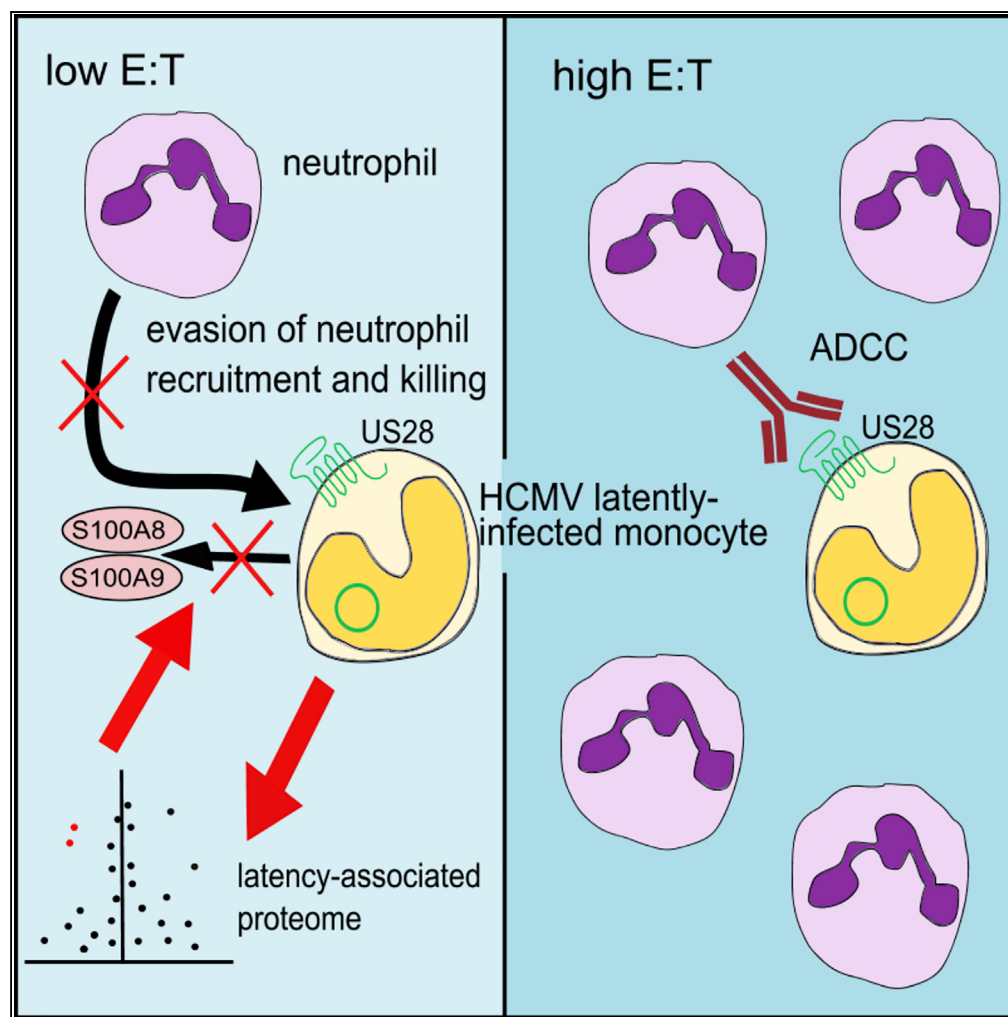


Article

Monocytes Latently Infected with Human Cytomegalovirus Evade Neutrophil Killing



Elizabeth Elder,
Benjamin Krishna,
James
Williamson, ...,
Paul J. Lehner,
John Sinclair,
Emma Poole

js152@cam.ac.uk (J.S.)
elp27@cam.ac.uk (E.P.)

HIGHLIGHTS

Neutrophils can target
HCMV latently infected
monocytes for ADCC-
mediated killing

Killing of latently infected
cells by neutrophils
requires high E:T ratios

Latent infection reduces
secretion of the neutrophil
chemoattractants
S100A8/A9

Decreased S100A8/A9
secretion prevents
neutrophil targeting of
latently infected cells

Elder et al., iScience 12, 13–26
February 22, 2019 © 2019 The
Authors.
<https://doi.org/10.1016/j.isci.2019.01.007>

Article

Monocytes Latently Infected with Human Cytomegalovirus Evade Neutrophil Killing

Elizabeth Elder,¹ Benjamin Krishna,² James Williamson,¹ Yusuf Aslam,¹ Neda Farahi,¹ Alexander Wood,¹ Veronika Romashova,¹ Kate Roche,³ Eain Murphy,³ Edwin Chilvers,¹ Paul J. Lehner,¹ John Sinclair,^{1,*} and Emma Poole^{1,4,*}

SUMMARY

One site of latency of human cytomegalovirus (HCMV) *in vivo* is in undifferentiated cells of the myeloid lineage. Although latently infected cells are known to evade host T cell responses by suppression of T cell effector functions, it is not known if they must also evade surveillance by other host immune cells. Here we show that cells latently infected with HCMV can, indeed, be killed by host neutrophils but only in a serum-dependent manner. Specifically, antibodies to the viral latency-associated US28 protein mediate neutrophil killing of latently infected cells. To address this mechanistically, a full proteomic screen was carried out on latently infected monocytes. This showed that latent infection down-regulates the neutrophil chemoattractants S100A8/A9, thus suppressing neutrophil recruitment to latently infected cells. The ability of latently infected cells to inhibit neutrophil recruitment represents an immune evasion strategy of this persistent human pathogen, helping to prevent clearance of the latent viral reservoir.

INTRODUCTION

Human cytomegalovirus (HCMV) is a human herpesvirus that can cause severe disease in immune-suppressed, immune-compromised, and immune-naïve individuals. For instance, it is the leading viral cause of birth defects in the developed world (Terrazzini and Kern, 2014). There is no effective vaccine against HCMV, and routinely used antivirals suffer from a number of drawbacks including poor bioavailability, toxic side effects, and the risk of emergence of drug resistance (Benzi et al., 2012; Hantz et al., 2010; Komatsu et al., 2014; Scott et al., 2007). One other aspect of HCMV biology also makes antiviral targeting difficult, and this is the ability of the virus to establish a latent infection in certain cell types *in vivo*. During latent infection, the maintenance of viral genome is underpinned by a latency-associated transcription program but in the absence of virus production. This likely helps the virus to avoid immune detection and clearance by host immune responses (Poole et al., 2014a, 2014b; Poole and Sinclair, 2015; Sinclair and Poole, 2014; Wills et al., 2015). However, it is now clear that a substantial level of HCMV disease in immunocompromised subjects results from reactivation of virus from these latently infected cells (Sissons and Wills, 2015).

One site of HCMV latency, *in vivo*, is in the myeloid cells such as CD14⁺ monocytes and their CD34⁺ progenitors. As these cells differentiate into dendritic cells (DCs), or macrophages, viral lytic gene expression reactivates leading to viral DNA replication and *de novo* production of infectious virions. Consequently, HCMV lifelong persistence likely results from constant reactivation of the virus from latency, but, in the immune-competent individuals, these reactivation events are kept sub-clinical by normal host immune responses (Poole et al., 2014a, 2014b; Poole and Sinclair, 2015; Sinclair and Poole, 2014; Wills et al., 2015).

Understanding latent carriage is clearly important for a full understanding of how this persistent human pathogen interacts with its host, and, lately, substantial progress has been made in identifying the effects of latent infection on the latently infected cell. For instance, although the transcription program of key lytic genes is heavily repressed during HCMV latency, a number of viral genes are known to be expressed in latently infected myeloid cells (Cheng et al., 2017; Dupont and Reeves, 2016; Shnyder et al., 2018) and the effects of some of these on latently infected cells have been reported (Humby and O'Connor, 2015; Keyes et al., 2013; Lau et al., 2016b; Poole et al., 2014a, 2014b; Weekes et al., 2013). This has uncovered a number of ways by which latency-associated viral gene expression manipulates the cell to optimize carriage and reactivation of latent viral genomes (Mason et al., 2012; Poole and Sinclair, 2015). Importantly, such studies have also led to proof of principals for chemotherapeutic (Krishna et al., 2017b; Weekes

¹Department of Medicine, University of Cambridge, Addenbrooke's Hospital, Hills Road, Cambridge CB2 0QQ, UK

²Genomic Medicine Institute, Lerner Research Institute, 9620 Carnegie Avenue, Cleveland, OH, USA

³Cleveland Clinic, Lerner Research Institute, Cleveland, OH, USA

⁴Lead Contact

*Correspondence: js152@cam.ac.uk (J.S.), elp27@cam.ac.uk (E.P.)

<https://doi.org/10.1016/j.isci.2019.01.007>



et al., 2013) and immunotherapeutic strategies to target the latent reservoir (Krishna et al., 2016) *in vitro*, although whether these will have clinical benefits is still under investigation.

Besides the known changes in the latently infected cell, which can modulate, e.g., cell survival (Krishna et al., 2016; Mason et al., 2012; Poole et al., 2008, 2015; Poole and Sinclair, 2015; Krishna et al., 2017a; Lau et al., 2016b), it is also clear that other latency-associated changes in cellular gene expression can manipulate the cellular microenvironment to also help latently infected cells evade T cell surveillance (Lau et al., 2016b; Mason et al., 2012, 2013). However, whereas there is a wealth of data regarding the effector functions of natural killer cells and neutrophils during lytic infection with HCMV (Bennett et al., 2010; Falk et al., 2002; Heo et al., 2015; Magri et al., 2011; Stern-Ginossar et al., 2008; Wills et al., 2005; Yamin et al., 2016), much less is known about whether these immune cells can detect and target latently infected cells and, if so, how latent infection combats such antiviral functions.

Neutrophils comprise about 50% of all leukocytes. These cells are rapidly recruited to sites of infection or inflammation by chemotaxis. Here, they shape the immune landscape through interactions with macrophages, DCs, and cells of the adaptive immune response by direct cell-cell contact or via soluble mediators (Mocsai, 2013; Moraes et al., 2006; Nani et al., 2015; Peters and Sacks, 2009; Ribeiro-Gomes and Sacks, 2012; Williams and Chambers, 2014). Once recruited, neutrophils become fully activated, characterized by their ability to release granule proteins, their acquisition of phagocytic capabilities, the production of reactive oxygen species (ROS), and their ability to produce neutrophil extracellular traps (NETs), all of which enhance the cells' effector capacity (Brinkmann et al., 2004; Kruger et al., 2015). In addition, neutrophils are able to mediate antigen-dependent cell cytotoxicity (ADCC) via Fc receptors on their cell surface, which allows recognition of pathogen-infected cells and target them for killing (Sionov et al., 2015; Sips et al., 2016; Yu et al., 2016). Recently, neutrophil-mediated ADCC killing has been the subject of intense research with clinical trials in place for using this function of neutrophils to target cancerous cells (Challacombe et al., 2006; Di Carlo et al., 2001a, 2001b; Matlung et al., 2018; Rajasekaran et al., 2015; Treffers et al., 2018). There is also an increasing body of evidence to suggest that neutrophils target virally infected cells (Fujisawa, 2008; Jegaskanda et al., 2013; Worley et al., 2018). Neutrophils have been shown to play antiviral roles via ROS for a number of viruses (Drescher and Bai, 2013), and herpesviruses, including HCMV, have been shown to induce ROS in phagocytic cells during lytic infection (Gonzalez-Dosal et al., 2011; Speir et al., 1996). Similarly, a number of viruses have also been implicated to be targeted by NETs (Saitoh et al., 2012) as well as ADCC (Sionov et al., 2015; Sips et al., 2016; Yu et al., 2016). Indeed, it is known that cells lytically infected with HCMV both induce interleukin (IL)-8 (Costa et al., 2013) and express a virally encoded CXCL1 homolog, UL146 (Luttichau, 2010), which both attracts neutrophils and then uses these attracted immune cells as a "Trojan horse" to help disseminate infectious virions (Pocock et al., 2017). Although it is clear that such a strategy could aid the dissemination of virus from lytically infected cells, this will not occur during a latent infection as no virion production occurs during this phase of the virus life cycle (Hargett and Shenk, 2011; Poole et al., 2014a, 2014b). In addition, previous reports have not identified UL146 as being expressed during latency (Goodrum et al., 2002; Shnyder et al., 2018; Slobedman and Cheung, 2008). Consequently, we have analyzed whether neutrophils can detect latently infected cells, whether any such response is antiviral, and, if so, how latently infected cells avoid this response. Our studies show that latently infected cells can, indeed, be recognized and killed by neutrophils at high Effector to Target (E:T) ratios and that this appears to be via ADCC, which is dependent on the presence of antibodies to the viral US28 protein in HCMV-seropositive serum. However, we also show that latently infected cells inhibit recruitment of neutrophils by decreasing the expression of S100A8 and S100A9, two secreted cellular proteins that are known to mediate neutrophil recruitment. Preventing this latency-associated downregulation of S100A8/A9 resulted in increased recruitment of neutrophils to latently infected cells and their subsequent killing by ADCC.

