TITLE

Human cytomegalovirus protein UL135 mediates myelosuppression of hematopoietic progenitor cells

ABSTRACT

Human cytomegalovirus (HCMV) is a main cause of morbidity and mortality for patients undergoing transplantation due to its suppression of hematopoiesis down the myeloid lineage (myelosuppression). The molecular mechanisms by which HCMV dysregulates hematopoietic progenitor cell (HPC) causing myelosuppression is largely unknown. We have identified a viral protein, UL135, that is required for HCMV-mediated myelosuppression of infected HPCs. A mutant virus lacking UL135 fails to induce myelosuppression compared to wildtype virus. We have previously shown that UL135 downregulates the epidermal growth factor receptor (EGFR) expression and alters EGFR endocytic trafficking by interacting with host adaptor proteins CIN85 and Abi-1. EGFR is a major signaling hub with many downstream signaling pathways that regulate lineage-specific transcription factors and are intricately involved in many aspects of HPC maintenance and differentiation. Therefore, *we hypothesize that UL135, through host interactor proteins CIN85 and Abi-1, downregulates EGFR downstream signaling and expression of myeloid-lineage-specific transcription factors, causing myelosuppression*. We will test this hypothesis through two specific aims: **Aim 1. Determine how UL135-CIN85/Abi1 interactions affect downstream EGFR signaling and HPC differentiation.** Since UL135 is suppressive towards EGFR expression, we will determine how downstream EGFR signaling is affected by UL135 and identify pathway(s) involved in myelopoiesis that are most affected. Successful completion of this aim will provide insight into specific host targets implicated in UL135-induced myelosuppression. **Aim 2. Determine the role of UL135 in modulating the expression of myeloid lineage transcription factors in HPCs during infection.** HPC myelopoiesis is tightly regulated by several myeloid lineage transcription factors. In this aim, we will identify the transcription factors that are differentially expressed in the presence of UL135, thereby altering the lineage decision process in HPCs leading to myelosuppression. We expect that these findings will provide some of the first mechanistic insights into HCMV-mediated myelosuppression.

SPECIFIC AIMS

Human cytomegalovirus (HCMV) is a leading cause of morbidity and mortality for patients undergoing solid organ transplantation and hematopoietic stem cell transplantation. This is in part due to HCMV-mediated suppression of hematopoiesis in the myeloid lineage (myelosuppression), where $CD34^+$ hematopoietic progenitor cells (HPCs) infected with HCMV produce significantly fewer myeloid progenies compared to uninfected HPCs. The molecular mechanisms by which HCMV causes myelosuppression are largely unknown.

We have identified a viral protein, UL135, that is required for HCMV-mediated myelosuppression of infected HPCs. Using colony forming unit (CFU) assay to measure differentiation potential of infected HPCs, we observed that HCMV fails to induce myelosuppression when UL135 expression is disrupted. UL135 has many host and viral protein interactors, including adaptor proteins CIN85 and Abi-1, which regulate the cytoskeleton, endocytic trafficking and signaling. Both adaptor proteins have roles in pathways important for HPC maintenance and differentiation, namely epidermal growth factor receptor (EGFR) signaling and trafficking. UL135 interactions with these adaptor proteins are required for UL135-mediated turnover of EGFR in HPCs. How these interactions modulate UL135-mediated myelosuppression is unknown.

The *long-term goal* of this proposal is to identify the mechanism by which HCMV modulates differentiation of hematopoietic cells leading to myelosuppression. *Our central hypothesis is that UL135, through host interactor proteins CIN85 and Abi-1, downregulates EGFR downstream signaling and expression of myeloid-lineage-specific transcription factors, causing myelosuppression*.

Aim 1: Determine how UL135-CIN85/Abi1 interactions affect downstream EGFR signaling and HPC differentiation

EGFR signaling is important for maintaining HPC stemness. EGFR total and surface expression is high in HPCs and downregulation of EGFR signaling is often coupled with differentiation. Since UL135 promotes EGFR turnover and downregulates EGFR signaling in HPCs, we will further determine how this affects EGFR downstream signaling and HPC differentiation. *We hypothesize that UL135 downregulates EGFR downstream signaling to drive HPC differentiation and viral replication, causing myelosuppression*. To directly measure EGFR and downstream signaling activity, namely PI3K/AKT, MEK/ERK, and STAT signaling, in primary $CD34^+$ HPCs during infection, we will use phospho-flow cytometry combined with immunoblotting of phosphorylated proteins. To elucidate the roles of these signaling pathways in myelopoiesis, we will use the CFU assay to determine the differentiation potential of HPCs under chemical inhibition of EGFR and downstream signaling, as well as HPCs infected with a virus defective in forming CIN85 and Abi-1 interactions (ΔUL135-CIN85/Abi-1). We expect that the completion of this aim will reveal the major signaling pathways downstream of EGFR that HCMV UL135 targets to induce myelosuppression in HPCs.

