV-infected monocytes. We reanalyzed our transcriptome databases of mock-infected versus HCMV-infected monocytes from previous studies (43, 55, 62) for changes in antiapoptotic Bcl-2 family transcripts following HCMV infection. We focused on three time points: 4 hpi (62) (an early time point at which we first begin to see significant transcriptional changes between mock-infected and HCMV-infected groups), 24 hpi (55) (a time point occurring just before the early viability checkpoint), and 48 hpi (43) (a time point co-

B)

FULL GENE NAME	GENE TITLE	PROTEIN	FOLD INDUCTION (HCMV vs. mock)		
			4h	24h	48h
Myeloid cell leukemia sequence 1	MCL1	Mcl-1	0.9	2.0*	1.1
B cell CLL/lymphoma 2	BCL2	Bcl-2	2.1*	1.7*	2.7*
Bcl2-like 1	BCL2L1	Bcl-xL	1.7*	4.6*	1.4
Bcl2-like 2	BCL2L2	Bcl-w	0.7	0.4	0.9

FIG 1 Bcl-2 family proteins are required for monocyte survival beyond the 48-h viability gate. (A) Isolated monocytes were mock infected or HCMV infected and treated with DMSO or with the indicated concentrations of ABT-737 (Bcl-2, Bcl-xL, Bcl-w antagonist). Cells were incubated under adherent conditions on 96-well plates at 37°C, and an MTT viability assay was performed at 0 and 72 h. Results are expressed as percentage of living cells at 72 h compared to that at 0 h. The graph shows the average of results of three replicates from three different blood donors. Paired Student's *t* tests were performed to determine statistically significant differences in cell survival between drug-treated groups and DMSO controls (indicated by asterisks). (B) Temporal transcriptome analyses of mock-infected versus HCMV-infected monocytes reveal that multiple antiapoptotic mRNAs from the Bcl-2 family are induced following HCMV infection. Data were obtained via reanalysis of Affymetrix gene data from previous studies (GSE19772 [43], GSE11408 [62], and GSE17948 [55]). Significantly upregulated transcripts (≥ 1.5 -fold) are marked with an asterisk.

inciding with navigation of the viability gate during which cells should be transcriptionally transitioning to the later survival strategy). We observed different degrees of induction of each of the antiapoptotic Bcl-2 transcripts in HCMV-infected versus mock-infected monocytes at the examined time points (Fig. 1B). Induction of the MCL1 transcript (Mcl-1 protein) is statistically significant at the 24-h time point only (Fig. 1B), consistent with the protein's already defined role as the primary antiapoptotic player prior to the early viability checkpoint in HCMV-infected monocytes (49). Of the remaining Bcl-2 family transcripts (BCL2, BCL2L1, and BCL2L2) corresponding to proteins that are inhibited by ABT-737 (Bcl-2, Bcl-xL, and Bcl-w, respectively), we found

that only BCL2 (Bcl-2 protein) was significantly induced at the critical 48-h time point (Fig. 1B).

HCMV infection enhances Bcl-2 expression via integrin/Src signaling. After identifying Bcl-2 as a potential candidate for mediating long-term survival in HCMV-infected monocytes, we used RT-PCR to confirm and expand the information gathered from our microarray analyses. Isolated monocytes were mock infected or HCMV infected, and Bcl-2 steady-state mRNA expression levels were examined over a 96-h course of infection. As expected, beginning at 24 hpi and continuing through 96 hpi, Bcl-2 mRNA levels were elevated in HCMV-infected monocytes compared to mock-infected controls (Fig. 2A). To determine whether elevated levels of Bcl-2 mRNA in HCMV-infected monocytes would translate to an increase in Bcl-2 protein, we performed a Western blot analysis of mock-infected versus HCMV-infected monocytes over 96 h (Fig. 2B). Densitometric analysis was also performed to quantify changes in Bcl-2 expression. Following the increase in Bcl-2 mRNA at 24 h in infected cells, Bcl-2 protein expression was upregulated in HCMV-infected monocytes to a greater extent than in mock-infected monocytes beginning at 48 hpi and continuing through 96 hpi (Fig. 2B). Because our previous data identified that HCMV-induced functional changes occurring in infected monocytes are driven by signaling events initiated by viral ligand/receptor binding to EGFR and/or cellular integrins (48, 49, 55–57), we next sought to determine whether activation of these signaling axes might be responsible for the HCMV-induced regulation of cellular Bcl-2 in infected monocytes. We pretreated HCMV-infected monocytes with an EGFR kinase inhibitor, AG1478 (49, 55), an Src inhibitor, PP2 (56, 57), or blocking antibodies against $\beta 1$ and $\beta 3$ integrins (56, 57) as previously performed for 1 h prior to infection and then monitored Bcl-2 levels by Western blotting (Fig. 2C). At 96 hpi (a time point at which we have previously observed high levels of Bcl-2 induction following HCMV infection), Bcl-2 expression is increased approximately 8-fold in HCMV-infected monocytes compared to mock-infected monocytes (Fig. 2C), consistent with our time course data (Fig. 2B). When monocytes were pretreated with AG1478 (EGFR inhibitor) for 1 h prior to infection, Bcl-2 expression was comparable (8.1-fold increase over mock-infected monocytes) to that observed in HCMV-infected monocytes pretreated with control DMSO (7.9-fold increase). In contrast, suppression of integrin/Src signaling by pretreatment with the inhibitor PP2 (1.5-fold increase over mock-infected monocytes) or blocking antibodies against $\beta 1$ and $\beta 3$ integrins (1.3-fold increase) reduced Bcl-2 expression to levels near those of mock-infected monocytes (Bcl-2 normalized to actin and set to 1) (Fig. 2C). Because viral binding and signaling through surface integrins occur within the first hour of HCMV infection but upregulation of Bcl-2 cannot be detected until 48 hpi, we wanted to further clarify whether an immediate signaling event does in fact trigger increased Bcl-2 expression. To address this question, we infected monocytes with HCMV BADwt, which lacks the gH/gL/UL128-131 pentameric complex, an important determinant of cellular tropism that we have shown binds to integrins on the surface of monocytes (57), and BADrUL131, in which the pentameric complex has been restored by repairing UL131 on the BADwt background. Following infection, we monitored changes in Bcl-2 expression at 72 and 96 h using Western blot analysis. Infection with the BADwt strain resulted in slightly higher levels of Bcl-2 (1.3-fold increase) than those observed in mock-infected cells (data not shown [DNS]),

