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Research Article

HCMV activation of ERK-MAPK drives a multi-factorial response promoting the survival of infected myeloid progenitors

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Abstract

Viral binding and entry provides the first trigger of a cell death response and thus how human cytomegalovirus (HCMV) evades this – particularly during latent infection where a very limited pattern of gene expression is observed – is less well understood. It has been demonstrated that the activation of cellular signalling pathways upon virus binding promotes the survival of latently infected cells by the activation of cell encoded anti-apoptotic responses. In CD34+ cells, a major site of HCMV latency, ERK signalling is important for survival and we now show that the activation of this pathway impacts on multiple aspects of cell death pathways. The data illustrate that HCMV infection triggers activation of pro-apoptotic Bak which is then countered through multiple ERK-dependent functions. Specifically, ERK promotes ELK1 mediated transcription of the key survival molecule MCL-1, along with a concomitant decrease of the pro-apoptotic BIM and PUMA proteins. Finally, we show that the elimination of ELK-1 from CD34+ cells results in elevated Bak activation in response to viral infection, resulting in cell death. Taken together, these data begin to shed light on the poly-functional response elicited by HCMV via ERK-MAPK to promote cell survival.

Introduction

Amongst the first host responses activated upon viral infection are cell autonomous immune responses. These functions are ubiquitous to every cell type and are normally triggered by pattern recognition receptors which detect various components of the incoming pathogen (Bieniasz 2004; Everett & Chelbi-Alix 2007; Randow *et al.* 2013). One aspect of the cell autonomous immune response is the activation of apoptotic and cell death pathways, which will ultimately eliminate both the pathogen and the infected cell (Clem *et al.* 1991; Everett & McFadden 1999; Levine *et al.* 1993). During lytic infection, human cytomegalovirus (HCMV) counters this with an arsenal of virally encoded anti-apoptotic functions that counter stress signals induced by viral binding and, subsequently, throughout the course of viral replication (Brune 2010; Goldmacher *et al.* 1999; Guo *et al.* 2015; Reeves *et al.* 2007; Skaletskaya *et al.* 2001; Stevenson *et al.* 2014; Terhune *et al.* 2007). However, a lack of expression of these functions in non-lytic infections raised the question of how cell survival is achieved – particularly during the initial phases of infection.

Current studies hypothesise that viral infection in cells non-permissive for lytic infection requires the generation of a pro-survival phenotype driven by virally induced up-regulation of key cellular anti-apoptotic proteins (Chan *et al.* 2010; Peppenelli *et al.* 2016; Reeves *et al.* 2012; Stevenson *et al.* 2014). This event relies on the regulation of a number of pro-survival and pro-death signals via the modulation of cellular signalling pathways initiated upon virus binding and entry (Chan *et al.* 2010; Reeves *et al.* 2012). It is the outcome of these signalling events which, ultimately, determines the fate of the cell during these very early stages of infection. Once latency is established, there are additional mechanisms activated which would be consistent with the latent virus propagating an anti-apoptotic state (Poole *et al.* 2011, 2015; Slobedman *et al.* 2004).

Apoptosis and cell death is an evolutionarily conserved process that is extensively regulated by Bcl-2 homology domain 3 (BH3) proteins (Doerflinger *et al.* 2015; Horvitz 1999; Puthalakath & Strasser 2002). The precise mechanism of action still remains equivo-

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cal although it is clear that the BH3 proteins trigger the activation of key apoptosis effector proteins (e.g. Bak and Bax) which exert their pro-apoptotic function predominantly at mitochondrial membranes (Wei *et al.* 2001; Westphal *et al.* 2014). A number of regulatory mechanisms have been suggested: The indirect activation model posits that Bax/Bak are required to be retained in an inactive form via direct sequestration by anti-apoptotic BCL-2 family members (Willis *et al.* 2005) and that BH3 proteins must engage with these to release the Bax/Bak to initiate apoptosis (Uren *et al.* 2007; Willis *et al.* 2007). The direct activation model hypothesises that BH3-only activators (e.g. PUMA and BIM) bind to Bak/Bax directly to activate them or, alternatively, other BH3 members (e.g. Bad) target BCL-2 members and inhibit their anti-apoptotic function through sequestration (Doerflinger *et al.* 2015; Erlacher *et al.* 2006; Kuwana *et al.* 2002; Villunger *et al.* 2003; Wei *et al.* 2000). The most recent evidence argues that these events are not mutually exclusive and that, instead, both mechanisms of regulation are likely to be active (Llambi *et al.* 2011; Westphal *et al.* 2014) although the strict delineation between activators and sensitisers in the BH3 family may not be entirely valid (Westphal *et al.* 2014).

Our own research interests have centred on the BCL-2-like protein myeloid cell leukaemia-1 (MCL-1). Unlike other BCL-2 proteins the regulation of MCL -1 is dynamic with a turnover of 30 minutes under certain experimental conditions (Adams & Cooper 2007; Warr & Shore 2008) and it is thus considered a highly responsive determinant of haematopoietic cell viability (Perciavalle & Opferman 2013) – the ablation of this protein from progenitor cells of the haematopoietic lineage being lethal (Opferman *et al.* 2005; Perciavalle & Opferman 2013). MCL-1 (along with BCL-XL) has been suggested to block Bak activity to exert an anti-apoptotic function (Willis *et al.* 2005) and is regulated by both the PI3K and ERK-MAPK pathways (Huang *et al.* 2000; Mills *et al.* 2008). Pertinently, MCL-1 is also regulated by HCMV through activation of the ERK and PI3K pathways in a cell type specific manner (Chan *et al.* 2010; Reeves *et al.* 2012). Depletion of MCL-1 from monocytes or THP1 cells renders them unable to prevent virally induced cell death upon infection (Chan *et al.* 2010; Reeves *et al.* 2012). In CD34+ cells, HCMV survival is also associated with MCL-1 (Reeves *et al.* 2012) although the absolute requirement for endogenous MCL-1 in normal CD34+ cells rendered similar genetic analysis through MCL-1 deletion intractable. The inducible survival effect in CD34+ cells required virus binding and was likely dependent on the engagement of glycoprotein B with an unknown receptor. Furthermore, the survival effect induced was transient (Reeves *et al.* 2012) and thus did not appear to be propagated for as long as was observed in the monocyte model of infection (Chan *et al.* 2010).