Aim 2: Determine the role of UL135 and UL135-CIN85/Abi1 interactions in modulating the expression of myeloid lineage transcription factors in HPCs during infection

HPC differentiation is tightly regulated by key lineage transcription factors (TFs) that are specifically expressed during the lineage commitment process of the progenitor cells. PU.1 and CEBPα are the two main myeloid lineage TFs that are essential to granulocyte-monocyte lineage differentiation. Since HCMV infected progenitor cells are specifically deficient in differentiation down the myeloid lineage, w*e hypothesize that UL135, through host interactions, downregulates the expression of myeloid lineage transcription factors, such as PU.1 and CEBPα, inducing myelosupression*. To test this hypothesis, we will measure the transcript and protein levels of PU.1 and CEBPα throughout a time course before and after HPC differentiation in HPCs infected with wildtype or mutant viruses defective in UL135 expression (UL135stop) and Abi1/CIN85 interactions (ΔUL135-CIN85/SH3cI). We expect that the expression levels of PU.1 and CEBPα will be lower in the myelosuppressed WT-infected HPCs compared to HPCs infected with UL135 mutant viruses that are not myelosuppressed. Next, we will use adenoviral vector constructs with Tet transactivator system to overexpress PU.1 and CEBPα in WT-infected HPCs to attempt restoration of myelopoiesis. We will measure the differentiation potential of the transduced and WT-infected HPCs using CFU assays to determine whether overexpression can restore myelopoiesis. If TFs can restore myelopoiesis in WT-infected HPCs, it will further confirm these TFs as primary host targets of UL135 in HCMV-induced myelosuppression.

At the completion of these aims, we *expect* to have defined the molecular mechanisms by which UL135 modulates HPC signaling to induce myelosuppression. These results will provide some of the first mechanistic insights into myelosuppression induced by HCMV, which could pave the way for the development of effective therapeutics for patients undergoing solid organ and blood stem cell transplantation.

SIGNIFICANCE & SCIENTIFIC PREMISE

Human cytomegalovirus (HCMV) is a ubiquitous beta-herpesvirus that infects between 40 and 99% of the world's population[1]. HCMV is the leading cause of disease-associated congenital infections in the United States, affecting 1 in every 150 children born every year[2]. The majority of the infected population consist of asymptomatic carriers, where the virus persists for life, often in a latent state where the viral genomes are maintained in the infected host cell without active production of viral progenies. However, the quiescent, latent viral genome can become reactivated. Reactivation from latency can lead to grave consequences for the immune-suppressed such as stem cell transplant (SCT)[3] and solid organ transplant (SOT) recipients[4]. HCMV infection is associated with a higher risk of acute graft rejection in SOT patients receiving heart, lung, kidney, and liver transplants[5] as well as bone marrow hyperplasia and myelosuppression in SCT patients[6]. Despite remaining high on the list of serious global health burdens and desginated top priority for vaccine development[7], there is no vaccine or cure. The currently available antivirals used for SCT patients such as ganciclovir can exacerbate myelosuppression and only target cells harboring actively replicating viruses[8]. *In order to develop effective therapeutics against HCMV-associated morbidity and mortality after transplantation, we need to address the gap in our knowledge of how HCMV dysregulates homeostasis of latently infected cells at the molecular level*.

HCMV is highly host-specific, thereby limiting the use of animal models. However, within its natural host, the virus can infect a wide variety of cell types, establishing different modes of infection depending on the permissiveness of the tissue. Certain primary cell types have emerged as excellent *in vitro* models that recapitulate viral expression patterns from clinical samples[9]. In permissive cells such as fibroblasts, there is a robust production of virions from the infected host cells. Infection in endothelial and epithelial cells exhibit a slower, active, but significantly decreased level of virus shredding. In $CD34^+$ hematopoietic progenitor cells (HPCs) and monocytes, HCMV establishes latent infection, characterized by the maintennance of viral genome with a low level of gene expression but no production of viral progeny[8]. These cells, especially HPCs, act as an important latent reservoir for the virus. As they undergo differentiation to produce myeloid and lymphoid cells, the virus often becomes reactivated, exiting latency and actively producing viral progeny[10]. Infected $CD34^+$ HPCs have been shown clinically[11] as well as through *in vitro*[12, 13] and *in vivo*[14] models to be myelosuppressed when differentiated, where infected progenitor cells have reduced potential to differentiate specifically down the myeloid cell lineage compared to uninfected progenitor cells. *Our laboratory has developed and established an in vitro assay that closely models HCMV latent infection and reactivation in primary human $CD34^+$ HPCs[15], which strongly positions us to investigate the molecular mechanisms behind HCMV-induced myelosuppression in infected HPCs.*