FIG 2 HCMV infection enhances Bcl-2 expression via integrin/Src signaling. (A) Isolated monocytes were mock infected or HCMV infected (MOI = 5) and incubated under adherent conditions at 37°C for 24, 48, 72, and 96 h. RNA was isolated at each time point, and RT-PCR was performed, using primers for Bcl-2 and 18S. Reverse transcriptase-negative (RT-) and nontemplate controls (NTC) were used to show primer specificity for the indicated mRNAs. The image is representative of three repeats from three separate blood donors. The image display has been inverted to show dark bands on a light background for ease of viewing. (B) Isolated monocytes were mock infected or HCMV infected (MOI = 5) and incubated under adherent conditions at 37°C for 24, 48, 72, and 96 h. Whole-cell lysates were collected at each time point, and Western blot analysis was performed using antibodies against Bcl-2 and actin. Densitometric analysis was performed using Quantity One Basic software (Bio-Rad); the numbers below each sample reflect a ratio of Bcl-2 expression normalized to control β -actin expression. The experiment was repeated three times with monocytes from three different donors, with similar results. (C) Isolated monocytes were pretreated for 1 h with DMSO, control IgG antibodies, a 25 μ M concentration of the EGFR inhibitor AG1478, a 25 μ M concentration of the Src inhibitor PP2, or blocking anti-integrin antibodies, washed in PBS, and then mock infected or HCMV infected (MOI = 5) for 96 h. The medium was supplemented with inhibitors and blocking antibodies every 24 h over the course of infection to ensure continuous blocking of signaling. Total lysates were harvested, and Western blot analysis was performed to examine Bcl-2 expression. Actin was used as a loading control. Densitometric analysis was performed using Quantity One Basic software (Bio-Rad) to obtain a ratio of Bcl-2 expression normalized to actin. The blots and densitometry values are representative of data from three repeats with three different donors.

similar to results seen when integrin signaling is blocked with PP2 or anti-integrin blocking antibodies (Fig. 2C). In contrast, infection with the repaired BAD rUL131 strain resulted in higher levels of Bcl-2 expression (~2-fold increase), which though not as robust as the increase observed following infection with a low passage Towne/E strain (Fig. 2B and C) are still higher than Bcl-2 expression in either mock-infected or BADwt-infected cells (DNS).

HCMV-induced upregulation of Bcl-2 promotes extended survival of infected monocytes beyond the 48-h viability gate. To address the role that Bcl-2 may play in promoting long-term

FIG 3 HCMV-induced upregulation of Bcl-2 promotes extended survival of infected monocytes beyond the 48-h viability gate. (A) Monocytes were mock infected or HCMV infected (MOI = 5) and treated with DMSO or with the indicated concentrations of ABT-199 (Bcl-2 antagonist). Cells were incubated under adherent conditions on 96-well plates at 37°C, and an MTT viability assay was performed at 0 and 72 h. Results are expressed as the percentage of living cells at 72 h compared to 0 h. The graph is the result of a single experiment performed in triplicate; similar results were seen in two additional repeats with different blood donors. Paired Student's *t* tests were performed to determine statistical significance (indicated by an asterisk) between the ABT-199-treated groups and the DMSO controls. (B) Mock-infected or HCMV-infected (MOI = 5) monocytes were treated with 32 μM ABT-199 (Bcl-2 antagonist) and incubated at 37°C on 96-well plates for 24, 48, 72, and 96 h before performance of an MTT viability assay. Results are expressed as the percentage of living cells at each time point compared to the viable cell count at 0 h. The graph is the average of results of three replicates from an experiment repeated with three different blood donors. Asterisks represent statistical

significance (as determined by paired Student's *t* test) between the ABT-199-treated groups and the DMSO-treated controls at each time point. (C) Isolated monocytes were mock infected or HCMV infected and treated with ABT-199 (16 μM or 32 μM) (Bcl-2 antagonist) for 24, 48, and 72 h. The frequency of apoptosis was determined by TUNEL analysis. Cells were visualized by microscopy; the numbers of TUNEL-positive cells and total cells were counted (three fields per well, three wells per experimental arm, and an average of 53 cells per field). Results are expressed as the percentage of apoptotic cells compared to the total number of cells counted at each time point. The graph is the average of results of three replicates from five repeated experiments with different donors (total of 15 data points for each experimental group). Paired Student's *t* tests were performed; asterisks are used to show statistical significance between the ABT-199-treated groups and the DMSO-treated controls in each time point.

survival of HCMV-infected monocytes, we used a Bcl-2-specific inhibitor, ABT-199, in conjunction with the MTT viability assay. ABT-199 is a more recently developed small-molecule Bcl-2 antagonist that functions similarly to ABT-737 (68, 69), with the exception that ABT-199 binds only Bcl-2 (not Bcl-xL or Bcl-w) (70). Mock-infected and HCMV-infected monocytes were treated with a DMSO solvent control or with various concentrations of ABT-199 before cell viability was assayed at 0 h and again at 72 h. As seen with ABT-737 treatment, both mock-infected and HCMV-infected monocytes displayed a decrease in cell viability when treated with the Bcl-2-specific antagonist ABT-199 (Fig. 3A). Again, however, higher concentrations of ABT-199 were required in HCMV-infected cells than in mock-infected cells to effect similar decreases in cell viability (Fig. 3A). We see a statistically significant decrease in cell survival following treatment with 1 μM ABT-199 in mock-infected monocytes compared to that after treatment with 8 μM ABT-199 in their HCMV-infected counterparts. We extrapolate that a dosage of ~5 μM ABT-199 in HCMV-infected cells, compared to ~1 μM in mock-infected cells, is required for a 50% reduction in survival, similar to the values observed in Fig. 1A for cells treated with ABT-737. When infected monocytes were treated with a high dose, 32 μM ABT-199, at 0 h and then assayed for cell viability at 24 to 96 h, a significant reduction in cell viability was not observed until around 48 h (no loss in cell viability was seen at 24 h), compared to the strong loss seen at 24 h in mock-infected cells (Fig. 3B). Because ABT-199 does not antagonize Mcl-1 activity (69, 70), these data support our model and hypothesis that only Mcl-1 controls infected-monocyte survival prior to the 48-h viability gate and that only Bcl-2 controls survival beyond 48 h. This is in contrast to what is seen in mock-infected cells, where both Mcl-1 and Bcl-2 appear to be required for early survival (Fig. 3B) (49), suggesting that a different regulation of Bcl-2 family proteins occurs in HCMV-infected monocytes. A TUNEL assay was performed next to detect DNA fragmentation on a per-cell basis. Isolated monocytes were mock infected or HCMV infected and then treated with DMSO or ABT-199 before TUNEL staining at various time points following infection. At the 24-h time point, we saw high rates (85% and 95%) of apoptosis with the addition of 16 μM or 32 μM ABT-199, respectively, to mock-infected monocytes, compared to low rates of apoptosis (<5%) seen in their ABT-199-treated, HCMV-infected counterparts (Fig. 3C). These data suggest that Bcl-2 (along with Mcl-1 [49]) contributes to early survival of normal monocytes, but the HCMV-induced upregulation of Mcl-1 is sufficient for early survival of HCMV-infected cells. At 48 hpi, we see an increase in the apoptotic baseline of both mock-