Taken together, our work shows that myeloid cells latently infected with HCMV can be targeted and killed by neutrophils through ADCC in a US28 antibody-dependent manner. However, a latency-associated decrease in the expression and secretion of cellular S100A8/A9 prevents recruitment of neutrophils to latently infected monocytes, thereby helping to avoid neutrophil-mediated targeting and elimination of the latent HCMV reservoir.

RESULTS

Neutrophils Target and Kill Latently Infected Monocytes but Only in the Presence of Serum from a Seropositive Donor

Previous analyses have shown that host T cell responses can recognize and target myeloid cells latently infected with HCMV. However, changes in the latency-associated secretome inhibit these T cell effector

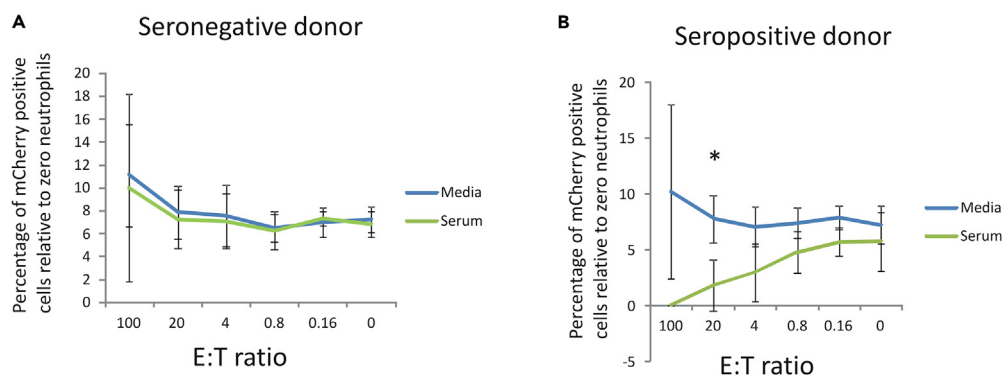


Figure 1. Neutrophils Decrease the Number of HCMV Latently Infected Cells at High E:T Ratios but Only in the Presence of HCMV+ Serum

(A and B) CD14+ monocytes and neutrophils were isolated from an HCMV-seronegative (A) or HCMV-seropositive donor (B). These CD14+ monocytes were then latently infected for 6 days with TB40E-GATA2-mCherry. Following this, the infected monocytes were pulsed with calcein and then co-cultured with autologous neutrophils at the indicated E:T ratio in the absence (Media) or presence of autologous serum (Serum). After 6-h co-culture, the numbers of latently infected cells (red cells) were counted relative to total monocytes (green cells) by fluorescence microscopy. (A) and (B) each show data from two independent experiments (from two separate donors) using eight replicates along with standard deviation error bars.

functions (Lau et al., 2016b; Mason et al., 2012, 2013). Whether other immune cells can also recognize and target latently infected cells is not known. For instance, it is becoming increasingly clear that neutrophils also target virally infected cells (Fujisawa, 2008; Jegaskanda et al., 2013; Worley et al., 2018) and, in the case of HCMV, neutrophils appear to be recruited to lytically infected cells and used to disseminate infectious virions (Pocock et al., 2017). However, it is not known if neutrophils recognize latently infected cells and, even if they did, it would be difficult to see how this could be pro-viral, as is seen during lytic infection, because no virion production occurs during latent infection (Hargett and Shenk, 2011; Poole et al., 2014a, 2014b). Consequently, we asked if latently infected cells are recognized by neutrophils and what effect this has on the latently infected cell.

To address this, we generated a recombinant HCMV carrying an mCherry expression cassette under the control of the cellular GATA-2 promoter (TB40E-GATA2-mCherry, detailed in the [Methods](#) section), which allowed detection of latently infected cells based on mCherry expression (Figure S1A). This virus showed no growth defects during lytic infection (Figure S1B), established latency normally (Figure S1C), produced infectious virus only after reactivation by differentiation (Figure S1D), and resulted in the detection of mCherry expressing latently infected cells for a much longer time frame compared with a recombinant SV40-GFP-HCMV (Figure S1A) in which GFP expression is known to wane after 3–4 days (Krishna et al., 2017b).

We then used this recombinant virus to analyze whether neutrophils were capable of targeting latently infected cells and what effect this would have on the cells. To this end, monocytes were latently infected with TB40E-GATA2-mCherry virus and the monocyte population, both latently infected (red) and bystander uninfected monocytes, were co-stained with calcein dye. The monocytes were then co-cultured with purified neutrophils at high E:T ratios; we used higher than physiological E:T ratios of neutrophils to monocytes (with physiological ratios in peripheral blood being approximately 1:5 monocytes:neutrophils) to overcome any potential inhibitors of neutrophil function that might have been produced by the latently infected monocytes.

Figure 1A shows that addition of neutrophils to experimentally latently infected monocytes from an HCMV-seronegative donor had little effect on the number of experimentally latently infected cells in the population compared with uninfected bystander cells at any E:T ratio in the presence (serum, green line) or absence (media, blue line) of serum from the same seronegative donor. However, we reasoned that, besides ROS- and NET-mediated killing, an important mechanism by which neutrophils are known to mediate cell killing of virally infected cells is by ADCC, an antibody-dependent mechanism (Sionov et al., 2015; Sips

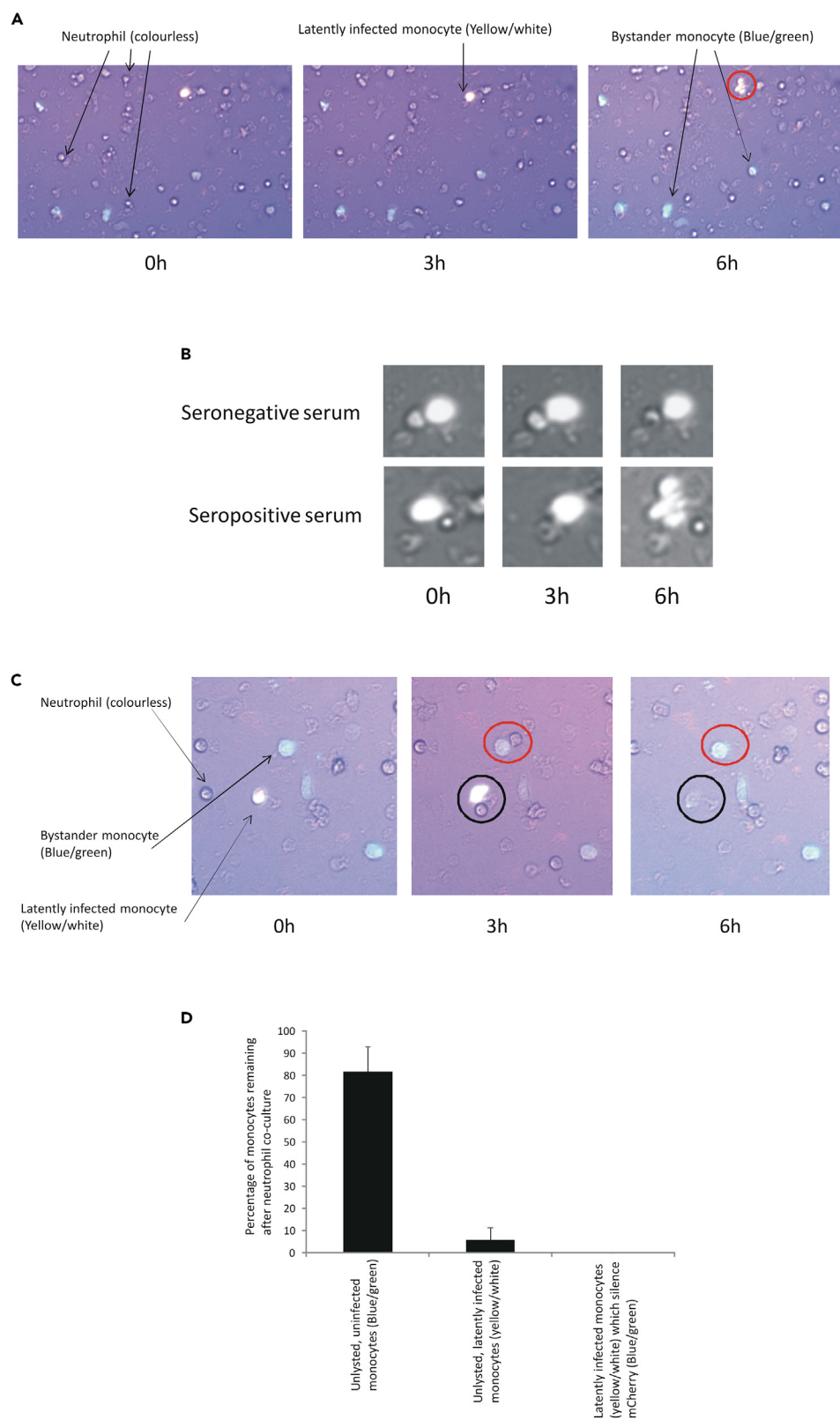


Figure 2. Neutrophils Mediate Lysis of Latently Infected CD14+ Monocytes in an HCMV-Seropositive Serum-Dependent Manner

(A) CD14+ monocytes from an HCMV-seropositive donor were latently infected with TB40E-GATA2-mCherry for 6 days before pulsing with calcein. These cells were then co-cultured with autologous neutrophils at an E:T ratio of 20:1 in the presence of autologous serum from the same donor. Cells were then analyzed by Cellomics live-cell imaging over a time course of 6 h. In these images, calcein-positive CD14+ monocytes latently infected with TB40E-GATA2-mCherry cells appear yellow/white, neutrophils appear colorless, and uninfected bystander CD14 monocytes appear green.

(B) The same as (A) except that in the top panel the CD14+ monocytes, neutrophils, and serum analyzed were from a seronegative donor (seronegative serum) and in the bottom panel, they were from a seropositive donor (seropositive serum) and cells are shown at a higher magnification to allow cell-cell contacts to be observed.

(C and D) (C) Same as (A) but also shows a higher magnification to allow cell-cell contacts to be observed. Again, latently infected monocytes appear yellow/white and bystander uninfected monocytes appear green. Neutrophils are colorless. Finally, 10 neutrophils per well from 6 wells in 3 different experiments, which were observed to have contact with neutrophils, were followed by live-cell imaging, and those cells which remained intact at the end of the neutrophil co-culture were enumerated (D). In addition, 10 neutrophils from 6 wells in 3 different experiments were analyzed for changes in cell color from yellow/white (green calcein-stained plus red mCherry) to green only (whereby the GATA2mCherry signal would have been silenced) (D). The graph represents standard deviation error bars and significance determined using the Student's t test; **p < 0.001.

et al., 2016; Yu et al., 2016). Consequently, we repeated the analysis using monocytes and serum from an HCMV-seropositive donor (Figure 1B).

Figure 1B shows that the number of experimentally latently infected monocytes (that are mCherry positive) from an HCMV-seropositive donor are profoundly reduced, particularly at high E:T ratios of neutrophil:monocytes, when cells were co-cultured in serum from the same HCMV-seropositive donor (serum, green line). No such decrease in latently infected monocytes was observed if the analysis was carried out in the presence of media only (media, blue line). These data were consistent with the view that neutrophils are able to target and kill latently infected cells but only in the presence of serum from an HCMV-seropositive donor suggesting that this killing may be mediated via ADCC.

Neutrophil Targeting of Latently Infected Monocytes Is Mediated by Cell-To-Cell Contacts and Results in Latently Infected Monocyte Killing

Clearly, addition of neutrophils to latently infected cells in the presence of HCMV serum from a seropositive donor resulted in decreases in the number of monocytes expressing mCherry after latent infection with TB40E-GATA2-mCherry virus. Although this was likely due to death of the latently infected cells, we wanted to rule out that this decrease was simply due to, e.g., neutrophil-mediated silencing of mCherry expression in these latently infected cells rather than their neutrophil-mediated killing.

