Title: Latency-associated expression of human cytomegalovirus US28 attenuates cell signalling pathways to maintain latent infection

Authors: B. A. Krishna[1,3★], E. L. Poole[1★], S. E. Jackson[1], M. J. Smit[2], M. R. Wills [1], J. H. Sinclair[1*]

Affiliations:

[1]Department of Medicine, Addenbrooke's Hospital, University of Cambridge, Cambridge, UK.

[2]Division of Medicinal Chemistry, University of Amsterdam, Amsterdam, The Netherlands.

[3] Now at the Department of Molecular Genetics, Lerner Research Institute, Cleveland Clinic, Cleveland, Ohio, USA.

*To whom correspondence should be addressed: js152@hermes.cam.ac.uk

B.A.K. and E.L.P. contributed equally to this work

Running title (54 characters): US28 attentuates signalling to maintain HCMV latency

Abstract word count: 187

Main text word count (introduction, methods, results and discussion): 7430

Abstract

Reactivation of human cytomegalovirus (HCMV) latent infection from early myeloid lineage cells constitutes a threat to immunocompromised or immune-suppressed individuals. Consequently, understanding the control of latency and reactivation to allow targeting and killing of latently infected cells could have far reaching clinical benefits. *US28* is one of the few viral genes that is expressed during latency and encodes a cell surface G protein-coupled receptor (GPCR) which, during lytic infection, is a constitutive cell-signalling activator. Here we now show that in monocytes, a recognised site of HCMV latency *in vivo*, US28 attenuates multiple cell signalling pathways, including MAP kinase and NFκB, and that this is required to establish a latent infection; viruses deleted for US28 initiate a lytic infection in infected monocytes. We also show that these monocytes now become potent targets for the HCMV-specific host immune response and that latently infected cells treated with an inverse agonist of US28 also reactivate lytic infection and, similarly, become immune targets. Consequently, we suggest that inhibitors of US28 could be a novel immunotherapeutic strategy to reactivate the latent viral reservoir allowing it to be targeted by pre-existing HCMV-specific T cells.

Importance

Human cytomegalovirus (HCMV) is a *betaherpesvirus* and a leading cause of morbidity and mortality among immunosuppressed individuals. HCMV can establish latent infection, where the viral genome is maintained in an infected cell, without production of infectious virus. A few genes are expressed by HCMV during latent infection, which includes *US28.* US28 has been shown to activate many cellular signalling pathways during lytic infection, promoting lytic gene expression and virus production. As such, the role of US28 remains unclear and seems at odds with latency. Here, the authors show that US28 has the opposite phenotype in cells that support latent infection – it attenuates cellular signalling, thereby maintaining latency. Inhibition of US28 with a small molecule inhibitor causes HCMV latent infection to reactivate, allowing latently infected cells to be

detected and killed by the immune system. This approach could be used to treat latent HCMV to clear it from human transplants.

Introduction

Human cytomegalovirus (HCMV) is a beta-herpesvirus which has characteristic lytic and latent stages to its lifecycle [1]. It is a widespread pathogen, establishing lifelong infection in 50-90% of the population [2]. Due to robust host T-cell and antibody immune responses, primary infection with HCMV is rarely symptomatic in healthy individuals. Despite this, HCMV infection is never cleared after primary infection but persists for the lifetime of the host and this is due, at least in part, to the ability of the virus to establish a latent infection which helps support immune evasion [3]. Although primary infection, as well as sporadic reactivation from latency, is asymptomatic in healthy individuals, it can be a severe clinical threat in immunocompromised individuals, such as transplant recipients and patients with AIDS [4].

Although many cell types become lytically infected upon primary HCMV infection [5], only cells of the early myeloid lineage have been shown to carry latent virus *in vivo*. These include CD34+ progenitor cells as well as their derivative CD14+ monocytes [4]. In these cells, the viral genome is maintained with a limited latency-associated transcription program which does not support the production of infectious virus. Differentiation of these latently infected early myeloid lineage cells to macrophages or dendritic cells triggers the lytic transcription program and full virus reactivation [6-12]. It is now relatively well established that the differentiation-dependent reactivation of HCMV in the myeloid lineage is associated with changes in post-translational modifications of histones around the major HCMV lytic promotor, the major immediate early promotor (MIEP). These changes drive expression of the major lytic IE72 and IE86 viral gene products, thereby initiating the lytic transcription program and the production of infectious virions [7,9,12-17]. Whilst the exact signals associated with myeloid differentiation, which induce HCMV reactivation, are far from clear, it is becoming apparent that orchestrated effects of both cellular and

viral factors are involved in this derepression of the MIEP and induction of the lytic transcription program [15,18-22] and that these effects are likely to involve ERK-MAP kinase signalling [23]. Consequently, these pathways, which are activated during myeloid cell differentiation and maturation are likely to play a prominent role in the differentiation-dependent activation of the MIEP [18-20,24-28].

Besides repression of the MIEP and expression of specific latency-associated viral gene products, [29-36], latent HCMV carriage has been shown to cause changes in the cellular microRNAome [37,38], the cellular secretome [39], and cell surface protein expression [40], and all of these are likely to be mediated by expression of latency-associated genes [41]. One of these, US28, is a G protein-coupled receptor (GPCR) and chemokine receptor (CCR) homologue whose expression has been detected in both natural and experimental models of HCMV latency [30,31,35,42]. US28 is one of four HCMV-encoded CCR homologues and is the only one to be expressed during both lytic and latent infection; the other CCR homologues, UL33, UL78, and US27 are only expressed during lytic infection [43-45]. Supporting this assertion, deletion of the US27, UL33 and UL78 genes does not affect the establishment of HCMV latency, whereas deletion of US28 has profound effects on latent infection in CD34+ progenitor cells and leads to lytic infection in these undifferentiated myeloid cells, due to a lack of MIEP repression [21].

US28 is the best characterised of the CCR homologues encoded by HCMV and, recently, its structure has been solved [46]. This viral GPCR can signal via multiple different G-alpha proteins and this signalling is modulated by cell type and cytokine binding [47-51], to activate a number of different signalling pathways [52-59]. US28 signalling can be modulated by binding of either CC or CX3C chemokines [52,59-61] and high affinity chemokine binding to US28 is known to be mediated by its N-terminal domain [51]; one point mutation to US28, US28-Y16F, greatly reduces chemokine binding for RANTES and fractalkine [59].

During lytic infection, US28 is known to promote proliferative signals including MAP kinase and NFκB [48,49,57,62], both of which are known to activate the MIEP [63-64] and its expression

has also been linked to vascular disease and oncomodulation [52,65,82]. This signalling by US28 during lytic infection requires G protein binding via the highly conserved DRY motif of US28 that is found in most GPCRs and all CCRs [66] and, consistent with this, a point mutation in this DRY motif of US28 (US28-R129A) greatly reduces G-protein binding and ablates US28 signalling capability [48,54,67,68]. Similarly, the US28 C-terminus is also heavily phosphorylated and this is also known to modulate US28 signalling [69-71]. All these known activatory functions of US28 during lytic infection, however, appear to be totally inconsistent with the observations that, during latency, US28 is required to enforce MIEP silencing [21] and suggests very different functions of US28 during latent and lytic infection.

In this work, we address this in detail by analysing the effects of US28 during HCMV latency. Firstly, we show that, as with CD34+ cells, US28 expression is also necessary for the maintenance of HCMV latency in CD14+ monocytes and that this US28 activity is not dependent on US28 binding of chemokines but requires constitutive G protein-coupled signalling. We also show that US28 has a completely different signalling profile in undifferentiated monocytic cells compared to differentiated, macrophage-like cells; in undifferentiated myeloid cells US28 attenuates multiple different cell signalling pathways, including MAP kinase and NFκB signalling, and it is the US28-mediated repression of these signalling pathways which help US28 to represses the MIEP, thereby maintaining latency. Consistent with this, treatment of latently infected cells with a small molecule inhibitor of US28 resulted in induction of IE expression and virus release. Finally, we demonstrate that monocytes infected with HCMV lacking US28 or treated with US28 inverse agonists, which now express viral IE proteins, are recognised and killed by pre-existing HCMV-specific T cells from HCMV seropositive donors and propose that small molecule inhibition of US28 could be a novel shock and kill approach towards targeting latent HCMV for existing host T cell responses.

Results

US28 is required for HCMV to establish latency in monocytes

Latent infection with HCMV is characterised by the expression of latency-associated genes, little concomitant lytic immediate early (IE) gene expression [32] and the absence of production of infectious virions. As expected, infection of monocytes after seven days with a clinical isolate of HCMV, Titan strain (Titan-WT) which has a UL32-GFP tag that is only expressed at late times of lytic infection, resulted in a characteristically latent infection - high levels of expression of viral UL138, a known latency-associated transcript, with little accompanying IE72 or late UL99 RNA (Figure 1A). Similarly, these Titan-WT infected cells showed no IE72 or late UL32-GFP protein expression (Figure 1B, left panels) nor did they produce infectious virions when analysed by co-culture on indicator fibroblasts (Figure 1C). In contrast, infection of monocytes with a virus in which US28 had been deleted (Titan-ΔUS28) showed high levels of IE72 RNA and concomitant expression of UL99 RNA, a true late gene transcript (Figure 1A), when infected with similar amounts of virus, as assessed by genome copy qPCR (Figure S1). To confirm the expression of IE72 and late proteins in these cells, we also stained the infected monocytes for IE72 protein and the late gene UL32-GFP protein and clearly observed the expression of IE and UL32 proteins (Figure 1B, right panels). Finally, we co-cultured these monocytes with indicator fibroblasts to quantify any virus release and also observed the presence of infectious virus in monocytes infected with Titan-ΔUS28 virus but not Titan-WT virus (Figure 1C). Consistent with viral lytic replication occuring only in Titan-ΔUS28 infected monocytes, an increase in viral genome copies were detected in Titan-ΔUS28 but not Titan-WT infected monocytes seven days post infection (Figure S1).

Taken together, these data argue for a requirement for US28 in either the establishment or maintenance of latent infection of CD14+ monocytes and show that, in the absence of US28, monocytes undergo full lytic infection.

Lytic infection of monocytes by Titan-ΔUS28 virus does not result from early induction of myeloid differentiation

Having established that infection of CD14+ monocytes with Titan-ΔUS28 for seven days resulted in a lytic rather than a latent infection, we wanted to rule out that US28 was simply maintaining a latent infection by actively suppressing myeloid cell differentiation. We reasoned that if this were the case, and an absence of US28 simply induces infected monocytes to differentiate and become fully permissive for HCMV lytic infection, there ought to be a temporal delay in the induction of lytic gene expression in monocytes infected with Titan-ΔUS28 to allow time for differentiation to occur. Consequently, we assayed for IE mRNA and protein expression in monocytes infected with Titan-WT or Titan-ΔUS28, but at very early time points post-infection. These analyses clearly showed that substantial levels of lytic IE gene expression were observed as early as 12 hours post-infection of monocytes with Titan-ΔUS28 (Figure 2A). We were also able to detect IE protein expression by immunofluorescent (IF) microscopy from as early as 24 hours post-infection (Figure 2B) and UL32-GFP from 48 hours post infection (Figure 2C). Additionally, supernatants from Titan-ΔUS28 infected monocytes, at 3 days post infection, showed the presence of infectious virus on indicator fibroblasts (Figure 2D). To further support this, we performed flow cytometry analysis on monocytes infected with Titan-WT or Titan-ΔUS28 for seven days, and observed little change in cell surface expression of CD14 or CD83, markers of differentiation (Figure S2). All these data were entirely consistent with the view that Titan-ΔUS28 infected monocytes, immediately undergo a lytic infection with little or no temporal delay, and make it unlikely that infection in the absence of US28 simply induced differentiation of monocytes to a cell phenotype that is permissive for lytic infection.

US28 signalling maintains latency independently of chemokine binding

On the basis that US28 appeared to be having a profound effect on the outcome of infection of monocytes, at least in part supporting the establishment of latency, we next decided to assess the effects of US28 expression on monocytic cells in detail. To do this, we used lentiviral vectors to over-express an N-terminally HA-tagged US28 (HA-US28-WT) [72] in isolation in the monocytic THP-1 cell line which we and others have used as a model of latent HCMV infection [42,73,74]. At the same time, we also over-expressed two HA-tagged US28 mutants: HA-US28-R129A and HA-US28-Y16F, which have ablated signalling and chemokine binding function, respectively (Figure 3A).

We then infected these THP-1 cell lines stably expressing HA-US28-WT, HA-US28-R129A or HA-US28-Y16F proteins, with Titan-ΔUS28 virus, to assess if supplying these US28 proteins in trans would affect the ability of Titan-ΔUS28 to undergo a lytic infection in these undifferentiated monocytic cells.

Figure 3B shows that, as expected, control THP-1 cells stably transduced with an empty vector underwent lytic infection when infected with Titan-ΔUS28 virus, in that IE and UL32-GFP protein were detectable. In contrast, expression of HA-US28-WT in THP-1 cells complemented the lack of US28 in Titan-ΔUS28 virus and this resulted in cells negative for IE and UL32-GFP expression - consistent with a latent infection. Interestingly, THP-1 cells expressing the HA-US28-R129A protein failed to complement the Titan-ΔUS28 virus mutation (these infected cells were IE and UL32-GFP positive), whereas infection of THP-1 cells stably expressing the HA-US28-Y16F mutant also complemented Titan-ΔUS28 virus and resulted in cells undergoing latent infection (as detected by a lack of IE and UL32-GFP expression) (Figure 3B). Also as expected, THP-1 cells infected with Titan-WT showed little lytic gene expression, regardless of expression of any HA-US28 construct (Figure S3).

We also tested whether any observed failure to complement Titan-ΔUS28 by these US28 constructs, which resulted in lytic gene expression, also resulted in production of infectious virus. Figure 3C shows that cells in which IE late gene expression could be detected also produced infectious virions, as expected.

Finally, we confirmed that the ability of HA-US28-WT and HA-US28-Y16F to complement Titan-ΔUS28 and to establish latent infection resulted in cells from which HCMV could be reactivated by differentiation (Figure S4). Taken together, these data suggest that the ability of US28 to suppress lytic infection likely resides in its downstream signalling, via G protein activation, and that this signalling occurs independently from chemokine binding.

US28 suppresses or activates the MIEP depending on differentiation status of the monocytic cell

As US28 signalling appeared to be necessary for the establishment of latency in monocytes, we hypothesised that US28 expression likely negatively regulates the MIEP in undifferentiated monocytic cells. To test this, we used THP-1 cell lines, that had been transduced with an MIEP-eGFP construct [75], and transfected these cells by nucleofection with three HA-US28 constructs, and the empty vector control (Figure S5). Two days post transfection, we measured eGFP expression in these cell lines by flow cytometry. Figure 4A shows that, consistent with a role for suppression of lytic infection in undifferentiated THP-1 cells, HA-US28-WT did, indeed, show a repression of MIEP activity as did the HA-US28-Y16F mutant. In contrast, the HA-R129A-US28 signalling mutant showed no such repression. We also repeated this analysis but, two days after nucleofection with the HA-US28 constructs and empty vector control we differentiated the THP-1 cells with PMA (Figure 4B). In contrast to the undifferentiated THP-1 cells, HA-US28-WT and the HA-US28-Y16F now activated the MIEP whereas HA-US28-R129A expression had no significant effect on MIEP activity. These data confirm that the effect of US28 on IE gene expression is differentiation-dependent; US28 appears to repress the MIEP in undifferentiated monocytic cells,

consistent with a role of US28 in maintaining latency, but activates the MIEP after cellular differentiation, likely to promote lytic infection.

US28 attenuates MAP kinase and NFκB cell signalling pathways

US28 expression during lytic infection is known to activate a number of cell signalling pathways, including the NFκB and MAP kinase pathways, both of which are known to activate the MIEP in fully permissive cells. To analyse the potential effect of US28 on such signalling during latent infection, we used phosphokinase antibody arrays to assess whether US28 mediates changes in phosphorylation of an array of different cellular signalling proteins (Figure 5). Specifically, we compared THP-1 cells expressing either HA-US28-WT or the HA-US28-R129A signalling mutant; we reasoned that comparing HA-US28-WT to the HA-US28-R129A signalling mutant would robustly control for potential non-specific effects of US28 protein over-expression. These analyses show that HA-US28-WT specifically decreased the phosphorylation of a number of cellular proteins, suggesting a general attenuation of cell signalling pathways by wild type US28 in undifferentiated monocytic cells. In particular, we noted significant reductions in phosphorylation levels of several key signalling proteins, chief among them being ERK1/2 of the MAP kinase pathway (Figure 5).