FIG 4 Bcl-2 promotes the extended survival of HCMV-infected monocytes by adopting the early role of Mcl-1 as a regulator of caspase-3 activation. (A) Isolated monocytes were mock infected or HCMV infected (MOI = 5) for 1 h and then nucleofected with nontargeting control siRNA or Bcl-2-specific siRNA and incubated at 37°C for 72 h. Whole-cell lysates were collected, and Western blot analyses were performed using antibodies against Bcl-2, caspase-3, and actin. The experiment was repeated in triplicate using three different donors, with similar results. Densitometric analyses were performed using Quantity One Basic software (Bio-Rad) to quantify the relative expression levels of Bcl-2, procaspase-3, and caspase-3 normalized to the actin control. Quantification is included in parentheses under each corresponding band. (B) Isolated monocytes were mock infected or HCMV infected (MOI = 5) for 1 h at 37°C and then seeded into 96-well plates. Following adhesion, cells were treated with DMSO, 6 μ M ABT-199 (Bcl-2 antagonist), 20 μ M Z-DEVD-FMK (caspase-3 inhibitor), or 20 μ M Z-VAD-FMK (pan-caspase inhibitor). An MTT assay was performed immediately following drug treatment at 0 h and again at 72 h. Results are expressed as percent cell survival at 72 h normalized to 100% survival at 0 h. The graph is representative of results of three replicates from a single experiment; similar results were seen when repeated with two additional donors. Asterisks are used to indicate statistical significance (as determined by paired Student's *t* tests) between the drug-treated groups and the DMSO-treated controls.

infected (from 18% to 30%) and HCMV-infected (from 3% to 7%) cells treated with the DMSO control (Fig. 3C). Mock-infected cells treated with either dose of ABT-199 display similar high rates of apoptosis, as seen at the 24-h time point, while the percentage of apoptotic cells among the HCMV-infected groups begins to increase above that which was observed at 24 h (from <5% to 30% and to 50% for the 16 μ M and 32 μ M concentrations, respectively) as the cells approach the initial viability checkpoint and the Mcl-1-to-Bcl-2 switch. By 72 hpi, we observe high levels of apoptosis (~50%) in mock-infected cells; these numbers increase to near 100% apoptotic cells with the addition of ABT-199 (Fig. 3C). In contrast, HCMV-infected monocytes retain their relatively low baseline rate of apoptosis (~10%) with DMSO treatment; however, the addition of ABT-199 results in a significant increase in apoptosis (80% at 16 μ M ABT-199 and 95% at 32 μ M ABT-199)

(Fig. 3C), indicating that Bcl-2 plays a dominant role in promoting long-term survival of HCMV-infected cells. This finding is consistent with expression patterns of Bcl-2 (Fig. 2A and B), Bcl-xL (Fig. 1B and DNS), and Bcl-w (Fig. 1B and DNS). Furthermore, a significant increase in percent apoptotic cells is seen at 24 h when mock-infected monocytes are treated with ABT-199 but not until 48 h in the HCMV-infected cohorts, consistent with the MTT viability analyses (Fig. 3B). Taken together, these data further support our model that HCMV-infected monocytes rely on a biphasic expression pattern of Bcl-2 family proteins in which Mcl-1 is the primary antiapoptotic player early in infection and Bcl-2 assumes this role after the initial viability checkpoint has been negotiated.

Bcl-2 promotes extended survival of HCMV-infected monocytes by adopting the early role of Mcl-1 as a regulator of caspase-3 activation. The role of cysteine-aspartic proteases (caspases) as arbiters of apoptosis is clearly defined in the literature (71, 72). Caspase-3 and caspase-7, in particular, are downstream executioner caspases whose role is to cleave cellular products as the cell prepares for the final stages of apoptotic death (71). Monitoring the proteolytic cleavage and subsequent activation of these caspases is therefore a good functional readout that may provide insight into HCMV's mechanism of inhibiting apoptosis. We previously reported that Mcl-1 promotes the early survival of HCMV-infected monocytes (49) by controlling the proteolytic processing and activation of caspase-3 so that no fully active caspase-3 is present in HCMV-infected cells prior to the 48-h viability gate (47). In this prior study, we observed a low-level activation of caspase-3 in HCMV-infected monocytes after 48 h (compared to mock-infected monocytes) and speculated that an unidentified cellular factor appears to take over Mcl-1's role as a caspase-3 regulator after Mcl-1 expression levels are extinguished at 48 hpi (47). To determine whether Bcl-2 may assume the role of Mcl-1 as a regulator of caspase-3 activation, we nucleofected mock-infected and HCMV-infected monocytes with a nontargeting control siRNA or with a Bcl-2-specific siRNA and collected whole-cell lysates at 72 hpi, a time point that occurs after the viability checkpoint and the Mcl-1-to-Bcl-2 regulatory shift, to examine caspase-3 activation by Western blotting. Caspase-3 is initially translated as a 32-kDa inactive procaspase (73). Procaspase-3 then undergoes two proteolytic cleavages, first to a 20-kDa intermediate and then to a 17-kDa fully active form (73). Knockdown of Bcl-2 in HCMV-infected monocytes resulted in a decrease in procaspase-3 expression coupled with an increase in active caspase-3 expression (Fig. 4A). These results suggest that increased expression of Bcl-2 in HCMV-infected monocytes blocks the proteolytic processing of procaspase-3 into its smaller, fully active form, thus assuming the early role of Mcl-1 in preventing apoptosis of HCMV-infected monocytes once the 48-h viability gate has been successfully negotiated. Knockdown of Bcl-2 had no effect on the proteolytic processing of caspase-7 (DNS), indicating that Bcl-2, like Mcl-1 (47), likely modulates infected-monocyte survival by controlling the activation of a single caspase, caspase-3.