Utilizing our established $CD34^+$ culturing and infection methods, we performed colony forming unit (CFU) assays on primary $CD34^+$ HPCs infected with WT and various mutant virus constructs to screen for viral genes that are important for myelosuppression. The CFU assay is a classic method to measure the differentiation potential of hematopoietic progenitor cells (HPCs), where HPCs are cultured as single cells suspended in a semi-solid medium rich in cytokines to induce differentiation. After an incubation period of 1-2 weeks, the morphology of the colony formed from each individual cell will be analyzed and quantified by observation under a microscope to determine the type of progenitor cells present in the initial population. We primarily focus on three types of colony forming units: GEMM colony representing the more immature, common myeloid progenitors, GM colony representing granulocyte/macrophage progenitors, and CFU/BFU-E colony representing erythroid progenitors. Using this technique, we have determined that HPCs infected with wildtype virus have a 50% reduction in GEMM/GM colonies compared with uninfected cells, while CFU/BFU-E colony counts are comparable. On the other hand, GEMM/GM

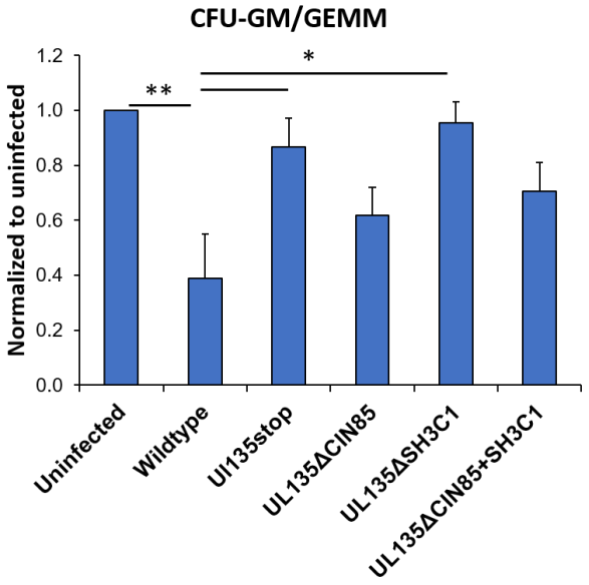


Figure 1. UL135 is required for HCMV-induced myelosuppression of $CD34^+$ HPCs. $CD34^+$ HPCs uninfected or infected with the indicated viruses (MOI=2) were isolated by FACS and plated for CFU assay. At 14 days post plating, the number of CFU-GEMM and CFU-GM colonies were scored for each sample. The total number of colonies were normalized to Uninfected. Asterisks indicate p-values:*, <0.05; **, <0.01.

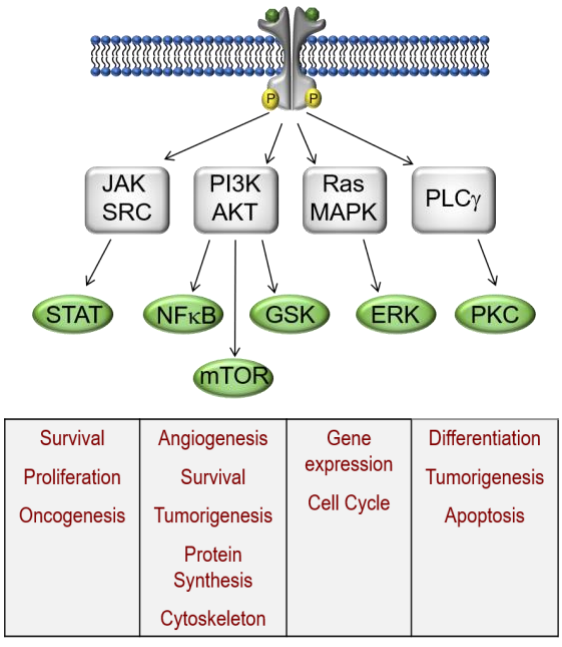


Figure 2. Major EGFR downstream signaling pathways and related cellular processes.

colony formation of HPCs infected with UL135stop virus are comparable to mock-infected cells, suggesting a restoration in myelopoiesis when UL135 is not expressed. (Figure 1.) This key preliminary results indicate that UL135 is required for HCMV-induced myelosuppression in infected HPCs.