ELK-1 is part of a 27 member superfamily of transcription factors that dictate a diverse range of processes including haematopoiesis, differentiation and survival, oncogenesis and inflammation (Sharrocks 2001). ELK-1 is a nuclear phosphoprotein that exhibits bimodal activity: redundant promoter binding via dimers with other Ets transcription factors and specific binding to a subset of genes through an interaction with serum response factor (SRF) (Odrowaz & Sharrocks 2012; Sharrocks 2001). Amongst these genes are MCL-1 and c-Fos, which, characteristically for this subset of genes, are dynamically regulated (Boros *et al.* 2009; Treisman *et al.* 1992). The activation of ELK-1 requires phosphorylation of serine residue 383 promoting the recruitment of transactivating co-factors to promoters in order to drive transcription (Boros *et al.* 2009; Gille *et al.* 1992, 1995). Furthermore, ELK-1 mediated activation of MCL-1 gene expression has been shown to be important for survival in a number of experimental models (Booy *et al.* 2011; Demir *et al.* 2011; Sun *et al.* 2013; Townsend *et al.* 1999) and likely contributes to the role of ELK-1 in cancer.

Herein we wanted to further explore the consequences of HCMV infection on the development of an anti-apoptotic phenotype in CD34+ cells based on our previous studies of MCL-1 and ERK-MAPK signalling. Here we show that the HCMV protection from cell death is concomitant with an inhibition of prolonged Bak activation in response to chemical and viral insult. Secondly, we show that the protective phenotype driven by virus induced ERK-MAPK signalling correlates with the down-regulation of pro-apoptotic BH3 proteins PUMA and BIM and, concomitantly, is dependent on the phosphorylation and thus activation of ELK-1 – a transcription factor important for MCL-1 expression and cell survival. Importantly, knock down of ELK-1 expression is sufficient to abrogate the protective effect elicited by HCMV infection. Taken together, these data suggest that HCMV infection drives a survival phenotype by simultaneously up-regulating MCL-1 proteins levels and reducing the levels of antagonistic interaction partners. We propose that the net effect tips the balance in favour of survival contributing to the successful establishment of latent infection.

Materials and Methods

Virus, cell lines, culture and reagents

The Merlin strain of HCMV was purified from infected human fibroblasts as previously described

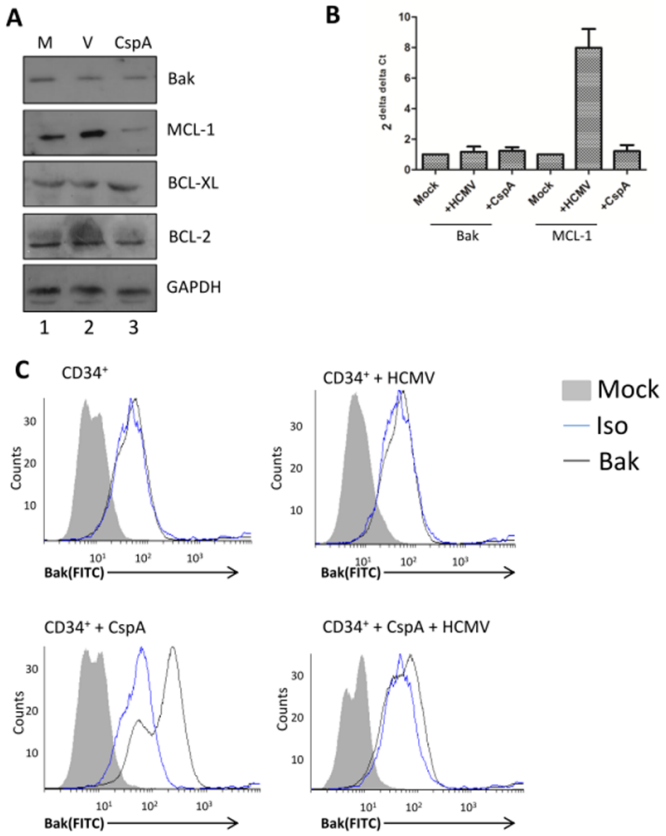

Figure 1. HCMV infection blocks cisplatin A mediated activation of Bak. (A) Western blot analysis of CD34+ cells either mock (M), HCMV infected (V) or cisplatin A treated (CspA) for Bak, MCL-1, BCL-XL, Bcl-2 or GAPDH expression 2 hours post-infection. (B) qRT-PCR analysis of RNA isolated from mock, HCMV infected or cisplatin A treated cells for Bak or MCL-1 expression 2 hours post-infection. Changes in gene expression were identified using GAPDH and $2^{-\Delta\Delta CT}$ method. n=3 (C) CD34+ cells were mock or HCMV infected and then incubated with DMSO or cisplatin A 3 hours post-infection. Cells were then permeabilised and stained with an antibody that specifically recognises an N terminal peptide exposed in activated Bak protein or with an isotype matched control. Fluorescent staining was achieved using a FITC-Goat anti-mouse antibody and then cells analysed by flow cytometry.

(Compton 2000). Primary CD34+ haematopoietic cells (Lonza, Slough, UK) were resuscitated for 24 hours prior to any studies of cell viability in X-vivo-15 (Biowhittaker, Lonza, Slough, UK) media supplemented with 10% human serum. For all subsequent studies, cells were switched into serum-free X-vivo media supplemented with 2mM L-Glutamine. For all infection experiments an MOI of 5 (based on titration in fibroblasts) was used.

Inhibition of the ERK-MAPK pathway was achieved using U0126 (final concentration 1uM; Calbiochem/Millipore, Darmstadt, Germany). The inhibitor was added directly to the culture media with 0.1% DMSO used as the solvent control 1 hour prior to virus infection.

Nucleic acid isolation, reverse transcription, PCR and Western Blot

Ten micrograms of DNase I treated RNA isolated using RNAeasy spin columns was reverse transcribed using ImpromII RT kit (Promega, Madison, WI) or Quantitect Reverse Transcription Kit (Qiagen, Hilden, Germany). Chromatin Immunoprecipitations were performed as previously described (Kew *et al.* 2014). Briefly, 10^6 cells were fixed and lysed and then subjected to sonication to shear DNA into 200-500bp fragments. DNA was then incubated with anti-ELK1 antibody (Cell Signaling, Danvers, MA; 1:100 dilution), anti-phospho ELK-1 antibody (Cell Signaling, Danvers, MA; 1:100 dilution) or an isotype matched control (mouse IgG1; SIGMA, St Louis, MO). DNA was rescued from the IP complexes and amplified with MCL-1 promoter specific primers.