Our result, showing that US28 mediated suppression of MIEP activity as well as inhibiting ERK1/2, fits well with the view that MAP kinase signalling is likely involved in HCMV reactivation [23]. Consequently, we validated the results of the phosphokinase array by performing western blotting analysis on three cellular proteins that are key to the MAP kinase signalling pathway: ERK1/2, MSK-1 and CREB. Figure 6A-C (left hand panels), show that all three proteins were hypo-phosphorylated in THP-1 cells expressing HA-US28-WT compared to control cells expressing HA-US28-R129A. As US28 is associated with activation of MAP kinase during lytic infection, we also repeated this analysis in these THP-1 cells after that had been differentiated to a macrophage-like phenotype, (which is permissive to HCMV lytic infection) [6,8-9,12]. Figure 6

shows that over-expression of US28 now showed the opposite effect on ERK1/2, MSK-1 and CREB phosphorylation; HA-US28-WT protein resulted in their hyperphosphorylation, compared to cells expressing HA-US28-R129A (Figure 6A-C).

Finally, we performed nuclear/cytoplasmic fractionation followed by western blotting to analyse the effect of US28 on NFκB activation (Figure 6D). These analyses showed that the NFκB pathway is also attenuated by US28 in undifferentiated monocytic THP-1 cells in that, in contrast to HA-US28-R129A or control vector, US28 expression resulted in a relative lack of nuclear localisation of p65 (Figure 6D, left hand panels). Whereas, as predicted, this was reversed in differentiated THP-1 cells (Figure 6D, right hand panels).

Inhibition of MAP kinase and NFκB cell signalling pathways can reduce lytic infection of monocytes by Titan-ΔUS28 virus

On the basis that US28 expression, in isolation in undifferentiated myeloid cells, attenuates MAP kinase and NFκB signalling pathways and that this correlates with the suppression of the MIEP and the ability of HCMV to establish latency, we reasoned that we should be able to mimic the action of US28 in undifferentiated CD14+ monocytes by inhibiting MAP kinase and/or NFκB signalling, in the context of HCMV infection; in essence, that we could compel Titan-ΔUS28 virus to establish latency in monocytes by pretreating these monocytes with inhibitors of either MSK-1 or IKKα (H89 and Bay11-7082, respectively), before infection. To test this, we treated cells with inhibitors prior to infection with Titan-ΔUS28, and then measured the number of UL32-GFP-positive cells three days post infection, as an indicator of full, lytic infection. Figures 7A and B show that neither inhibitor alone was able to prevent Titan-ΔUS28 virus from undergoing lytic infection. However, infection in the presence of both inhibitors together did, indeed, lead to an absence of UL32-GFP gene expression in Titan-ΔUS28 infected monocytes in a dose-dependent manner and this could not be attributed to non-specific cell toxicity effects (Figure 7C, black squares).

Our observations in figure 2 that Titan-ΔUS28 virus initiated IE expression in infected monocytes 6-12 hours post infection also suggested that US28 may be required at very early times of HCMV infection of monocytes to suppress the MIEP and help establish latency. Therefore, we hypothesised that, during infection with HCMV virus, US28 protein expression likely blocks activatory signals for the MIEP in monocytes, which could occur due to virus binding or internalisation. In order to test this, we delayed inhibitor treatment of monocytes infected with Titan-ΔUS28 until either one hour or one day post infection, assuming that by delaying inhibition of MAP kinase and NFκB, would lead to the activation of the MIEP by viral entry, and that this would no longer by preventable once MIEP activity is fully established. Treatment of monocytes with H89 and BAY11-7082, one hour post infection, was still effective at blocking lytic infection by Titan-ΔUS28. However, if treatment was delayed until one day post infection, the inhibitors were no longer as effective at blocking lytic infection (Figures 7D and E). These data suggest that signals triggered within the first 24 hours of infection of monocytes by HCMV activate MIEP activity but that US28 attenuates these signals to suppress IE expression, in order to establish latent infection.

US28 attentuation of cellular signalling prevents phosphorylation of histone H3 and subsequent activation of the MIEP

It is well established that in undifferentiated myeloid cells the viral MIEP is associated with histone marks of transcriptional repression, including methylated histones and presence of repressor proteins such as heterochromatin protein 1 (HP1). In contrast, when this repression is relieved during myeloid differentiation, the MIEP becomes associated with histone marks of transcrituonal activation such as acetylated histones and histone H3 phosphorylation [7,9,12-17]. It has also been established, more recently, that myeloid differentaition activates CREB binding to the MIEP causing co-recruitment of mitogen and stress activated kinase (MSK1) and subsequent phosphorylation of histone H3 which is known to de-stabilise the binding of HP1 [23]. We therefore

hypothesised that US28-mediated attenuation of MSK and CREB signalling could prevent histone phosphorylation and activation of the MIEP, thereby maintaining latency. Consequently, we infected monocytes with Titan-WT and Titan-ΔUS28 and performed chromatin immunoprecipiation assays against HP-1, and H3-S10p, markers of histone repression and activation, respectively. As expected, the MIEP was associated with HP-1 in monocytes latently infected with Titan-WT, but associated with phosphorylated H3 in monocytes lytically infected with Titan- ΔUS28 (Figure 8). US28, therefore, appears to mediate parts of its repressive function via preventing de-stabilisation of HP1 binding.

The US28 inhibitor VUF2274 can induce lytic infection in monocytes infected with wild type HCMV

VUF2274 (BX 513 hydrochloride) is an antagonist of CCR1 and an inverse agonist of US28 [76]. Given that our results, so far, showed that US28 signalling was required to help establish latency in monocytes, we predicted that monocytes infected with wild-type HCMV in the presence of VUF2274, would trigger viral lytic gene expression, and possibly virus reactivation. To test this, we latently infected monocytes for three days with an isolate of HCMV, RV1164, which has an IE2-YFP tag, and then treated them with a titration of VUF2274 and quantified IE protein expression by counting YFP-positive monocytes three days after drug treatment (Figure 9A). We also quantified any production of infectious virus in these cultures treated with VUF2274 by removing media from monocytes three days post drug treatment and titrating this media onto indicator fibroblasts (Figure 9B). As predicted, VUF2274 did induce IE gene expression (Figure 9A), and resulted in measurable release of virus from these reactivated cells (Figure 9B). Consistent with this, equivalent experiments using infection with Titan-WT, which has a UL32-GFP tag, also confirmed late gene expression (UL32) in these VUF2274 treated cells (Figure 9C). Taken together, these data argue that inhibition of US28 signalling by VUF2274 appears to reactivate full lytic gene expression and lytic infection in monocytes latently infected with wild type HCMV. It should be pointed out,

however, that VUF2274 did show significant toxicity (as measured by trypan blue staining) towards primary blood monocytes, even at concentrations below an approximate Ki value of 10μM (Figure 9D). Additionally, as expected, although attenuation of cellular signalling by US28 can be inhibited by VUF2274, which leads to activation of the MIEP and IE protein expression, this signaling to the MIEP can be blocked by simultaneous inhibition of MSK-1 and NFkB (Figure S6).

Monocytes infected with Titan-ΔUS28 virus are targets for killing by pre-existing HCMV-specific donor cytotoxic T cells

After primary infection, it is well established that healthy HCMV carriers maintain extremely high frequencies of HCMV-specific CD8+ cytotoxic T cell (CTLs) in their peripheral blood which are often dominated by CTLs which recognise IE antigens; up to 10% of effector memory CD8+ CTLs can recognise IE72 in some donors [77-79]. However, because latently infected cells express little of these lytic antigens, they escape these HCMV-specific CTL responses. We, therefore, reasoned that the inability of Titan-ΔUS28 infected monocytes to undergo latent infection, and their resulting high level of lytic gene expression, should make them targets for pre-existing HCMV specific CTLs in the peripheral blood of healthy HCMV carriers. Similarly, the same would be observed in monocytes latently infected with wild-type virus if US28 was inhibited by VUF2274. Figure 10A, shows that co-culture of monocytes, infected with Titan-ΔUS28, with donor-matched IE72-specific T cell clones resulted in a reduction in the frequency of reactivation of latently infected cells from these infected monocytes after their differentiation and maturation to mature dendritic cells (mDCs). As expected, we also found that treatment with IE-specific T cells resulted in a reduction in virus release from monocytes infected with Titan-ΔUS28, due to killing of these lytically infected cells in the absence of their differentiation and maturation (Figure 10B). We also repeated this analysis with donor-matched total PBMCs, instead of IE-72 specific T cell clones, in order to show that the *in vivo* HCMV-specific host immune response is also able to kill Titan-ΔUS28-infected monocytes; we saw similar reductions in virus reactivation events from monocytes infected with Titan-ΔUS28

compared to those infected with Titan-WT after their subsequent differentiation and maturation (Figure 10C).

To confirm that these observations were a result of T cell killing rather than, for instance, repression of GFP expression, we also incubated monocytes infected with Titan-WT or Titan-ΔUS28 with isolated CD8+ T cells from matched donors and analysed T cell specific increases in two markers of T cell activation, CD69 and 4-1BB, by flow cytometry, to assay for CMV specific CD8+ T cell response. The CD8+ T cells exposed to Titan-ΔUS28 infected monocytes were more activated than those exposed to monocytes latently infected with Titan-WT (Figure 10D). As a read out of the functional capacity of these CMV specific CD8+ T cells, we also analysed their production of the cytokines IFN-γ and TNF-α as well as upregulation of the degranulation marker CD107a and expression of Granzymes A, B and K. Figure 10E shows that, using these functional effector markers, CD8+ T cells clearly recognised Titan-ΔUS28 infected monocytes but not Titan-WT infected monocytes. We also confirmed that monocytes latently infected with Titan-WT virus can act as targets for CTLs; the expression of CD107a as well as the production of Granzymes, IFN-γ and TNF-α by CMV specific CD8+ T cells in response to the Titan-ΔUS28 virus, were equivalent to levels seen in response to monocytes which had been infected with Titan-WT virus and pulsed with IE1/2 peptides as positive control targets for CMV specific CD8+ T cell responses. These data, taken together, argue that monocytes infected with Titan-ΔUS28 are robustly detected by pre-existing CD8+ T cells in HCMV seropositive donors.

Finally, we tested whether treatment of latently infected cells with VUF2274 also made them targets for IE72-specific CTLs. Figure 10F shows that, consistent with previous analyses [37,72,90], monocytes latently infected with SV40-GFP-TB40E were detectable as GFP+ cells and their numbers remained relatively constant when cultured with IE72-specific CTLs. In contrast, when these latently infected monocytes (GFP+ cells) were treated with VUF2274, a steady loss of latently infected cell number was observed in these IE72-specific CTL co-cultures (Figure 10F). Unfortunately, due to the long-term toxicity of VUF2274, we were not able to show that this also

resulted in a subsequent reduction in reactivation events after monocyte differentiation and maturation to mDCs.

Taken together, these data suggest that the pre-existing CTL response to HCMV, in healthy carriers, is able to target and kill monocytes infected with Titan-ΔUS28 and the treatment of latently infected monocytes with the US28 inhibitor, VUF2274, also makes them novel CTL targets. On this basis, we suggest that inhibition of US28 with, for instance, small molecule inhibitors could result in untimely reactivation of latent virus and allow their targeting by pre-existing HCMV-specific host T cell responses.

Discussion

HCMV latency and reactivation of virus from latency pose a significant clinical threat to immunosuppressed transplant recipients and other immunocompromised individuals [80]. However currently, there are only a few published strategies to treat HCMV latency [17,40,72]. HCMV establishes latent infection in early myeloid lineage cells [4], where its latent life-cycle is characterised by expression of only a small subset of viral genes independent of viral IE gene expression. This includes expression of the viral chemokine receptor homologue US28 [35,81]. US28 expression during HCMV lytic infection is well established to activate multiple cell signalling pathways which can activate the viral MIEP [48,52-57]. These signals have previously been linked to vascular diseases as well as oncomodulation [53,65,82]. However, this powerful signal activation appears contrary to the recently identified requirement for US28 expression to establish a latent infection in CD34+ stem cells, likely by repressing viral IE gene expression [21].

Here, we confirm this important function of US28 during latent infection in CD14+ monocytes and, in part, solve this paradox by showing that US28 appears to have very different effects on cell signalling between undifferentiated and differentiated cells. In undifferentiated cells, US28 attenuates cellular signalling of MAP kinase and NFκB which supports epigenetic suppression of the MIEP to prevent lytic infection. In contrast, in differentiated myeloid cells, US28 activates these

same signalling pathways to help drive IE expression and virus reactivation. Our analysis, using a viral isolate with a deletion for the *US28* gene, showed that US28 was necessary for the maintenance of HCMV latency in monocytes; monocytes infected with Titan-ΔUS28, underwent full lytic infection and produced infectious virus.

These initial analyses were made seven days post infection. Interestingly, repeating this analysis at early times post-infection indicated that this ability of Titan-ΔUS28 to initiate a lytic infection in monocytes was immediate and did not require time for monocytes to become differentiated to a macrophage/DC phenotype, arguing that US28 does not function by suppressing myeloid differentiation and subsequent viral reactivation. We observed IE mRNA expression at 12 hours post-infection, IE protein expression 24 hours post-infection and UL32-GFP expression 48 hours post-infection, indicating that infection of monocytes by Titan-ΔUS28 undergoes a similar time course of gene expression as lytic infection of fibroblasts. Additionally, we did not see changes in cell surface markers of myeloid differentiation, 7 days post infection with Titan-ΔUS28. This suggests that, in the absence of US28 protein, monocytes themselves can support lytic HCMV infection and that differentiation of monocytes is not necessary for lytic infection under these conditions.

Complementation analyses in THP-1 cells stably expressing US28 functional mutations gave us substantial insight into mechanisms by which US28 suppressed lytic infection of monocytes. Infection of THP-1 cell lines stably expressing different US28 mutants, including wild type US28 protein (HA-US28-WT), US28 protein which cannot signal (HA-US28-R129A), and US28 protein which cannot bind chemokines (HA-US28-Y16F), with Titan-ΔUS28 shows that US28 maintains latency by G protein-mediated signalling, and that this signalling occurs in a constitutive manner, independently of chemokine binding. These observations fitted well with analyses of the effect of US28 on the MIEP in transfection assays. Nucleofection of our HA-US28 constructs into THP-1 cells, which had been transduced by lentivirus to express eGFP driven by the MIE promotor, confirmed that HA-US28-WT repressed the MIEP in a signalling-dependent manner and further

showed that in differentiated THP-1 cells, the activity of US28 switched from a repressor, as seen in undifferentiated monocytic cells, to an activator of the MIEP and this, too, was dependent on the US28 signalling capacity. This was entirely consistent with data in other permissive cell types which have consistently shown the ability of US28 to activate IE expression [63,64]. Our view, that the regulation of IE expression by US28 was likely signalling dependent, was confirmed by our observations that US28 profoundly affected the level of a number of cellular phosphokinases. In undifferentiated monocytic cells, HA-US28-WT significantly attenuated the MAP kinase pathway; in particular, ERK1/2, MSK1 and CREB were all less phosphorylated. Similarly, consistent with the observation that in cells that are permissive for HCMV lytic infection US28 has an activatory effect on the MIEP [52,63,64], in differentiated monocytic cells US28 activated MAP kinase signalling and this is diametrically opposed to its suppressive effect in undifferentiated monocytic cells. US28 also differentially affected NFκB localisation in undifferentiated and differentiated monocytic cells; in undifferentiated cells, US28 resulted in increased cytoplasmic NFκB localisation but in differentiated cells it enhanced NFκB nuclear localisation. This, again, is consistent with the known NFκB-mediated activation of the MIEP [63] and helps explain the differentiation-dependent reversal of US28 activity on the chromatin-mediated control of MIEP activity and IE gene expression in undifferentiated or differentiated monocytic cells which suppress or support IE gene expression, respectively. Taken together, it appears that US28 activity in early myeloid lineage cells serves to maintain latency by attenuating reactivation signals, such as MAP kinase [23] and NFκB which are both known to activate the MIEP either directly or via release of chromatin-mediated suppression around the MIEP [23,47,63,83,84]. We were able to observe this change in activatory and repressive chromatin marks on the MIEP by ChIP assay at the level of H3 phosphorylation and HP1 recruitmnet. This radical differentiation-dependent reversal of US28 activity helps resolve the problem of why US28, considered to be a strong activator of cellular signalling in lytic infection, is also expressed during latency, when lytic infection is known to be actively suppressed.

How US28 apparently reverses its signalling properties, between undifferentiated and differentiated myeloid cells, in such a significant manner, remains unclear. US28 has been investigated in a range of different cell types, but its effects between different cell types have not always been consistent [56,65]. Our observations appear to be the first evidence that US28 can attenuate cell signalling, independently from other viral GPCRs, in a constitutive manner, at least in undifferentiated myeloid cells. Two models may explain these observations: firstly, phosphorylation of the C terminal tail of US28 is known to modify its signalling properties [69-71] and US28 is known to be phosphorylated by PKC [70], which show isoform changes during myeloid cell differentiation [85]. It is, therefore, possible that changes in cellular kinase expression during myeloid differentiation alter US28 signalling by its differentiation-dependent phosphorylation. Alternatively, US28 is known to interact promiscuously with a range of different G-alpha proteins [47,49,50], and similarly, changes in cellular G-alpha protein expression are known to occur during myeloid differentiation. Consequently, differentiation-specific changes in the G-alpha protein interactions with US28, could lead to changes in the signalling properties of US28.