We next wanted to examine whether the Bcl-2-mediated block on caspase-3 activation has functional effects on infected-monocyte survival. To address this question, we performed an MTT viability assay using the Bcl-2 antagonist ABT-199 in combination with Z-DEVD-FMK (a caspase-3 inhibitor) or Z-VAD-FMK (a pan-caspase inhibitor). A concentration of 6 μ M ABT-199 (ex-

trapolated from previous dose curves) was used to reduce the survival of HCMV-infected monocytes to ~50% at 72 h (Fig. 4B, lane 2); this concentration reduces the survival of mock-infected monocytes to 5% at this same time point (Fig. 4B, lane 2). Addition of the caspase-3 inhibitor Z-DEVD-FMK along with ABT-199 at 0 h resulted in a significant increase in cell survival and thus a partial rescue of HCMV-infected cells from apoptosis (survival increased from 50% [lane 2] to 78% [lane 3]) (Fig. 4B). In contrast, addition of Z-DEVD-FMK to the mock-infected groups did little to combat the proapoptotic effects of Bcl-2 antagonism (survival increased from 5% [lane 2] to 8% [lane 3]) (Fig. 4B). These data suggest that activation of an additional caspase (likely caspase-7) contributes to apoptosis in the mock-infected population, while control of caspase-3 activation alone significantly enhances survival of HCMV-infected monocytes. In support, when mock-infected cells are treated with ABT-199 and the pan-caspase inhibitor Z-VAD-FMK, survival increases from 5% (lane 2) to 40% (lane 4) (Fig. 4B). Interestingly, treatment of HCMV-infected monocytes with the pan-caspase inhibitor Z-VAD-FMK in the presence of ABT-199 results in only a modest rescue in cell viability, from 50% (lane 2) to 60% (lane 4) (Fig. 4B). Additionally, treatment of HCMV-infected monocytes with Z-VAD-FMK, even in the absence of ABT-199, appears to reduce their overall viability as a population, from 85% survival (lane 1) to 55% survival (lane 6) (Fig. 4B). These surprising results could be due to a low-level activation of caspase-3 occurring as a result of general caspase (and not caspase-3-specific) targeting so that not every caspase-3 molecule is inhibited. Nonetheless, these data corroborate our model of differential regulation and function of Bcl-2 family proteins and caspases in mock-infected versus HCMV-infected monocytes, and these differences may have implications for both HCMV-induced monocyte survival and HCMV-directed monocyte-to-macrophage differentiation. Cellular differentiation has, in recent years, come to be viewed as an incomplete apoptotic process involving many of the same players (caspases and Bcl-2 family proteins) that function in the apoptotic pathway (48, 74). Specifically, it has been shown that activation of caspase-3 and caspase-9 contributes to macrophage colony-stimulating factor (M-CSF)-induced monocyte-to-macrophage differentiation without causing cell death (74). Our previous work suggests a role for caspase-3 (but not caspase-9) in promoting HCMV-induced monocyte-to-macrophage differentiation (47); however, it is possible that activation of some other caspase contributes to the survival of HCMV-infected monocytes either directly or by promoting monocyte-to-macrophage differentiation through an additional function.

An upregulation of proapoptotic Bax parallels the Mcl-1-to-Bcl-2 shift seen at 48 hpi. Our data indicate that Bcl-2 adopts the early functional role of Mcl-1 in preventing caspase-3 activation and, thus, apoptosis. Mcl-1 and Bcl-2 appear to have similar downstream functional activities at the caspase level of apoptotic regulation in that they both appear to target a single caspase (caspase-3) to control cell survival, at least in HCMV-infected cells (mock-infected cells appear to utilize a different process). As such, these data do not provide a clear biological mechanism for the distinctive shift in antiapoptotic Bcl-2 family proteins from Mcl-1 early to Bcl-2 late during infection. To decipher why there is a biphasic regulation of Mcl-1 and Bcl-2 that occurs in HCMV-infected monocytes but a requirement for both Mcl-1 and Bcl-2 in mock-infected monocytes, we next sought to examine potential

FIG 5 An upregulation of proapoptotic Bax parallels the Mcl-1-to-Bcl-2 shift seen at 48 hpi. (A) The potential binding interactions among various Bcl-2 family proteins are depicted (75). Specifically, the potential binding interactions of the antiapoptotic proteins (Mcl-1, A1, Bcl-2, Bcl-xL, and Bcl-w) with the proapoptotic proteins (Bax and Bak) are shown. (B) Isolated monocytes were mock infected, HCMV infected (MOI = 5), or treated with an alternative activating agent, LPS (20 μ M). RNA was isolated at 4, 24, 48, 72, and 96 hpi and used to complete RT-PCR analyses using primers specific for Bax and control 18S RNA. Reverse transcriptase negative (RT-) and nontemplate controls (NTC) were used to ensure that the bands represent proper amplification of the indicated mRNAs. The image display has been inverted to show dark bands on a light background for ease of viewing. The experiment was repeated three times with three different donors; similar results were seen. (C) Monocytes were mock infected or HCMV infected (MOI = 5) for 24, 48, 72, and 96 h. At these time points, whole-cell lysates were collected for a Western blot analysis of cellular Bax expression. Actin was also probed as a loading control. Densitometric analysis was performed using Quantity One Basic software (Bio-Rad). The relative expression of Bax normalized to actin is indicated below each individual sample. Results are representative of data from three repeats with different donors.