Gene and promoter specific primers were then used to amplify target sequences by real time PCR using SYBR green amplification kit (Qiagen, Hilden, Germany). MCL-1 (gene): 5'-TGC AGG TGT GTG CTG GAG TAG and 5'-GCT CTT GGC CAC TTG CTT TTC', GAPDH: 5'-GAG TCA ACG GAT TTG GTC GT and 5'-TTG ATT TTG GAG GGA TTC TCG, 18S: 5'- GTA ACC CGT TGA ACC CCA and 5'- CCA TCC AAT CGG TAG TAG CG, Bak: 5'-GCC CAG GAC ACA GAG GAG GTT TTC and 5'-AAA GTG GCC CAA CAG AAC CAC ACC, Bim: 5'-CAC AAA CCC CAA GTC CTC CTT and 5'-TTC AGC CTG CCT CAT GGA A, Puma: 5'-ACG ACC TCA ACG CAC AGT ACG and 5'-TGG GTA AGG GCA GGA GTC C, UL138: 5'- GAG CTG TAC GGG GAG TAC GA and 5'- AGC TGC ACT GGG AAG ACA CT; MCL-1 (promoter): 5′-TAG GTG CCG TGC GCA ACC CT and 5′-ACT GGA AGG AAG CGG AAG TGA GAA (Booy *et al.* 2011).

For Western Blot, 10^5 cells were lysed in Laemmli buffer and subjected to SDS-PAGE electrophoresis. Following transfer, blots were incubated with anti-MCL-1 (Cell Signaling, Danvers, MA; 1:500), anti-ELK-1 or anti-phospho-ELK-1 (phosphor-serine 383; Cell signaling, Danvers, MA; both 1:750), anti-Bak (#06-536, Millipore, Darmstadt, Germany; 1:500), anti-PUMA (Santa Cruz, CA, 1:200) anti-actin (Abcam, Cambridge, UK; 1:1000) or anti-GAPDH (Abcam, Cambridge, UK; 1:2000) for 1 hour followed by detection with the appropriate HRP-conjugated secondary antibody. Specific bands were visualized by ECL detection (Amersham, Horsham, UK).

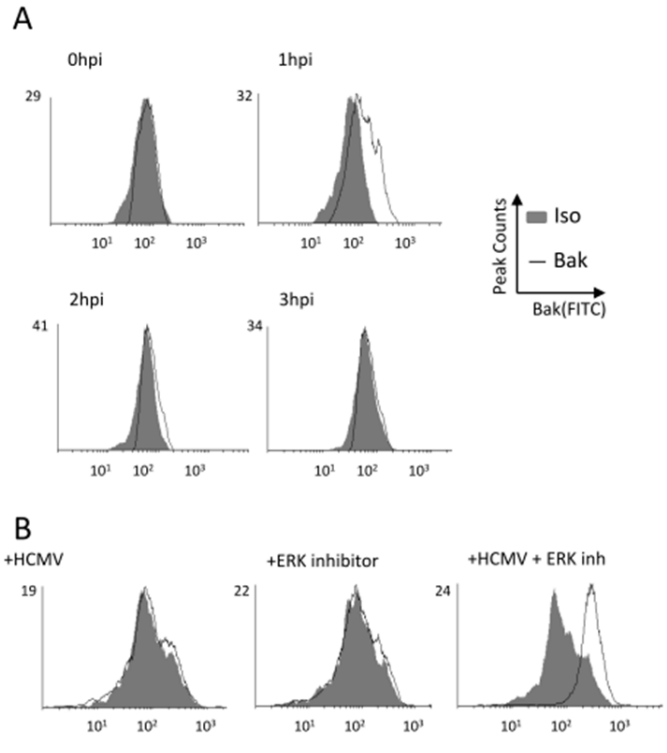

Figure 2. HCMV infection induces a transient activation of Bak which is not reversed when ERK responses are inhibited. (A) CD34+ cells were infected with HCMV and then at times 0 to 3 hours post infection cells were permeabilised and stained for evidence of Bak activation by flow cytometry. (B-C) CD34+ cells were either pre-treated with an ERK inhibitor (B) or subjected to ELK1 or control siRNA knockdown (C) and then either mock of HCMV infected. Cells were then permeabilised and stained for evidence of Bak activation by flow cytometry.

siRNA knockdown

CD34+ cells were transfected with Silencer® ELK-1 siRNAs or Silencer® negative control (Thermofisher, Waltham, MA) using Viromer Green transfection reagent as described by the manufacturer (lipocalyx/ Cambridge Biosciences, UK). CD34+ cells were transfected in bulk in X-vivo serum free media and then plated at the required density for downstream analyses at 48 hours post-transfection. Prior to plating, the viromer:siRNA mix was removed from the cell culture 4 hours post-transfection by pelleting the CD34+ cells at 300g followed by resuspension in fresh X-vivo 15 media.

Cell Death assay and Bak activation

Induction of cell death was achieved using the chemotherapeutic drug cisplatin A (Rosenberg *et al.* 1965). Cisplatin A (25uM-250uM; SIGMA-Aldrich, St Louis, MO) or 0.1% DMSO (mock) control was added for 4 hours to trigger the apoptotic pathway. Since the effects of cisplatin A induced cell death are not evident until at least 12-24 hours post treatment (Barry *et al.* 1990) cell viability was performed 21 hours after cisplatin A addition. To determine levels of cell death, cells were stained with a TUNEL detection kit (Roche, Basel, Switzerland) as described by the manufacturer and analysed for apoptotic cell death by immune-fluorescent microscopy.

Alternatively, cell death was induced using the virus as a ligand through blockade of ERK-MAPK signaling (U0126; 1uM, Calbiochem) 1 hour prior to infection with HCMV. 0.1% DMSO was used as solvent control.

