Published in final edited form as:

Science. 2013 April 12; 340(6129): 199–202. doi:10.1126/science.1235047.

Latency-Associated Degradation Of The MRP1 Drug Transporter During Latent Human Cytomegalovirus Infection[‡]

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Abstract

Reactivation of latent human cytomegalovirus (HCMV) infection following transplantation is associated with high morbidity and mortality. In vivo, myeloid cells and their progenitors are an important site of HCMV latency, whose establishment and/or maintenance requires expression of UL138. Using SILAC (stable isotope labeling by amino acids in cell culture)-based mass spectrometry, we found a dramatic UL138-mediated loss of cell surface Multidrug Resistance-associated Protein-1 (MRP1), and reduction of substrate export by this transporter. Latency-associated loss of MRP1 and accumulation of the cytotoxic drug vincristine, an MRP1 substrate, depleted virus from naturally latent CD14⁺ and CD34⁺ progenitors, all in vivo sites of latency. The UL138-mediated loss of MRP1 provides a marker for detecting latent HCMV infection and a therapeutic target for eliminating latently-infected cells prior to transplantation.

Human cytomegalovirus (HCMV) is a ubiquitous beta-herpesvirus that infects 60-90% of individuals (1). Following primary infection, HCMV establishes a latent infection under the control of a healthy immune system. Reactivation from viral latency to productive infection causes serious disease in immunocompromised individuals, such as transplant recipients and AIDS patients (1,2).

Cells of the myeloid lineage such as CD34⁺ bone marrow progenitors and CD14⁺ monocytes represent sites of latent HCMV infection (3-5). Viral genome persists in these cells with little gene expression and no detectable virus production (6,7). Reactivation from

[‡]This manuscript has been accepted for publication in Science. This version has not undergone final editing. Please refer to the complete version of record at <http://www.sciencemag.org/>. The manuscript may not be reproduced or used in any manner that does not fall within the fair use provisions of the Copyright Act without the prior, written permission of AAAS.

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latency occurs upon myeloid differentiation, resulting in chromatin-mediated activation of the lytic gene expression cascade, virus DNA replication and production of infectious virions (8). Latent viral infection is thus required for viral persistence. Establishing how latency is maintained, and how latently-infected cells avoid immune recognition, is crucial to understanding how HCMV persists in vivo. Furthermore, the elimination of latently-infected cells represents a key target in preventing recurrent HCMV infection in immunocompromised individuals.

A limited number of viral transcripts have been identified during natural latency in myeloid cells (6,7) and include UL138 (9,10) which encodes a 21kDa transmembrane Golgi-associated protein (10). UL138 is expressed with early-late kinetics during productive HCMV infection (10), but is also required for efficient latent carriage in vitro (9,10). Expression of UL138 during lytic infection results in increased tumour necrosis factor receptor 1 (TNFR1) cell surface expression (11,12), but little is known about UL138 during latency.

To address how UL138 affects host cell surface receptor expression during latent HCMV infection, we used 'plasma membrane profiling' (PMP) (13), a proteomic technique that employs SILAC-based differential analysis to compare the expression of plasma membrane (PM) proteins in the presence and absence of UL138 in undifferentiated myeloid cells. Of the 592 plasma membrane proteins isolated from the monocytic cell line (THP-1) only three were reproducibly affected more than two-fold (Fig. 1A, Table S1-S2). Most notable was MRP1 (downregulated 6.7–10.3 fold in three independent experiments), while Notch-ligand Delta-like protein 1 (DLL1) was downregulated 2.1–2.6 fold. As expected, cell surface expression of tumour necrosis factor receptor 1 (TNFR1) increased (2.4–2.8 fold) (11,12).

These cell surface changes were confirmed by cell surface flow cytometry (DLL-1, TNFR1 and CD36), or intracellular FACS (MRP1), while expression of the control protein (CCR7) was unaffected (Fig. 1B). UL138 downregulated MRP1 in all four cell lines tested, including fibroblasts (Fig. 1C), HL60-ADR cells, a promyelocytic leukaemia cell line that overexpresses MRP1 (14), and HeLas (Fig. S1).