differences in apoptotic regulation upstream at the mitochondrial membrane. We examined the expression of the proapoptotic Bcl-2 family proteins that are known to interact with Mcl-1 and/or Bcl-2 at the mitochondrial membrane (Fig. 5A) (75). Two Bcl-2 family proapoptotic proteins (Bak and Bax) are directly responsible for inducing the apoptotic response by forming dimers that open channels in the mitochondrial membrane, releasing cytochrome *c* into the cytoplasm and beginning the caspase activation cascade that ultimately results in the cleavage of cellular products and cell death (71, 75, 76). The ratio of these proapoptotic proteins to their antiapoptotic counterparts (Mcl-1, Bcl-2, Bcl-xL, and Bcl-w) determines the apoptotic fate of a given cell, as binding of Bak and Bax by the antiapoptotic proteins prevents dimerization (75, 76). The potential binding interactions among the various proapoptotic and antiapoptotic Bcl-2 family proteins have been well characterized in the literature and are compiled in Fig. 5A (75). As indicated in Fig. 5A, proapoptotic Bak can be bound and inactivated by each of the known antiapoptotic Bcl-2 family proteins, while proapoptotic Bax can be bound and inactivated by all except Mcl-1. To determine whether an upregulation of proapoptotic Bax in HCMV-infected monocytes at later times

postinfection might explain the biphasic regulation of Mcl-1 and Bcl-2, we performed both mRNA (Fig. 5B) and protein (Fig. 5C) analyses of Bax in mock-infected and HCMV-infected monocytes at various time points between 4 and 96 hpi to examine its expression before and after the 48-h viability gate. In addition, we asked whether regulation of Bax follows a particular expression program in response to general inflammatory stimuli by treating monocytes with the proinflammatory bacterial protein LPS. These analyses revealed that Bax is upregulated in HCMV-infected monocytes compared to mock-infected cells and those treated with the alternative activating agent, LPS (Fig. 5B and C). At 4 hpi, low levels of Bax steady-state mRNA were detected in both mock-infected monocytes and those stimulated with LPS (Fig. 5B). In comparison, we were unable to detect any Bax mRNA in the HCMV-infected population (Fig. 5B). However, by 24 hpi, we saw an increase in Bax mRNA levels in the HCMV-infected monocytes, a trend that continued through 96 hpi (Fig. 5B). This dramatic increase was not observed in the mock-infected or LPS-stimulated cohorts (Fig. 5B). Bax mRNA levels slowly declined in the LPS-stimulated groups over the course of infection, while mock-infected monocytes exhibited a slight increase in Bax mRNA through the first 48 h of infection, followed by a decrease in expression beginning at 72 h (Fig. 5B). These results were also mirrored in the subsequent Bax protein analysis; we see that Bax expression is upregulated in HCMV-infected versus mock-infected monocytes beginning at 48 hpi (Fig. 5C), consistent with an increase in Bax mRNA beginning at 24 h.

HCMV-induced upregulation of Bax drives the increase in Bcl-2 expression, an event which is required to inactivate Bax and rescue infected cells from apoptosis. We next asked if the increased expression of Bax beginning at 48 hpi and the Mcl-1-to-Bcl-2 regulatory shift during that same time frame were parallel events occurring during HCMV infection, or whether Bax upregulation might be driving the upregulation of Bcl-2. Thus, we examined the regulation of Bcl-2 in HCMV-infected monocytes in the absence of high levels of Bax expression. We performed an siRNA knockdown of Bax and then examined Bcl-2 expression by Western blotting at 72 hpi, a time point at which Bcl-2 expression in HCMV-infected monocytes is increased in comparison to that in mock-infected monocytes. As expected, Bcl-2 was upregulated in the HCMV-infected monocytes nucleofected with a control siRNA in comparison to their mock-infected counterparts (Fig. 6A, panel 1). When HCMV-infected cells were nucleofected with a Bax-specific siRNA, however, Bcl-2 expression levels were abrogated to near mock-infected levels (Fig. 6A, panel 1), suggesting that Bcl-2 is upregulated in response to an induction of Bax. The counterexperiment (Fig. 6A, panel 2), in which we knocked down Bcl-2, had no effect on Bax expression. To further test our model that Bcl-2 is upregulated at later times postinfection to specifically counter the proapoptotic effect of Bax, we next sought to evaluate the functional effects of Bcl-2 antagonism in the absence of Bax. Monocytes were mock infected, HCMV infected, or stimulated with the proinflammatory agent LPS before proapoptotic Bax was knocked down and/or the binding activity of Bcl-2 was inhibited with ABT-199. We used an MTT assay to monitor cell survival over a 96-h time course of infection. When the survival of mock-infected monocytes is compared to that of HCMV-infected monocytes over time, we see that knocking down proapoptotic Bax in HCMV-infected monocytes rescues them from the apoptotic effects of inhibiting antiapoptotic Bcl-2. The survival

rate of HCMV-infected monocytes lacking both increased Bax expression and active Bcl-2 (78% to 90% at all time points) is restored to near that seen in the DMSO control-treated HCMV-infected groups bearing the HCMV-induced ratio of Bcl-2 to Bax (85% to 90% at all time points) (Fig. 6C). Indeed, we observe an increase from 60% survival in HCMV-infected cells treated with ABT-199 alone to 80% survival in ABT-199-treated Bax knockdown groups at 48 hpi (Fig. 6C), the time point at which we first see a sizeable increase in Bax protein expression (Fig. 5C). This trend continues through the later time points, with an increase from 40% survival to 78% survival at 72 hpi and from just under 40% survival to 75% survival at 96 hpi (Fig. 6C). The rescue effect achieved by depleting Bax in addition to inhibiting Bcl-2 activity is less pronounced in the mock-infected cohorts at later times postinfection. When comparing Bcl-2-deficient, mock-infected monocytes that are either mock nucleofected or nucleofected with a Bax-specific siRNA, we observe an increase in survival from ~20% in the presence of Bax at both 72 and 96 hpi to only 45% and 40%, respectively, when Bax is depleted. There is, however, a similar rescue effect to what is seen in the HCMV-infected groups at the earlier time points (survival increased from 45% to 80% at 24 hpi and from 20% to 60% at 48 hpi) (Fig. 6B). These data serve to highlight the apparent requirement for both Mcl-1 and Bcl-2 prior to the 48-h viability gate in normal monocytes in comparison to a requirement for only Mcl-1 early and Bcl-2 late in HCMV-infected cells, consistent with our findings in Fig. 3B and C. These data are also consistent with the higher levels of Bax mRNA observed in mock-infected cells at early time points than at later time points (Fig. 5B). In LPS-stimulated groups, neither Bcl-2 inactivation nor Bax depletion appears to have a substantial effect on monocyte survival (Fig. 6D), suggesting that the biphasic regulation of antiapoptotic Bcl-2 family proteins and the interplay between Bax and Bcl-2 are not the result of a general inflammatory response in monocytes.