To do this, we employed live-cell imaging using monocytes latently infected with TB40E-GATA2-mCherry virus and co-stained with calcein such that uninfected bystander monocytes would stain blue/green (calcein positive) and mCherry-positive latently infected cells would stain yellow/white. These cultures were co-cultured for 6 h with neutrophils (unstained) at an E:T ratio of 1:20 (this E:T ratio was chosen as it gave good statistically significant neutrophil-mediated reduction of latently infected cells in Figure 1A). Figure 2A shows that when experimentally latently infected cells (mCherry) were co-cultured with neutrophils (colorless), in the presence of serum from the HCMV-seropositive donor, uninfected monocytes (green) maintained their cellular integrity as expected at all time points shown. In contrast, calcein-positive latently infected cells (yellow/white) in the same field of view (circled in the 6-h panel) clearly blebbed and died. This killing of experimentally latently infected cells, easily detectable by 6 h post co-culture, only occurred in the presence of HCMV-seropositive donor serum (Figure 2B, lower panel), but not in the presence of serum from a seronegative donor (Figure 2B, upper panel). Interestingly, killing of experimentally latently infected cells by neutrophils in the presence of HCMV-seropositive donor serum only appeared to occur upon close contact between neutrophils and the latently infected monocyte (Figure 2C, black circles 3-h and the 6-h panels). In addition, Figure 2C shows that similarly close contact between uninfected monocytes (blue/green) in the same population and neutrophils (colourless) showed no such killing (a monocyte in contact with a neutrophil is circled red in the 3-h panel, and this contact is lost as shown in the 6-h panel, where the monocyte alone is circled in red). Quantification of these data from multiple fields of view in Figure 2D shows that, of the latently infected (mCherry) and calcein-stained monocytes (yellow/white) that had contacted a neutrophil only 6% survived. However, of the uninfected monocytes in the same population (green) that contacted a neutrophil 84% survived. None of the latently infected monocytes (mCherry)

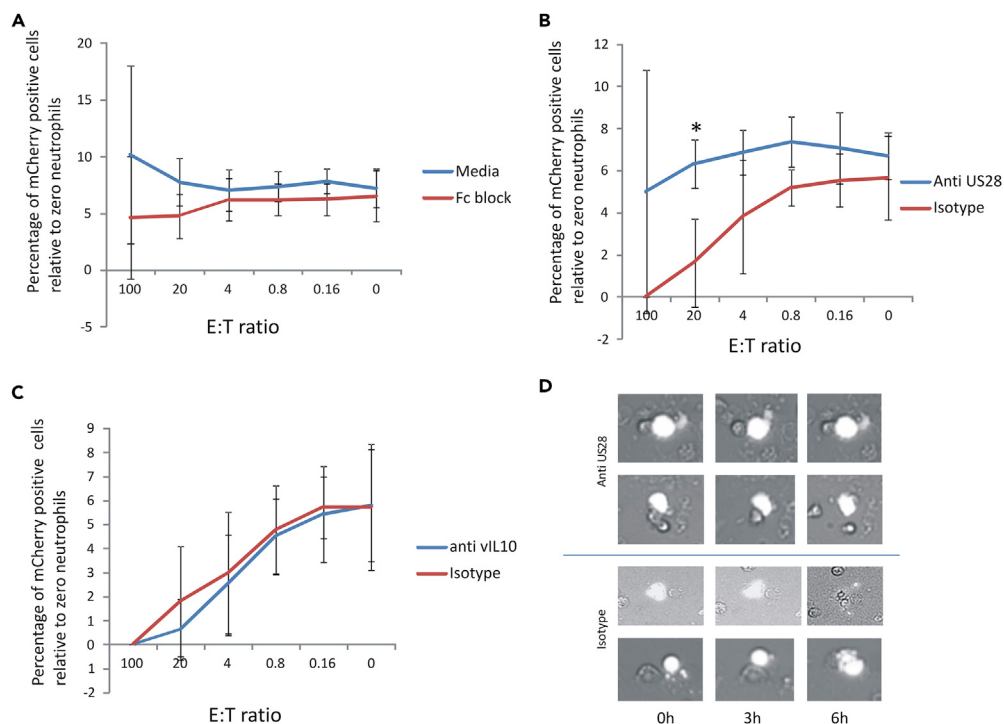


Figure 3. Neutrophil Killing of Latently Infected CD14+ Monocytes Is Due, At Least in Part, to ADCC Mediated by Viral Latency-Associated Protein US28

(A and B) CD14+ monocytes from an HCMV-seropositive donor were latently infected with the TB40E-GATA2-mCherry for 6 days before preincubation with media (Media) or an Fc-blocking antibody (Fc block). After this, cells were pulsed with calcein. CD14+ monocytes were then co-cultured with autologous neutrophils in the presence of serum from the same seropositive donor (red line) or media (blue line), as indicated. Data represent two independent experiments each with eight replicates. Latent monocytes (red) were enumerated relative to total monocytes (green), and standard deviation error bars are shown. (B) The same as (A) except the CD14+ monocytes were preincubated with either F(ab')₂ isotype control antibody (isotype, red line) or an F(ab')₂ antibody specific for US28 (anti-US28, blue line) before pulsing with calcein and co-culture with neutrophils. Data represent two independent experiments each with eight replicates. Latent monocytes (red) were enumerated relative to total monocytes (green) and standard deviation error bars are shown; ***p < 0.0001.

(C) The same as (B) except that a neutralizing F(ab')₂ antibody to viral IL-10 was used (blue line) with an isotype control (red line).

(D) Cells taken from the 20:1 ratio condition shown in (B) and analyzed by Cellomics live-cell imaging to show cell-cell contacts between latently infected CD14+ monocytes (yellow/white) and autologous neutrophils (colorless).

and those co-stained with calcein (yellow/white) became green over time, suggesting that the decrease in the number of latently infected monocytes was due to cell death and not silencing of the mCherry cassette (Figure 2D).

Taken together, these observations suggest that, in the presence of HCMV-seropositive donor serum, neutrophils can target and kill latently infected monocytes and that this requires cell-to-cell contact between the neutrophil and the latently infected cell.

The Requirement for HCMV-Seropositive Donor Serum in the Neutrophil Killing of Latently Infected Monocytes, in Part, Involves US28-Specific Antibodies

Our data, so far, had shown that the ability of neutrophils to target and kill experimentally latently infected monocytes required serum from an HCMV-seropositive donor; this suggested that the killing involved ADCC. To examine this in more detail, we first tested whether blocking all Fc receptors on the neutrophils had any impact on their ability to kill latently infected monocytes. Figure 3A shows that co-culture of latently infected monocytes with neutrophils in control media (with no HCMV-seropositive serum) showed no

latently infected cell killing, as expected (media, blue line). Importantly, co-culture of latently infected monocytes with neutrophils in the presence of autologous HCMV-seropositive serum, now also showed no killing if the neutrophils were pretreated with media containing an Fc receptor block before incubation with the latently infected CD14⁺ monocytes (Fc block, red line) when compared with the media-only control (blue line).

As serum from an HCMV-seropositive donor and not a seronegative donor mediated killing of latently infected monocytes by neutrophils, it seemed likely that this effect was due to an HCMV-specific antibody and one that recognized antigen on the surface of latently infected cells. Although it is now becoming clear that the complexity of HCMV latency-associated gene expression is higher than first thought (Goodrum et al., 2002; Rossetto et al., 2013; Shnayder et al., 2018; Slobedman and Cheung, 2008), one well established latency-associated viral gene product, which is likely to be expressed on the surface of latently infected cells, is US28 (Avidic et al., 2016; Krishna et al., 2017b). Indeed, although the levels of US28 expression by latently infected CD14⁺ were variable, US28 was detectable on non-permeabilized cells (Figure S2). This confirmed cell surface expression of US28 and argued that US28-specific antibodies in HCMV-seropositive donor serum might recognize US28 on the surface of latently infected cells. To test whether recognition of this viral antigen was involved in the observed neutrophil-mediated killing of latently infected cells, we tested whether blocking of US28-specific antibodies in the serum from HCMV-seropositive donors could prevent neutrophil-mediated killing of latently infected monocytes. To do this, we removed the Fc portion from the US28-specific rabbit antibody (to prevent any potential contamination of the rabbit Fc regions of the antibodies interfering in the assay), and the resulting F(ab')₂ fragment of the US28 antibody was then used to pretreat latently infected monocytes before repeating the killing assay in the presence of serum from a seropositive donor. Figure 3B shows that when this F(ab')₂ fragment specific to US28 was used, neutrophil-mediated killing of latently infected cells in the presence of serum from a seropositive individual was inhibited. In contrast, an F(ab')₂ fragment from the relative isotype control had no such effect. Similarly, Figure 3C shows that if an F(ab')₂ fragment specific to viral IL-10 (another well-established latency-associated viral gene product) was used to pretreat the latently infected monocytes, there was also no inhibition of neutrophil-mediated killing. Finally, Figure 3D shows microscopy of four representative cells from the experiment shown in Figure 3B and confirms that pretreatment of latently infected monocytes with US28-specific F(ab')₂ fragment, but not the relevant isotype-matched control, prevented their killing despite being physically contacted by neutrophils.

Taken together, these data show that neutrophils can detect and kill HCMV latently infected CD14⁺ monocytes, at least in part, mediated by US28-specific antibody.

Cellular Proteins S100A8/A9 Are Downregulated during HCMV Latency

Thus far, our results had shown that neutrophils could kill latently infected cells but only at high E:T ratios. This suggested to us that latently infected cells may be suppressing neutrophil targeting/killing, which could be overcome by using high levels of neutrophils in the assay. To investigate this further, we decided to assess changes in total cellular protein expression during latent infection using an unbiased proteomic screen of latently infected CD14⁺ monocytes in an attempt to identify latency-associated changes in monocyte gene expression, which might be involved in such neutrophil evasion. Most previous studies, analyzing global changes in cellular gene expression during HCMV latency, have been carried out against a background of bystander uninfected cells (Mason et al., 2012; Poole et al., 2011, 2014a, 2015; Poole and Sinclair, 2015; Rossetto et al., 2013; Slobedman and Cheung, 2008), which can confound the identification of true latency-associated changes and their effects on cellular gene expression. To analyze global changes in cellular proteins accompanying HCMV latent infection of CD14⁺ monocytes, we initially enriched for the latent HCMV population by infecting monocytes with a clinical isolate of HCMV, TB40E, which expresses GFP from an SV40-GFP expression cassette, and isolated latently infected GFP-positive cells 72 h following infection (Lau et al., 2016a). GFP-positive and GFP-negative populations were cultured for a further 3 days (6 days latency, in total) before harvesting for proteome analysis. We initially confirmed that the GFP-positive population was latently infected by RT-qPCR for UL138 transcription in the absence of detectable lytic immediate early (IE) gene expression (Figure 4A). As further proof of latent infection, we also inoculated indicator fibroblasts with supernatants from latently infected cells. These fibroblasts showed no evidence of the presence of infectious virus. In contrast, supernatants from latently infected monocytes, which had been reactivated by differentiation into DCs, showed clear evidence of infectious virus production