We focused on MRP1, the most dramatically downregulated protein. In the presence of UL138, not only did MRP1 cell surface expression decrease but the protein was undetectable (Fig. 1C-D). UL138 expression is not restricted to latent HCMV infection, is detected 6-hours after lytic infection and accumulates over 48 hours (10). We analysed the temporal relationship between UL138 expression and MRP1 degradation during lytic infection in human fibroblasts (HFFs) with the TB40 isolate of HCMV. The observed loss of MRP1 at 48 hours coincided with high levels of UL138 expression (Fig. 2A). UL138 is encoded at the 3' end of a polycistronic transcript that also encodes genes UL133, UL135 and UL136 (10,15). Consequently, we used Toledo UL133-UL138 and UL138 ORF deletion mutants of HCMV (12) to determine whether this region is necessary for MRP1 downregulation. As expected, these viruses replicated similarly to wild-type (12) (Fig 2B). 48 hours after HFF infection with the deletion viruses, no UL138 expression was detected by RT-PCR and MRP1 expression was restored (Fig. 2B). Thus UL138 is necessary for MRP1 downregulation and degradation, although other HCMV genes might also target MRP1. Human monocyte-derived macrophages infected with wtTB40 but not TB40ΔUL138 (an additional UL138-deletion mutant) (11) also showed decreased cell surface MRP1 expression (Fig. 2C).

To determine the functional consequence of UL138-mediated MRP1 downregulation, we examined export of the fluorescent reporter 5-carboxyseminaphtharhodafleur (SNARF-1), an MRP1-specific substrate (16) and leukotriene C4 (LTC4), an endogenous MRP1 substrate

(17). The loss of SNARF-1 from cells loaded with the SNARF-1 ester, is a robust measure of MRP1 activity (Fig. S2A). Pre-incubation with the MRP1-specific inhibitor MK571 allowed accumulation of SNARF-1 and slowed export (Fig. S2B). In THP-UL138 cells, SNARF-1 was exported more slowly than THP-1 controls (Fig. 3A, S2C); by 8 hours, 97% of control cells, compared with 35% of THP-UL138 cells had unloaded dye. SNARF-1 was also exported significantly more slowly from HCMV- than mock-infected fibroblasts (Fig. 3B) and export of LTC₄ was inhibited in THP-UL138 cells (Fig 3C).

Because HA-tagged UL138 did not alter MRP1 mRNA levels, despite loss of MRP1 protein (Fig. 3D), MRP1 downregulation by UL138 was likely to be post-transcriptional. In the presence of UL138, [³⁵S]-methionine radiolabeled 170kDa MRP1 matured normally through the secretory pathway prior to rapid degradation (Fig. 3E), with a reduction in half-life from 16–20 hours (18) to less than 3 hours, suggesting that MRP1 targets UL138 for degradation in the late secretory pathway. Consistent with this, the loss of MRP1 was inhibited by the vacuolar ATPase inhibitor Concanamycin A (CcmA) but was insensitive to proteasome inhibition (Fig. 3E-F). Furthermore, UL138-HA interacted with MRP1 in cells pre-incubated with CcmA, due to the increased MRP1 expression (Fig. 3G). Thus, UL138 associates with MRP1 and induces its lysosomal degradation.

CD34+ bone marrow progenitors and CD14+ monocytes are key sites of latent HCMV infection in vivo (3-5,19). Experimental models of latent infection in both cell types are routinely used to analyse HCMV latency in vitro (4,19-23). To address how UL138 expression affects latent HCMV infection, we latently infected primary CD34+ progenitor cells with a GFP-labeled recombinant HCMV (TB40gfp) and found that latently-infected cells (GFP-positive) also showed specific loss of cell surface MRP1 (Fig. 4A).

MRP1 exports a number of cytotoxic agents including vincristine, a Vinca alkaloid mitotic inhibitor which is relatively MRP1-specific (24). We initially tested the ability of vincristine to reduce the latent load of HCMV by killing experimentally-latent CD14+ monocytes in vitro. Monocytes latently infected with TB40gfp and then treated with vincristine for 4 days, showed a reduced number of latently-infected (GFP-positive) cells (Fig. 4B, S4) as well as a concomitant reduction in detectable latently-expressed UL138 RNA (Fig. 4C), consistent with vincristine-mediated killing of latently-infected cells and a reduction in latency-associated viral load.