DISCUSSION

To effectively use monocytes as viral carriers, HCMV must extend the survival of infected cells. This virally directed process of creating long-lived, HCMV-infected monocytes/macrophages requires HCMV to overcome at least two distinct biological hurdles: the intrinsic apoptotic programming of monocytes to undergo cell death within 48 to 72 h of entering the circulation (49, 50), and the cellular antiviral proapoptotic response to infection (58, 59). In the absence of viral antiapoptotic proteins, a consequence of monocytes being initially nonpermissive for viral gene replication, HCMV appears to have evolved a mechanism whereby it alters the expression of cellular Bcl-2 family proteins in order to navigate these critical cellular checkpoints and escape apoptosis. By comparing the long-term survival of infected monocytes to that of mock-infected and LPS-stimulated control populations, we have uncovered a number of exciting findings that point to an HCMV-specific survival strategy by which the virus uses receptor-ligand signaling to establish a unique expression pattern of cellular proteins involved in cell survival (Fig. 7). (i) We have identified a critical 48-h viability checkpoint in monocytes and the existence of two distinct survival strategies on either side of that gate. (ii) We see a biphasic regulation of cellular Bcl-2 family proteins where Mcl-1 promotes early survival of infected cells prior to the 48-h checkpoint and Bcl-2 promotes late survival. (iii) We have demonstrated a level of HCMV specificity in the biphasic regulation of

FIG 6 HCMV-induced upregulation of Bax drives the increase in Bcl-2 expression, an event which is required to inactivate Bax and rescue infected cells from apoptosis. (A) Monocytes were mock infected or HCMV infected (MOI = 5) for 1 h and then nucleofected with a nontargeting control siRNA, a Bax-specific siRNA (panel 1), or a Bcl-2-specific siRNA (panel 2). Whole-cell lysates were collected after 72 h of incubation at 37°C. Western blot analysis was performed to analyze expression levels of Bax and Bcl-2. Actin was used as a loading control. Results are representative of three repeats using monocytes from different donors. (B to D) Isolated monocytes were mock infected (B), HCMV infected (MOI = 5) (C), or stimulated with 20 μ M LPS for 1 h (D) and then nucleofected with nontargeting control siRNA (circles and triangles) or a Bax-specific siRNA (squares and inverted triangles) and plated in 96-well microtiter plates. Following adhesion, cells were treated with DMSO (circles and squares) or 6 μ M ABT-199 (Bcl-2 antagonist) (triangles and inverted triangles). MTT analysis was used to assess cell viability at each of the indicated time points. Data are expressed as percent cell survival normalized to 100% at 0 h (immediately following ABT-199 treatment). Results are the average of three replicates from a single experiment and are representative of data analyzed from two additional repeats with different blood donors.

Bcl-2 family proteins by comparing HCMV-infected monocytes with those governed by classical adhesion-dependent survival signals or stimulated with an alternative inflammatory agent, LPS. (iv) We have shown that the individual early and late survival mechanisms are controlled by divergent signaling events (EGFR early and β 1/ β 3 integrins late) involving two cellular receptors that must work in concert for viral entry and for many of the functional and molecular changes we have previously described in HCMV-infected monocytes (55–57). (v) We have discovered that the Mcl-1-to-Bcl-2 regulatory shift is influenced by the potential

binding interactions of cellular Bcl-2 family proteins and that, specifically, an increase in proapoptotic Bax at later times postinfection prompts the HCMV-induced late upregulation of cellular Bcl-2.

In vivo studies indicate that monocytes have a life span of 1 to 3 days in the blood (50). Within the first 72 h of entering the circulation, the monocyte must make a critical cell fate decision, either committing to undergo monocyte-to-macrophage differentiation or fulfilling its natural “default” biological programming to undergo apoptosis (49, 50). The relatively short duration of this cell

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FIG 7 Our model of the role of cellular Bcl-2 family proteins in the survival of HCMV-infected monocytes. Viral binding to EGFR enhances Mcl-1 expression levels in infected monocytes (49), allowing cells to successfully navigate the early cell viability checkpoint by blocking the cleavage of proapoptotic caspase-3 (47). HSP-27 is also upregulated in response to viral infection to ensure complete control over caspase-3 activation at early times postinfection, when cell survival is of primary importance. At later times postinfection, when survival and differentiation become equally important to the viral persistence strategy, Mcl-1 expression is extinguished (49), and integrin-mediated signaling controls Bcl-2 expression in infected monocytes. Bcl-2 is upregulated in HCMV-infected monocytes beginning at 48 hpi to assume the role of Mcl-1 as an inhibitor of caspase-3 activation; however, without additional inhibition by HSP-27, a low-level activation of caspase-3 allows monocyte-to-macrophage differentiation without causing apoptosis. This shift from Mcl-1 to Bcl-2 as the principal antiapoptotic player occurs in response to an upregulation of the proapoptotic protein, Bax, beginning at around 48 hpi. We speculate that this increase in Bax occurs as part of the antiviral proapoptotic response to HCMV infection. In contrast, the majority of mock-infected cells undergo apoptosis at the 48-h checkpoint. For those that survive due to adhesion-dependent signals, it appears that both Mcl-1 and Bcl-2 are required prior to the viability gate for continued survival. LPS-stimulated monocytes appear to engage an unknown alternative survival mechanism, as our data show that Bax/Bcl-2 interactions do not affect survival outcomes in these cells.

type is likely a crucial control measure meant to prevent any potentially harmful effects of having an unchecked population of activated proinflammatory immune cells. Nonetheless, the limited life span of monocytes represents a significant hurdle that HCMV must overcome in order to utilize the cells as carriers for viral dissemination. We are able to model the biological realities of a short-lived monocytic life span with our *ex vivo* system in order to address questions that are central to HCMV's viral survival and persistence strategy. Indeed, we have identified a critical mono-

cyte viability checkpoint at 48 h following isolation from the peripheral blood that mirrors the *in vivo* 24- to 72-h life span (49). We see that distinct survival strategies are engaged by HCMV on either side of this 48-h viability gate and the separate mechanisms involve distinct virus-induced signaling events as well as the usage of varied cellular players.