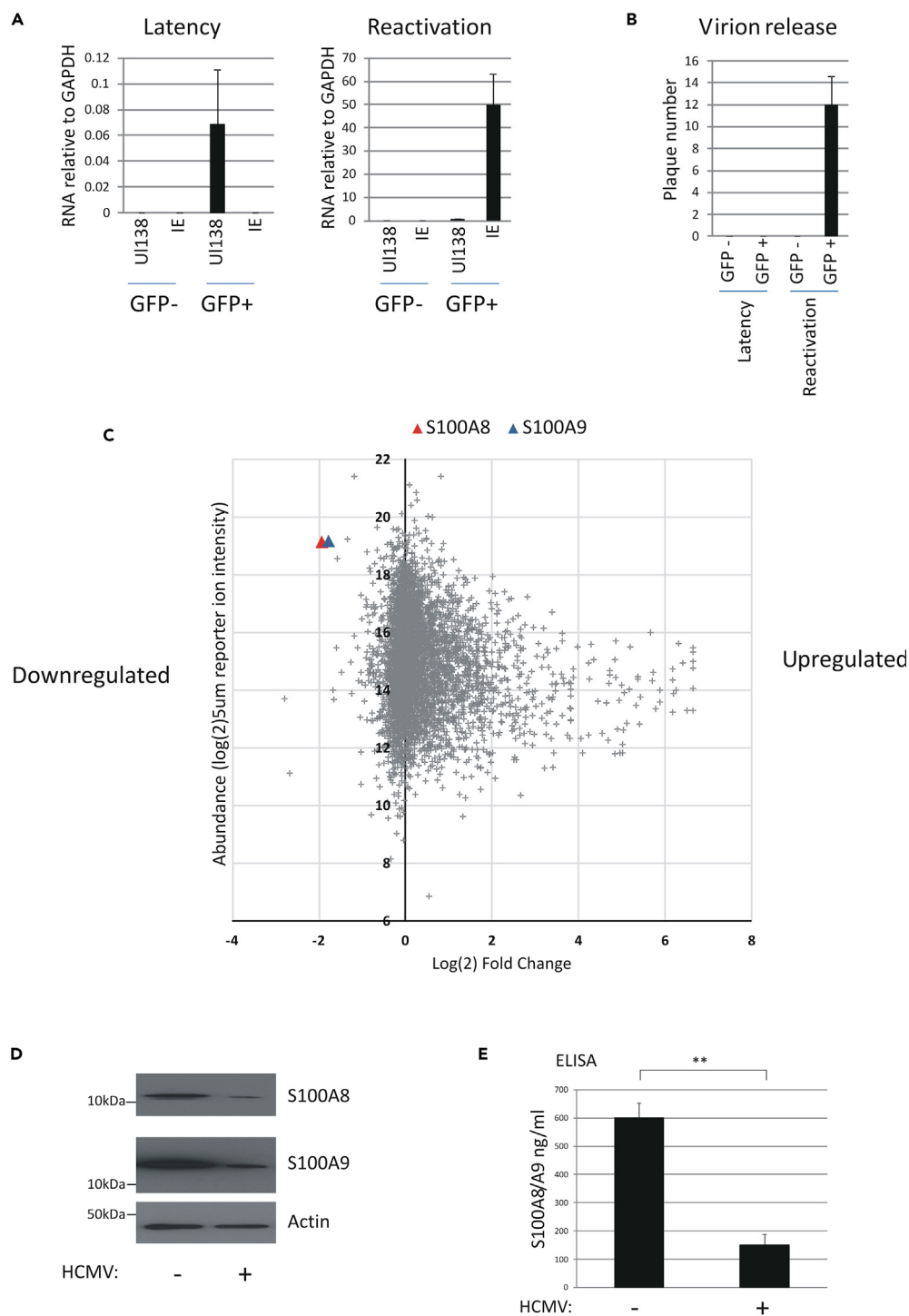


Figure 4. S100A8/A9 Are Downregulated during HCMV Latency

(A–C) CD14⁺ monocytes were isolated from an apheresis cone before infecting with the SV40-GFP-TB40E isolate of HCMV. After infection, cells were washed thoroughly and cultured for 3 days in suspension before sorting into GFP-positive (GFP⁺) and GFP-negative (GFP⁻) populations. Cells were then cultured for a further 3 days following plastic adherence. On day 6 post infection, subsets of these sorted cells were cultured for an additional 6 days in X-VIVO 15 alone (latency) or in differentiation media containing IL-4/GM-CSF and LPS to reactivate virus (reactivation) and processed for RT-qPCR analysis (A). Supernatants from these cells were also transferred to indicator fibroblasts to assay for virion production (B). In all cases in (A) and (B), data shown are from triplicate samples and bars represent standard errors.

Figure 4. Continued

Alternatively, on day 6 post-infection, the bulk of the GFP⁺ sorted cells were directly lysed and processed for total cell proteome analysis by TMT; 5,000 proteins are shown with a minimum of three unique peptides in log(2) fold changes. The S100A8 (red triangle) and A9 (blue triangle) proteins are highlighted (C).

(D and E) CD14⁺ monocytes were also mock infected (HCMV⁻) or latently infected with TB40E-SV40-GFP virus (HCMV⁺) for 3 days before sorting and then cultured for a further 3 days before harvesting and analyzed for S100A8 and A9 proteins by western blot (D). Supernatants from (D) were further analyzed for S100A8/A9 levels by ELISA (E). Data represent two experiments of six replicates plotted with standard deviation error bars and significance determined using the Student's t test where ****p** < 0.001.

(Figure 4B). As expected, we observed no viral gene expression or virion production from the GFP-negative population (Figures 4A and 4B).

Once we had confirmed that our target cells were latent, they were lysed and proteins analyzed by tandem mass tagging (TMT) and mass spectrophotometry proteomics (Figure 4C). We compared GFP-positive and GFP-negative cells (Figure 4C), and as a control, we also compared mock infected cells with cells treated with UV-inactivated virus (Figure S3), which allowed the identification of specific changes in cellular proteins resulting from latency-associated viral gene expression rather than just interactions with virions. Total changes in cellular proteins from 2×10^6 cells per condition were quantified by Mascot and Proteome Discoverer. This analysis identified the robust induction and repression of a number of cellular proteins in latently infected cells. The graph in Figure 4C represents 1,993 proteins detected (the full list of targets is shown in Table S1). This analysis resulted in the identification of a number of cellular proteins that changed as a result of latent infection of monocytes and included S100A8 and A9 (detected with 14 and 11 unique peptides, respectively), which were two of the most robustly downregulated proteins during latent infection (Figure 4C). No such downregulation of S100A8 and S100A9 was observed by infection with UV-inactivated virus when compared with mock infected cells (Figure S3).

Consistent with our proteomic analyses, western blot analysis of GFP-positive latently infected monocytes confirmed the downregulation of S100A8 and S100A9 (Figure 4D). As S100A8 and S100A9 are secreted as heterodimers (Edgeworth et al., 1991), we tested supernatants of these latently infected cells for secreted S100A8/A9 by ELISA (Figure 4E). Consistent with downregulation of both proteins in latently infected cells, a significant decrease in secreted S100A8/A9 was observed in supernatants from latently infected CD14⁺ monocytes. We also observed identical results if monocytes were latently infected with TB40EmCherry-GATA2 virus (Figures S1E and S1F). Together, these results show that S100A8/A9 is downregulated during latent infection with HCMV both intra- and extracellularly. Intriguingly, S100A8/A9 is a known neutrophil chemoattractant (Gomes et al., 2013; Newton and Hogg, 1998; Ryckman et al., 2003). Consequently, we reasoned that downregulation of these proteins during latency could be part of a mechanism to help the latently infected cell avoid neutrophil-mediated killing.

S100A8/A9 Acts as a Neutrophil Chemoattractant That Is Downregulated during HCMV Latency

We first tested whether S100A8/A9 could act as a neutrophil chemoattractant in our system using transwell assays. These assays showed that recombinant S100A8/A9 did attract neutrophils and that this was decreased in a dose-dependent manner upon addition of neutralizing antibody to S100A8/A9 in the assays (Figure 5A). On the basis that S100A8/A9 is routinely secreted at high levels by myeloid cells, but that its secretion is decreased in latently infected monocytes, we next tested whether supernatants from latently infected CD14⁺ monocytes had reduced ability to recruit neutrophils compared with uninfected monocytes. To do this, CD14⁺ monocytes were latently infected with TB40E-SV40-GFP virus for 3 days and then GFP⁺ cells were sorted by fluorescence-activated cell sorting. These GFP⁺ cells were then cultured for a further 3 days (6 days latency) before supernatants were harvested and used to assess their ability to attract neutrophils. Figure 5B shows that, as expected, supernatants from control monocytes were capable of recruiting neutrophils, whereas this recruitment was severely impaired if supernatants from latently infected monocytes were used and this impairment could be partially rescued by the addition of recombinant S100A8/A9. In essence, the ability of monocytes to recruit neutrophils through the S100A8/A9 axis was severely compromised if these cells were latently infected with HCMV.

Finally, we asked if addition of S100A8/A9 to latently infected monocyte cultures (to replete the downregulated S100A8/A9) would overcome the low level of neutrophil recruitment to latently infected cells we

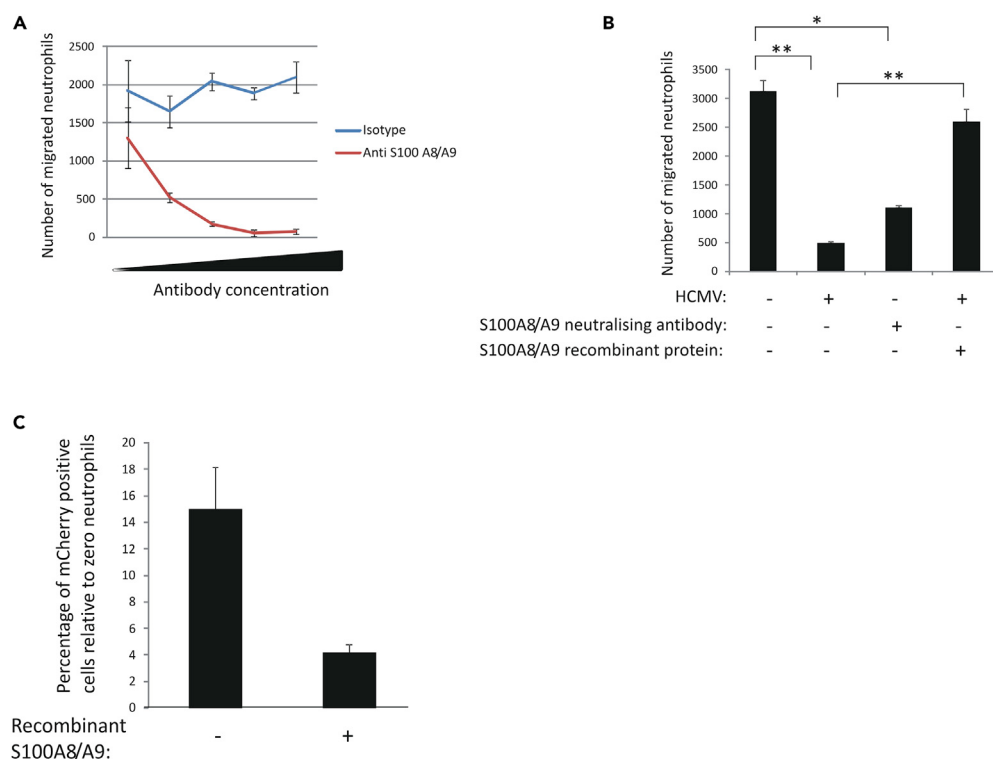


Figure 5. Neutrophil Migration to S100A8/A9 Can Be Blocked with Neutralizing Antibodies to These Proteins

(A and B) Recombinant S100A8/A9 (500 pg/mL in cell culture medium) was incubated with increasing concentrations of isotype control antibody (blue line) or antibody specific for S100A8/A9 (red line), and this was added to the bottom of a transwell plate. Calcein-pulsed neutrophils were then added to the top of the transwell filter, and after 2 h the number of migrated neutrophils were enumerated (A). The graph shows two independent experiments with triplicate samples. Standard deviation error bars are shown. (B) Supernatants from control uninfected monocytes (HCMV-), monocytes

latently infected with TB40E-GATA2-mCherry virus (HCMV+), or media supplemented (+) or not supplemented (–) with recombinant S100A8/A9 were either incubated with neutralizing antibody to S100A8/A9 (+) or the equivalent isotype control antibody (–) before placing in the bottom chamber of a transwell plate. Calcein-pulsed neutrophils were then added to the top of the transwell filter, and after 2 h migrated neutrophils were enumerated. Finally, the same experiment (as detailed in B, above) was repeated, but the monocytes were latently infected with TB40E-GATA2-mCherry virus and all monocytes pulsed with calcein and placed in the bottom of a transwell plate in the presence or absence of recombinant S100A8/A9. Neutrophils were then added to the transwells at an E:T ratio of 20. The cells were then left for 6 h before analyzing for viral cell killing relative to the absence of neutrophils (0 neutrophils).

(C) The Graphs represent two independent experiments with triplicate samples. Standard deviation error bars and significance determined using the Student's t test where * $p < 0.01$ and ** $p < 0.001$ are shown.

had previously seen. To do this we latently infected monocytes (with TB40E-GATA2-mCherry) that had also been pulsed with calcein. These were then added to the lower wells of a transwell plate, and these cells were supplemented with recombinant S100A8/A9. We then added neutrophils to the top of the transwell plate (at an E:T ratio of neutrophils that was able to elicit killing of latently infected monocytes as shown in Figures 1 and 3). Figure 5C shows that when recombinant S100A8/A9 was added to the latently infected cells to overcome latency-associated S100A8/A9 downregulation, neutrophils now migrated to the latently infected cells, resulting in their killing. These results show that the latency-associated reduction in neutrophil migration to, and killing of, latently infected monocytes can be overcome by the addition of recombinant S100A8/A9 to the latently infected cultures.

Taken together, these data show that neutrophils are able to target and kill HCMV latently infected monocytes via ADCC. However, in culture, efficient killing only occurs at high E:T ratios as high numbers of neutrophils are required to overcome the latency-associated downregulation of the neutrophil chemoattractants S100A8/A9, which prevent neutrophil recruitment to the latently infected cell (at low E:T ratios), which results in them being less efficiently targeted.

DISCUSSION

It is becoming increasingly clear that latent infection of myeloid cells with HCMV is far from silent or quiescent but results in profound changes in the latently infected cell to optimize viral carriage and reactivation. It is also clear that these changes in the cell act, in part, as pro-survival signals (Poole et al., 2011, 2015; Poole and Sinclair, 2015; Slobedman and Cheung, 2008) as well as to prevent surveillance and targeting of the latently infected cell by host immune responses, particularly CD4+ and CD8+ T cells by regulating the latency-associated cell secretome of latently infected CD34+ cells (Mason et al., 2012). Exactly where in the host such changes in secreted cell proteins would be most effective is unclear, and there is no tractable animal model for HCMV, so *in vivo* studies are difficult. However, although we do not rule out that such latency-associated changes during latent infection could affect, e.g., CD4+ and CD8+ T cell effector functions in the periphery, we think it likely that such latency-associated changes could aid T cell evasion in, e.g., the microenvironment around latently infected cells in tissues such as bone marrow. By the same argument, we feel that latently infected CD14+ cells may also create a microenvironment in sites of latency, and we, therefore, favor the view that this likely occurs in the bone marrow or other tissue sites of latency.