More recently, US28 has been shown to activate PLC-β in monocytes [58], via G protein-coupling, in a chemokine-independent manner, which suggests that attenuation of the MAP kinase and NFκB signalling pathways may not be the only mechanism by which US28 may affect IE expression differently in undifferentiated and differentiated monocytic cells. Given that HDAC inhibitors induce IE gene expression in otherwise latently infected monocytes, but do not trigger full, lytic gene expression or virus production in treated monocytes [17], it appears that major IE protein expression alone in monocytes is not sufficient to induce full virus production. Since deletion of the US28 gene causes full lytic gene expression, US28 is likely to have functions beyond just repression of the MIEP.

Our attempts to reproduce US28-mediated attenuation of lytic cycle in undifferentiated cells, using small molecule inhibitors to either MAP kinase or NFκB in isolation had only a limited effect on the ability of Titan-ΔUS28 to establish lytic infection of monocytes. However, inhibition of both

MAP kinase and NFκB signalling pathways, concomitantly, profoundly reduced lytic gene expression in Titan-ΔUS28 infected monocytes. Our view is that lytic infection, via activation of the MIEP, can be stimulated by either MAP kinase or NFκB signalling but requires activation of at least one of these two pathways. Our observation that delaying this treatment of inhibitors, until 24 hours post infection, no longer prevented lytic infection demonstrates that US28 has to act at a very early time point post infection to suppress activatory signals to the MIEP and thus prevent lytic infection. We believe that these activatory signals are likely triggered by viral binding or entry into the cell, perhaps triggering innate immune responses, which could lead to the activation of the MIEP [86,87] and that US28 may serve to attenuate this response and thereby stifle IE activation to help initiate viral latency.

Finally, based on our findings that US28 is crucial to establish HCMV latency in monocytes by suppressing IE gene expression and subsequent lytic infection, we predicted that inhibition of US28 activity, using its inverse agonist VUF2274, would stimulate lytic gene expression in normally latently infected monocytes. This was, indeed, the case and led to proof of principle that inducing lytic infection in monocytes could lead to their targeting by pre-existing host HCMV-specific CTL responses. Firstly, IE72-specific CD8+ T cell clones reduced viral reactivation from monocytes infected with Titan-ΔUS28 compared to monocytes infected with Titan-WT virus and, similarly, treatment of experimentally latent monocytes with VUF2274 also made latently infected cells targetable by these IE72-specific CD8+ T cell clones. We also demonstrated that monocytes infected with Titan-ΔUS28 are targets for PBMCs from healthy HCMV-positive donors and confirmed that this was mediated by classical CTL killing on the basis of staining for markers of T cell activation and degranulation.

Although this approach of "shock and kill", using HDAC inhibitors, has already been demonstrated to be effective against latent HCMV [17], HDACs have a wide range of biological functions and the inhibition of HDACs could have significant off-target effects. Consequently an inhibitor of US28 to "reactivate" IE expression could be an attractive alternative, particularly in

healthy seropositive tissue donors where reactivation events are thought to be subclinical [88,89]. We do note that VUF2274 did show some cytotoxicity, likely due to off-target effects, possibly including the inhibition of CCR1. However, the structure of the US28 protein has recently been solved [46] and could aid the development of more specific small-molecule inverse agonists of US28.

Taken together, our observations point to a crucial role for viral US28 in the establishment of HCMV latency in monocytes which is mediated by differentiation-dependent US28 signalling and that inhibition of US28, resulting in the induction of IE expression in normally latently infected cells, could aid in novel immunotherapeutic strategies to target and clear the HCMV latent reservoir in certain clinical settings.

Methods

Cell culture and virus infection

Viral isolates of Titan wild type (Titan-WT) and the equivalent isolate with a deletion in the US28 gene (Titan-ΔUS28), which have a UL32-GFP tag, have been described previously [67] as has an isolate of TB40/E a wild type clinical isolate of HCMV carrying an SV40-GFP expression cassette tag (termed SV40-GFP-TB40E) which allows the detection of latently infected cells [90], as well as an isolate of TB40/E with an IE2-YFP tag (termed RV1164) [40]. Primary CD14+ monocytes were isolated from apheresis cones (NHS blood and transfusion service, UK), as described previously [89] and cultured in X-vivo15 (Lonza) at 37°C in 5% CO_2. Cells were infected with all HCMV viral isolates at a predicted multiplicity of infection (MOI) of 5 (based on infection of RPE-1 cells) leading to 10% latently infected cells as determined by GFP expression upon infection with SV40-GFP-TB40E. Monocytes were activated to immature dendritic cells by GM-CSF and IL-4 (Peprotech) stimulation at 1000U/ml for five days. Mature dendritic cells were produced by stimulation for two further days with lipopolysaccharide at 500 ng/ml.

THP-1 cells were infected for 3 days with Titan-WT, Titan-ΔUS28 or SV40-GFP-TB40E at a predicted MOI of 5 (based on infection of RPE-1 cells) and differentiated using 50ng/μl phorbol myristate acetate (PMA) as previously described [91]. To quantify reactivation/virus release, cells were co-cultured with $3x10^3$ fibroblast cells per cm^2 of growth area in a 50:50 mixture of DMEM-10 and RPMI-20 or X-vivo15 (Lonza).

RT-qPCR

Monocytes were infected with Titan-WT or Titan-ΔUS28. After three hours incubation, cultures were citrate washed to remove cell-associated virus. Samples for each condition were then harvested immediately into TRIzol (Life technologies) to act as control wells for input mRNA from incoming virions. Other cultures were incubated for the duration of time indicated in each experiment (between 1 and 7 days), before also being harvested in TRIzol and mRNA was then isolated using miRNeasy mini kit (Qiagen), following manufacturer's instructions. UL138, IE and UL99 were quantified using a one-step RT-qPCR using Quantitect Virus kit (Qiagen) as previously described [92]. Values were calculated using the ΔΔCT method, as a relative change between the input control and the relevant time point. Samples were normalised to GAPDH, as described previously [92].

IF staining and microscopy

Cells were fixed and stained as described previously [93]. In brief, following fixation in paraformaldehyde and permabilisation in 70% ethanol, cells were washed in PBS and stained with mouse anti-IE (Argene 11-003) diluted at 1 in 1000 in PBS containing 10% goat serum. This was followed by detection with goat anti-mouse (Alexafluor 594). Where indicated, goat anti-GFP (FITC conjugated, Abcam) and Hoechst 33258 nuclear stain were added simultaneously.

Virus release assays

Media was harvested from infected monocyte cultures and titrated onto HFFs. Following three hours of incubation with rocking at room temperature the cultures were then washed, media

replaced, and incubated for a further 24 hours at 37°C in 5% CO_2. Fibroblasts were then stained for immediate early antigen, using the protocol above and the virus titre was calculated as the number of infected cells per μL of monocyte supernatant media added to the fibroblast culture.

Lentiviral transduction of US28 expression, and confirmation of expression by western blot

Lentiviral US28 expression constructs, where US28 expression is driven by an SFFV promotor, with an N-terminal haemagglutinin tag (HA) were kindly provided by Daniel Streblow (Oregan Health and Science University) and transduced into THP-1 cells, followed by confirmation by western blot, as previously described [72].

Detection of phosphoproteins by immunoblot

THP-1 cells, transduced to express various constructs of HA-US28, were lysed in RIPA buffer and nuclei and cell debris were removed by centrifugation at 13,000 × g for 10 min at 4 °C. Proteins were separated on 10% SDS-polyacrylamide gels and transferred to nitrocellulose membranes (Axygen, Corning). Incubations with primary and secondary antibodies were in 5% skimmed milk for 1 h each at room temperature. Proteins were detected using the following antibodies: anti-p42/p44 or phosphor-anti-p42/p44 antibodies, anti-MSK1 or anti-phosphor-MSK1 (serine 360) (all 1:1000; Cell Signalling Technology, Danvers, MA); anti-CREB or phosphor-CREB (S360) (both Merck). The secondary antibody used was chicken anti-rabbit horseradish peroxidase (Santa Cruz Biotech). Blots were developed with the use of enhanced chemiluminescence (GE Healthcare) and visualized with autoradiography film.

To detect cellular localisation of NFkB, cells were fractionated using REAP (Rapid, Efficient and Practical, described in [94]), and proteins detected using the following antibodies: NFκB (Abcam), p84 (Thermo), GAPDH (Millipore). Secondary antibodies used were chicken anti-rabbit and bovine anti-mouse horseradish peroxidase (both Santa Cruz Biotech).

MIEP activation/repression assays using

THP-1 cells expressing an MIEP-eGFP construct have been previously described [75]. These cells were transfected by nucleofection with various US28 constructs, using an Amaxa Cell Line Nucleofector® kit R (Lonza) and MIEP driven eGFP expression was detected and measured using a BD Accuri C6 flow cytometer, where dead cells were excluded from analysis by staining with Zombie Red^{TM} Fixable Viability Dye (BioLegend) and mean fluorescence intensities for eGFP expression were analysed within the same experiment using FlowJo software (Tree Star Inc).

Phosphokinase arrays

For phosphokinase antibody arrays, THP-1 cells were transduced with lentiviral US28 expression vectors, as described previously [72]. Cells were harvested and lysed following the manufacturer's protocol (Proteome Profiler Human Phospho-Kinase Array Kit; R&D systems) and spot intensity was analysed using ImageJ software.

IE-specific T cell, and PBMC killing assays

CD14+ monocytes were plated at $1x10^5$ cells per well of a 96 well plate and infected with either Titan-WT, Titan-ΔUS28 or SV40-GFP-TB40E. Three days post infection, the monocytes were then co-cultured with HLA-matched (HLA-A2) immediate-early-specific CD8+ T cells [95] at an E:T ratio of 5:1 or donor PBMCs at an E:T ratio of 1:3. VUF2274 was added, at a concentration of $6x10^{-7}$ M to the relevant wells. In order to measure the killing of latently infected monocytes, the number of HCMV-infected, GFP-expressing, or UL32-GFP-expressing monocytes were enumerated, by fluorescence microscopy, over several days as indicated. Media from these cultures was collected to titre any virus that was produced by Titan-ΔUS28 infected monocytes before being were differentiated to dendritic cells by cytokine treatment. To quantify virus reactivation events, these monocyte-derived dendritic cells were co-cultured with indicator fibroblasts for two weeks and reactivation events enumerated by IF staining for IE, and counting IE-positive foci.

Flow cytometry

Experimentally infected monocytes were co-incubated with CD8+ T cells from the same, seropositive donor, overnight in the presence of CD107a Alexa fluor 647, 5µg/ml, Brefeldin A and 2µM Monensin (all from BioLegend) at 37°C. CD8+ T cells were harvested and washed, then stained with a combination of surface antibodies (CD3 brilliant violet 650, CD14 Brilliant Violet 510 and CD19 Brilliant Violet 510 BioLegend)) and LIVE/DEAD Fixable Aqua Dead cell stain (Invitrogen) at 4°C. Cells were fixed and permeabilised using FIX&PERM (ADG, Kaumberg, Austria) and stained intracellularly with antibodies (CD69 Pacific Blue, 4-1BB PE-Cy5, CD8 Brilliant Violet 570, Granzyme A FITC (BioLegend), Granzyme B FITC (Miltenyi Biotec), Granzyme K FITC (Santa Cruz Biotechnology, Texas, USA), TNFα Brilliant Ultra Violet 395 and IFN-γ Brilliant Violet 786). Responding CD8+ T cell populations were identified by the expression of CD69 and 4-1BB, above background and their expression of CD107a, TNF-α and IFN-γ were then measured. In all cases, cell doublets, monocytes, B cells and dead cells were eliminated from the analysed populations.

To analyse differentiation markers, monocytes were labelled with anti-CD14 and anti-CD83 antibodies (both APC-conjugated from Biolegend).

Chromatin immunoprecipitation

Chromatin immunoprecipitation of the MIEP was performed using a Sigma Imprint ChIP kit, and antibodies against S10P and HP1 (HP1 Antibody (FL-191): sc-28735) anti-ser-10-H3 (Phospho-Histone H3 (Ser10) Antibody (9H12L10), ABfinity™ Rabbit Monoclonal). The MIEP was quantitated against a standard curve of viral DNA, analysed by qPCR and then plotted as a percentage of input DNA with each sample run in triplicate. The primers used were as follows: forward: CCAAGTCTCCACCCCATTGAC, reverse: GACATTTTGGAAAGTCCCGTTG and probe: (FAM)TGGGAGTTTGTTTTGGCACCAAA(TAM).

Ethics Statement

All human samples were obtained under ethical approval and after approval of protocols from the Cambridgeshire 2 Research Ethics Committee (REC reference 97/092) conducted in accordance with the Declaration of Helsinki. Informed written consent was obtained from all of the volunteers included in this study before providing blood samples and all experiments were carried out in accordance with the approved guidelines.

Acknowledgements

The authors would like to thank Linda Teague and Georgina Brown (Department of Medicine, Cambridge, UK) for technical assistance. We would like to thank D. Streblow (Oregan Health and Science University, USA) for the US28 gene, P. Lehner (CIMR, Cambridge, UK) for the lentivirus vector and D. Michel (University of Ulm, Germany) for the Titan-WT an Titan-ΔUS28 isolates. Funding: This work was funded by the British Medical Research programme grant G0701279 (JS), Wellcome Research Studentship Grant (BK), and the Cambridge NIHR BRC Cell Phenotyping Hub. Author contributions: BK, and SJ performed experiments and analysed data. MS provided reagents and feedback. BK, EP, MW and JS designed experiments, BK and JS wrote the

manuscript. All authors read and edited the manuscript. Competing interests: The authors declare no competing interests.

Bibliography

1. Rook, A. H. 1988, 'Interactions of cytomegalovirus with the human immune system', *Review of Infectious Diseases* **10**(Supplement 3), S460–S467.
2. Cannon, M. J. 2009, 'Congenital cytomegalovirus (CMV) epidemiology and awareness', *Journal of Clinical Virology* **46**, S6–S10.
3. Wills, M. R., Poole, E., Lau, B., Krishna, B. Sinclair, J. H. 2014, 'The immunology of human cytomegalovirus latency: could latent infection be cleared by novel immunotherapeutic strategies?', *Cellular and molecular immunology*. **12**(2):128-38
4. Sinclair, J. Sissons, P. 2006, 'Latency and reactivation of human cytomegalovirus', *The Journal of general virology* **87**(Pt 7), 1763–1779.
5. Sinzger, C. Jahn, G. 1996, 'Human cytomegalovirus cell tropism and pathogenesis', *Intervirology* **39**(5-6), 302–319.
6. Taylor-Wiedeman, J., Sissons, J. G., Borysiewicz, L. K. Sinclair, J. H. 1991, 'Monocytes are a major site of persistence of human cytomegalovirus in peripheral blood mononuclear cells', *The Journal of general virology* **72 (Pt 9)**, 2059–2064.
7. Reeves, M. B., MacAry, P. A., Lehner, P. J., Sissons, J. G. Sinclair, J. H. 2005, 'Latency, chromatin remodeling, and reactivation of human cytomegalovirus in the dendritic cells of healthy carriers', *Proceedings of the National Academy of Sciences of the United States of America* **102**(11), 4140–4145.
8. Soderberg-Naucler, C., Fish, K. N. Nelson, J. A. 1997, 'Reactivation of latent human cytomegalovirus by allogeneic stimulation of blood cells from healthy donors', *Cell* **91**(1), 119–126.

9. Söderberg-Nauclér, C., Streblow, D. N., Fish, K. N. et al. 2001, 'Reactivation of latent human cytomegalovirus in CD14+ monocytes is differentiation dependent', *Journal of Virology* **75**(16), 7543–7554.
10. Reeves, M., Sissons, P. Sinclair, J. 2005, 'Reactivation of human cytomegalovirus in dendritic cells', *Discovery medicine* **5**(26), 170–174.
11. Reeves, M. B. Sinclair, J. H. 2013, 'Circulating dendritic cells isolated from healthy seropositive donors are sites of human cytomegalovirus reactivation in vivo', *Journal of virology* **87**(19), 10660–10667.
12. Poole, E., Juss, J. K., Krishna, B., Herre, J., Chilvers, E.R., Sinclair, J. 2015, 'Alveolar macrophages isolated directly from human cytomegalovirus (hcmv)–seropositive individuals are sites of hcmv reactivation *in vivo*', *Journal of Infectious Diseases* **211**(12), 1936–1942.
13. Murphy, J. C., Fischle, W., Verdin, E. Sinclair, J. H. 2002, 'Control of cytomegalovirus lytic gene expression by histone acetylation', *The EMBO journal* **21**(5), 1112–1120.
14. Bain, M., Mendelson, M. Sinclair, J. 2003, 'Ets-2 repressor factor (ERF) mediates repression of the human cytomegalovirus major immediate-early promoter in undifferentiated non-permissive cells', *Journal of general virology* **84**(1), 41–49.
15. Wright, E., Bain, M., Teague, L., Murphy, J. Sinclair, J. 2005, 'Ets-2 repressor factor recruits histone deacetylase to silence human cytomegalovirus immediate-early gene expression in non-permissive cells', *The Journal of general virology* **86**(Pt 3)
16. Taylor-Wiedeman, J., Sissons, P. Sinclair, J. 1994, 'Induction of endogenous human cytomegalovirus gene expression after differentiation of monocytes from healthy carriers', *Journal of virology* **68**(3), 1597–1604.
17. Krishna, B., Lau, B., Jackson, S., Wills MR., Sinclair JH., Poole E., 2016, 'Transient activation of human cytomegalovirus lytic gene expression during latency allows cytotoxic T cell killing of latently infected cells', *Scientific Reports* **6**.