In this work, we describe a distinct biphasic regulation of cellular antiapoptotic Bcl-2 family proteins that promotes long-term survival of HCMV-infected monocytes (Fig. 7). We have previ-

ously shown that HCMV promotes early survival of infected monocytes by upregulating the antiapoptotic protein Mcl-1 (49). Our previous work shows that freshly isolated monocytes under homeostatic conditions initially exhibit high levels of Mcl-1, followed by a rapid decline in its expression around 48 h following isolation (49), consistent with the *in vivo* life span of these cells (50). Indeed, we speculate that Mcl-1 functions as a molecular clock in this cell type to ensure a controlled population of unstimulated monocytes (49). This idea is consistent with what is known about the antiapoptotic role of Mcl-1 in hematopoietic cells (77) and the way in which dysregulation of Mcl-1 contributes to the development of myeloid (78–80) and other cancers (81–86). Mcl-1 was first identified as a result of its overexpression during TPA (12-O-tetradecanoyl phorbol 13-acetate)-induced differentiation of a human myeloid leukemia cell line (ML-1) (87). Subsequent studies of Mcl-1 have classified the protein as a member of the Bcl-2 family of mitochondrial membrane permeability regulators and defined a dual role for Mcl-1 in myeloid cells: regulating both cell survival and differentiation (77, 88). It is the prosurvival function of Mcl-1 that appears to drive the development of myeloid leukemias and other cancers (79, 81–85, 89) in addition to contributing to their drug resistance (89–91). We have shown that HCMV upregulates Mcl-1 in a transient manner, effectively usurping its prosurvival function to promote early survival of HCMV-infected monocytes (49). Mcl-1, in concert with heat shock protein 27 (HSP-27), prevents the proteolytic cleavage and activation of caspase-3 (47) during the early hours of HCMV infection, ensuring strict control of the apoptosome and allowing infected cells to bypass the critical viability checkpoint at 48 h (49). Mcl-1 expression levels decline through the 48-h checkpoint (49), allowing basal levels of caspase-3 activation (below a threshold that induces cell death), which our data show is required for commencement of monocyte-to-macrophage differentiation (47). The present study addresses the following question: what molecular player(s) is involved in promoting long-term enhanced survival of HCMV-infected monocytes once Mcl-1 expression is extinguished? Our new data show that Bcl-2 expression is increased during the time frame surrounding the 48-h viability window (Fig. 2) to promote the long-term survival of HCMV-infected monocytes (Fig. 3). Our data demonstrate an increase in Bcl-2 steady-state mRNA beginning at 24 h (Fig. 2A) and an increase in Bcl-2 protein beginning at 48 h (Fig. 2B) in HCMV-infected monocytes. Although the upregulation of Bcl-2 mRNA appears to be far more pronounced than the increase in Bcl-2 protein expression, these differences can be accounted for by our observation that Bcl-2, in addition to being upregulated following HCMV infection, is also cleaved into a smaller, inactive form (DNS), an event which we believe serves to balance two potential roles for Bcl-2 in HCMV-infected monocytes: survival and monocyte-to-macrophage differentiation (48). Although there is also an upregulation of Bcl-2 in mock-infected cells (Fig. 2B), this is likely caused by a low-level activation of cellular integrins as a result of monocyte adhesion to fibronectin-coated cell culture plates. Even so, the increase in Bcl-2 expression is more pronounced in the HCMV-infected cohorts (6-fold versus 2-fold in mock infected), indicative of higher activation levels of $\beta 1/\beta 3$ integrin signaling following viral binding. This transition from Mcl-1 to Bcl-2 as the principal mediator of infected-cell survival allows for continued tight regulatory control over the proteolytic processing and activation of caspase-3, resulting in basal expression levels of active

caspase-3 (Fig. 4) that we propose are balanced in a mechanism that allows monocyte-to-macrophage differentiation without inducing cell death.

Although the functional roles of Bcl-2 and caspase-3 as governors of the apoptotic process are described in the literature, we believe that the unique findings from this study are the distinct biphasic regulation of Bcl-2 family proteins in HCMV-infected monocytes and the mechanisms of action that seem to promote this specific expression pattern. The shift from Mcl-1 to Bcl-2 as the primary antiapoptotic player in HCMV-infected monocytes is in contrast to what we observe in normal monocytes that receive only adhesion-dependent survival and differentiation signals or in alternatively activated monocytes where LPS is used as a general inflammatory response modulator (Fig. 6B to D). By comparing HCMV-infected monocytes to both mock-infected and LPS-treated cells, we are able not only to highlight differences between normal cells and infected cells but also to demonstrate a level of specificity that occurs during HCMV infection. The degree of specificity seen in HCMV-induced survival of infected monocytes is consistent with our observations from a number of previous functional and molecular studies in which we have compared HCMV as a stimulus among a variety of alternative activating agents. We have shown that HCMV induces a rapid proinflammatory response in infected monocytes, as characterized by increases in the production of inflammatory cytokines/chemokines (46, 62) and cellular adhesion molecules (42, 43), as well as by enhanced monocyte motility and transendothelial migration (42, 43, 55, 56). We have long suggested that HCMV evolved to induce the inflammatory response as a means to facilitate viral spread, and although a number of these proinflammatory functional changes are similar to those seen following activation with other proinflammatory agents, many are unique to HCMV, further suggesting that HCMV twists the inflammatory response to maximize proviral effects. For example, we have previously described an enhanced motility of HCMV-infected monocytes (compared to mock-infected monocytes) that surpasses even the level of motility observed following activation with proinflammatory PMA (42, 43). In subsequent studies, we reported that monocytes infected with HCMV produced high levels of an actin nucleator, N-WASP, that is not normally expressed in myeloid cells (55). We propose that upregulation of N-WASP contributes to the greater levels of motility observed in HCMV-infected than in PMA-treated monocytes, as knockdown of N-WASP reduced the motility of HCMV-infected, but not PMA-treated, cells (55). The molecular differences between HCMV-infected monocytes and those treated with alternative activators are perhaps even more pronounced when examining differences in the monocyte-to-macrophage differentiation program (48, 62, 63). The result of monocyte-to-macrophage differentiation is a range of macrophage phenotypes that share some functional characteristics and cytokine expression profiles while differing in other select traits that confer specialized functionality for various tissues and pathogenic responses. We propose that HCMV takes advantage of the inherent plasticity of the monocyte/macrophage system to create an ideal cell type to serve as a viral carrier. In support, we have reported that HCMV-induced monocyte-to-macrophage differentiation creates a macrophage with a distinct transcriptional profile in comparison to that of macrophages differentiated with either LPS or the classical macrophage differentiating agent, M-CSF (48), with some 2,500 genes differentially regulated in HCMV-infected versus LPS- or

M-CSF-treated monocytes. We believe that the distinct monocyte/macrophage created by HCMV infection serves to promote effective viral dissemination while maximizing viral replication and immune evasion. Congruent with these previous reports, we see that the biphasic regulation of Mcl-1 and Bcl-2 does not occur in unstimulated monocytes or as part of a general inflammatory response following aberrant activation of these cells by LPS and may therefore represent a unique mechanism by which HCMV-infected monocytes escape apoptosis.