The routine secretion of S100A8/A9 by monocytes (Ryckman et al., 2003 and Figure 4E) suggests that neutrophils may well be routinely chemoattracted to monocytes. Our view is that, because of this, neutrophils may well be constantly sampling potential targets but these would only be routinely killed if they were expressing recognizable signals for neutrophil-mediated killing. This would be consistent with neutrophils playing a role in routine surveillance and removal of cancerous (Challacombe et al., 2006; Di Carlo et al., 2001a, 2001b; Matlung et al., 2018; Rajasekaran et al., 2015; Treffers et al., 2018) or virally infected cells (Sionov et al., 2015; Sips et al., 2016; Yu et al., 2016) during normal surveillance. However, downregulation of S100A8/A9 from monocytes during HCMV latency could help to reduce this neutrophil surveillance and decrease the likelihood of their killing.

The ability of a pathogen to limit its visibility to multiple branches of the innate immune system is one immune evasion strategy often employed by pathogens and, in particular, those pathogens that establish latent or persistent infections, and this also includes avoidance of neutrophil killing. ADCC-mediated killing of virally infected cells by neutrophils has been reported for a number of viruses (Ackerman et al., 2016; Ashkenazi and Kohl, 1990; Bradford et al., 1992; Chai et al., 2017; Ihara et al., 1986; Siebens et al., 1979; Smalls-Mantey et al., 2013; Veillette et al., 2015). However, except for vaccinia virus, which is known to express a protein that interferes with this (Al-Mohanna et al., 2001), little has been reported on the strategies by which other viruses evade neutrophil killing. Our results now show that HCMV employs a strategy during latent infection to prevent targeting and killing of latently infected cells by neutrophils. This neutrophil targeting is combated by the ability of latent infection to suppress the secretion of the neutrophil chemoattractants S100A8/A9, thereby preventing neutrophil attraction, which otherwise would result in high levels of neutrophil recruitment to, and killing of, latently infected cells.

The ability of latently infected monocytes to target neutrophils is likely to have far-reaching implications. Neutrophils are rapidly recruited to sites of infection or inflammation by chemotaxis where they shape the immune landscape through direct and indirect interactions with macrophages, DCs, and cells of the adaptive immune response. Although it is clear that neutrophils play a central role in the control of HCMV lytic replication (Heo et al., 2015; Yamin et al., 2016), what role, if any, neutrophils play in the context of a latent infection has so far been unclear. We now show that, at high density, neutrophils are able to detect and kill latently infected cells via ADCC by targeting US28 expression on the latently infected cell, as this killing was blocked by anti-Fc receptor antibody as well as by US28-specific antibodies. The ability of HCMV to target S100A8/A9 during latent infection may have additional roles beside evasion of neutrophil targeting. For example, it is known that S100A9 prevents DC differentiation (Cheng et al., 2008), and, therefore, any latency-associated reduction of S100A8/A9 in monocytes might modulate their responses to differentiation signals, ensuring efficient reactivation only when conditions are optimal.

It is now clear that latent infection of CD14+ monocytes results in major changes in cellular gene expression at the protein level and that this includes changes in cellular proteins that interfere with neutrophil-mediated killing of latently infected cells by decreasing their expression of the neutrophil-chemoattractant S100A8/A9. Therapies to target virus-infected cells with neutrophils are currently ongoing for HIV-1 and influenza infection (Chai et al., 2017; Stephenson et al., 2016; Wines et al., 2017). Knowing that monocytes, which carry latent HCMV, can be targets of host neutrophils, but that this is combated by

latency-associated changes in the cell, opens up the possibility that this viral immune evasion mechanism during latency could, itself, be exploited by strategies to help target the latent reservoir.

Limitations of the Study

This work shows that avoidance of neutrophil recruitment is an immune evasion strategy during experimental latency of HCMV. However, as there is no tractable animal model for HCMV latent infection the findings cannot, as yet, be verified *in vivo*.

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 the volunteers included in this study before providing blood samples, and all experiments were carried out in accordance with the approved guidelines.

METHODS

All methods can be found in the accompanying [Transparent Methods supplemental file](#).

SUPPLEMENTAL INFORMATION

Supplemental Information includes Transparent Methods and three figures and one table and can be found with this article online at <https://doi.org/10.1016/j.isci.2019.01.007>.

ACKNOWLEDGMENTS

We would like to thank Linda Teague and Roy Whiston for their continued technical support as well as Arlette Vassallo for providing neutrophils and Esther Perez for cell sorting. This work was funded by a program grant from the British Medical Research Council (grant G0701279 to J.S.) and the Cambridge NIHR BRC Cell Phenotyping Hub.

AUTHOR CONTRIBUTIONS

E.E., Y.A., J.W., B.K., and E.P. performed experiments. J.W., N.F., A.W., V.R., K.R., E.C., and E.P. performed technical procedures and provided reagents. N.F., V.R., K.R., E.M., P.J.L., J.S., and E.P. designed experiments. E.M., E.C., and P.L. contributed to manuscript preparation. J.S. and E.P. wrote the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

Received: October 1, 2018

Revised: December 13, 2018

Accepted: January 3, 2019

Published: February 22, 2019

REFERENCES

- Ackerman, M.E., Mikhailova, A., Brown, E.P., Dowell, K.G., Walker, B.D., Bailey-Kellogg, C., Suscovich, T.J., and Alter, G. (2016). Polyfunctional HIV-specific antibody responses are associated with spontaneous HIV control. *PLoS Pathog.* 12, e1005315.
- Al-Mohanna, F., Parhar, R., and Kotwal, G.J. (2001). Vaccinia virus complement control protein is capable of protecting xenoendothelial cells from antibody binding and killing by human complement and cytotoxic cells. *Transplantation* 71, 796–801.
- Ashkenazi, M., and Kohl, S. (1990). Reduced antibody-dependent cellular cytotoxicity to herpes simplex virus-infected cells of salivary polymorphonuclear leukocytes and inhibition of peripheral blood polymorphonuclear leukocyte cytotoxicity by saliva. *J. Immunol.* 144, 4781–4787.
- Avdic, S., McSharry, B.P., Steain, M., Poole, E., Sinclair, J., Abendroth, A., and Slobedman, B. (2016). Human cytomegalovirus-encoded human interleukin-10 (IL-10) homolog amplifies its immunomodulatory potential by upregulating human IL-10 in monocytes. *J. Virol.* 90, 3819–3827.
- Bennett, N.J., Ashiru, O., Morgan, F.J., Pang, Y., Okecha, G., Eagle, R.A., Trowsdale, J., Sissons, J.G., and Wills, M.R. (2010). Intracellular sequestration of the NKG2D ligand ULBP3 by human cytomegalovirus. *J. Immunol.* 185, 1093–1102.
- Benzi, F., Vanni, I., Cassina, G., Ugolotti, E., Di Marco, E., Cirillo, C., Cristina, E., Morreale, G., Melioli, G., Malnati, M., et al. (2012). Detection of ganciclovir resistance mutations by pyrosequencing in HCMV-infected pediatric patients. *J. Clin. Virol.* 54, 48–55.
- Bradford, H.E., Adair, B.M., McNulty, M.S., and Foster, J.C. (1992). Cytotoxicity of bovine leucocytes for parainfluenza type-3 virus-infected cells. *Vet. Immunol. Immunopathol.* 31, 115–127.
- Brinkmann, V., Reichard, U., Goosmann, C., Fauler, B., Uhlemann, Y., Weiss, D.S., Weinrauch, Y., and Zychlinsky, A. (2004). Neutrophil extracellular traps kill bacteria. *Science* 303, 1532–1535.