18. Lee, S. H., Albright, E. R., Lee, J.-H., Jacobs, D. Kalejta, R. F. 2015, 'Cellular defense against latent colonization foiled by human cytomegalovirus UL138 protein', *Science advances* **1**(10), e1501164.
19. Buehler, J., Zeltzer, S., Reitsma, J. Petrucelli A., Umashankar M., Rak M., Zagallo P., Schroeder J., Terhune S., Goodrum F,. 2016, 'Opposing regulation of the egf receptor: A molecular switch controlling cytomegalovirus latency and replication', *PLoS Pathog* **12**(5), e1005655.
20. Rauwel, B., Jang, S. M., Cassano, M., Kapopoulou, A., Barde, I., Tronto, D. 2015, 'Release of human cytomegalovirus from latency by a KAP1/TRIM28 phosphorylation switch', *Elife* **4**, e06068.
21. Humby, M. S. O'Connor, C. M. 2016, 'Human cytomegalovirus US28 is important for latent infection of hematopoietic progenitor cells', *Journal of virology* **90**(6), 2959–2970.
22. Liu, R., Baillie, J., Sissons, J. G. Sinclair, J. H. 1994, 'The transcription factor YY1 binds to negative regulatory elements in the human cytomegalovirus major immediate early enhancer/promoter and mediates repression in non-permissive cells', *Nucleic acids research* **22**(13), 2453–2459.
23. Kew, V. G., Yuan, J., Meier, J. Reeves, M. B. 2014, 'Mitogen and stress activated kinases act co-operatively with CREB during the induction of human cytomegalovirus immediate-early gene expression from latency', *PLoS Pathog* **10**(6), e1004195.
24. Macagno, A., Napolitani, G., Lanzavecchia, A., Sallusto, F. 2007, 'Duration, combination and timing: the signal integration model of dendritic cell activation', *Trends in immunology* **28**(5), 227–233.
25. Meier, J. L. Stinski, M. F. 1996, 'Regulation of human cytomegalovirus immediate-early gene expression', *Intervirology* **39**(5-6), 331–342.
26. Reeves, M. Sinclair, J. 2013, 'Regulation of human cytomegalovirus transcription in latency: beyond the major immediate-early promoter', *Viruses* **5**(6), 1395–1413.

27. Pizzorno, M. C. 2001, ‘Nuclear cathepsin B-like protease cleaves transcription factor YY1 in differentiated cells’, *Biochimica et Biophysica Acta (BBA)-Molecular Basis of Disease* **1536**(1), 31–42.
28. Thomas, M. J. Seto, E. 1999, ‘Unlocking the mechanisms of transcription factor YY1: are chromatin modifying enzymes the key?’, *Gene* **236**(2), 197–208.
29. Bego, M., Maciejewski, J., Khaiboullina, S., Pari, G. Jeor, S. S. 2005, ‘Characterization of an antisense transcript spanning the UL81-82 locus of human cytomegalovirus’, *Journal of virology* **79**(17), 11022–11034.
30. Cheung, A. K., Abendroth, A., Cunningham, A. L. Slobedman, B. 2006, ‘Viral gene expression during the establishment of human cytomegalovirus latent infection in myeloid progenitor cells’, *Blood* **108**(12), 3691–3699.
31. Goodrum, F. D., Jordan, C. T., High, K. Shenk, T. 2002, ‘Human cytomegalovirus gene expression during infection of primary hematopoietic progenitor cells: a model for latency’, *Proceedings of the National Academy of Sciences of the United States of America* **99**(25), 16255–16260.
32. Goodrum, F., Reeves, M., Sinclair, J., High, K. Shenk, T. 2007, ‘Human cytomegalovirus sequences expressed in latently infected individuals promote a latent infection in vitro’, *Blood* **110**(3), 937–945.
33. Jenkins, C., Abendroth, A. Slobedman, B. 2004, ‘A novel viral transcript with homology to human interleukin-10 is expressed during latent human cytomegalovirus infection’, *Journal of virology* **78**(3), 1440–1447.
34. Reeves, M. B. Sinclair, J. H. 2010, ‘Analysis of latent viral gene expression in natural and experimental latency models of human cytomegalovirus and its correlation with histone modifications at a latent promoter’, *The Journal of general virology* **91**(Pt 3), 599–604.
35. Poole, E., Walther, A., Raven, K., Benedict, C.A., Mason, G.M., Sinclair, J. 2013, ‘The myeloid transcription factor GATA-2 regulates the viral UL144 gene during human

cytomegalovirus latency in an isolate-specific manner', *Journal of virology* **87**(8), 4261–4271.

36. Rossetto, C. C., Tarrant-Elorza, M. Pari, G. S. 2013, 'Cis and trans acting factors involved in human cytomegalovirus experimental and natural latent infection of CD14 (+) monocytes and cd34 (+) cells', *PLoS pathogens* **9**(5), e1003366.
37. Lau, B., Poole, E., Krishna, B., Sellart I., Wills MR., Murphy E., Sinclair J., 2016 , 'The expression of human cytomegalovirus microrna mir-ul148d during latent infection in primary myeloid cells inhibits activin a-triggered secretion of il-6', *Scientific Reports* **6**.
38. Poole, E., Dallas, S. R. M., Colston, J., Joseph, R. S. Sinclair, J. 2011, 'Virally induced changes in cellular micrornas maintain latency of human cytomegalovirus in cd34(+) progenitors', *The Journal of general virology* **92**(Pt 7), 1539–1549.
39. Mason, G. M., Poole, E., Sissons, J. G., Wills, M. R. Sinclair, J. H. 2012, 'Human cytomegalovirus latency alters the cellular secretome, inducing cluster of differentiation (cd)4+ t-cell migration and suppression of effector function', *Proceedings of the National Academy of Sciences of the United States of America* **109**(36), 14538–14543.
40. Weekes, M. P., Tan, S. Y., Poole, E., Talbot, S. Antrobus, R. Smith, D. Montag, C. Gygi, S. Sinclair, J. Lehner, P. 2013, 'Latency-associated degradation of the mrp1 drug transporter during latent human cytomegalovirus infection', *Science (New York, N.Y.)* **340**(6129), 199–202.
41. Poole, E., Avdic, S., Hodkinson, J., Jackson, S., Wills, M., Slobedman, B., Sinclair, J. 2014, 'Latency-associated viral interleukin-10 (il-10) encoded by human cytomegalovirus modulates cellular il-10 and ccl8 secretion during latent infection through changes in the cellular microrna hsa-mir-92a', *Journal of virology* **88**(24), 13947–13955.
42. Beisser, P. S., Laurent, L., Virelizier, J. L. Michelson, S. 2001, 'Human cytomegalovirus chemokine receptor gene US28 is transcribed in latently infected thp-1 monocytes', *Journal of virology* **75**(13), 5949–5957.

43. Chee, M. S., Bankier, A. T., Beck, Bohni S., Brown C., Cerny R., Horsnell T., Hutchison C., Kouzarides T., Martignetti J. A., 1990, 'Analysis of the protein-coding content of the sequence of human cytomegalovirus strain ad169', *Current topics in microbiology and immunology* **154**, 125–169.
44. Margulies, B. J., Browne, H. Gibson, W. 1996, 'Identification of the human cytomegalovirus g protein-coupled receptor homologue encoded by ul33 in infected cells and enveloped virus particles', *Virology* **225**(1), 111–125.
45. Welch, A. R., McGregor, L. M. Gibson, W. 1991, 'Cytomegalovirus homologs of cellular g protein-coupled receptor genes are transcribed', *Journal of virology* **65**(7), 3915–3918.
46. Burg, J. S., Ingram, J. R., Venkatakrishnan, A., Jude K., Dukkipati A., Feinburg E,. Angelini A., Waghray D., Dror R., Ploegh H., Garcia K. 2015, 'Structural basis for chemokine recognition and activation of a viral g protein–coupled receptor', *Science* **347**(6226), 1113–1117.
47. Casarosa, P., Bakker, R. A., Verzijl, D. Navis M., Timmerman H., Luers R., Smit MJ., 2001, 'Constitutive signaling of the human cytomegalovirus-encoded chemokine receptor US28', *The Journal of biological chemistry* **276**(2), 1133–1137.
48. Miller, W. E., Zagorski, W. A., Brenneman, J. D.. Avery, D., Miller, J.L., O'Connor, C.M. 2012, 'US28 is a potent activator of phospholipase C during hcmv infection of clinically relevant target cells', *PloS one* **7**(11), e50524.
49. Moepps, B., Tulone, C., Kern, C., Minisini, R., Vatter, P., Wieland, T., Gierschik, P. 2008 , 'Constitutive serum response factor activation by the viral chemokine receptor homologue pUS28 is differentially regulated by Gα q/11 and Gα 16', *Cellular signalling* **20**(8), 1528–1537.
50. Vischer, H. F., Leurs, R. Smit, M. J. 2006, 'Hcmv-encoded g-protein-coupled receptors as constitutively active modulators of cellular signaling networks', *Trends in pharmacological sciences* **27**(1), 56–63.

51. Vomaske, J., Nelson, J. A. Streblow, D. N. 2009, 'Human cytomegalovirus US28: a functionally selective chemokine binding receptor', *Infectious disorders drug targets* **9**(5), 548.

52. Billstrom, M. A., Johnson, G. L., Avdi, N. J. Worthen, G. S. 1998, 'Intracellular signaling by the chemokine receptor US28 during human cytomegalovirus infection', *Journal of virology* **72**(7), 5535–5544.

53. Streblow, D. N., Soderberg-Naucler, C., Vieira, J., Smith, P., Wakabayashi, E., Ruchti, F., Mattison, K., Altschuler, Y., Nelson, J.A. 1999, 'The human cytomegalovirus chemokine receptor US28 mediates vascular smooth muscle cell migration', *Cell* **99**(5), 511–520.

54. Maussang, D.I., Langemeijer, E., Fitzsimons, C.P., Stigter-van Walsum, M., Dijkman, R., Borg, M.K., Slinger, E., Schreiber, A., Michel, D., Tensen, C.P., van Dongen, G.A., Leurs, R., Smit, M.J. 2009, 'The human cytomegalovirus–encoded chemokine receptor US28 promotes angiogenesis and tumor formation via cyclooxygenase-2', *Cancer research* **69**(7), 2861–2869.

55. Minisini, R., Tulone, C., Lüske, A., Michel, D., Mertens, T., Gierschik, P., Moepps, B. 2003, 'Constitutive inositol phosphate formation in cytomegalovirus-infected human fibroblasts is due to expression of the chemokine receptor homologue pUS28', *Journal of virology* **77**(8), 4489–4501.

56. Vomaske, J., Melnychuk, R.M., Smith, P.P., Powell, J., Hall, L., DeFilippis, V., Früh, K., Smit, M., Schlaepfer, DD., Nelson, J.A., Streblow, D.N. 2009, 'Differential ligand binding to a human cytomegalovirus chemokine receptor determines cell type-specific motility', *PLoS pathogens* **5**(2), e1000304.

57. Langemeijer, E. V., Slinger, E., de Munnik, S., Schreiber A., Maussang D., Vischer H., Verkaar F., Leurs R., Siderius M., Smit MJ., 2012, 'Constitutive beta-catenin signaling by the viral chemokine receptor US28', *PloS one* **7**(11), e48935.

58. Wu, S.-e. Miller, W. E. 2016, 'The hcmv US28 vgpcr induces potent gαq/plc-β signaling in

monocytes leading to increased adhesion to endothelial cells', *Virology* **497**, 233–243.

59. Casarosa, P., Waldhoer, M., LiWang, P. J. Vischer H., Kledal T., Timmerman H., Schwartz T., Smit M., Leurs R., 2005, 'Cc and cx3c chemokines differentially interact with the n terminus of the human cytomegalovirus-encoded US28 receptor', *The Journal of biological chemistry* **280**(5), 3275–3285.
60. Gao, J.-L. Murphy, P. M. 1994, 'Human cytomegalovirus open reading frame US28 encodes a functional beta chemokine receptor.', *Journal of Biological Chemistry* **269**(46), 28539–28542.
61. Kledal, T. N., Rosenkilde, M. M. Schwartz, T. W. 1998, 'Selective recognition of the membrane-bound cx3c chemokine, fractalkine, by the human cytomegalovirus-encoded broad-spectrum receptor US28', *FEBS letters* **441**(2), 209–214.
62. Slinger, E., Maussang, D., Schreiber, A., Siderius, M., Rahbar, A., Fraile-Ramos, A., Lira, S.A., Söderberg-Nauclér, C., Smit, M.J. 2010, 'Hcmv-encoded chemokine receptor US28 mediates proliferative signaling through the il-6-stat3 axis', *Science signaling* **3**(133), ra58.
63. Boomker, J. M., The, T. H., de Leij, L. F. Harmsen, M. C. 2006, 'The human cytomegalovirus-encoded receptor US28 increases the activity of the major immediate-early promoter/enhancer', *Virus research* **118**(1-2), 196–200.
64. Wen, D.-Q., Zhang, Y.-Y., Lv, L.-P. Zhou, X.-P., Yan, F., Ma, P., Xu, J.-B. 2009, 'Human cytomegalovirus-encoded chemokine receptor homolog US28 stimulates the major immediate early gene promoter/enhancer via the induction of creb', *Journal of Receptors and Signal Transduction* **29**(5), 266–273.
65. Vischer, H. F., Siderius, M., Leurs, R. Smit, M. J. 2014, 'Herpesvirus-encoded gpcrs: neglected players in inflammatory and proliferative diseases?', *Nature reviews Drug discovery* **13**(2), 123–139.

66. Rovati, G. E., Capra, V. Neubig, R. R. 2007, 'The highly conserved dry motif of class ag protein-coupled receptors: beyond the ground state', *Molecular pharmacology* **71**(4), 959–964.

67. Maussang, D., Verzijl, D., van Walsum, M., Leurs, R., Holl, J., Pleskoff, O., Michel, D., van Dongen, G.A., Smit, M.J. 2006, 'Human cytomegalovirus-encoded chemokine receptor US28 promotes tumorigenesis', *Proceedings of the National Academy of Sciences* **103**(35), 13068–13073.

68. Waldhoer, M., Kledal, T. N., Farrell, H. Schwartz, T. W. 2002, 'Murine cytomegalovirus (cmv) m33 and human cmv US28 receptors exhibit similar constitutive signaling activities', *Journal of virology* **76**(16), 8161–8168.

69. Miller, W. E., Houtz, D. A., Nelson, C. D., Kolattukudy, P. Lefkowitz, R. J. 2003, 'G-protein-coupled receptor (gpcr) kinase phosphorylation and β-arrestin recruitment regulate the constitutive signaling activity of the human cytomegalovirus US28 gpcr', *Journal of Biological Chemistry* **278**(24), 21663–21671.

70. Mokros, T., Rehm, A., Droese, J., Oppermann, M., Lipp, M., Höpken, U.E. 2002 , 'Surface expression and endocytosis of the human cytomegalovirus-encoded chemokine receptor US28 is regulated by agonist-independent phosphorylation', *Journal of Biological Chemistry* **277**(47), 45122–45128.

71. Sherrill, J. D. Miller, W. E. 2006, 'G protein-coupled receptor (gpcr) kinase 2 regulates agonist-independent gq/11 signaling from the mouse cytomegalovirus gpcr m33', *Journal of Biological Chemistry* **281**(52), 39796–39805.

72. Krishna, B., Spiess, K., Poole, E. Lau B., Voigt S., Kledal TN., Rosenkilde MM., Sinclair JH., 2017, 'Targeting the latent cytomegalovirus reservoir with an antiviral fusion toxin protein', *Nature Communications* **8**, 14321.

73. Albright, E. R. Kalejta, R. F. 2013, 'Myeloblastic cell lines mimic some but not all aspects of human cytomegalovirus experimental latency defined in primary cd34+ cell populations', *Journal of virology* **87**(17), 9802–9812.

74. Keyes, L. R., Hargett, D., Soland, M., Bego M., Rossetto C., Almeida-Porada G., St Jeor S., 2012, 'Hcmv protein luna is required for viral reactivation from latently infected primary CD14+ cells', *PloS one* **7**(12), e52827.

75. Van Damme, E., Sauviller, S., Lau, B., Kesteleyn, B., Griffiths, P., Burroughs, A., Emery, V., Sinclair, J., Van Loock, M. 2015, 'Glucocorticosteroids trigger reactivation of human cytomegalovirus from latently infected myeloid cells and increase the risk for hcmv infection in D+R+ liver transplant patients', *Journal of General Virology* **96**(1), 131–143.