To further highlight the distinctiveness of the biphasic regulation of Mcl-1 and Bcl-2, which constitutes the survival strategy of HCMV-infected monocytes, we also report a signaling mechanism by which HCMV regulates the expression of these cellular proteins. Specifically, we see that viral binding and signaling through EGFR is responsible for the early upregulation of Mcl-1 (49), while the later upregulation of Bcl-2 is mediated via $\beta 1/\beta 3$ integrin signaling (Fig. 2C and DNS). Our previous data suggest that both distinct and coincidental signaling events along the EGFR and integrin signaling axes are required for viral entry into monocytes and for the biological changes we have previously described as occurring in HCMV-infected monocytes (45, 49, 55–57). For example, viral binding and activation of both EGFR and integrins are required for viral entry into monocytes as well as for the enhanced motility observed in HCMV-infected monocytes (55–57). The overall functional consequence of enhanced motility, however, can be bifurcated into distinct EGFR versus integrin signaling events when examined at the molecular level. HCMV activation of EGFR alone is responsible for upregulation of the actin nucleator N-WASP, an important contributor to the enhanced motility of HCMV-infected monocytes (55). In contrast, the actin cytoskeleton regulator paxillin is upregulated in HCMV-infected monocytes as a result of integrin signaling alone and is an additional mediator of HCMV-infected monocyte motility, as well as a key player in the viral entry process (56). Similarly, we report here that although binding and activation of both EGFR and integrins are required for enhanced survival of HCMV-infected monocytes, the temporally distinct upregulation of Mcl-1 (49) and Bcl-2 (Fig. 2) is the result of differential signaling through either EGFR or integrins alone. Importantly, both EGFR-dependent upregulation of Mcl-1 and integrin-dependent upregulation of Bcl-2 occur largely as a result of viral binding and signaling through cellular surface receptors, an event which we have shown occurs within minutes of infection; however, continued integrin signaling and/or signaling via an unknown non-EGFR/nonintegrin pathway may marginally contribute to the observed increase in Bcl-2 (DNS), as infection with BAD_{wt} lacking the pentameric complex required to efficiently bind integrins resulted in a slight increase in Bcl-2 expression above that in mock-infected cells with low levels of integrin signaling due to adherence. Nonetheless, these data are consistent with our model that Bcl-2 is upregulated via integrin signaling, as infection with BAD rUL131 resulted in a 2-fold increase in Bcl-2 protein. Despite this single binding event, we see differences in Mcl-1 expression in mock-infected versus HCMV-infected monocytes as early as 4 hpi (49) but no remarkable differences in Bcl-2 expression until 48 hpi (Fig. 2B). It is currently unclear to us what causes the delayed regulation of Bcl-2. At the most simplistic level, Bcl-2 expression may be delayed due to differences in translation efficiency between Mcl-1 and Bcl-2 mRNAs. In support, there is an induction of Bcl-2 mRNA in HCMV-infected monocytes over that in mock-infected

controls beginning as early as 4 hpi (Fig. 1B and 2A) but no increase in protein until 48 h (Fig. 2B). Alternatively, the temporal differences in expression could be the result of distinct methods of regulation. For example, concurrent studies in our laboratory demonstrate that the Signal Transducer and Activator of Transcription factor, Stat-1, plays an important dual role in regulating both the survival and differentiation of HCMV-infected monocytes. Perhaps the rapid upregulation of Mcl-1 occurs directly via initial EGFR/PI(3)K signaling events, while Bcl-2 is upregulated as a result of the integrin-dependent activation of Stat-1, thus requiring more time for Stat-1 to translocate to the nucleus and activate target genes.

We have so far reported that the transition from Mcl-1 to Bcl-2 as the principal prosurvival effector is a distinct event occurring during HCMV infection that is regulated via differential signaling events through the EGFR and integrin signaling axes. In addition, we have discovered that the Mcl-1-to-Bcl-2 shift seems to be a consequence of differential binding potentials among proapoptotic and antiapoptotic Bcl-2 family proteins. Specifically, we report that the late upregulation of antiapoptotic Bcl-2 appears to be driven by an increase in proapoptotic Bax expression (Fig. 6A). Bax is expressed at high levels in response to viral infection beginning at around 48 hpi (Fig. 5C), with an increase in steady-state Bax mRNA as early as 24 hpi (Fig. 5B). In response, Bcl-2 is upregulated in HCMV-infected monocytes to combat the Bax-mediated antiviral proapoptotic cellular response (Fig. 6). Although we see some changes in Bak expression following infection, preliminary data suggest that Bak is not the apoptotic regulator following infection that Bax is in our system (DNS). Perhaps Bak dictates changes in the LPS-treated cells (future experiments will address this issue). We propose that HCMV-infected monocytes must undergo a shift from Mcl-1 (which cannot bind and inactivate Bax) to Bcl-2 (which can bind and inactivate Bax) as the principal antiapoptotic player in order to combat the antiviral proapoptotic cellular host defense response that occurs following HCMV infection. Of note, Bax could also potentially be bound and inactivated by multiple other antiapoptotic Bcl-2 family proteins (A1, Bcl-xL, and Bcl-w), yet Bcl-2 is the only such protein that is upregulated at later times postinfection (Fig. 2B; and confirmed by PCR and Western blot analysis [DNS]). As such, it stands to reason that Bcl-2 may be the preferential antiapoptotic player later during the course of HCMV infection because it is able to fulfill a second functional role other than promoting cell survival. We speculate that Bcl-2 may play a dual role in the HCMV-infected monocyte: promoting extended survival and driving monocyte-to-macrophage differentiation. Recent studies hint that the monocyte-to-macrophage differentiation program parallels an incomplete apoptotic process, at least from a molecular standpoint, and that Bcl-2, along with various caspases, may play an important role in the myeloid differentiation process (74, 92). As such, it is not surprising that a number of cellular factors seem to be involved in both of these interconnected processes (47, 49, 71, 74). Future studies in our laboratory will be directed at exploring the intimate link between survival and differentiation of HCMV-infected monocytes as well as the potential for players like Mcl-1, Bcl-2, and the caspases to function as dual regulators of both monocyte survival and monocyte-to-macrophage differentiation.

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