- Chai, N., Swem, L.R., Park, S., Nakamura, G., Chiang, N., Estevez, A., Fong, R., Kamen, L., Kho, E., Reichelt, M., et al. (2017). A broadly protective therapeutic antibody against influenza B virus with two mechanisms of action. *Nat. Commun.* 8, 14234.
- Challacombe, J.M., Suhrbier, A., Parsons, P.G., Jones, B., Hampson, P., Kavanagh, D., Rainger, G.E., Morris, M., Lord, J.M., Le, T.T., et al. (2006). Neutrophils are a key component of the antitumor efficacy of topical chemotherapy with ingenol-3-angelate. *J. Immunol.* 177, 8123–8132.
- Cheng, P., Corzo, C.A., Luetke, N., Yu, B., Nagaraj, S., Bui, M.M., Ortiz, M., Nacken, W., Sorg, C., Vogl, T., et al. (2008). Inhibition of dendritic cell differentiation and accumulation of myeloid-derived suppressor cells in cancer is regulated by S100A9 protein. *J. Exp. Med.* 205, 2235–2249.
- Cheng, S., Caviness, K., Buehler, J., Smithey, M., Nikolich-Zugich, J., and Goodrum, F. (2017). Transcriptome-wide characterization of human cytomegalovirus in natural infection and experimental latency. *Proc. Natl. Acad. Sci. U S A* 114, E10586–E10595.
- Costa, H., Nascimento, R., Sinclair, J., and Parkhouse, R.M. (2013). Human cytomegalovirus gene UL76 induces IL-8 expression through activation of the DNA damage response. *Plos Pathog.* 9, e1003609.
- Di Carlo, E., Forni, G., Lollini, P., Colombo, M.P., Modesti, A., and Musiani, P. (2001a). The intriguing role of polymorphonuclear neutrophils in antitumor reactions. *Blood* 97, 339–345.
- Di Carlo, E., Iezzi, M., Pannellini, T., Zaccardi, F., Modesti, A., Forni, G., and Musiani, P. (2001b). Neutrophils in anti-cancer immunological strategies: old players in new games. *J. Hematother. Stem Cell Res.* 10, 739–748.
- Drescher, B., and Bai, F. (2013). Neutrophil in viral infections, friend or foe? *Virus Res.* 171, 1–7.
- Dupont, L., and Reeves, M.B. (2016). Cytomegalovirus latency and reactivation: recent insights into an age old problem. *Rev. Med. Virol.* 26, 75–89.
- Edgeworth, J., Gorman, M., Bennett, R., Freemont, P., and Hogg, N. (1991). Identification of p8,14 as a highly abundant heterodimeric calcium binding protein complex of myeloid cells. *J. Biol. Chem.* 266, 7706–7713.
- Falk, C.S., Mach, M., Schendel, D.J., Weiss, E.H., Hilgert, I., and Hahn, G. (2002). NK cell activity during human cytomegalovirus infection is dominated by US2-11-mediated HLA class I down-regulation. *J. Immunol.* 169, 3257–3266.
- Fujisawa, H. (2008). Neutrophils play an essential role in cooperation with antibody in both protection against and recovery from pulmonary infection with influenza virus in mice. *J. Virol.* 82, 2772–2783.
- Gomes, L.H., Raftery, M.J., Yan, W.X., Goyette, J.D., Thomas, P.S., and Geczy, C.L. (2013). S100A8 and S100A9-oxidant scavengers in inflammation. *Free Radic. Biol. Med.* 58, 170–186.
- Gonzalez-Dosal, R., Horan, K.A., Rahbek, S.H., Ichijo, H., Chen, Z.J., Mieyal, J.J., Hartmann, R., and Paludan, S.R. (2011). HSV infection induces production of ROS, which potentiate signaling from pattern recognition receptors: role for S-glutathionylation of TRAF3 and 6. *PLoS Pathog.* 7, e1002250.
- Goodrum, F.D., Jordan, C.T., High, K., and Shenk, T. (2002). Human cytomegalovirus gene expression during infection of primary hematopoietic progenitor cells: a model for latency. *Proc. Natl. Acad. Sci. U S A* 99, 16255–16260.
- Hantz, S., Cotin, S., Borst, E., Couvreur, A., Salmier, A., Garrigue, I., Merville, P., Mengelle, C., Attal, M., Messerle, M., et al. (2010). Novel DNA polymerase mutations conferring cytomegalovirus resistance: input of BAC-recombinant phenotyping and 3D model. *Antiviral Res.* 98, 130–134.
- Hargrett, D., and Shenk, T.E. (2011). Experimental human cytomegalovirus latency in CD14+ monocytes. *Proc. Natl. Acad. Sci. U S A* 107, 20039–20044.
- Heo, J., Dogra, P., Masi, T.J., Pitt, E.A., de Kruijff, P., Smit, M.J., and Sparer, T.E. (2015). Novel human cytomegalovirus viral chemokines, vCXCL-1s, display functional selectivity for neutrophil signaling and function. *J. Immunol.* 195, 227–236.
- Humby, M.S., and O'Connor, C.M. (2015). Human cytomegalovirus US28 is important for latent infection of hematopoietic progenitor cells. *J. Virol.* 90, 2959–2970.
- Ihara, T., Ito, M., and Starr, S.E. (1986). Human lymphocyte, monocyte and polymorphonuclear leucocyte mediated antibody-dependent cellular cytotoxicity against varicella-zoster virus-infected targets. *Clin. Exp. Immunol.* 63, 179–187.
- Jegaskanda, S., Weinfurter, J.T., Friedrich, T.C., and Kent, S.J. (2013). Antibody-dependent cellular cytotoxicity is associated with control of pandemic H1N1 influenza virus infection of macaques. *J. Virol.* 87, 5512–5522.
- Keyes, L.R., Hargrett, D., Soland, M., Bego, M.G., Rossetto, C.C., Almeida-Porada, G., and St Jeor, S. (2013). HCMV protein LUNA is required for viral reactivation from latently infected primary CD14(+) cells. *PLoS One* 7, e52827.
- Komatsu, T.E., Pikis, A., Naeger, L.K., and Harrington, P. (2014). Resistance of human cytomegalovirus to ganciclovir/valganciclovir: a comprehensive review of putative resistance pathways. *Antiviral Res.* 101, 12–25.
- Krishna, B.A., Lau, B., Jackson, S.E., Wills, M.R., Sinclair, J.H., and Poole, E. (2016). Transient activation of human cytomegalovirus lytic gene expression during latency allows cytotoxic T cell killing of latently infected cells. *Sci. Rep.* 6, 24674.
- Krishna, B.A., Poole, E.L., Jackson, S.E., Smit, M.J., Wills, M.R., and Sinclair, J.H. (2017a). Latency-associated expression of human cytomegalovirus US28 attenuates cell signaling pathways to maintain latent infection. *MBio* 8, <https://doi.org/10.1128/mBio.01754-17>.
- Krishna, B.A., Spiess, K., Poole, E.L., Lau, B., Voigt, S., Kledal, T.N., Rosenkilde, M.M., and Sinclair, J.H. (2017b). Targeting the latent cytomegalovirus reservoir with an antiviral fusion toxin protein. *Nat. Commun.* 8, 14321.
- Kruger, P., Saffarzadeh, M., Weber, A.N., Rieber, N., Radsak, M., von Bernuth, H., Benarafa, C., Roos, D., Skokowa, J., and Hartl, D. (2015). Neutrophils: between host defence, immune modulation, and tissue injury. *PLoS Pathog.* 11, e1004651.
- Lau, B., Poole, E., Krishna, B., Montanuy, I., Wills, M.R., Murphy, E., and Sinclair, J. (2016a). Corrigendum: the expression of human cytomegalovirus MicroRNA MiR-UL148D during latent infection in primary myeloid cells inhibits activin A-triggered secretion of IL-6. *Sci. Rep.* 6, 33771.
- Lau, B., Poole, E., Van Damme, E., Bunkens, L., Sowash, M., King, H., Murphy, E., Wills, M., Van Loock, M., and Sinclair, J. (2016b). 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. *J. Gen. Virol.* 97, 2387–2398.
- Luttichau, H.R. (2010). The cytomegalovirus UL146 gene product vCXCL1 targets both CXCR1 and CXCR2 as an agonist. *J. Biol. Chem.* 285, 9137–9146.
- Magri, G., Muntasell, A., Romo, N., Saez-Borderias, A., Pende, D., Geraghty, D.E., Hengel, H., Angulo, A., Moretta, A., and Lopez-Botet, M. (2011). NKP46 and DNAM-1 NK cell receptors drive the response to human cytomegalovirus infected myeloid dendritic cells overcoming viral immune evasion strategies. *Blood* 117, 848–856.
- Mason, G.M., Jackson, S., Okecha, G., Poole, E., Sissons, J.G., Sinclair, J., and Wills, M.R. (2013). Human cytomegalovirus latency-associated proteins elicit immune-suppressive IL-10 producing CD4(+) T cells. *PLoS Pathog.* 9, e1003635.
- Mason, G.M., Poole, E., Sissons, J.G., Wills, M.R., and 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. *Proc. Natl. Acad. Sci. U S A* 109, 14538–14543.
- Matlung, H.L., Babes, L., Zhao, X.W., van Houdt, M., Treffers, L.W., van Rees, D.J., Franke, K., Schornagel, K., Verkuijlen, P., Janssen, H., et al. (2018). Neutrophils kill antibody-opsonized cancer cells by trogoptosis. *Cell Rep.* 23, 3946–3959.e6.
- Mocsai, A. (2013). Diverse novel functions of neutrophils in immunity, inflammation, and beyond. *J. Exp. Med.* 210, 1283–1299.
- Moraes, T.J., Zurawska, J.H., and Downey, G.P. (2006). Neutrophil granule contents in the pathogenesis of lung injury. *Curr. Opin. Hematol.* 13, 21–27.
- Nani, S., Fumagalli, L., Sinha, U., Kamen, L., Scapini, P., and Berton, G. (2015). Src family kinases and Syk are required for neutrophil extracellular trap formation in response to beta-glucan particles. *J. Innate. Immun.* 7, 59–73.
- Newton, R.A., and Hogg, N. (1998). The human S100 protein MRP-14 is a novel activator of the beta 2 integrin Mac-1 on neutrophils. *J. Immunol.* 160, 1427–1435.
- Peters, N.C., and Sacks, D.L. (2009). The impact of vector-mediated neutrophil recruitment on cutaneous leishmaniasis. *Cell Microbiol.* 11, 1290–1296.

- Pocock, J.M., Storisteanu, D.M.L., Reeves, M.B., Juss, J.K., Wills, M.R., Cowburn, A.S., and Chilvers, E.R. (2017). Human cytomegalovirus delays neutrophil apoptosis and stimulates the release of a pro-survival secretome. *Front. Immunol.* 8, 1185.
- Poole, E., Wills, M., and Sinclair, J. (2014a). Human cytomegalovirus latency: targeting differences in the latently infected cell with a view to clearing latent infection. *New J. Sci.* 2014, 10.
- Poole, E., Atkins, E., Nakayama, T., Yoshie, O., Groves, I., Alami, A., and Sinclair, J. (2008). NF- κ B-mediated activation of the chemokine CCL22 by the product of the human cytomegalovirus gene UL144 escapes regulation by viral IE86. *J. Virol.* 82, 4250–4256.
- Poole, E., Avdic, S., Hodgkinson, J., Jackson, S., Wills, M., Slobedman, B., and Sinclair, J. (2014b). 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. *J. Virol.* 88, 13947–13955.
- Poole, E., Lau, J.C., and Sinclair, J. (2015). Latent infection of myeloid progenitors by human cytomegalovirus protects cells from FAS-mediated apoptosis through the cellular IL-10/PEA-15 pathway. *J. Gen. Virol.* 96, 2355–2359.
- Poole, E., McGregor Dallas, S.R., Colston, J., Joseph, R.S., and Sinclair, J. (2011). Virally induced changes in cellular microRNAs maintain latency of human cytomegalovirus in CD34 progenitors. *J. Gen. Virol.* 92, 1539–1549.
- Poole, E., and Sinclair, J. (2015). Sleepless latency of human cytomegalovirus. *Med. Microbiol. Immunol.* 204, 421–429.
- Rajasekaran, N., Chester, C., Yonezawa, A., Zhao, X., and Kohrt, H.E. (2015). Enhancement of antibody-dependent cell mediated cytotoxicity: a new era in cancer treatment. *Immunotargets Ther.* 4, 91–100.
- Ribeiro-Gomes, F.L., and Sacks, D. (2012). The influence of early neutrophil-Leishmania interactions on the host immune response to infection. *Front. Cell Infect. Microbiol.* 2, 59.
- Rossetto, C.C., Tarrant-Elorza, M., and 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 Pathog.* 9, e1003366.
- Ryckman, C., Vandal, K., Rouleau, P., Talbot, M., and Tessier, P.A. (2003). Proinflammatory activities of S100: proteins S100A8, S100A9, and S100A8/A9 induce neutrophil chemotaxis and adhesion. *J. Immunol.* 170, 3233–3242.
- Saitoh, T., Komano, J., Saitoh, Y., Misawa, T., Takahama, M., Kozaki, T., Uehata, T., Iwasaki, H., Omori, H., Yamaoka, S., et al. (2012). Neutrophil extracellular traps mediate a host defense response to human immunodeficiency virus-1. *Cell Host Microbe* 12, 109–116.
- Scott, G.M., Weinberg, A., Rawlinson, W.D., and Chou, S. (2007). Multidrug resistance conferred by novel DNA polymerase mutations in human cytomegalovirus isolates. *Antimicrob. Agents Chemother.* 51, 89–94.
- Shnyder, M., Nachshon, A., Krishna, B., Poole, E., Boshkov, A., Binyamin, A., Maza, I., Sinclair, J., Schwartz, M., and Stern-Ginossar, N. (2018). Defining the transcriptional landscape during cytomegalovirus latency with single-cell RNA sequencing. *MBio* 9, <https://doi.org/10.1128/mBio.00013-18>.
- Siebens, H., Tevethia, S.S., and Babior, B.M. (1979). Neutrophil-mediated antibody-dependent killing of herpes-simplex-virus-infected cells. *Blood* 54, 88–94.
- Sinclair, J., and Poole, E. (2014). Human cytomegalovirus latency and reactivation in and beyond the myeloid lineage. *Future Virol.* 6, 7.
- Sionov, R.V., Fridlender, Z.G., and Granot, Z. (2015). The Multifaceted roles neutrophils play in the tumor microenvironment. *Cancer Microenviron.* 8, 125–158.
- Sips, M., Krykbaeva, M., Diefenbach, T.J., Ghebremichael, M., Bowman, B.A., Dugast, A.S., Boesch, A.W., Strecek, H., Kwon, D.S., Ackerman, M.E., et al. (2016). Fc receptor-mediated phagocytosis in tissues as a potent mechanism for preventive and therapeutic HIV vaccine strategies. *Mucosal Immunol.* 9, 1584–1595.
- Sissons, J.G., and Wills, M.R. (2015). How understanding immunology contributes to managing CMV disease in immunosuppressed patients: now and in future. *Med. Microbiol. Immunol.* 204, 307–316.
- Slobedman, B., and Cheung, A.K. (2008). Microarrays for the study of viral gene expression during human cytomegalovirus latent infection. *Methods Mol. Med.* 141, 153–175.
- Smalls-Mantey, A., Connors, M., and Sattentau, Q.J. (2013). Comparative efficiency of HIV-1-infected T cell killing by NK cells, monocytes and neutrophils. *PLoS One* 8, e74858.
- Speir, E., Shibutani, T., Yu, Z.X., Ferrans, V., and Epstein, S.E. (1996). Role of reactive oxygen intermediates in cytomegalovirus gene expression and in the response of human smooth muscle cells to viral infection. *Circ. Res.* 79, 1143–1152.
- Stephenson, K.E., D'Couto, H.T., and Barouch, D.H. (2016). New concepts in HIV-1 vaccine development. *Curr. Opin. Immunol.* 41, 39–46.
- Stern-Ginossar, N., Gur, C., Biton, M., Horwitz, E., Elboim, M., Stanietsky, N., Mandelboim, M., and Mandelboim, O. (2008). Human microRNAs regulate stress-induced immune responses mediated by the receptor NKG2D. *Nat. Immunol.* 9, 1065–1073.
- Terrazzini, N., and Kern, F. (2014). Cell-mediated immunity to human CMV infection: a brief overview. *F1000prime Rep.* 6, 28.
- Treffers, L.W., Zhao, X.W., van der Heijden, J., Nagelkerke, S.Q., van Rees, D.J., Gonzalez, P., Geissler, J., Verkuiljen, P., van Houdt, M., de Boer, M., et al. (2018). Genetic variation of human neutrophil Fc γ receptors and SIRP α in antibody-dependent cellular cytotoxicity towards cancer cells. *Eur. J. Immunol.* 48, 344–354.
- Veillette, M., Coutu, M., Richard, J., Batrville, L.A., Dagher, O., Bernard, N., Tremblay, C., Kaufmann, D.E., Roger, M., and Finzi, A. (2015). The HIV-1 gp120 CD4-bound conformation is preferentially targeted by antibody-dependent cellular cytotoxicity-mediating antibodies in sera from HIV-1-infected individuals. *J. Virol.* 89, 545–551.
- Weekes, M.P., Tan, S.Y., Poole, E., Talbot, S., Antrobus, R., Smith, D.L., Montag, C., Gygi, S.P., Sinclair, J.H., and Lehner, P.J. (2013). Latency-associated degradation of the MRP1 drug transporter during latent human cytomegalovirus infection. *Science* 340, 199–202.
- Williams, A.E., and Chambers, R.C. (2014). The mercurial nature of neutrophils: still an enigma in ARDS? *Am. J. Physiol. Lung Cell Mol. Physiol.* 306, L217–L230.
- Wills, M.R., Ashiru, O., Reeves, M.B., Okecha, G., Trowsdale, J., Tomasec, P., Wilkinson, G.W., Sinclair, J., and Sissons, J.G. (2005). Human cytomegalovirus encodes an MHC class I-like molecule (UL142) that functions to inhibit NK cell lysis. *J. Immunol.* 175, 7457–7465.
- Wills, M.R., Poole, E., Lau, B., Krishna, B., and Sinclair, J.H. (2015). The immunology of human cytomegalovirus latency: could latent infection be cleared by novel immunotherapeutic strategies? *Cell. Mol. Immunol.* 12, 128–138.
- Wines, B.D., Billings, H., McLean, M.R., Kent, S.J., and Hogarth, P.M. (2017). Antibody functional assays as measures of Fc receptor-mediated immunity to HIV - new technologies and their impact on the HIV vaccine field. *Curr. HIV Res.* 15, 202–215.
- Worley, M.J., Fei, K., Lopez-Denman, A.J., Kelleher, A.D., Kent, S.J., and Chung, A.W. (2018). Neutrophils mediate HIV-specific antibody-dependent phagocytosis and ADCC. *J. Immunol. Methods* 457, 41–52.
- Yamin, R., Lecker, L.S., Weisblum, Y., Vitsenshtein, A., Le-Trilling, V.T., Wolf, D.G., and Mandelboim, O. (2016). HCMV vCXCL1 binds several chemokine receptors and preferentially attracts neutrophils over NK cells by interacting with CXCR2. *Cell Rep.* 15, 1542–1553.
- Yu, Z., Chen, T., and Cao, X. (2016). Neutrophil sensing of cytoplasmic, pathogenic DNA in a cGAS-STING-independent manner. *Cell Mol. Immunol.* 13, 411–414.