In order to further define the complex interactions between HCMV and the host leading to myelosuppression, we will investigate potential host factors involved in HPC differentiation that are targeted by UL135. Epidermal growth factor receptor (EGFR) has recently emerged as a major signaling hub targeted by many herpesviruses including HCMV. EGFR downstream signaling is complex and implicated in virtually all cellular processes such as proliferation, survival, and stem cell differentiation. (Figure 2.) We and others have shown that HCMV targets EGFR signaling pathways in many *in vitro* models (Figure 3.), alternating between positive and negative regulation of EGFR to fine tune signaling processes to facilitate viral establishment of latency as well as productive infection.[16-20] Even though there is growing evidence of viral programs targeting EGFR and its downstream signaling, the specific viral factors and host interactors targeted by HCMV to modulate EGFR signaling are still poorly understood. *This proposal aims to identify specific molecular determinants at the nexus of EGFR signaling, HCMV latency and reactivation programs, and HPC differentiation.*

Toward this aim, we have previously shown that UL135 downregulates EGFR expression[17] and alters EGFR endocytic trafficking by interacting with host adaptor proteins CIN85 and Abi-1[18]. The UL135 protein sequence contains various consensus binding motifs for host interactors. Through a combination of yeast-two-hybrid screening, IP-mass spec, and co-immunoprecipitation experiments, we have identified and validated interactions between UL135 and host proteins CIN85, and Abi-1 (Figure 4). These UL135 host interactors are important for UL135-mediated downregulation of EGFR surface levels in primary $CD34^+$ HPCs (Figure 5), suggesting that they might also affect downstream EGFR signaling in HPCs and related processes of stemness maintenance and differentiation. Therefore, we propose to continue investigating the role of these interactions more specifically in the context of downstream EGFR signaling and HPC differentiation.

APPROACH

AIM 1. Determine how UL135-CIN85/Abi1 interactions affect downstream EGFR signaling and HPC differentiation

EGFR signaling is an essential signaling pathway for the maintenance of HPC stemness. Our laboratory has previously identified viral genes that alter EGFR expression and signaling in HCMV-infected HPCs. Combined with our preliminary data, UL135 has emerged as a viral gene that is implicated in both EGFR signaling and myelosuppression of infected progenitor cells. A virus defective in UL135 protein expression (UL135stop, where the start codon for UL135 is mutated to a stop codon) fails to downregulate EGFR expression (Figure 3) and fails to induce myelosuppression compared to wildtype virus (Figure 1). We have further shown that UL135 regulates the endocytic trafficking and signaling of EGFR through its interactions with CIN85 and Abi-1 adaptor proteins. A mutant viruses with UL135 mutated to eliminate interactions with Abi-1 and CIN85 (Figure 4) failed to downregulate EGFR surface expression in

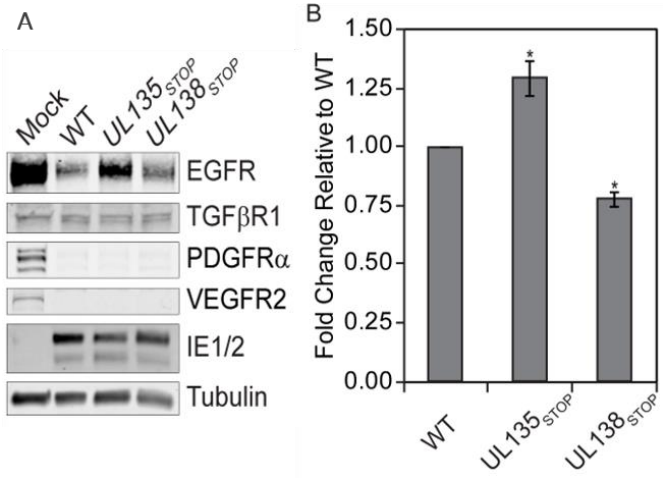


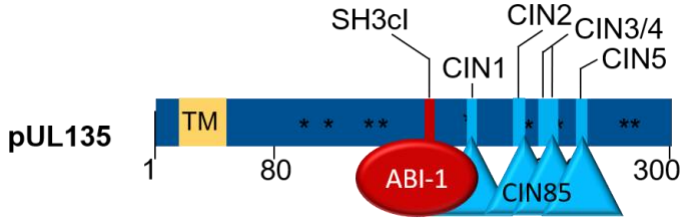
Figure 3. UL135 downregulates EGFR expression in MRC-5 fibroblasts. (A) Total EGFR expression in MRC-5 cells infected with the indicated virus at MOI of 1 by immunoblotting. $UL135_{STOP}$ mutant virus fails to downregulate EGFR expression compared to WT an $UL138_{STOP}$. (B) EGFR surface level expression in infected MRC-5 determined by fluorescently labeled EGF_{647} ligand and flow cytometry. $UL135_{STOP}$ mutant virus also fails to downregulate EGFR surface expression. Asterisks indicate p-values: *, <0.05.