76. Casarosa, P., Menge, W. M., Minisini, R. Otto C., van Heteren J., Jongejan A., Timmerman H., Moepps B., Kirchhoff F., Mertens T., Smit MJ., Leurs R., 2003, 'Identification of the first nonpeptidergic inverse agonist for a constitutively active viral-encoded g protein-coupled receptor', *Journal of Biological Chemistry* **278**(7), 5172–5178.

77. Jackson, S. E., Mason, G. M. Wills, M. R. 2011, 'Human cytomegalovirus immunity and immune evasion', *Virus research* **157**(2), 151–160.

78. Khan, N., Shariff, N., Cobbold, M., Bruton R., Ainsworth J., Sinclair A., Nayak L., Moss P., 2002, 'Cytomegalovirus seropositivity drives the CD8 t cell repertoire toward greater clonality in healthy elderly individuals', *The Journal of Immunology* **169**(4), 1984–1992.

79. Sylwester, A.W., Mitchell, B.L., Edgar, J.B., Taormina, C., Pelte, C., Ruchti, F., Sleath, P.R., Grabstein, K.H., Hosken, N.A., Kern, F., Nelson, J.A., Picker, L.J. 2005, 'Broadly targeted human cytomegalovirus-specific CD4+ and CD8+ t cells dominate the memory compartments of exposed subjects', *The Journal of experimental medicine* **202**(5), 673–685.

80. Takenaka, K., Nishida, T., Asano-Mori, Y., Oshima, K., Ohashi, K., Mori, T., Kanamori, H., Miyamura, K., Kato, C., Kobayashi, N., Uchida, N., Nakamae, H., Ichinohe, T.,

Morishima, Y., Suzuki, R., Yamaguchi, T., Fukuda, T. 2015, 'Cytomegalovirus reactivation after allogeneic hematopoietic stem cell transplantation is associated with a reduced risk of relapse in patients with acute myeloid leukemia who survived to day 100 after transplantation: the japan society for hematopoietic cell transplantation transplantation-related complication working group', *Biology of Blood and Marrow Transplantation* **21**(11), 2008–2016.

81. Sinclair, J. Poole, E. 2014, 'Human cytomegalovirus latency and reactivation in and beyond the myeloid lineage', *Future Virology* **9**(6), 557–563.
82. Streblow, D.N., Vomaske, J., Smith, P., Melnychuk, R., Hall, L., Pancheva, D., Smit, M., Casarosa, P., Schlaepfer, D.D., Nelson, J.A. 2003, 'Human cytomegalovirus chemokine receptor US28-induced smooth muscle cell migration is mediated by focal adhesion kinase and src', *Journal of Biological Chemistry* **278**(50), 50456–50465.
83. DeMeritt, I. B., Milford, L. E. Yurochko, A. D. 2004, 'Activation of the NF-kappaB pathway in human cytomegalovirus-infected cells is necessary for efficient transactivation of the major immediate-early promoter', *Journal of virology* **78**(9), 4498–4507.
84. Keller, M., Wheeler, D., Cooper, E. Meier, J. 2003, 'Role of the human cytomegalovirus major immediate-early promoter's 19-base-pair-repeat cyclic amp-response element in acutely infected cells', *Journal of virology* **77**(12), 6666–6675.
85. Chang, Z. Beezhold, D. 1993, 'Protein kinase C activation in human monocytes: regulation of PKC isoforms.', *Immunology* **80**(3), 360.
86. Evers, D. L., Wang, X. Huang, E.-S. 2004, 'Cellular stress and signal transduction responses to human cytomegalovirus infection', *Microbes and infection* **6**(12), 1084–1093.
87. Yurochko, A. 2008, Human cytomegalovirus modulation of signal transduction, in 'Human Cytomegalovirus', Springer, pp. 205–220.

88. Bolovan-Fritts, C. A., Mocarski, E. S. Wiedeman, J. A. 1999, 'Peripheral blood CD14+ cells from healthy subjects carry a circular conformation of latent cytomegalovirus genome', *Blood* **93**(1), 394–398.

89. Poole, E., Reeves, M. Sinclair, J. H. 2014, 'The use of primary human cells (fibroblasts, monocytes, and others) to assess human cytomegalovirus function', *Methods in molecular biology (Clifton, N.J.)* **1119**, 81–98.

90. O'Connor, C. M. Murphy, E. A. 2012, 'A myeloid progenitor cell line capable of supporting human cytomegalovirus latency and reactivation, resulting in infectious progeny', *Journal of Virology* **86**(18), 9854–9865.

91. Lau, B., Poole, E., Van Damme, E., Bunkens L., Sowash M., King H., Murphy E., Wills M., Van Loock M., Sinclair J., 2016, 'Human cytomegalovirus mir-UL112-1 promotes the down-regulation of viral immediate early-gene expression during latency to prevent T-cell recognition of latently infected cells', *Journal of General Virology* **97**(9), 2387–2398.

92. Poole, E., Avdic, S., Hodkinson, J., Jackson, S., Wills, M., Slobedman, B., Sinclair, J. 2014, 'Latency-associated viral IL-10 encoded by human cytomegalovirus modulates cellular IL-10 and CCl8 secretion during latent infection through changes in the cellular mirna, hsa-mir-92a', *Journal of virology* **88**(24), 13947–13955.

93. Poole E., Groves, I., MacDonald, A., Pang, Y., Alcami, A., Sinclair, J. 2009, 'Identification of TRIM23 as a cofactor involved in the regulation of nf-κb by human cytomegalovirus', *Journal of virology* **83**(8), 3581–3590.

94. Suzuki, K., Bose, P., Leong-Quong, R. Y., Fujita, D. J., and Riabowol, K. 2010, 'REAP: A two minute cell fractionation method.' *BMC research notes*, 3(1):294.

95. Jackson, S., Mason, G., Okecha, G., Sissons, J. Wills, M. 2014, 'Diverse specificities, phenotypes, and antiviral activities of cytomegalovirus-specific CD8+ t cells', *Journal of virology* **88**(18), 10894–10908.

Figure 1: Infection of monocytes with Titan-ΔUS28 results in lytic infection. CD14+ peripheral blood monocytes were isolated and experimentally infected at an MOI of 5 with HCMV Titan-WT or Titan-ΔUS28. A) Seven days post-infection, RNA from these cultures was harvested and analysed for expression of the latency-associated gene UL138, the major immediate early lytic gene IE1, and the major late gene UL99. Data were normalised to GAPDH RNA. B) Seven days post-infection, monocytes were also fixed and stained for IE protein and UL32-GFP (using an antibody against the GFP tag). C) These monocyte cultures were then co-cultured with HFFs and the number of infected HFFs was measured, by staining for cells expressing HCMV IE protein, 72 hours post-co-culture. All data points show means from at least four independent experiments, error bars show standard deviations, and p values * = 0.05 were calculated using Student's T test and were considered significant

Figure 2: Titan-ΔUS28 virus initiates lytic infection immediately after infection of CD14+ monocytes. CD14+ peripheral blood monocytes were isolated and experimentally infected at an MOI of 5 with HCMV Titan-WT or Titan-ΔUS28. A) 12 hours post infection, RNA from these cultures was harvested and analysed by RT-qPCR for the latent gene UL138 and the lytic genes, IE and UL99. Data was normalised to GAPDH RNA. B) One day post-infection, monocytes were fixed and stained for IE protein. C) Two days post-infection, monocytes were fixed and stained for IE or UL32-GFP protein. D) Each day post infection, media was harvested from monocytes infected with Titan-WT or Titan-ΔUS28 and titrated onto indicator HFFs. These were subsequently stained for HCMV IE protein, as a measure of viral titres, 24 hours post infection. All data points show means from at least four independent experiments and error bars show standard deviations.

Figure 3: Ectopic US28 expression in THP-1 cells can complement for a deletion of US28 from the virus. THP-1 cells stably expressing an N-terminally HA- tagged US28 (HA-US28-WT), US28 with a disrupted G protein binding DRY motif (HA-US28-R129A) and US28 with a disrupted chemokine binding region (HA-US28-Y16F) were generated by lentiviral transduction and puromycin selection. A) western blot analysis using an antibody against the N terminal HA tag was carried out on an empty vector transduced cell line and the three cell lines expressing HA-US28 constructs. B) These THP-1 cells, expressing different HA-US28 constructs and empty vector control cells, were infected with Titan-ΔUS28 and fixed five days post infection. Fixed samples were stained for immediate early or UL32-GFP and nuclei were also stained. C) Media from these infected cells was titrated on indicator fibroblasts and the number of infectious virions quantified by IE staining. Data are means from at least three independent experiments, error bars show standard deviations.

Figure 4: US28 represses the MIEP in undifferentiated myeloid cell lines, but activates it in differentiated myeloid cells. A) THP-1 cells which had been transduced with an MIEP-eGFP construct were then transfected by nucleofection with HA-US28-WT, HA-US28-R129A or HA-US28-Y16F constructs. Three days after nucleofection, cells were analysed for eGFP expression by flow cytometry. B) Additionally, cells were treated with PMA two days after nucleofection and two days after treatment, analysed by flow cytometry. Data shows percentage change in mean fluorescent intensities from four technical replicates, after selecting for single cells and excluding dead cells using Zombie red dye. Error bars show standard deviations and p values * = 0.05 and ** =0.01 were calculated using Student's T test and were considered significant.

Figure 5: US28 expression, in isolation, in THP-1 cells attenuates cellular signalling. THP-1 cells which had been induced to express either US28-WT or US28-R129A (which cannot maintain latency) were lysed and analysed for changes in cellular kinase phopshorylation levels by antibody array. Data represent fold change in dephopshorylation of each kinase from THP-1 cells expressing US28-WT over the levels induced by THP-1 cells expressing US28-R129A. Data points in red had a change in intensity of +/- log2(0.5) arbitrary units and/or a p value greater than log10(1.5).

Figure 6: US28 expression, in THP-1 cells, in isolation, attenuates MAP kinase and NFκB cellular signalling. A) THP-1 cells expressing either HA-US28-WT or HA-US28-R129A, (which cannot maintain latency) or an empty vector control were lysed and analysed by western blot for phospho- and total ERK1/2 and beta-actin (left panels). THP-1 cells were also differentiated with PMA treatment and, four days post treatment, were analysed by western blot for phospho- and total ERK1/2 and beta-actin (left panels). B) The same analysis was performed for phospho- and total MSK-1 in undifferentiated cells (left panels) or four days after differentiation with PMA treatment (right panels), and also for phospho- and total CREB (panel C). D) Cells were also fractionated into nuclear and cytoplasmic fractions and these fractions were analysed by western blot analysis for NFκB protein (p65) before (left) and after (right) differentiation. The nuclear protein p84 and the cytoplasmic protein GAPDH were used as loading and fractionation controls for the respective fractions. E) These blots were analysed by densitometry analysis using ImageJ software, and the relative amount of phospho-protein (for panels A-C) or NFκB localisation (for panel D) was quantified against actin, GAPDH or p84 loading controls.

Figure 7: Inhibition of MAP kinase and NFκB pathways can prevent lytic infection of monocytes by Titan-ΔUS28. A) CD14+ peripheral blood monocytes were isolated and infected at an MOI of 5 with Titan-ΔUS28 in the presence of an increasing concentration of H89 (an inhibitor of MSK-1). Subsequently, GFP-positive cells were counted three days post-infection and cell survival was measured by trypan blue exclusion staining. B) Monocytes were infected with Titan-ΔUS28 in the presence of titrations of BAY11-7082 (an inhibitor of IKKα). Subsequently, GFP-positive cells were counted three days post-infection and cell survival was measured by trypan blue exclusion staining. C) Monocytes were treated with both H89 and BAY11-7082 and then infected with Titan-ΔUS28 in the presence of both inhibitors. Subsequently, GFP-positive cells were counted three days post-infection and cell survival was measured by trypan blue exclusion staining. D) Monocytes were infected with Titan-ΔUS28 but treatment with H89 and BAY11-7082 was delayed until one hour post-infection. Subsequently, GFP-positive cells were counted three days post-infection and cell survival was measured by trypan blue exclusion staining. E) Monocytes were infected with Titan-ΔUS28 but treatment with H89 and BAY11-7082 was delayed until one day post-infection. Subsequently, GFP-positive cells were counted three days post-infection and cell survival was measured by trypan blue exclusion staining. All data points show means from at least three independent experiments, error bars show standard deviations.

Figure 8: The MIEP is associated with phosphorylated Serine-10-H3 in the absence of US28. CD14+ monocytes were infected with either Titan-WT or Titan-ΔUS28. The cells were harvested 6 days post infection for ChIP analysis (using Sigma Imprint ChIP kit). The MIEP was precipitated with either anti-HP1 (HP1 Antibody (FL-191): sc-28735) anti-ser-10-H3 (Phospho-Histone H3 (Ser10) Antibody (9H12L10), ABfinity™ Rabbit Monoclonal) or the isotype control. The MIEP was quantitated against a standard curve of viral DNA, analysed by qPCR and then plotted as a percentage of input DNA with each sample run in triplicate.

Figure 9: VUF2274 is able to induce reactivation HCMV from latently infected monocytes. CD14+ peripheral blood monocytes were isolated and infected at an MOI of 5 with RV1164, which has an IE2-YFP fluorescent tag. Three days post infection, an increasing concentration of the US28 inhibitor, VUF2274 was added to cells. A) IE2-YFP positive cells were counted by immunofluorescent microscopy, 72 hours post-treatment. B) Five days post drug treatment, media was removed from these cells and titrated onto HFFs. Three days post infection, HFFs were fixed and stained for IE protein, and IE positive cells were counted by immunofluorescence microscopy. C) CD14+ peripheral blood monocytes were isolated and infected at an MOI of 5 with Titan-WT. Three days post infection, an increasing concentration of the US28 inhibitor, VUF2274 was added to cells. Five days post-drug treatment, UL32-GFP positive cells were counted by fluorescence microscopy. D) Cell survival of monocytes in the presence of VUF2274 for 4 days was measured using trypan blue exclusion staining. All data points show means from three replicates and error bars show standard deviations, data were analysed by ANOVA followed by Tukey's post hoc test, and p-values * ($p=0.05$) were considered significant.

Figure 10: Monocytes infected with Titan-ΔUS28, or monocytes infected with SV40-GFP-TB40E in the presence of US28 inhibitors, are targets for HCMV-specific T cell responses. A) CD14+ monocytes were infected with either Titan-WT or Titan-ΔUS28. Three days post infection, monocytes were co-cultured with IE72-specific T cells for a further three days. After this, monocytes were washed to remove T cells and monocytes were then differentiated and matured to induce virus reactivation. Reactivated virus was quantified by fibroblast co-culture and staining for IE foci. B) From the experiment in A), media from monocytes was removed three days after T cell treatment and titrated onto fibroblasts to quantify virus release from Titan-ΔUS28-infected monocytes. C) From seropositive donors, CD14+ monocytes were infected with either Titan-WT or Titan-ΔUS28 and, three days post infection, the non-monocyte PBMCs were added back to the infected monocytes for four days and then removed by washing. The remaining adherent monocytes were then differentiated and matured to induce virus reactivation. Reactivated virus was quantified by fibroblast co-culture and staining for IE foci. D) From seropositive donors, CD14+ monocytes were infected with either Titan-WT or Titan-ΔUS28, and five days post infection, latent monocytes were left untreated or co-cultured overnight with isolated CD8+ T cells from the same donor and analysed for expression of the activation markers CD69 and 4-1BB, and degranulation markers: CD107a, Granzymes A, B and K, TNFα and IFN-γ expression. D) shows the percentage of CD8+ T cells expressing both activation markers, above background stimulation, in response to either Titan-WT or Titan-ΔUS28 infected monocytes. E) Of these activated monocytes, the proportion of HCMV-specific CD8+ T cells expressing degranulation markers are shown, as are CD8+ T cells stimulated with virus infected monocytes and pulsed with IE1/2 peptides as antigen specific positive controls. F) Monocytes from an HLA-A2-positive donor were latently infected with an

SV40-GFP-TB40E and three days post infection were left untreated or treated with VUF2274. Two days post treatment with drug, monocytes were co-cultured with IE72-specific T cells following which monocytes expressing GFP were counted over the next four days. Data points for all panels show means from at least three independent experiments, error bars show standard deviations.

Figure S1: Titan-ΔUS28 infected monocytes replicate viral genomes. CD14+ peripheral blood monocytes were isolated from the PBMCs of healthy donors and experimentally infected at an MOI of 5 with Titan-WT or Titan-ΔUS28. Either 1 day, or 7 days post infection, DNA was collected from cultures and genomes quantified by qPCR analysis against the MIEP region. Values were corrected to GAPDH qPCR values, all data points show means from three independent experiments, error bars show standard deviations.

Figure S2: CD14+ monocytes infected with Titan- ΔUS28 showed no changes in phenotypic markers associated with myeloid differentation. CD14+ peripheral blood monocytes were isolated from the PBMCs of healthy donors and experimentally infected at an MOI of 5 with Titan-WT or Titan-ΔUS28. 7 days post infection, cells were stained with anti-CD14 (A) or anti-CD83 (B) antibodies and analysed by flow cytometry.