Bak activation in CD34+ cells by flow cytometry was measured as previously described (Griffiths *et al.* 1999). Briefly, 10^5 cells were incubated with rabbit serum for 20 minutes and then with an anti-Bak antibody directed against the N terminal region (#06-536, Millipore, Darmstadt, Germany; 1:50 dilution in PBS) or an isotype matched control for 15 minutes at 4°C. Cells were washed, incubated with goat serum for 20 minutes and then incubated with a goat anti-rabbit FITC conjugated secondary antibody (1:100 dilution in PBS) for 15 minutes at 4°C. Unstained, isotype stained and Bak stained cells were then analysed by flow cytometry.

Assays for latent infection

CD34+ cells were infected with HCMV 48hrs post treatment with siRNAs in X-vivo-15 media for 3 hours. Cells were pelleted, supernatant removed, washed in PBS and then re-suspended in fresh X-vivo media and cultured for 3 days. RNA was then isolated, converted to cDNA and amplified in a UL138 (viral gene) and 18S (cellular gene) specific PCR. Changes in gene expression were calculated using $2^{-\Delta\Delta CT}$ method (UL138 and 18S RNA).

Results

HCMV infection reduces the level of activated Bak in cisplatin A treated cells

Our previous study had shown that HCMV infection could block the induction of CD34+ cell death in response to the chemotherapeutic drug, cisplatin A (Reeves *et al.* 2012). Cisplatin A-induced degradation of survival molecules and the triggering of cell death are functionally entwined events (Yang *et al.* 2007). Here we show that in CD34+ cells, cisplatin A promotes the degradation of pro-survival MCL-1, but not the related BCL-XL or Bcl-2 proteins (Figure 1A). As expected, HCMV infection promoted MCL-1 up-regulation but, interestingly, no effect on BCL-2 or BCL-XL was again observed (Figure 1A). A key target of MCL-1 is Bak. However, no impact on the total lev-

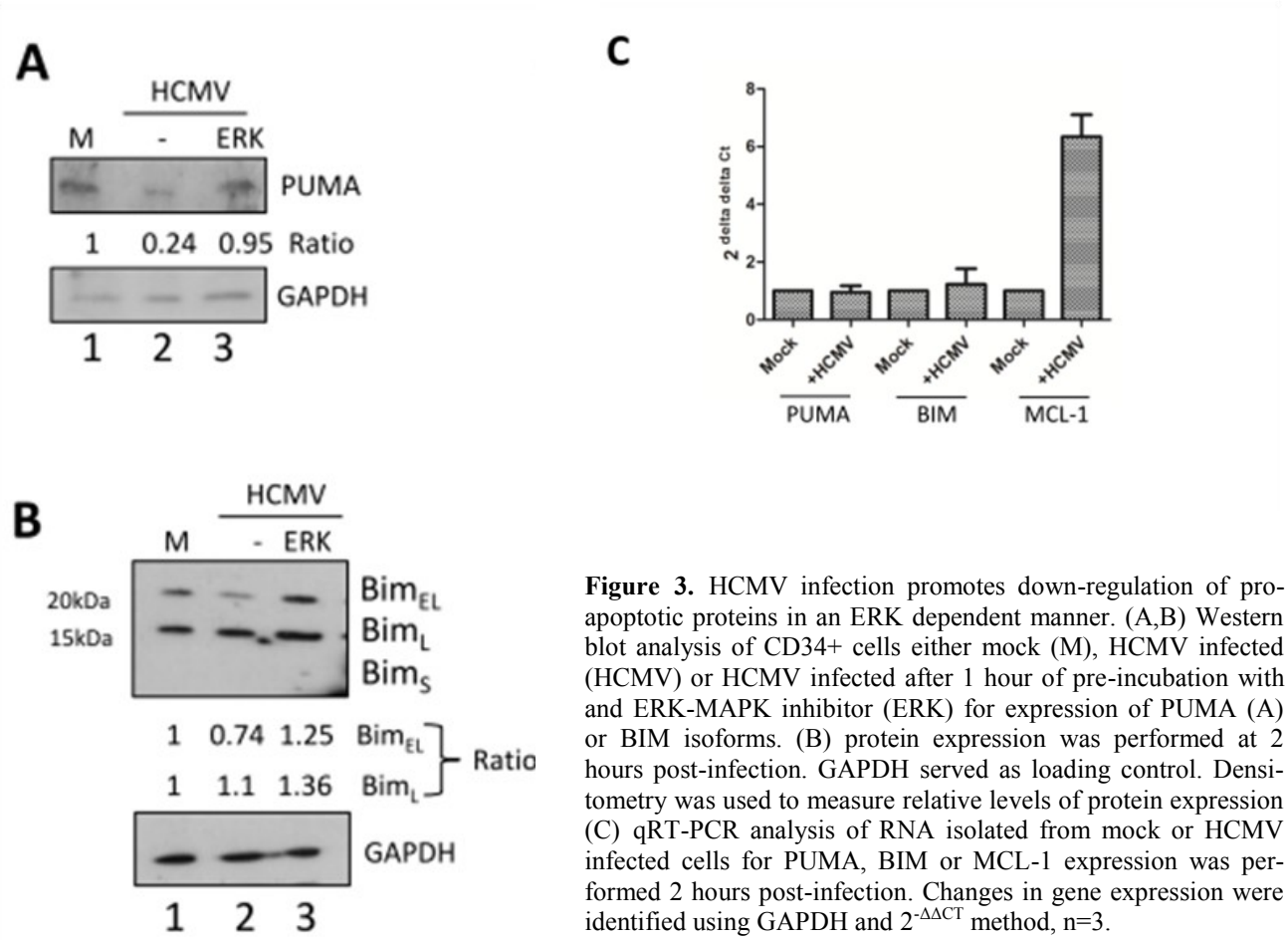

Figure 3. HCMV infection promotes down-regulation of pro-apoptotic proteins in an ERK dependent manner. (A,B) Western blot analysis of CD34+ cells either mock (M), HCMV infected (HCMV) or HCMV infected after 1 hour of pre-incubation with and ERK-MAPK inhibitor (ERK) for expression of PUMA (A) or BIM isoforms. (B) protein expression was performed at 2 hours post-infection. GAPDH served as loading control. Densitometry was used to measure relative levels of protein expression (C) qRT-PCR analysis of RNA isolated from mock or HCMV infected cells for PUMA, BIM or MCL-1 expression was performed 2 hours post-infection. Changes in gene expression were identified using GAPDH and $2^{-\Delta\Delta CT}$ method, n=3.