CD34$^+$ HPCs (Figure 5).[18] We will next determine whether these UL135-CIN85/Abi1 interactions play a role in downstream EGFR signaling thereby affecting HPC differentiation.

1.1 Measuring EGFR and downstream signaling activity in HPCs during infection.

Rationale: Previously, our group has tested how productive HCMV infection affects EGFR signaling pathways downstream of EGFR such as the PI3K/AKT, MEK/ERK, and STAT signaling. We found that EGFR and downstream signaling is attenuated in fibroblasts during productive infection with WT virus[16]. Given that UL135 downregulates EGFR expression, we hypothesize that it is also responsible for the observed attenuation of EGFR and downstream signaling during HCMV infection. In this subaim, we will test the role of UL135 and its interactions with Abi-1 and CIN85 in EGFR and downstream signaling during productive infection in fibroblasts as well as latent infection and reactivation in HPCs.

Primary fibroblasts infection experiments: To determine the role of UL135 in modulating EGFR signaling during productive infection and validate our methods, we will use MRC-5 cells as our *in vitro* model for productive replication. We will serum starve the cells for 24 hours then mock infect or infect with wildtype or ΔUL135-CIN85/SH3cI mutant virus at a 1:1 cell numbers to plaque forming unit (PFU) ratio (MOI of 1). At 24, 48, and 72 hours post infection, we will pulse the starved cells with 10 nM EGF (GoldBio) for 15 minutes then collect intact cells detached by Accustase for flow cytometry, or collect whole cell lysates for immunoblotting. These timepoints and pulse durations are chosen based on our previous results from the WT virus experiments. For flow cytometry, cells will be fixed with 2% paraformaldehyde, permeablized with BD Perm/Wash Buffer, then stained with a panel of phospho-antibodies: EGFR (pY1068), PI3K p85 (pTyr458)/p55 (pTyr199), AKT (pS473 and pT308), MEK1/2 (pS217/221), ERK1/2 (pT202/pY204), and STAT3 (pTyr705). In parallel, we will also measure the same phosphorylated species as well as total protein levels (total EGFR, PI3K, AKT1/2/3, MEK1/2, ERK1/2, and STAT3) using immuboblots. A no-pulse control will be included for all samples to determine basal signal level. The combination of these two techniques to measure signaling in fibroblasts will both strengthen our results and validate our approach in a more readily available cell type.



SH3 Ligand Class	Consensus Ligand	UL135 residues	WT Sequence	Mutated Sequence
SH3cI	(R/K)xxPxxP	187-193	KRPPTPP	**R**RPPTP**A**
CIN85	PxxxPR	209-214 238-243 251-256 254-259 277-282	PIPAPR PPVTPR PQKPPR PPRNPR PCPRPR	PIPA**AA** PPVT**AA** PQKP**AA** P**AA**N**AA** PCPR**AA**

Figure 4. Schematic of CIN85 and Abi-1 binding motifs within UL135 and table indicating the mutation changes in the ΔUL135-CIN85/Abi-1 mutant virus. CIN85 has 5 binding sites that are involved in cooperative binding. SH3cI is the binding motif for Abi-1. These interactions have been validated by co-immunoprecipitation experiments (data not shown). TM: transmembrane domain.

CD34$^+$ HPC infection experiments: After establishing these approaches in fibroblasts, we will employ the validated phospho-flow protocol to measure EGFR and downstream signaling in primary CD34$^+$ HPCs during infection, before and after HPC differentiation. We will mock-infected or infect HPCs with wildtype, UL135stop, or ΔUL135-CIN85/SH3cI viruses. All of our virus constructs contain eGFP gene sequence under control of a SV40 promoter leading to expression of GFP upon infection of the host cell. Using GFP as infection marker, we will perform FACS (fluoresence-activated cell sorting) at 1 day post infection to retain only cells that are HCMV-positive. We will then plate sorted cells in long-term culture with supportive mouse stromal cells secreting human cytokines to maintain stemness as previously described[15]. At 10 days post infection (dpi), we will remove cells from long-term culture and add differentiation cytokine cocktail to induce HPC differentiation and viral replication. Throughout this time course, phosphorylated protein levels will be measured by flow at 1, 3, 5, 10, 11, 13, 15 days post infection. The day 1, 3, 5, and 10 timepoints serve to measure change in EGFR and downstream signaling during the establishment of latent infection in HPCs, while day 11, 13, and 15 timepoints will measure signaling during reactivation of virus from latency and HPC differentiation.