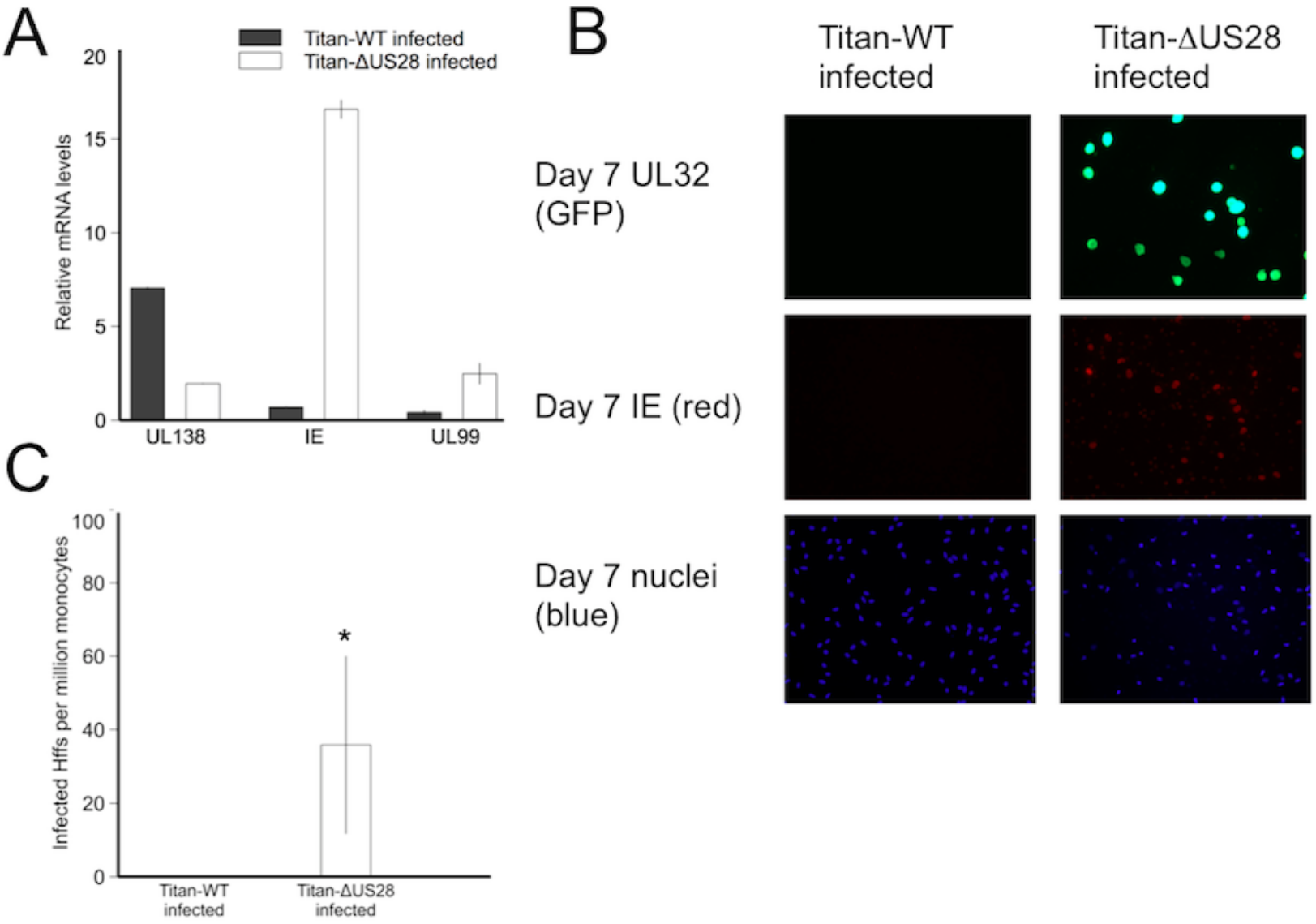
Figure S3: Ectopic US28 expression in THP-1 cells does not affect the establishment of latency when infected with Titan-WT virus. THP-1 cells stably expressing HA-US28-WT, HA-US28-R129A or HA-US28-Y16F (see Figure **3**) were were infected with Titan-WT for five days. Cells were then fixed and stained for IE proteins or UL32-GFP and nuclei were also stained.

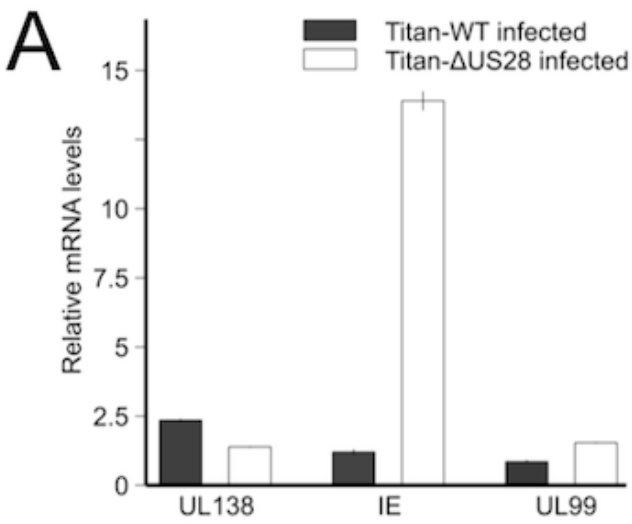
Figure S4: Ectopic US28 expression in THP-1 cells complements for a deletion of US28 and virus can be reactivated from these cells. THP-1 cells stably expressing HA-US28-WT, HA-US28-R129A or HA-US28-Y16F (see Figure **3**) were infected for three days with Titan-ΔUS28 and then subsequently treated with PMA. Four day post PMA treatment, cells were fixed and stained for immediate early or UL32-GFP and nuclei were also stained.

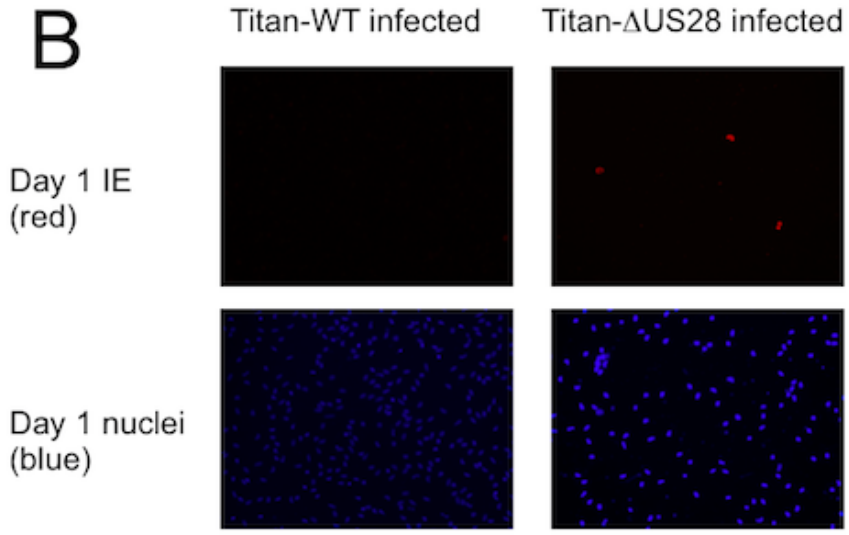
Figure S5: US28 represses the MIEP in undifferentiated myeloid cell lines, but activates it in differentiated myeloid cells. THP-1 cells which had been transduced by lentivirus to stably express an MIEP-eGFP construct were then transfected by nucleofection with HA-US28-WT, HA-US28-R129A or HA-US28-Y16F constructs. Western blot analysis using an antibody against the N terminal HA tag was carried out on an empty vector transfected cell line and the three cell lines transfected with HA-US28 constructs.

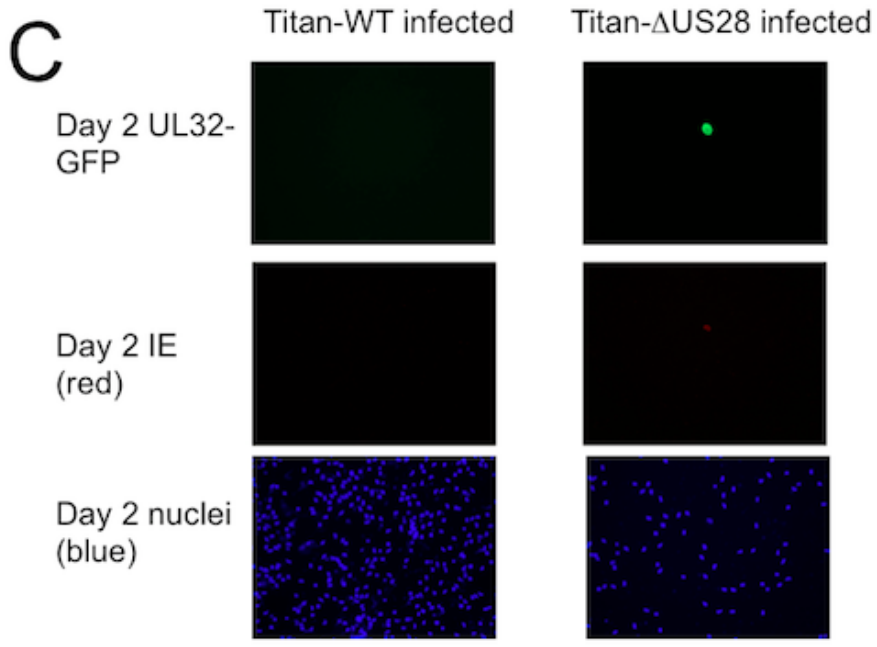
Figure S6: The inhibitors of MAP kinase and NFkB: Bay11-7082 and H89, can block VUF2274-induced IE gene expression in latent cells. CD14+ monocytes were infected with IE2-YFP and then treated with inhibitors (VUF2274 in a concentration gradient, and Bay11-7082 and H89 at the fixed concentration 5uM) as indicated, 24h post infection.

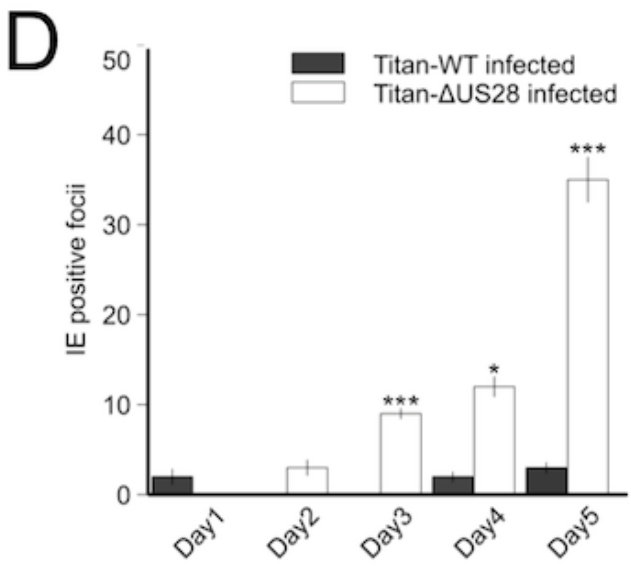
Three days later, IE positive cells were enumerated in triplicate wells of a 96 well plate. All data points show means from three replicates and error bars show standard deviations, data were analysed by ANOVA followed by Tukey's post hoc test, and p-values * ($p=0.05$) were considered significant.

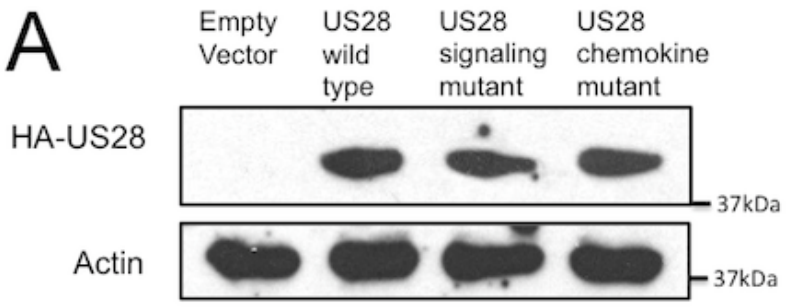

A
Titan-WT infected
Titan-ΔUS28 infected
Relative mRNA levels
20
15
10
5
0
UL138
IE
UL99
B
Titan-WT infected
Titan-ΔUS28 infected
Day 7 UL32 (GFP)
Day 7 IE (red)
Day 7 nuclei (blue)
C
Infected Hffs per million monocytes
100
80
60
40
20
0
*
Titan-WT infected
Titan-ΔUS28 infected

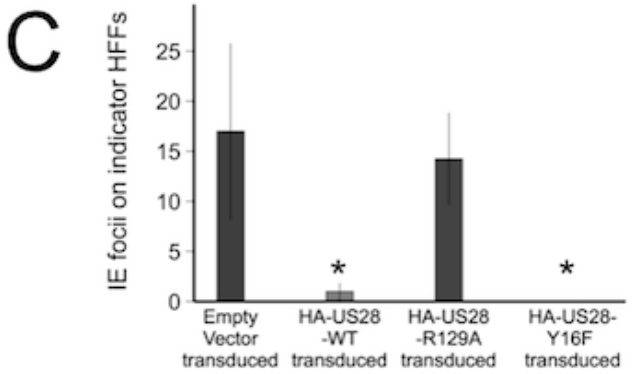

A
Titan-WT infected
Titan-ΔUS28 infected
Relative mRNA levels
15
10
7.5
5
2.5
0
UL138
IE
UL99

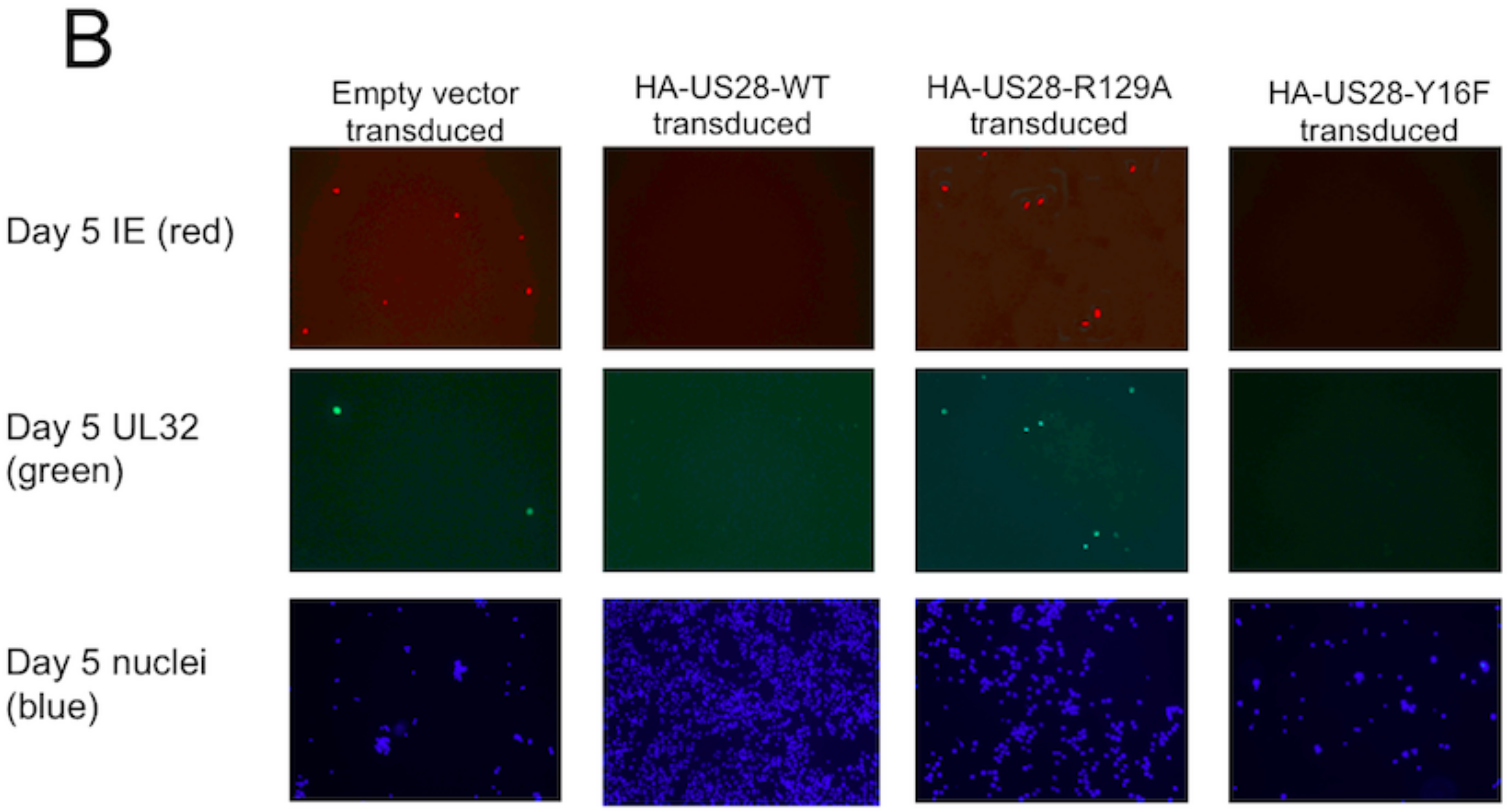

B
Titan-WT infected
Titan-ΔUS28 infected
Day 1 IE (red)
Day 1 nuclei (blue)