els of Bak were observed whether treated with cisplatin A or infected with HCMV (Figure 1A). Similarly, no impact on the transcription of Bak was detected at these early times post-infection (Figure 1B). In contrast, HCMV infection was a clear inducer of MCL-1 transcription (Figure 1B). We next investigated the activation of Bak and although levels in the total protein did not change, evidence of increased activation of Bak in response to cisplatin A was detected (Figure 1C). The specific activation of Bak can be detected by flow cytometry utilising the conformational change that exposes Bak epitopes when active (i.e. pro -apoptotic) Bak is released from the inactive complex it is normally sequestered in. As expected, resting CD34+ cells show very little evidence of Bak activation (Figure 1C) consistent with their viability status. In contrast, stimulation of CD34+ cells with cisplatin A resulted in a substantial increase in the level of activated Bak detectable in the cells (Figure 1C). Pertinently, minimal levels of active Bak were detectable in HCMV infected cells at 3hpi. Crucially, pre-infection with HCMV prior to cisplatin A treatment markedly reduced the detection of active Bak (Figure 1C) - consistent with the protective phenotype of HCMV against cisplatin A. Taken together, these data suggested HCMV engendered a cellular phenotype that was directly antagonistic to cisplatin A induced cell death.

HCMV activation of ERK-MAPK is necessary to prevent prolonged activation of Bak upon infection

To investigate this further we first asked whether Bak activation occurred during viral infection – which would underpin the need to promote a cellular environment that antagonises Bak activation under normal infection conditions. Intriguingly, a time course analysis revealed that Bak was clearly activated in infected cells at 1hpi and that, over time, the level of Bak activation was reversed by 3hpi (Figure 2A).

To explore this further we next exploited the knowledge that inhibition of survival pathways triggered by HCMV promoted the death phenotype (Reeves *et al.* 2012). CD34+ cells were pre-incubated with ERK-MAPK inhibitor for 1 hour prior to infection (Figure 2B). Cells were then stained for Bak activation. As expected, there was little evidence of Bak activation at 3hpi in infected cells or cells treated with ERK inhibitor alone. However, the infection of cells with inhibited ERK-MAPK signalling resulted in a

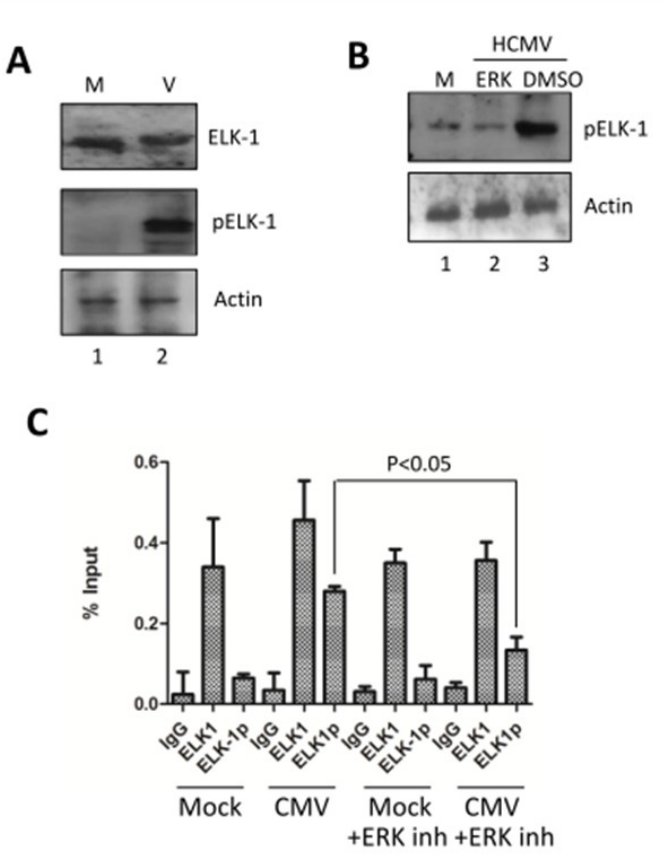

Figure 4. HCMV targets ELK1 for phosphorylation in an ERK dependent manner. (A) Western blot analysis of CD34+ cells, either mock (M) or HCMV infected (V) for ELK1 and ELK1 phosphorylation, 2 hours post-infection. Actin served as loading control. (B) Western blot analysis of CD34+ cells, either mock (M) or HCMV infected (HCMV) cells, with or without prior incubation with ERK-MAPK inhibitor (ERK) or DMSO control for 1 hour at 2 hours post-infection. Actin served as loading control. (C) Chromatin immuno-precipitation assays were performed on CD34+ cells with isotype (IgG), ELK1 or phosphor-ELK1 (ELK-1p) antibodies. ChIPs were performed on CD34+ cells, HCMV infected CD34+ cells or the equivalent but with prior incubation with U0126 ERK inhibitor for 1 hour. Cells were analysed at 2 hours post infection, n=3. Students t-test was used to test for significance at $p<0.05$.

clear Bak activation phenotype (Figure 2B) and thus clearly pheno-copied the cisplatin effect. Taken together, the data show that HCMV infection activates Bak which it then reverses via the concomitant activation of ERK-MAPK signalling.

HCMV modulates the activity of multiple Bak-interacting functions in protected cells

The activation of Bak in the mitochondrial membrane is elicited through a complicated interplay between effector and inhibitor mechanisms (Llambi *et al.* 2011). Having already observed changes to the levels of the MCL-1 regulator we next asked whether any impact on activators of Bak were triggered by HCMV. Two key pro-apoptotic molecules reported to be upstream of Bak in this pathway are the pro-apoptotic PUMA and BIM BH3 proteins (Westphal *et al.* 2014). Western blot analyses indicated that basal levels of both PUMA and 2 of 3 BIM isoforms (Bim_{EL} and Bim_L) were detectable in uninfected cells. Furthermore, HCMV infected cells analysed at 1hpi suggested that a down-regulation of both PUMA and Bim_{EL} was evident. In contrast no changes in Bim_L were detected (Figure 3A, B). We also noted that the highly pro-apoptotic isoform Bim_S (Marani *et al.* 2002) was not detectable nor was expression induced upon infection. The down-regulation of both PUMA and BIM_{EL} products was specific to the protein as no effect on RNA levels was observed (Figure 3C). Finally, the reduction in both PUMA and BIM_{EL} levels by HCMV was dependent on ERK-MAPK activity (Figure 3A, B).