Expected outcomes: We have previously demonstrated that during productive infection in fibroblasts, wildtype virus attenuates EGFR signaling and EGF pulse response slowly dampens as infection progresses from day 1 to day 3. Here with the investigation focus on UL135 mutant viruses, we expect that EGFR signaling will not be attenuated and will be sustained for longer due to a defective UL135 protein unable to downregulate EGFR. Similarly, in HPCs, for wildtype virus, we expect to observe sustained EGFR and downstream signaling prior to

HPC differentiation (day 1, 3, 5, 10 time points) as opposed to attenuated EGFR signaling after viral reactivation and HPC differentiation (day 11, 13, 15 time points). The trends for UL135 mutant viruses in HPCs might be more difficult to predict, but we would expect EGFR signaling to be maintained throughout the time course without significant differences between pre- and post-differentiation.

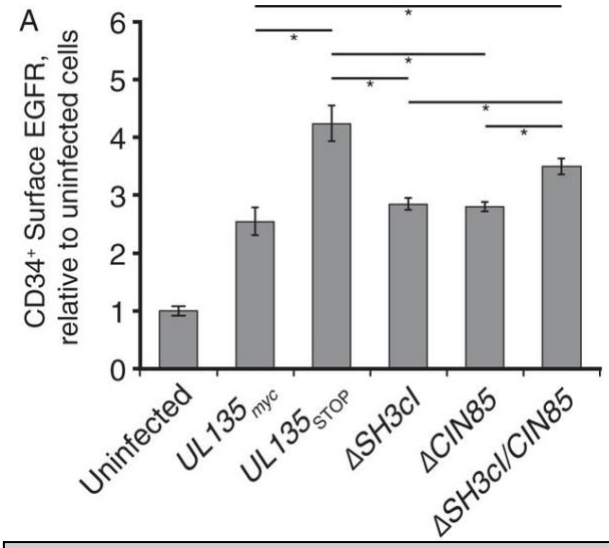


Figure 5. UL135 interactions with CIN85 and Abi-1 regulates EGFR surface levels and reactivation in CD34⁺ HPCs. PrimaryCD34⁺ cells were infected with the indicated viruses at an MOI of 2. 24 hours post-infection, cell surface proteins were stained with EGF_{647} and BV_{421} anti-CD34 antibody prior to analysis by flowcytometry. Asterisks indicate p-values: *, <0.05.

Pitfalls & alternatives: Our previously published data and preliminary experiments strongly support the proposed hypothesis in this aim. However, it is possible that we will not find significant effects of UL135 and its host interactions on EGFR and downstream signaling. In that event, we will explore other pathways that UL135, CIN85 and Abi-1 are known to be involved in that are important to hematopoiesis. For example, UL135 interaction with Abi-1 mediates actin cytoskeleton remodeling, leading to a defect in the formation of the immune synapse[21]. Regulation of the actin cytoskeleton is also implicated in hematopoiesis[22]. Abi-1 has also been shown to be a key mediator of hematopoietic differentiation through the Src/NFkB signling pathway[23]. These are all important signaling avenues that could be explored to identify key molecular components modulated by UL135 during HPC myelopoiesis.

1.2 Determining the differentiation potential of HPCs under chemical inhibition of EGFR and downstream signaling

Rationale: Our previous work investigating the role of signaling pathways downstream of EGFR such as the PI3K/AKT, MEK/ERK, and STAT signaling in HCMV infection has revealed that both PI3K/AKT and MEK/ERK pathways are suppressive toward HCMV replication[16]. To elucidate whether these pathways are also implicated in HPC differentiation, potentiating their roles in HCMV-mediated myelosuppression, we will determine the effects of these pathways on HPC differentiation in uninfected HPCs.

Experiments: In this subaim, we will use the CFU assay developed by STEM CELL Technologies (RRID: SCR_013642) to measure the myeloid differentiation potential of HPCs treated with a range of inhibitors specifically blocking EGFR (Gefitinib), PI3K (LY294002), AKT (MK-2206), MEK (Binimetinib), ERK (SCH772984), and STAT (S3I-201) signaling to elucidate the role of these signaling pathways in HPC differentiation. We have previously used these inhibitors to treat CD34⁺ HPCs in another published work (Figure 6) and a dose-response curve has been determined for each inhibitor[16]. We will plate sorted uninfected CD34⁺ HPCs in the CFU differentiation medium mixed with the appropriate concentration of each inhibitor or DMSO control. Each inhibitor or control treament will have three technical replicates with 250 HPCs in each well, similar to our previous assays. After the recommended 14-day period to allow for differentiation to occur, we will observe each sample under a light microscope and score each type of colonies (myeloid or erythroid) formed to determine the effects of each inhibitor on differentiation outcomes.