Supplemental Information

Monocytes Latently Infected with Human

Cytomegalovirus Evade Neutrophil Killing

Elizabeth Elder, Benjamin Krishna, James Williamson, Yusuf Aslam, Neda Farahi, Alexander Wood, Veronika Romashova, Kate Roche, Eain Murphy, Edwin Chilvers, Paul J. Lehner, John Sinclair, and Emma Poole

TRANSPARENT METHODS, SUPPLEMENTARY FIGURES AND SUPPLEMENTARY FIGURE LEGENDS

Transparent Methods

Cells and viruses

Primary CD14⁺ monocytes were isolated from total peripheral blood mononuclear cells (PBMC) from apheresis cones or venous blood by MACS separation using CD14⁺ microbeads (Miltenyi) as described previously (Krishna et al., 2016; Mason et al., 2012). Primary neutrophils were isolated from venous blood as described previously (Farahi et al., 2017). Human foetal foreskin fibroblasts (HFFFs) were obtained from ECACC and cultured as described previously (Poole et al., 2006). All cells were incubated at 37°C in a 5% CO₂ atmosphere unless specifically stated.

TB40E-SV40-GFP has been described previously (Krishna et al., 2016) and details of the TB40E-GATA2-mCherry cloning and validation follows later in this section.

RT-qPCR

Total RNA was isolated from cells using miRNeasy mini RNA extraction kits (Qiagen) following the manufacturer's instructions from 1x10⁵ cells in a well of a 24 well plate. Viral transcript analyses were performed using the Quantitect virus kit (Qiagen) using primers and probes as previously described (Krishna et al., 2016).

Preparation of cells carrying latent virus for cell sorting

Primary CD14⁺ monocytes were infected in suspension and latency established for 3 days using TB40E-SV40-GFP virus. Latently infected cells were then sorted using live cell FACS isolation using the FITC channel and then immediately washed with x-vivo 15 (Lonza) and plated on plastic for a further 3 days with media changes every 24 hours. Particulars of the proteome analysis are described below.

Transwell assays

Transwell assays were carried out using 5µm pore size transwells and allowing 2h for migration as described previously (Mason et al., 2012) except that neutrophils were pulsed with calcein (BD) and analysed for the ability to migrate.. For the neutralisation assay, 500pg/ml of recombinant S100A8/A9 (R&D) was mixed with x-vivo 15 in the presence or absence of S100A8/A9 neutralising antibody (R&D) at 200ng/ml of neutralising antibody in 5-fold decreasing concentrations of antibody

for 1 hour at RT before placing in the transwell assay. For the neutralisation of supernatants from HCMV latency, the supernatants were pre-incubated with 200ng/ml of neutralising antibody to S100A8/A9 for 1h at RT before placing in the transwell assay. The number of cells which had migrated were enumerated using Image J software with size exclusion to allow specific counting of calcein stained neutrophils.

S100A8/A9 ELISA

Detection of S100A8/A9 heterodimers in supernatant was via ELISA (BioLegend) following instructions from the manufacturer.

Neutrophil killing assay

For all analyses all cells and sera were autologous. CD14⁺ monocytes were isolated from venous blood and plated in 96 well plate format at 1×10^5 cells per well. Cells were then incubated overnight and either left uninfected or were latently infected with HCMV isolates, as described in the text, for 6 days. Neutrophils were then isolated from fresh venous blood obtained from the same donor 7 days after the initial bleed. Monocytes were pulsed with calcein, as described previously (Mason et al., 2012) and washed thoroughly before the neutrophils were titrated onto the monocytes at the described E:T ratios in the presence or absence of autologous serum (1:1 in x-vivo 15 media). Cells were left for 6h with or without Cellomics live cell imaging and then analysed by fluorescence microscopy and ImagePro software. For the graphical presentation, the data were analysed as follows: Initially the number of virally infected cells (as determined by red fluorescence) were counted per well in the absence of the addition of neutrophils (0 neutrophils) and worked out as a percentage relative to the total number of monocytes (stained green with calcein). This value was then taken as the 100% value (i.e. no killing), as the efficiency of virus infection can differ from independent experiment to independent experiment and donor to donor, thereby allowing a baseline of “no killing” to be determined. The level of latent cell killing by neutrophils was then determined by assessing the number of red cells relative to uninfected cells (green due to calcein pulsing) as a percentage of the number of latently infected cells in the absence of neutrophils. So, if in 1×10^4 monocytes there were 1000 latently infected cells (i.e. 10%) in the absence of neutrophils, then if addition of neutrophils resulted in only 650 latently infected cells in a well of 9.5×10^3 monocytes (due to e.g. some low level cell death of uninfected cells), this would give a value of 6.8% latently infected cells. In all cases, to account for any low level non-specific killing at high levels of neutrophil addition, the value is corrected to the 0 neutrophils number.

HCMV serostatus determination

To determine the serostatus of an individual, serum was isolated from venous blood and tested by ELISA using the Capital Cytomegalovirus IgG kit (Trinity Biotech) in accordance with the manufacturer's protocol.

Immunofluorescence and western blotting

CD14⁺ monocytes were fixed with 4% PFA for 20 min and then blocked for 1h in 1% BSA/PBS before the addition of rabbit anti-US28 specific antibody (Source Bioscience) at a concentration of 1:50 or the equivalent isotype control in 1% BSA/PBS overnight at 4°C. After this time cells were washed for 3 times for half an hour in PBS before staining with anti-rabbit TRITC and visualising by fluorescence microscopy and analysing with ImagePro software.

Cell lysates were analysed by SDS-PAGE and western blotting. Blots were probed with actin (Abcam) and S100A8/A9 (R&D systems) primary antibodies followed by anti-rabbit or anti-mouse HRP respectively before chemiluminescence detection with ECL reagent using the manufacturer's instructions (Thermo Fisher).

F(ab')₂ purification

F(ab')₂ regions of Isotype control antibody and antibody specific for US28 (Source Bioscience) were purified from total antibody using the Pierce F(ab')₂ Preparation Kit (Pierce) following the manufacturer's protocol.

Proteomic screen

Lysis, digestion and clean-up of protein preps

From 6 well plates, cells were washed 2x with cold PBS before scraping cells into 200µL 8Murea/50mM TEAB pH 8.5. Samples were quantified by BCA assay and 28µg of each sample was taken and adjusted to the same volume with lysis buffer. Reduction and alkylation was achieved by addition of 10mM TCEP and 20mM iodoacetamide for 20mins at room temperature in the dark followed by quenching with 10mM DTT. 3µg of rLysC (Promega) was added to each sample and incubated at 30 degrees for 3h before diluting the 1:5 with 50mM TEAB and addition of 3µg trypsin

(Proteomics Grade, Thermo Fisher Scientific) and incubation at 37 degrees overnight. Samples were acidified by adding 1 volume of 0.2% TFA and formic acid until pH was $\sim$ 2. Samples were desalted using in-house prepared microcolumns consisting of Oligo R3 resin (SCIEX) packed behind a C18 plug (Empore, 3M) in p200 pipette tips. Columns were washed with 100uL ACN and equilibrated with 2x100uL 0.1% TFA before loading of samples (3 passes over the column). Columns were washed with 3x 50uL 0.1% TFA before sequential elution with 20uL each of 40% ACN, 70% ACN and 70% ACN with 1% FA. Samples were dried in a vacuum centrifuge.

TMT Labelling

Samples were resuspended in 20uL 100mM TEAB and to each tube 0.2ug of a unique TMT label for each sample was added in 8.5uL acetonitrile and incubated for 1h at room temperature. Labels were as follows: TMT reactions were quenched by addition of 3uL of 200mM ammonium formate, pooled and dried in a vacuum centrifuge. The sample was then Resuspended in 800uL 0.1% TFA and acidified to $\sim$ pH2 with formic acid before performing a C18-SPE cleanup using a Sep-Pak cartridge (Waters) attached to a vacuum manifold. C18 Eluate was dried in a vacuum centrifuge and resuspended in 40uL 200mM ammonium formate, pH10.

High pH Reversed Phase Fractionation

Sample was injected onto an Ultimate 3000 RSLC UHPLC system (Thermo Fisher Scientific) equipped with a 2.1 i.d x25cm, 1.7uM particle Kinetix Evo C18 column (Phenomenex). Mobile phase consisted of A: 3% ACN, B:ACN and C: 200mM ammonium formate pH 10. Isocratic conditions were 90% A/10%C and C was maintained at 10% throughout the gradient elution. Separations were carried out at 45 degrees. After loading at 200uL/min for 5 mins and ramping the flow rate to 400uL/min over 5mins the gradient elution proceed as follows: 0-19% B over 10 minutes (curve 3), 19-34%B over 14.25mins (curve 5), 34-50%B over 8.75mins (curve 5), followed by a 10 min wash at 90% B. UV absorbance was monitored at 280nm and 15s fractions were collected into 96 well microplates using the integrated fraction collector. Peptide containing fractions were then orthogonally recombined into 12 fractions and dried in a vacuum centrifuge and resuspended in 10uL 5% DMSO 0.5% TFA for analysis.

LC-MS analysis

All samples were injected onto an Ultimate 3000 RSLC nano UHPLC equipped with a 300uM i.d. x 5mm Acclaim PepMap u-Precolumn (Thermo Fisher Scientific) and a 75uM i.d. x50cm 2.1uM particle Acclaim PepMap RSLC analytical column. Loading solvent was 0.1% TFA, analytical solvent A: 0.1% FA and B: ACN+0.1% FA. All separations were carried out at 55 °C. Samples were loaded at 10uL/min for

5 mins in loading solvent before beginning the analytical gradient. For High pH RP fractions a gradient of 3-5.6% B over 4 mins, 5.6 – 32%B over 162mins, followed by a 5 minute wash at 80%B and a 5 minute wash at 90%B and equilibration at 3%B for 5mins. During the gradient the Orbitrap Fusion mass spectrometer (Thermo Fisher Scientific) was set to acquire spectra.

Data Processing

All Raw files were searched by Mascot within Proteome Discoverer 2.1 (Thermo Fisher Scientific) against the Swissprot Human database and a database of common contaminants.

For TMT labelled samples the search parameters were as follows. Enzyme: Trypsin. MS1 tol: 10ppm. MS2 tol: 0.6Da. Fixed modifications: Carbamidomethyl Cysteine, TMT peptide N-termini and Lysine. Variable modification oxidised methionine. MS3 reporter ion tol: 20ppm, most confident centroid. Mascot Percolator was used to calculate PSM FDR.

Search results were further processed and filtered as follows: Peptides below a percolator FDR of 0.01% and proteins below the 0.01% protein FDR (calculated from a built in decoy database search) were rejected. Protein groups were then generated using the strict parsimony principle. Peptides both unique and razor with a co-isolation threshold of 50 and an average s/n threshold of 10 were used for quantification and a normalisation of these values to the total peptide amount in each channel was applied. Instances where a protein was identified but not quantified in all channels were rejected from further analysis. “Scaled” abundances of proteins provided by Proteome Discoverer were used to derive ratios of abundance.