Thus HCMV activation of ERK-MAPK was promoting several related effects that would be conducive for cell survival – BIM isoform and PUMA degradation alongside an up-regulation of MCL-1 levels. MCL-1 protein levels within the cell are regulated by multiple processes. As well as increased transcription, MCL-1 is also regulated post-translationally with ERK hypothesised to promote a stabilising phosphorylation event of MCL-1 that antagonises degradation. Thus to investigate the potential contribution of transcription and translation of MCL-1 in response to HCMV, we reasoned we were required to understand the basis of HCMV induced up-regulation of MCL-1 mRNA expression.

Key elements responsible for the regulation of the MCL-1 promoter include binding sites for the ELK family of proteins (Booy *et al.* 2011). Western blot analysis of infected cells revealed that although HCMV binding did not increase the levels of ELK-1 in the cell, there was an apparent mobility shift which would be indicative of ELK-1 phosphorylation. This was confirmed using a phosphor-ELK1 specific antibody (Figure 4A). We next showed that the phosphorylation, and thus activation of ELK-1, was dependent on ERK-MAPK signalling (Figure 4B). To link this clear activation of ELK-1 upon infection with MCL-1 expression we performed chromatin immunoprecipitation analyses. These revealed that the ELK1 protein was bound to the MCL-1 promoter in CD34+ cells irrespective of whether they were virally infected or not (Figure 4C). Although the data suggested that more ELK1 was bound in virally infected cells, it was the analysis of phosphorylated ELK1 bound to the MCL-1 promoter which exhibited the clearest phenotype. Here, viral infection promoted an increase in the

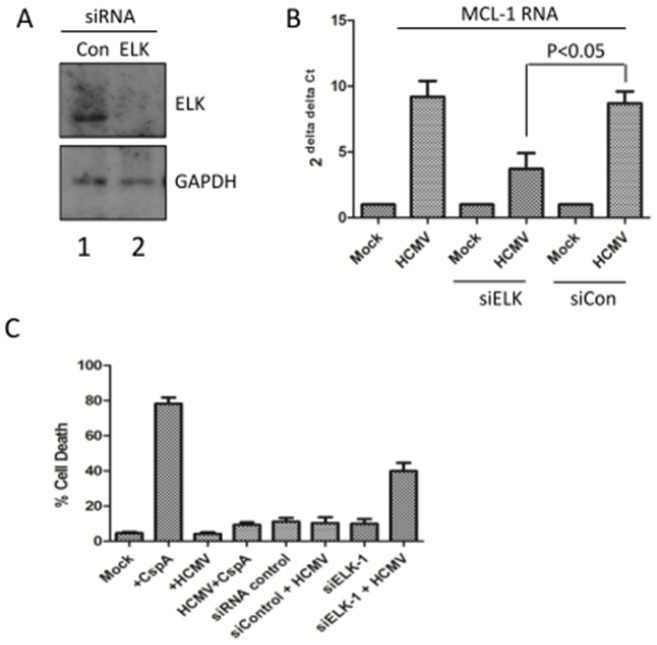

Figure 5. Depletion of ELK1 from CD34+ cells abrogates the HCMV survival response. (A) Western blot analysis of CD34+ cells 48 hours post-transfection with a control (Con), ELK1-specific (ELK) siRNA for ELK1 and GAPDH expression. (B) qRT-PCR analysis of RNA isolated from mock or HCMV infected cells 2 hours post-infection that have first been treated with either mock, control (Scr KD) or ELK1 (ELK KD) siRNAs. Changes in gene expression were identified using GAPDH and $2^{-\Delta\Delta CT}$ method, n=3. Students t -test was used to test for significance at $p<0.05$. (C) CD34+ cells were either mock or HCMV infected and then, 3 hours post-infection, incubated with cisplatin A or DMSO control. Alternatively, CD34+ cells were transfected with control or ELK-1 specific siRNAs and then either mock or HCMV-infected. In all experiments cell viability was measured 24 hours post-infection. Graph is the average of 2 independent experiments analysed in triplicate.

detectable levels of pELK1 at the MCL-1 promoter and this event was significantly dependent on ERK signalling (Figure 4C).

To test whether ELK-1 was directly involved in the pro-survival phenotype we used siRNA knockdown in CD34+ cells to address this. Delivery of ELK-1 siRNAs significantly reduced ELK-1 protein present in the cell (Figure 5A) but did not result in any overt effects on cell viability over the short time frame of analysis (Figure 5C). Consistent with the ChIP data (Figure 4C), ELK-1 knockdown in the CD34+ cells dramatically impacted on the ability of HCMV to up-regulate MCL-1 RNA expression (Figure 5B). Together with the phenotypic impact on MCL-1 gene expression, there was a clear abrogation of the virally induced survival response (Figure 5C). Although the delivery of ELK-1 siRNAs alone was not deleterious to the cell, viral infection triggered a marked increase in cell death (Figure 5C) indicating that the elimination of ELK-1 from CD34+ cells renders them more sensitive to HCMV induced cell death.

ELK-1 knockout cells show elevated levels of Bak activation upon HCMV infection resulting in a less efficient establishment of latency

Next, we revisited our studies of Bak activation using our ELK-1 knock down cells (Figure 6A). Unsurprisingly, ELK-1 depletion from cells had little impact on levels of Bak activation. However, infection of ELK-1 knock down cells again resulted in an increased detection of activated Bak (Figure 6A). Although the analysis suggested that not all cells displayed evidence of activated Bak – which may reflect relative efficiency of siRNA knock down – the data clearly suggested that removal of ELK-1 from CD34+ cells promoted Bak activation in response to HCMV infection.

Finally, to assess if this was having any impact on the virus we assessed the ability of HCMV to establish latency (Figure 6B). Control or ELK-1 siRNA treated cells were infected with HCMV and analysed 3 days post-infection for evidence of latent viral gene expression. It was evident that the failure to prevent virally induced cell death manifested with significantly reduced levels of UL138 expression, which would be consistent with a failure to establish a latent infection.