Experimentally-latent CD14+ monocytes or CD34+ progenitors can be induced to reactivate latent virus by differentiation to dendritic cells (DCs) and subsequent maturation (8). If vincristine was reducing latent viral load by killing latently-infected cells, this should also be reflected in a reduction in reactivating virus. Indeed, treatment of experimentally latently-infected cells with vincristine reduced reactivation of latent HCMV from CD14+ monocytes after their differentiation to DCs (Fig. S5). CD14+ monocytes and CD34+ progenitors isolated from latently-infected donors routinely reactivate infectious HCMV after differentiation and maturation to mature DCs, detected by co-culture with indicator fibroblasts (Fig. 4D-E, S6) (5). Vincristine treatment of CD14+ monocytes, from 7/7 healthy HCMV-seropositive donors, as well as CD34+ cells, showed reduced reactivation of infectious virus after differentiation and maturation (Fig. 4D-E). Thus, MRP1 is a potential therapeutic target for eliminating latent HCMV-infected cells from bone marrow prior to transplantation.

The study of HCMV latency has been hampered by the inability to identify low frequency latently-infected cells ex vivo. The downregulation of MRP1 by UL138 provides a novel marker of latent infection, but why is MRP1 targeted? DC from MRP1-deficient mice fail to respond to chemotactic stimuli or migrate into afferent lymphatics (25), as the endogenous

MRP1 substrate LTC4 (17) sensitizes the CCR7 chemokine receptor to CCL19 (25). UL138-mediated downregulation of MRP1 reduced cellular LTC4 export, suggesting that UL138 could inhibit migration of infected DCs to draining lymph nodes and impair the generation of an HCMV-specific immune response. Decreased MRP1 expression could also help maintain latent infection by inhibiting premature terminal differentiation of DC progenitors until conditions for reactivation are established, as reported for other HCMV latency proteins (UL111.5A) (26), and the terminal differentiation of DC progenitors is dependent upon functional MRP1 (27).

UL138-mediated downregulation of MRP1 was functionally significant, leading to a dramatic reduction in the export of MRP1-specific substrates and predicted that MRP1-transported cytotoxic drugs would accumulate and kill UL138-expressing cells. Indeed, vincristine treatment of experimentally-latent myeloid cells, naturally-latent CD14+ cells and their CD34+ progenitors decreased the latent CMV viral load. Importantly, vincristine treatment dramatically reduced levels of reactivated virus after myeloid cell differentiation and maturation to mature DCs, a well-established signal for virus reactivation (5).

Our results open up the possibility of developing strategies using MRP1-specific reagents to clear bone marrow or haematopoietic stem cells of latently-infected cells prior to transplantation, either based on the selection of HCMV-negative cell sub-populations or the targeted killing of latently-infected cells using cytotoxic agents normally exported by MRP1.

One Sentence Summary: HCMV-encoded UL138 degrades the cell surface MRP1 drug transporter in latent HCMV infection.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We are grateful to Matt Reeves for discussions and cells. All data are tabulated in the main paper and the supplementary materials. This work was supported by a Next Generation Fellowship from Cambridge Institute for Medical Research and a Wellcome Trust Fellowship (093966/Z/10/Z) to M.P.W.; a grant from the Agency for Science, Technology and Research, Singapore to S.Y.L.T.; an MRC Programme grant (G0701279) to J.H.S. and a Wellcome Trust Senior Fellowship (084957/Z/08/Z) to P.J.L. This work was also supported by the NIHR Cambridge Biomedical Research Centre and the Cambridge Institute for Medical Research is in receipt of a Wellcome Trust Strategic Award. Patent application PCT/GB2012/051094 was filed to cover 'Detection and depletion of HCMV infected cells'.

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Fig. 1. HCMV UL138 downregulates cell surface MRP1 and other targets

(A) Scatterplot of proteins identified in PMP and quantified by >2 unique peptides. The summed ion intensity (y-axis) is shown as log_{10} . Significance A was used to estimate p-values (28).

(B) Cytofluorometric analysis of the indicated proteins in THP-1 cells stably expressing HCMV-encoded UL138 (THP-UL138) and control THP-1 cells.

(C/D) Immunoblot for MRP1/UL138 in control or UL138-transduced fibroblasts (C), or UL138-transduced THP-1 cells (D).