Expected outcomes: We expect that inhibition of pathways important for myelopoiesis in HPCs will lead to the myelosuppression phenotype, where the counts of myeloid colonies are significantly reduced, similar to HPCs infected with WT virus. Such results will allow us to identify host pathways and specific targets that might be implicated in HCMV-induced myelosuppression and modulated by UL135. Since EGFR signaling is important for maintaining stemness, we also expect that inhibition of EGFR signaling would reduce differentiation potential of HPCs in uninfected HPCs.

Pitfalls & alternatives: A potential problem is the possible inconsistency of the CFU assay due to its subjective method of counting and identifying colony morphology by observation under the microscope. Should we encounter problems with inconsistent data from this method, we can mitigate the issue by culturing and

inducing HPC differentiation in liquid culture with cytokines instead of the semi-solid medium. Differentiation phenotypes can then be analyzed by staining with lineage-specific cell surface marker antibodies and processed on a flow cytometer. Secondly, if we are unable to identify any specific EGFR downstream effector that is important for hematopoiesis, we will broaden our search using the antibody phospho array such as the Full Moon BioSystems (RRID: SCR_000215). This array has a broad range of detection with 304 antibodies detecting phosphospecies across 16 signaling pathways in the sample. This screening approach will allow us to rapidly identify key molecular species in myelopoiesis.

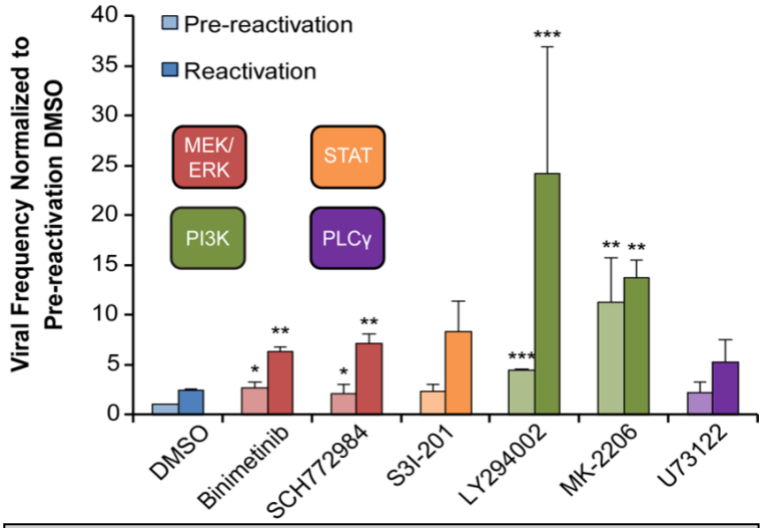


Figure 6. Chemical inhibition of downstream EGFR signaling pathways in WT-infected CD34⁺ HPCs. The inhibition of MEK/ERK and PI3K/AKT signaling pathways stimulates viral reactivation in primary HPCs infected with WT virus at an MOI of 2.

Summary: Together, the experiments proposed in aim 1 will elucidate the mechanisms behind HCMV-induced myelosuppression in HPCs by defining the interplay between UL135, host interactors Abi-1/CIN85, EGFR signaling pathways and HPC differentiation. With EGFR and downstream signaling as the centerpiece, the first subaim will address the role of viral factor UL135 in modulating EGFR signaling, while the second subaim will investigate EGFR signaling in HPC differentiation to identify specific host factors potentially targeted by UL135 in the infected and myelosuppressed HPCs.

AIM 2. Determine the role of UL135 in driving expression of myeloid lineage transcription factors in HPCs during infection

HPCs differentiation is tightly regulated by key lineage transcription factors that are specifically expressed during diffentiation of multipotent progenitor cells toward a more committed lineage. PU.1 and CEBPα are the two main myeloid lineage transcription factors that had been shown to significantly alter the fate of HPC differentiating down the myeloid lineage. Knock-down of these two transcriptional factors using short-hairpin RNA lead to a reduced myeloid-to-erythroid ratio in the differentiated cell population[24]. Since HCMV infected progenitor cells are specifically deficient in differentiation down the myeloid lineage while other lineages such as the erythroid lineage is not affected[25], UL135 might be targeting EGFR signaling or other pathways to downregulate the expression of specific myeloid lineage transcription factors, such as PU.1 and CEBPα, to induce myelosupression.

2.1 *Measuring the transcript and protein levels of PU.1 and CEBPα transcription factors*

Rationale: Given that the expression of PU.1 and CEBPα are important for HPC differentiation into myeloid cells, we hypothesize that the transcript and protein levels of these two TFs are significantly lower in infected HPCs, leading to myelosuppression. If these two TFs are targeted and downregulated by HCMV infection, and more specifically by UL135, then their expression levels will be altered accordingly in each infection context.