Discussion

In this study we have investigated the phenotype of the pro-survival environment elicited by infection of CD34+ cells with HCMV. This and previous studies provide accumulating evidence that HCMV is required to promote an anti-apoptotic environment through the activation of signalling pathways in non-permissive cells in order to ensure long term survival (Chan *et al.* 2010; Peppenelli *et al.* 2016; Reeves *et al.* 2012; Stevenson *et al.* 2014). These events are likely critical for the pathogenesis and dissemination of virus in monocytes and the establishment of latency in CD34+ cells.

The requirement for a pro-survival signalling cascade is necessitated by the evident induction of pro-death responses which likely represent anti-viral responses to infection. This induction of pro-death responses is assumed to be due to the highly pro-apoptotic phenotype associated with viral infection when the activity of the survival factors is impaired (Chan *et al.* 2010; Reeves *et al.* 2012). Thus HCMV cannot necessarily completely eliminate pro-death signalling but, instead, relies on the concomitant up-regulation of survival factors to counter-balance this. It is of interest that a pan up-regulation of the anti-

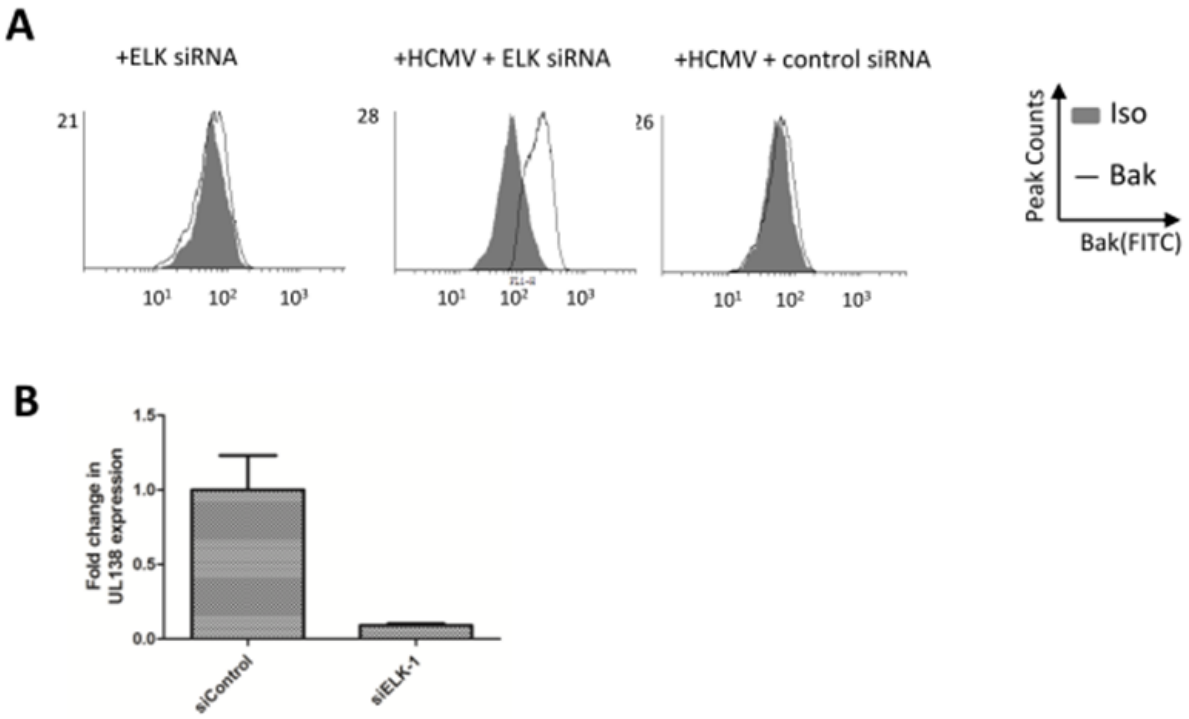

Figure 6. ELK-1 is required for the reversal of Bak activation and the establishment of HCMV latency. (A,B) CD34+ cells were subjected to ELK1 or control siRNA knockdown and then either mock of HCMV-infected. Cells were then either permeabilised and stained for evidence of Bak activation by flow cytometry at 3hpi (A) or analysed 3 days post infection for evidence of viral latent gene expression (B).

apoptotic machinery is not evident in the infected CD34+ cells. An analysis of 3 major players MCL-1, BCL-XL and BCL-2 revealed that only MCL-1 levels were affected by HCMV infection during the initial phases of latent infection in this cell type. However, we cannot dismiss a role for the other proteins completely, as the localisation or binding to target pro-apoptotic proteins of these other BCL-2 family members may be modified to augment the virally induced survival observed. Furthermore, our studies have really focussed on the role of MCL-1 as a transitory regulator during the very initial stages of infection. It is important to note that data from studies performed in the monocyte model have shown that BCL-2 activity becomes increasingly important to maintain the viability of the infected cells (Collins-McMillen *et al.* 2015), suggesting that different molecules are required at different stages of viral infection. Consistent with this, reports in progenitor model systems have suggested that PEA-15 up-regulation via the activity of interleukin-10 could play a role in long term latency in CD34+ cells (Poole *et al.* 2011, 2015; Slobedman *et al.* 2004). In this regard, the apparent targeting of the MCL-1 member of the BH3 family during the very early stages could be consistent with the central role that MCL-1 plays in the regulation of apoptosis in haematopoietic cells (Opferman *et al.* 2005) and also reflective of its more dynamic regulation in the cell (Adams & Cooper 2007). Unlike counterparts (e.g. Bcl -2, Bcl-Xl), MCL-1 has a much shorter half-life (Adams & Cooper 2007). Ablation of MCL-1 results in spontaneous cell death in a number of cell lineages, indicating that, firstly, endogenous levels of other BCL -2 proteins are not sufficient to compensate and secondly, it has a central role in haematopoietic cell survival (Dzhagalov *et al.* 2008; Opferman 2007; Opferman *et al.* 2005). This exquisite sensitivity of primary cells to MCL-1 levels may also involve the complex interaction of MCL-1 with mitochondria (Huang & Yang-Yen 2010; Perciavalle *et al.* 2012). As well as a classical role in apoptosis, MCL-1 has also been shown to regulate ATP biogenesis (Perciavalle *et al.* 2012) which we know, from studies of lytic HCMV infection, has a profound effect on the viability of infected cells (Reeves *et al.* 2007). Whether the impact of MCL-1 on mitochondrial bioenergetics is important here is not clear but, given the relatively short lived nature of the protection in the experimental conditions, it is perhaps unlikely. It is more plausible to have a role either in the MCL-1 mediated protection of monocytes where elevated MCL-1 expression persists for 72 hours (Chan *et al.* 2010) or, it could possibly augment the function of beta 2.7 during lytic HCMV infection.