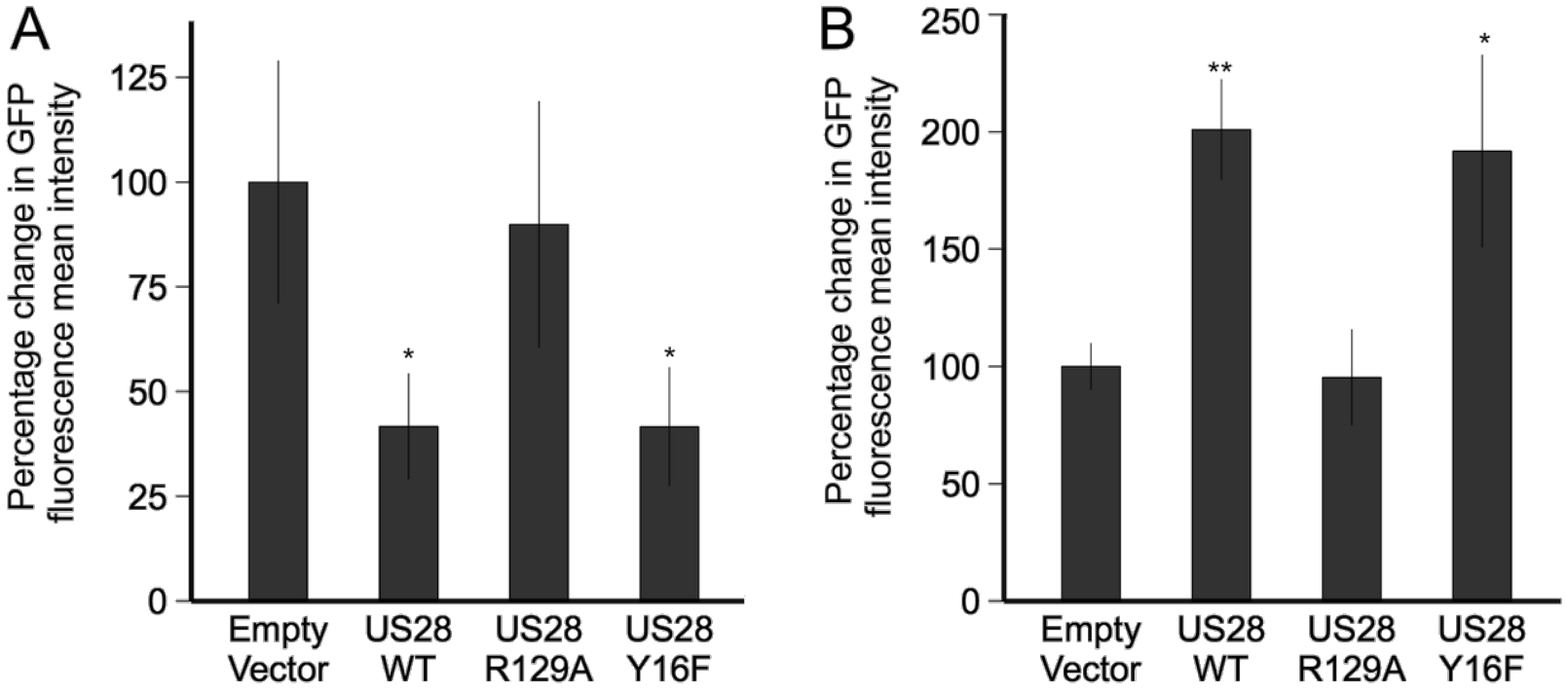

C
Titan-WT infected
Titan-ΔUS28 infected
Day 2 UL32-GFP
Day 2 IE (red)
Day 2 nuclei (blue)

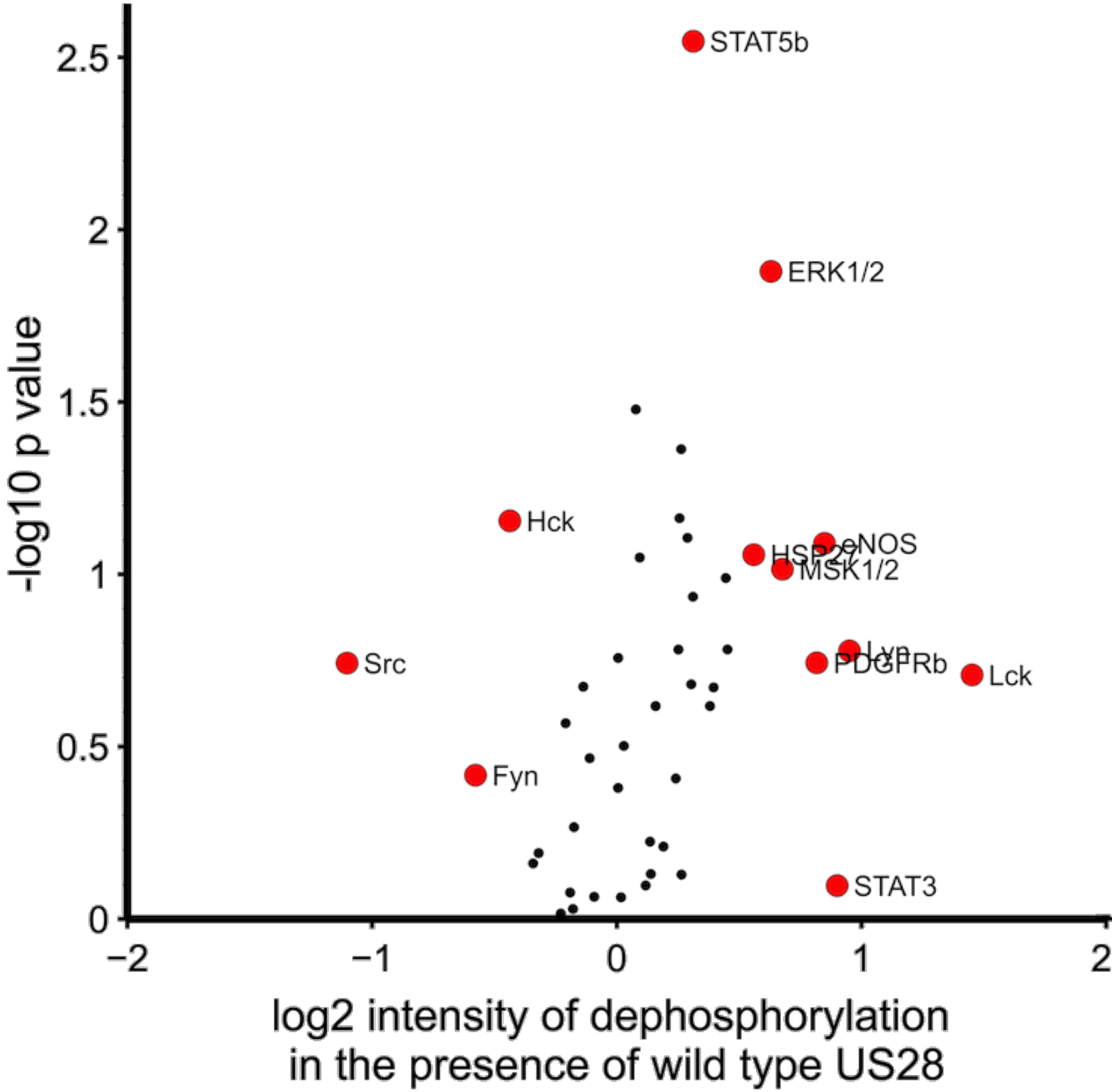

D
Titan-WT infected
Titan-ΔUS28 infected
IE positive focii
50
40
30
20
10
0
***
*
***
Day1
Day2
Day3
Day4
Day5

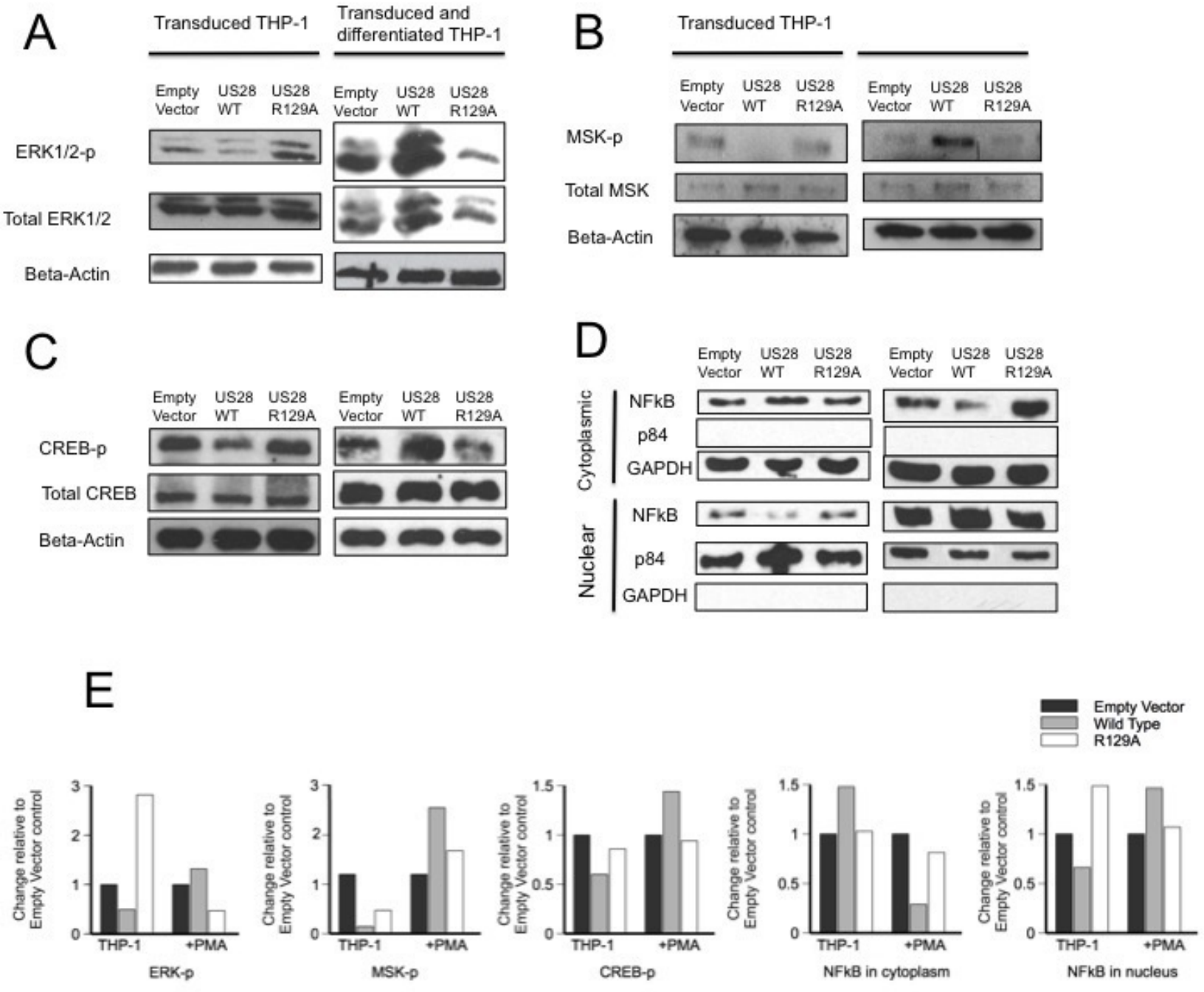

A
Empty Vector
US28 wild type
US28 signaling mutant
US28 chemokine mutant
HA-US28
37kDa
Actin
37kDa

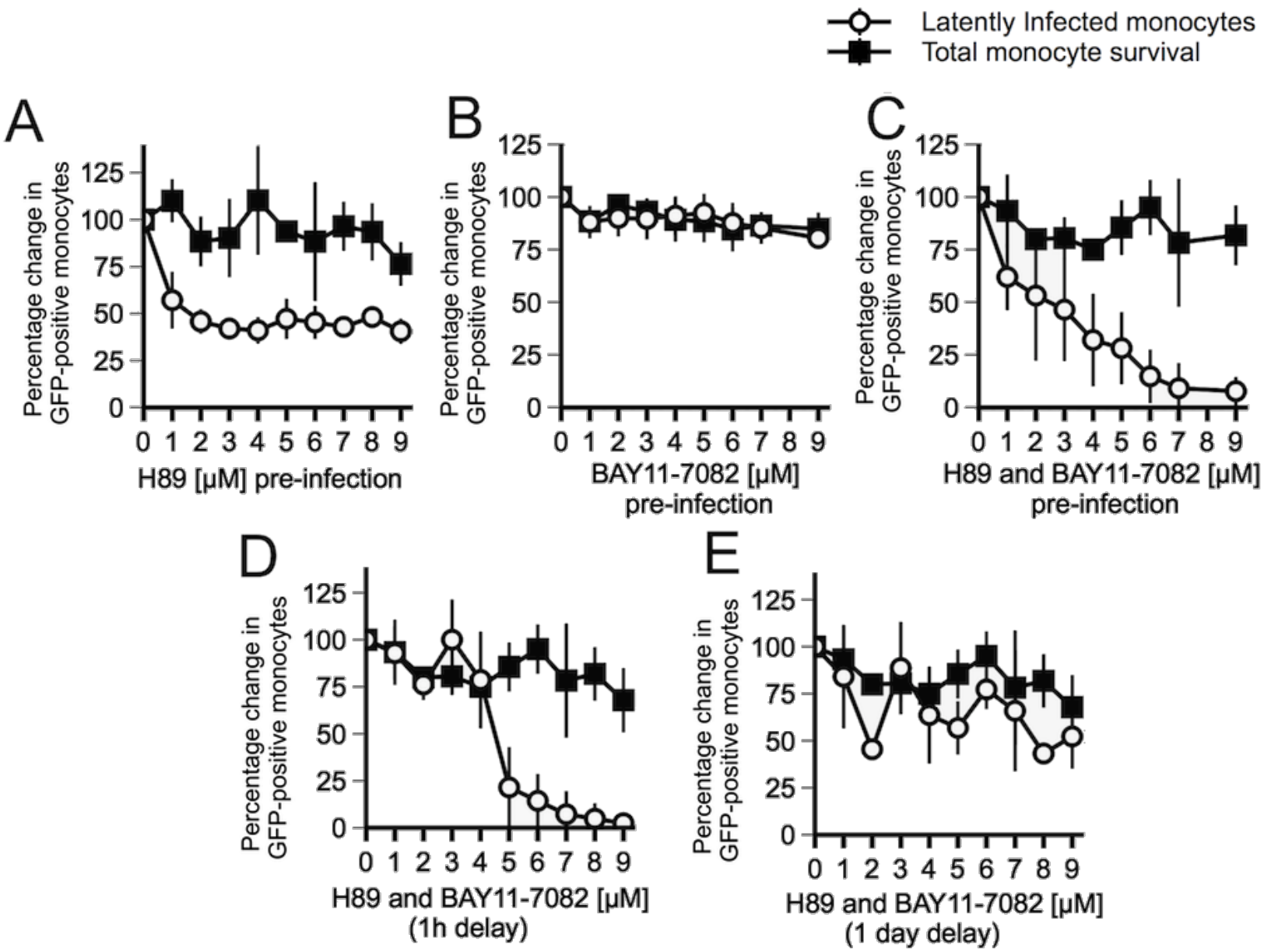

C
IE focii on indicator HFFs
25
20
15
10
5
0
*
*
Empty Vector transduced
HA-US28 -WT transduced
HA-US28 -R129A transduced
HA-US28-Y16F transduced