Experiments: To test whether the expression of these two transcription factors are affected by HCMV infection and/or expression of UL135, we will infect primary human CD34⁺ HPCs with wildtype, UL135stop, or ΔUL135-CIN85/SH3cI viruses and utilize FACS to sort for infected cells expressing GFP similar to the approach in subaim 1.1. Throughout a time course of 14 day post infection, we will collect cells for transcript and protein analysis every 4-5 days (day 1, 5, 10, and 14). We will use quantitative real-time PCR (qPCR) to determine the transcript levels and immunoblotting to determine the protein levels of PU.1 and CEBPα before and after HPC differentiation, in WT infected or UL135 mutant virus, which represent the myelosuppressed and the myelorestored phenotypes of infected HPCs.

Expected outcomes: We expect that WT-infected HPCs will have a reduced transcript and protein levels of both transcription factors compared to uninfected HPCs as well as HPCs infected with UL135 mutant viruses. We also expect that HPCs infected with UL135stop or ΔUL135-CIN85/SH3cI viruses will have similar levels of transcripts and proteins of these two transcription factors compared to the uninfected group, directly implicating

UL135, and more specifically, UL135 interactions with CIN85 and Abi-1 in the expression of these myeloid lineage transcription factors.

Pitfalls & alternatives: Even though both PU.1 and CEBPα are important transcription factors for myelopoiesis, there are many other transcription factors involved in myelopoiesis. In the event that we do not observe a difference in the transcript and protein expression levels of these two transcription factors using our approach, we can broaden our search using a transcriptomic approach with RNA sequencing to analyze all differentially expressed genes between WT- versus UL135stop- infected HPCs. This approach can better identify specific hematopoietic transcription factors that are altered by UL135 during infection. Our laboratory have previously conducted similar transcriptomic experiments in primary $CD34^+$ HPCs focusing on viral gene expression[9] and are confident that we can expand on this approach to investigate host factors as well.

2.2 *Determining the effects of overexpressing PU.1 and CEBPα in infected HPC differentiation*
Rationale: Since downregulation of PU.1 and CEBPα leads to reduced myelopoiesis, we hypothesize that overexpressing these two TFs in WT-infected HPCs might compensate for the myelosuppression caused by infection. Such observations will also further indicate that these two TFs are being targeted by the viral program during infection leading to myelosuppression.
Experiments: In order to test if reduced expression levels of myeloid transcription factors in infected HPCs are the cause of myelosuppression, we will transduce infected HPCs to overexpress myeloid transcription factors. This increase in transcription factor expression will elucidate whether such overexpression can rescue and restore myelopoiesis. Specifically, we will overexpress PU.1 and CEBPα in infected HPCs using an adenoviral vector construct with a Tet-transactivator enhancer sequence[26, 27]. We will first validate that overexpression is successful by transducing uninfected primary $CD34^+$ HPCs followed by measuring the transcript and protein levels of the transcription factors in transduced cells using qPCR and immunoblotting. Once overexpression is validated, we will infect the transduced HPCs with WT, and the non-transduced HPCs with UL135stop, or ΔUL135-CIN85/SH3cI viruses. Then, we will plate all samples in the CFU assay medium described in subaim 1.2. to measure their differentiation potentials and test whether overexpression of these transcription factors will restore myelopoiesis in the myelosuppressed WT-infected HPCs.

Expected outcomes: We expect that overexpression of these myeloid transcription factors will restore myelopoiesis in wildtype-infected HPCs to a comparable level observed in UL135stop and ΔUL135-CIN85/SH3cI infected HPCs (Figure 1). In our overexpression validation experiments, we expect to observe a statistically significant increase in the transcript and protein levels of the TFs in the transduced cells compared to the control transduced with an empty vector.

Pitfalls & alternatives: A potential problem with the proposed approach is the technical challenge of adenoviral vector in HPCs and cell viability due to HPCs being very sensitive to environmental stimuli. In the case where we encounter inconsistent results with overexpression in HPCs, we will utilize primary monocytes as a replacement *in vitro* model, which might respond better to transduction and is a cell type that can further differentiate. Instead of using the CFU assay, we will employ flow cytometry, staining for specific surface markers of monocytes versus differentiated macrophages to measure levels of differentiation. Secondly, as we will first validate the importance of PU.1 and CEBPα in subaim 2.1, in the event where we find that the expression of these two transcription factors are not implicated in myelosuppression, we will use results from the alternative RNA-seq screening to choose new target transcription factors for these overexpression experiments.

Summary: Together, the proposed experiments in aim 2 will define the potential roles of myeloid-lineage-specific transcription factors in HCMV-induced myelosuppression. These transcription factors might be targeted by HCMV through EGFR and downstream signaling, connecting with our first aim, or independently through other mechanisms. Nevertheless, through this aim, we will identify specific host factors in HPCs and how HCMV might be modulating them to alter hematopoiesis.

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