Cisplatin A mediated MCL-1 degradation is a

trigger for apoptosis (Yang *et al.* 2007) and thus tumours exhibiting resistance to cisplatin A often display elevated levels of MCL-1 that is resistant to drug induced degradation (Michels *et al.* 2014). It is hypothesised that the degradation of MCL-1 exposes Bak to the activity of pro-apoptotic proteins like PUMA and BIM, ultimately resulting in Bak-mediated mitochondrial dysfunction (Letai *et al.* 2002). The precise nature of the inhibitory effect of MCL-1 on Bak is debated: MCL-1 could bind to Bak directly to block activation or could block activation by sequestering pro-apoptotic BH3 proteins like PUMA and BIM or, in fact, perform both functions (Erlacher *et al.* 2006, Letai *et al.* 2002; Marani *et al.* 2002; Willis & Adams 2005).

Another possibility is that although PUMA and BIM are pro-apototic proteins themselves and thus viral induced degradation is a mechanism to eliminate a direct activator, another consequence of the down-regulation of these proteins could be contribute to increasing the level of 'free' MCL-1 in the cell. Thus the up-regulation of MCL-1 and the down-regulation of a binding partner that sequesters it increase the likelihood of an interaction with Bak. Additionally, very recent data suggests that the interaction of MCL-1 with Bak is induced under conditions that promote Bak oligomerisation/activation (Dai *et al.* 2015). Thus, viral infection – an apparent trigger of Bak activation – promotes a concomitant increase in MCL-1 levels which likely counters this. Indeed, it is interesting to note that when Bak activation was analysed at multiple points during the initial infection, we detected evidence of Bak activation at very early times which was quickly reversed.

Many of these observations were dependent on virally induced ERK-MAPK signalling. Viral induced activation of ERK-MAPK signalling and subsequent survival is a recurring theme suggesting that this pathway is a common target (Dai *et al.* 2016; Liu & Cohen 2013; Pleschka 2008; Pontes *et al.* 2015). The multifactorial response driven by ERK may also partially explain the observation that ELK-1 activity, whilst important, was never as detrimental as inhibition of upstream ERK signalling. Despite this, the induction of ELK-1 phophorylation was another virally induced response that was dependent on ERK-MAPK signalling. Again, this had a pro-survival effect, whereby elimination of ELK-1 phosphorylation, and thus activation (Cruzalegui *et al.* 1999), either through inhibition of ERK-MAPK or via a siRNA depletion of ELK-1 was deleterious, specifically upon virus infection. We note that evidence of ELK-1 binding to the MCL-1 promoter prior to any stimulation was observed and, although viral infection provided evidence of increased occupancy of ELK-1, phosphorylation of ELK-1 was most important for MCL-1 transcription (Booy *et al.* 2011; Cruzalegui *et al.* 1999). The observed occupancy of ELK-1 in an unphosphorylated (presumably inactive) form at the promoter may suggest only a partial involvement of ELK-1 in basal MCL-1 expression in the CD34+ cells but, instead, being important for dynamic responses to further stimuli. It would also be consistent with ELK-1 activation representing a mechanism for rapidly inducing MCL-1 expression (Booy *et al.* 2011; Vickers *et al.* 2004). This stimulus-specific activity would explain the virally induced effects on MCL-1 and, also, the transient nature of the elevated MCL-1 expression (Reeves *et al.* 2012). The virus triggers a burst of ERK-MAPK activity upon entry promoting ELK-1 phosphorylation but once ERK signalling is down-regulated through normal feedback mechanisms (Fritsche-Guenther *et al.* 2011) coupled with a loss of the initial virus binding induced signal as the virus enters the cell, then MCL-1 expression returns to basal levels. The occupancy of inactive or even inhibitory (e.g. p50 homodimers) forms of transcription factors at promoters represents a mechanism for rapid induction of gene expression and has been observed at multiple promoters (Altarejos & Montminy 2011; Baer *et al.* 1998; Herrera *et al.* 1989) including in our own studies of CREB and HCMV reactivation (Kew *et al.* 2014). Finally, although our analyses focused on the regulation of MCL-1 we cannot preclude the possibility that ELK-1 activates multiple responses (Boros *et al.* 2009) that contribute to survival upon viral infection. Indeed, pathogens often utilise central components in pathways, thus it would not be unsurprising to detect further ELK-1 controlled responses that are important.

Prescient to this study is that ERK-MAPK signalling under certain conditions can, for example, be pro-apoptotic (Cagnol & Chambard 2010; Cagnol *et al.* 2006; Lu & Xu 2006) and thus in future studies it will be interesting to determine how HCMV directs the ERK response - possibly via the activation of concomitant pathways - to generate the pro-survival phenotype observed. Put simply, the kinase pathway that implements the final effector function (i.e. ERK-MAPK) is not dictating the outcome alone - that decision is defined by the nature of the signalling milieu activating the ERK-MAPK module upstream and the signalling and molecular context that those pathways are being activated in. Thus an aim of future studies is to address the identity of the death signals that are activated by the host cell in response to viral infection.

What this study illustrates is that viruses are the master regulators of signalling cascades and pathways with an impressive ability to hijack, re-direct or partition them to enhance infection. Understanding

these events has greatly improved our understanding of cell biology and shed new light on the mechanisms that govern the activity of cellular functions required in key biological processes. Furthermore, it also illustrates how pleiotropic signalling pathways are modified to generate very specific outputs downstream. Greater understanding of these events and how they contribute to cell survival in the context of pathogen infection also has broader implications on our knowledge regarding the decision a cell makes to live or die.

Conflicts of interest

The authors declare no conflicts of interest associated with this work.

Authors' contributions

VGK, MRW and MBR performed experiments and MRW and MBR analysed the data and wrote the paper.

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