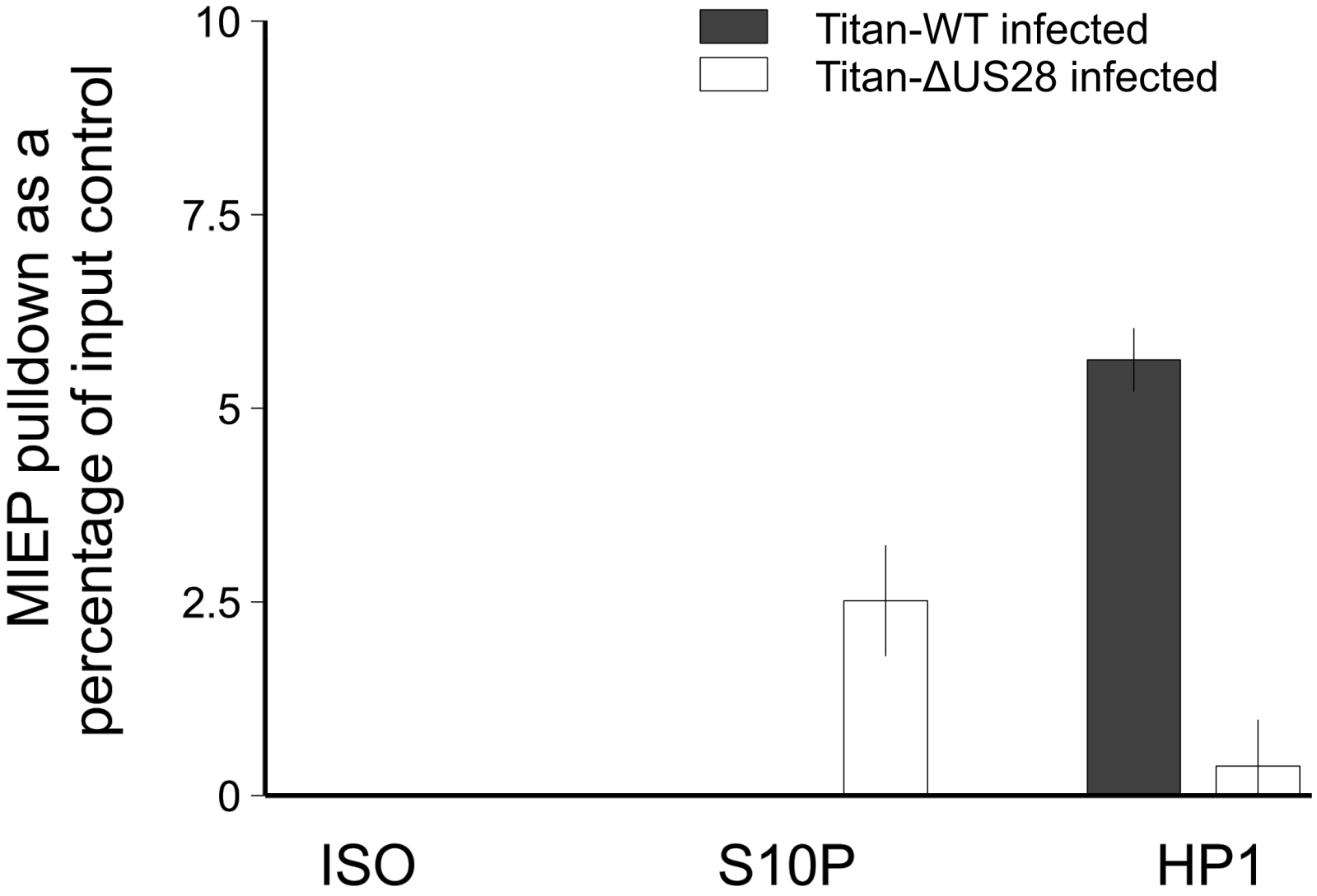

B
Empty vector transduced
HA-US28-WT transduced
HA-US28-R129A transduced
HA-US28-Y16F transduced
Day 5 IE (red)
Day 5 UL32 (green)
Day 5 nuclei (blue)

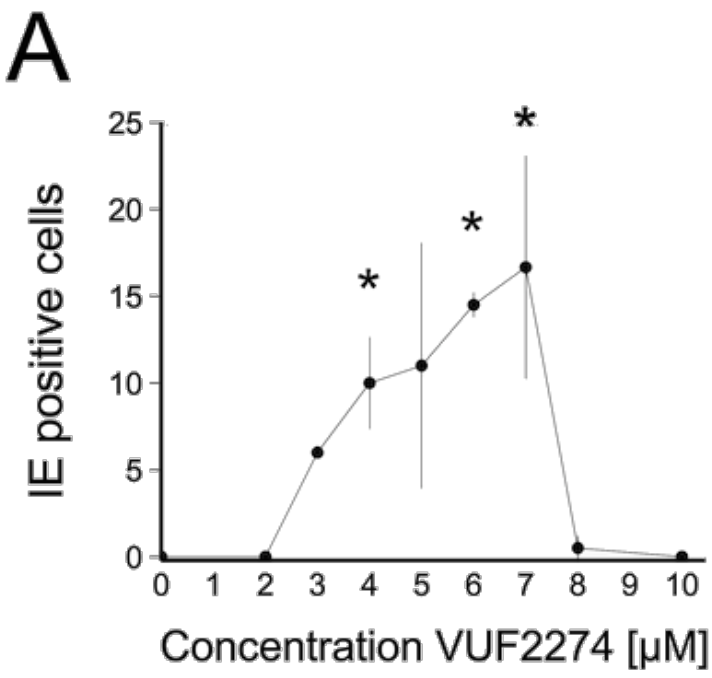

A
Percentage change in GFP fluorescence mean intensity
125
100
75
50
25
0
*
*
Empty Vector
US28 WT
US28 R129A
US28 Y16F
B
Percentage change in GFP fluorescence mean intensity
250
200
150
100
50
0
**
*
Empty Vector
US28 WT
US28 R129A
US28 Y16F

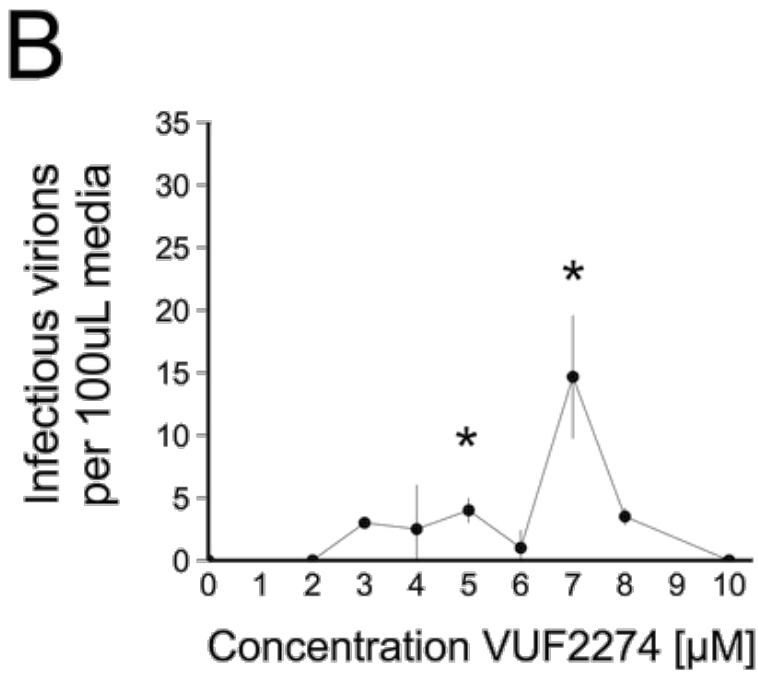

STAT5b
ERK1/2
Hck
eNOS
HSP27
MSK1/2
Src
Lyn
PDGFRb
Lck
Fyn
STAT3
2.5
2
1.5
1
0.5
0
−2
−1
0
1
2
-log10 p value
log2 intensity of dephosphorylation
in the presence of wild type US28

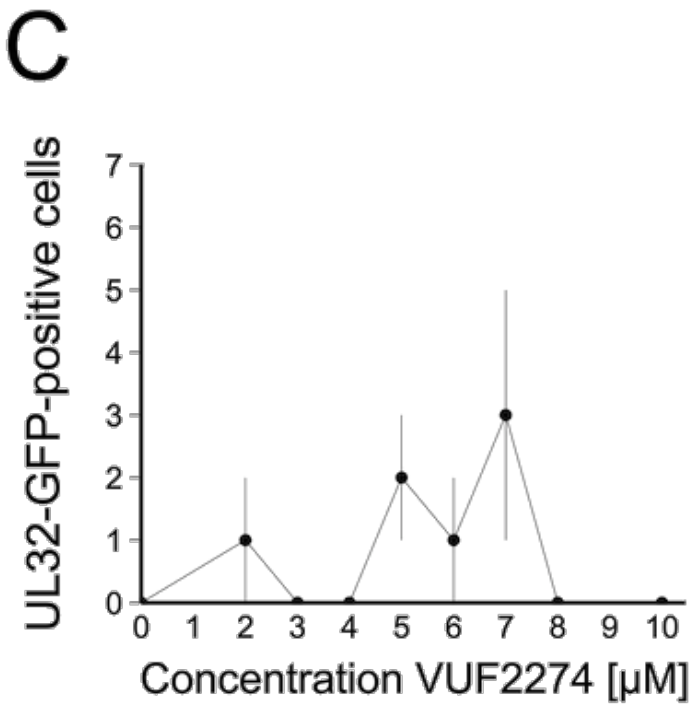

A
Transduced THP-1
Transduced and differentiated THP-1
Empty Vector
US28 WT
US28 R129A
ERK1/2-p
Total ERK1/2
Beta-Actin
B
Transduced THP-1
MSK-p
Total MSK
Beta-Actin
C
CREB-p
Total CREB
Beta-Actin
D
Cytoplasmic
Nuclear
NFkB
p84
GAPDH
E
Empty Vector
Wild Type
R129A
Change relative to Empty Vector control
THP-1
+PMA
ERK-p
MSK-p
CREB-p
NFkB in cytoplasm
NFkB in nucleus

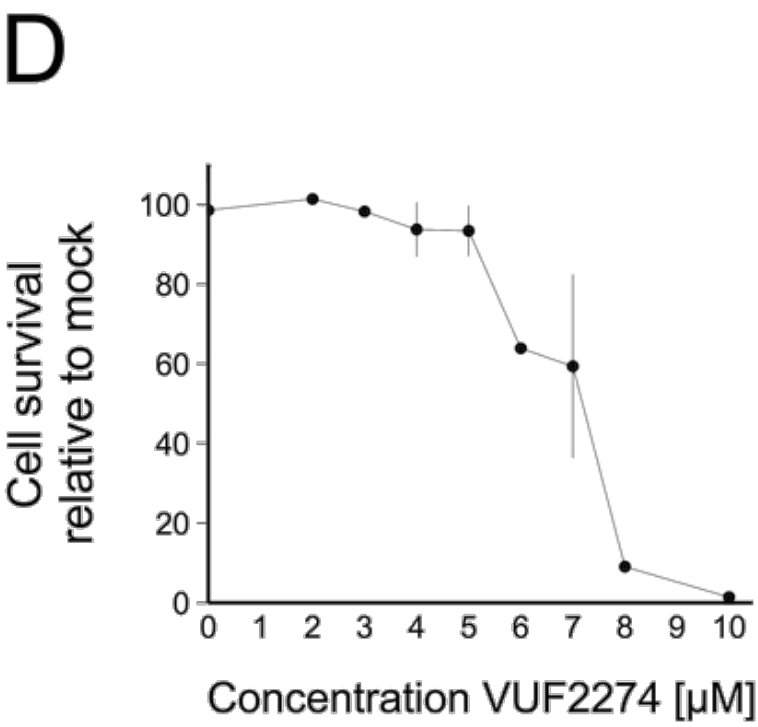

Latently Infected monocytes
Total monocyte survival
A
B
C
D
E
Percentage change in GFP-positive monocytes
0
25
50
75
100
125
0 1 2 3 4 5 6 7 8 9
H89 [μM] pre-infection
BAY11-7082 [μM] pre-infection
H89 and BAY11-7082 [μM] pre-infection
H89 and BAY11-7082 [μM] (1h delay)
H89 and BAY11-7082 [μM] (1 day delay)

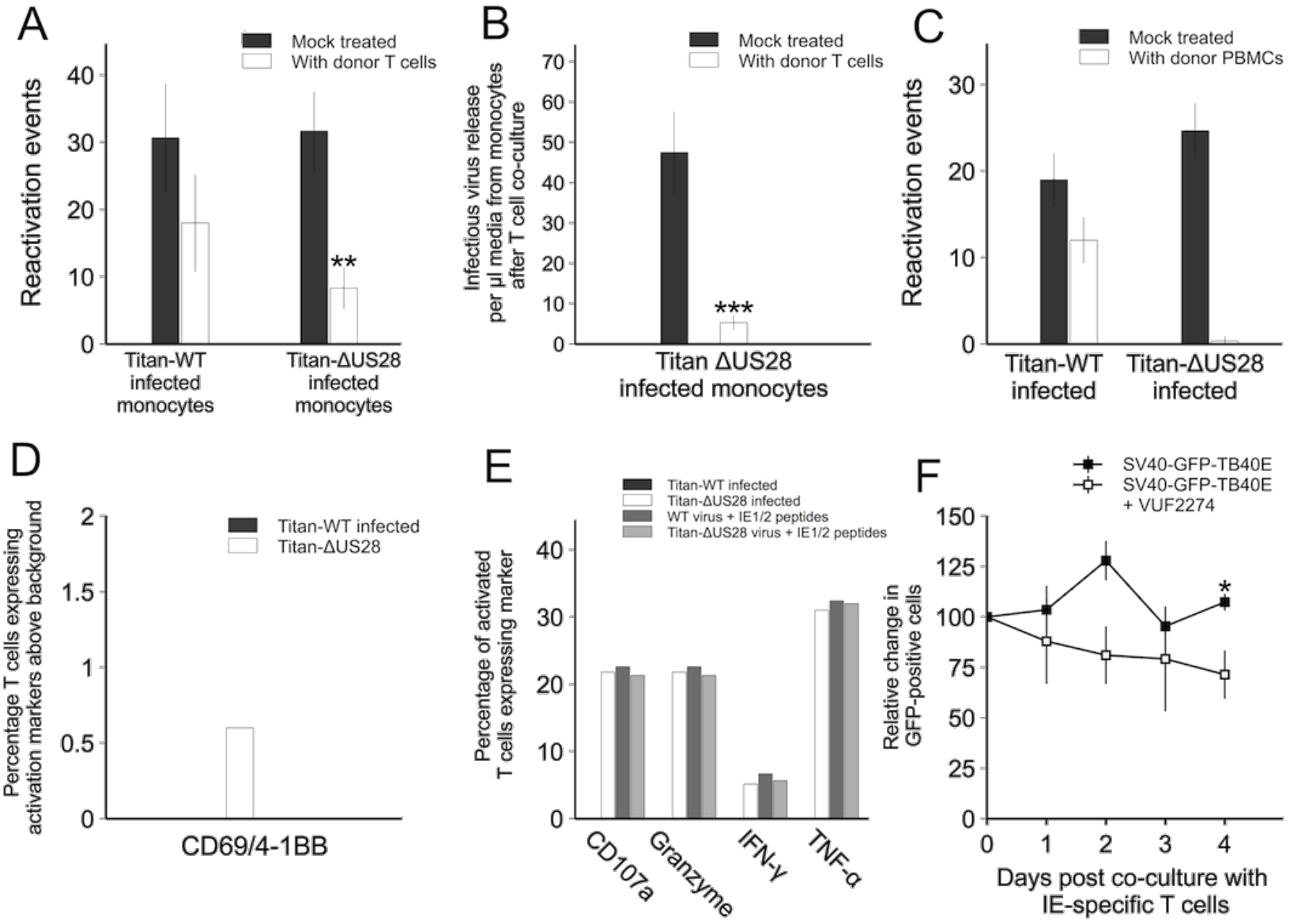

MIEP pulldown as a percentage of input control
10
7.5
5
2.5
0
Titan-WT infected
Titan-ΔUS28 infected
ISO
S10P
HP1


A
IE positive cells
25
20
15
10
5
0
0 1 2 3 4 5 6 7 8 9 10
Concentration VUF2274 [µM]
*
*
*


B
Infectious virions per 100uL media
35
30
25
20
15
10
5
0
0 1 2 3 4 5 6 7 8 9 10
Concentration VUF2274 [µM]
*
*


C
UL32-GFP-positive cells
7
6
5
4
3
2
1
0
0 1 2 3 4 5 6 7 8 9 10
Concentration VUF2274 [µM]


D
Cell survival relative to mock
100
80
60
40
20
0
0 1 2 3 4 5 6 7 8 9 10
Concentration VUF2274 [µM]


A
Mock treated
With donor T cells
Reactivation events
40
30
20
10
0
**
Titan-WT infected monocytes
Titan-ΔUS28 infected monocytes
B
Mock treated
With donor T cells
Infectious virus release per µl media from monocytes after T cell co-culture
70
60
50
40
30
20
10
0
***
Titan ΔUS28 infected monocytes
C
Mock treated
With donor PBMCs
Reactivation events
30
20
10
0
Titan-WT infected
Titan-ΔUS28 infected
D
Titan-WT infected
Titan-ΔUS28
Percentage T cells expressing activation markers above background
2
1.5
1
0.5
0
CD69/4-1BB
E
Titan-WT infected
Titan-ΔUS28 infected
WT virus + IE1/2 peptides
Titan-ΔUS28 virus + IE1/2 peptides
Percentage of activated T cells expressing marker
40
30
20
10
0
CD107a
Granzyme
IFN-γ
TNF-α
F
SV40-GFP-TB40E
SV40-GFP-TB40E + VUF2274
Relative change in GFP-positive cells
150
125
100
75
50
25
0
*
0
1
2
3
4
Days post co-culture with IE-specific T cells