Fig. 2. UL138 downregulates MRP1 during productive HCMV infection

(A) Mock or TB40 IE2-eYFP infected HFFs (m.o.i. 5). At 24 and 48h post-infection cells were harvested, and analysed by immunoblot.

(B) Mock, Toledo wt, Toledo Δ UL133-138 or Toledo Δ UL138 virus infected HFFs were analysed by immunoblot 48h post infection (top 3 panels). UL138 and IE mRNA was analysed (RT-PCR) with GAPDH (internal mRNA control) (bottom 3 panels).

(C) Differentiated primary monocytes were infected with wtTB40 or TB40 Δ UL138. 72h post infection, cells were stained for MRP1, IE and DAPI prior to confocal microscopy.

Fig. 3. UL138 targets mature MRP1 for lysosomal degradation, and inhibits export of MRP1-specific substrates

(A) THP-1 or THP-UL138 cells were loaded with SNARF-1 ester and intracellular SNARF-1 measured by cytofluorometry. The proportion of cells retaining SNARF-1 were plotted.

(B) 15h post infection, HCMV-infected HFFs (m.o.i 5) were analysed for intracellular SNARF-1 (left panel) and immunoblot (right panel). Three independent replicates were used per time point. Plotted: mean $\pm$ SEM, relative to the post-load HCMV-infected sample. Two-tailed p-values (*p<0.05).

(C) LTC4 export assayed in A23187-stimulated cells (Fig. S3) (28), with three independent replicates per condition. Plotted: mean $\pm$ SEM and two-tailed p-values (*p<1 $\times$ 10⁻⁶, **p<0.0005).

(D) RT-qPCR analysis of MRP1 and GAPDH (28).

(E) MRP1 immunoprecipitations (QCRL3 antibody) from cells radiolabeled and chased as indicated, with CcmA included at the 5 hour time point (5*). Total MRP1 is quantified as percentage of MRP1 at time 0.

(F) Cells incubated with MG132, CcmA, or DMSO (28) and immunoblotted as indicated.

(G) HA immunoprecipitation from ADR-UL138HA cells pre-incubated with CcmA for 24 hours to increase MRP1 expression. Anti-FLAG beads were used as a control.

Fig. 4. Selective vincristine-mediated depletion of HCMV-infected cells from experimental and natural latent infection

(A) CD34+ progenitors were latently infected with TB40gfp. 72h post-infection, cells were examined (28) by confocal microscopy (left panels). GFP signal in latently-infected cells was boosted with anti-GFP FITC antibody (28). RT-PCR confirmed latent infection (right panel).

(B - C) Treatment of experimentally latently-infected monocytes with vincristine reduced latent viral load as determined by detection of latently-expressed UL138 mRNA by RT-qPCR, and the relative number of latently-infected cells. Primary CD14+ monocytes were latently infected with TB40gfp. After 3 days, vincristine was added at the indicated concentration (28). 4 days later, the GFP+ cells were counted in 5 independent replicates. 3 further independent replicates were analysed by RT-qPCR for UL138, IE and GAPDH (C).

IE RT-qPCR was always below the limit of detection. Plotted: mean+/-SEM and two-tailed p-values (*p<0.005, **p<0.001, ***p<0.05).

(D) Primary CD14+ monocytes from HCMV-seropositive donor D were treated for 4 days with vincristine (28). Endogenous HCMV was reactivated by differentiation and maturation to mature DC (22), cocultured with fibroblasts for 2 weeks (4 replicates/condition) which were examined for viral IE protein, and foci counted. Plotted are mean+/-SEM %IE+ foci compared to 0ng/ml vincristine and two-tailed p-values

(*p<0.005, **p<0.001, ***p<0.0005).

(E) Primary CD34+ progenitors were treated for 4 days with vincristine (28). Endogenous HCMV was reactivated by differentiation and maturation to mature DC (22) then cocultured with fibroblasts for 2 weeks. Cell supernatants were transferred onto fresh fibroblasts (8 replicates/condition) and 100 cells/replicate examined after 4 days for viral IE protein with DNA counterstaining. Plotted: mean+/-SEM and two-tailed p-values (each treatment vs 0ng/ml vincristine): *p<5×10⁻⁸, **p<5×10⁻⁹.