Cellomics live cell imaging

Live cells were automatically imaged and analysed using Thermo Scientific Array Scan (formerly Cellomics) XTI High Throughput Screening Microscope equipped with Live Cell Module, Objective Module, CCD Camera, solid-state 7 colour LED light engine providing 386 nm, 438 nm, 485 nm, 549 nm, 560 nm, 650 nm and 740 nm excitation wavelengths and 5 or 6 position emission filter wheel for Widefield fluorescence detection and a Brightfield Module with a single colour LED light source. To maintain cell health throughout, all imaging experiments the Array Scan plate acquisition chamber temperature and CO₂ level were kept at 37°C and 5% retrospectively using Live Cell Module.

All images were acquired with Zeiss 20x/0.4 Korr LD Plan NEOFLUAR objective and X1 highly sensitive CCD camera with 4.5 micron/pixel resolution. The X1 camera was set to 2x2 binning mode. The instrument is supplied with Thermo Scientific HCS Studio Cell Analysis Software for further cellular image quantitative analysis.

For fluorescence detection 5 multiband wavelength dichroic and emitter filter set was used. GFP (or Cell Track Green dye) and mCherry signals from infected cells were detected in 485-20 BGRFRN (BGRFRN states for Blue, Green, Red, Far Red and Near-IR emission) and 560-25 BGRFRN channels retrospectively. White light images were obtained in BGRFRN – BRIGHTFIELD channel. Cells were acquired simultaneously in all channels. The cells were cultured, treated and imaged in 96 well micro plates. The entire area of each well for all 96 wells in all experiments was imaged to acquire a statistically significant amount of a low frequency of GFP expressing cells for future analysis.

Generation of TB40E-GATA2-mCherry virus

TB40E-GATA2-mCherry was generated by replacing the SV40-GFP cassette with a GATA2-mCherry cassette on the basis that latency-associated gene promoters are rich in GATA-2 binding sites and depend on GATA-2 in the absence of lytic IE72 expression (Poole et al., 2013). To generate TB40E-GATA2-mCherry virus gBLOCKs from IDT (Coralville, IA) were utilised with primers for recombineering using the GalK/2-DOG selection method. The GATA promoter has high GC content therefore Phusion Polymerase was used with standard protocols from the manufacturer in proprietary buffer (Phusion GC buffer). The GalK insertion used the following two primers to generate the insertion construct with the pGalK plasmid as a PCR template:

GATA galk ins 5'

TGG GGA TGA AAT ATA TCC AGA TAC GCA GTT TTG TTA TCC TAA CAA AAC CCG TGT CAT GCC CTG
TTG ACA ATT AAT CAT CGG CA

GATA galk ins 3'

ACC AGG ATG GGC ACC ACC CCG GTG AAC AGC TCC TCG CCC TTG CTC ACC ATG GTG TCA GCA CTG
TCC TGC TCC TT

Excision of GalK (by 2-DOG selection) was achieved with a PCR product made using the GATA2promoter gBLOCK as template and using the following 2 primers:

GATA galk reversion 5'

TGT CGC ATA AAA GGC GGT GGG ATG TGG GGA TGA AAT ATA TCC AGA TAC GCA GTT T

GATA galk reversion 3'

GCC GTT TAC GTC GCC GTC CAG CTC GAC CAG GAT GGG CAC CAC CCC GGT G

Supplementary figures

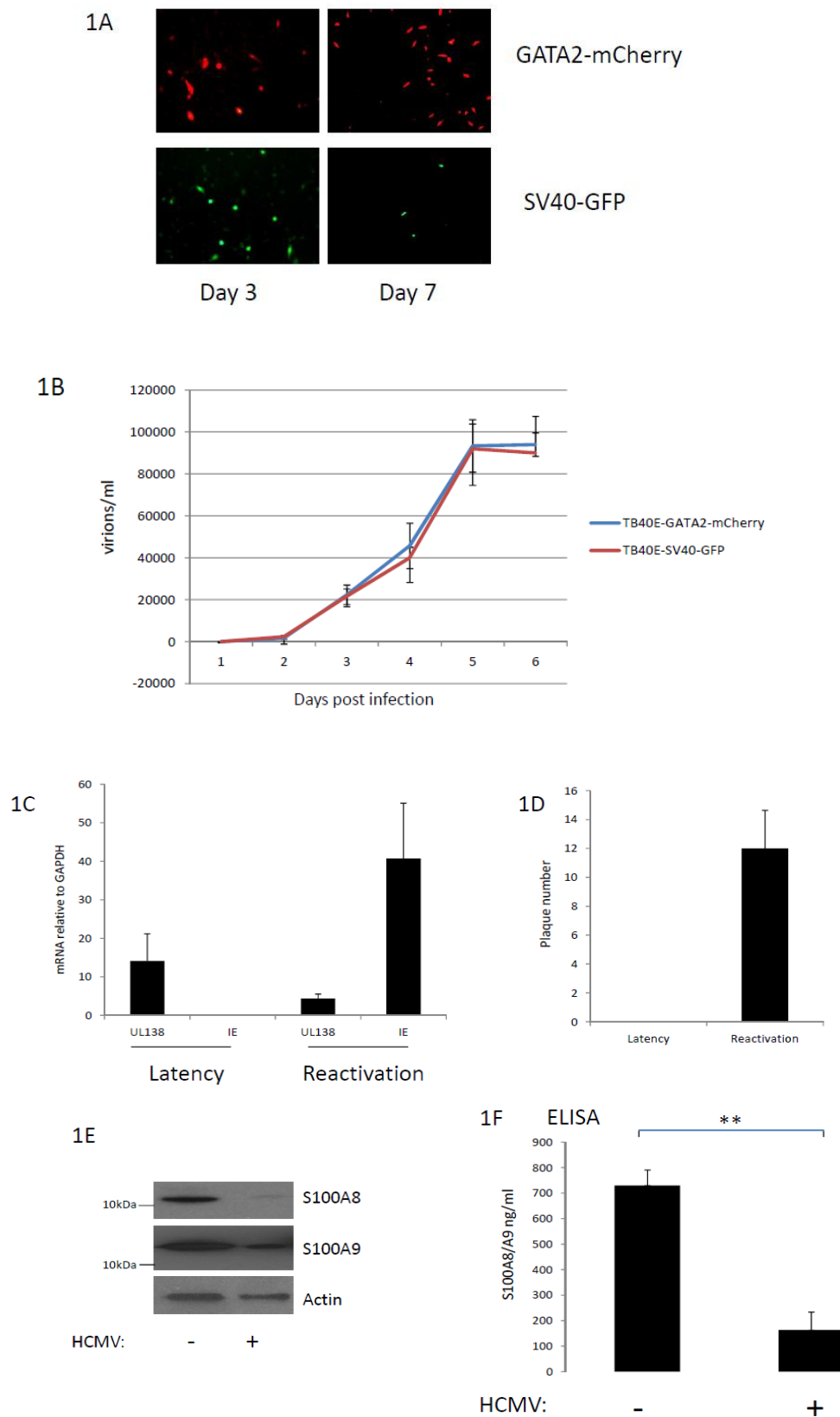


Figure S1 relates to all figures throughout the paper. **Characterisation of the TB40E-GATA2-mCherry virus.** (A) CD14⁺ monocytes were latently infected with TB40E-SV40-GFP virus (SV40-GFP) or TB40E-

GATA2-mCherry (GATA2-mCherry) virus for 3 and 7 days. (B) HFF cells were infected with either TB40E-SV40-GFP or TB40E-GATA2-mCherry and supernatants from 1-6 days post-infection were analysed for infectious virus production by inoculation of fresh fibroblasts followed by indirect immunofluorescence staining for IE1 positive cells allowed numbers of infectious units/mL of supernatants to be quantified. (C) CD14⁺ monocytes latently infected with TB40E-GATA2-mCherry were analysed by RTqPCR analysis for cellular GAPDH, UL138 or IE RNA (Latency) as well as after their differentiation and into mature dendritic cells with GM-CSF/IL-4 and LPS (reactivation). (D) Supernatants from these cells were also transferred onto indicator fibroblasts to test for virus production. (E) CD14⁺ monocytes were latently infected for 3 days with TB40E-GATA2-mCherry virus (HCMV⁺) or mock infected (HCMV⁻), sorted according to mCherry expression and then cultured for a further 3 days (6 days latency) before harvesting and analysis by western blot for S100A8, S100A9 or actin. (F) Supernatants from (E) were also analysed for S100A8/A9 by ELISA. Data represent two independent experiments each with 6 replicates plotted with standard deviation error bars and significance determined using the student's T test where **= $p < 0.001$

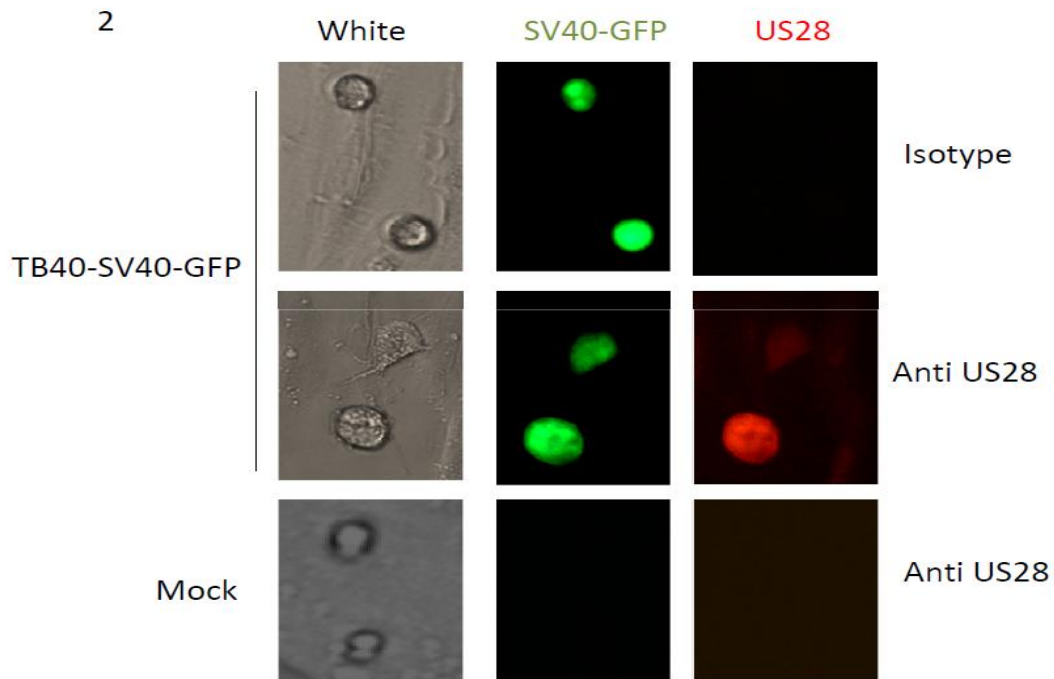


Figure S2 relates to figure 3 in the paper. **US28 antibody detects US28 during on the surface of latently infected CD14⁺ monocytes.** CD14⁺ monocytes were left uninfected (mock) or latently infected for 6 day with TB40E-SV40-GFP (SV40-GFP) before fixing and then staining with anti-US28 antibody or an isotype matched control antibody.

3

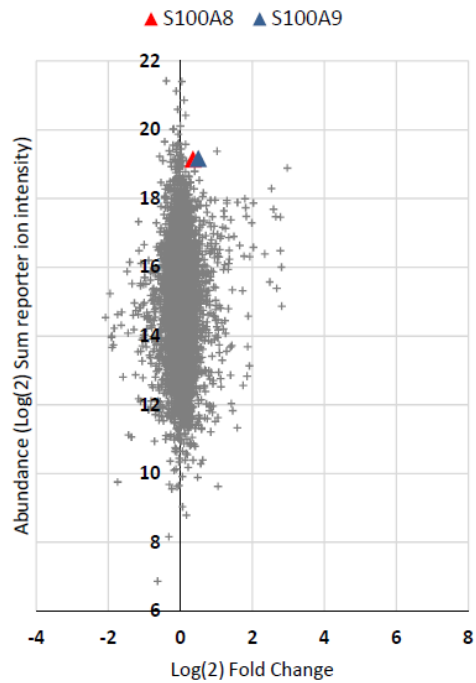


Figure S3 relates to figure 4 in the paper. **UV inactivated virus has no effect on S100A8/9 levels.** Monocytes were either untreated or infected with uv-inactivated virus. On day six post-infection, the cells were directly lysed and processed for total cell proteome analysis by Tandem Mass Tagging (TMT); 5000 proteins are shown with a minimum of 3 unique peptides in Log(2) fold-changes. The S100A8 and A9 proteins are highlighted (triangles).