Human cytomegalovirus-induced host protein citrullination is crucial for viral replication

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Citrullination is the conversion of arginine-to-citrulline by protein arginine deiminases (PADs), whose dysregulation is implicated in the pathogenesis of various types of cancers and autoimmune diseases. Consistent with the ability of human cytomegalovirus (HCMV) to induce post-translational modifications of cellular proteins to gain a survival advantage, we show that HCMV infection of primary human fibroblasts triggers PAD-mediated citrullination of several host proteins, and that this activity promotes viral fitness. Citrullinome analysis reveals significant changes in deimination levels of both cellular and viral proteins, with interferon (IFN)-inducible protein IFIT1 being among the most heavily deiminated one. As genetic depletion of IFIT1 strongly enhances HCMV growth, and in vitro IFIT1 citrullination impairs its ability to bind to 5’-ppp-RNA, we propose that viral-induced IFIT1 citrullination is a mechanism of HCMV evasion from host antiviral resistance. Overall, our findings point to a crucial role of citrullination in subverting cellular responses to viral infection.
**Human cytomegalovirus (HCMV)** is a β-herpesvirus infecting 40–90% of the adult human population. Even though HCMV infection is frequently harmless in healthy patients, it can lead to serious health consequences in individuals with a deficient immune system, such as transplant recipients and AIDS patients. In addition, congenital HCMV infection is the most common cause of fetal and neonatal malformations in developed countries. More recently, HCMV has been linked to autoimmune diseases and degenerative disorders, such as atherosclerosis, vascular disease, and immune aging as well as to certain types of tumors.

One of the strategies devised by HCMV to favor its replication consists in modifying host cellular proteins at the post-translational level, thereby altering their localization, interaction, activation, and/or turnover.

A post-translational modification (PTM) that is increasingly recognized to play an essential role in immune-related diseases is citrullination, also called deimination, a process where the guanidinium group of arginine is hydrolyzed to form citrulline, a non-genetically encoded amino acid. This PTM is catalyzed by the calcium-dependent protein arginine deiminase (PAD) family of enzymes, which in humans is composed of five isoforms (PADs 1–4 and 6), with different tissue-specific expression and substrate specificities. Although aberrant citrullination has been detected in several inflammatory conditions, such as rheumatoid arthritis (RA), systemic lupus erythematosus (SLE), Alzheimer’s disease (AD), multiple sclerosis (MS), Parkinson’s disease, cancer, and atherosclerosis, suggesting that it may play a pathogenic role in inflammation-related diseases, a direct correlation between citrullination and viral infections has only recently emerged. In particular, human rhinovirus (HRV)-induced citrullination of LL37 was found to impair the antiviral activity of this host defense protein. Consistent with the role of citrullination in viral infections, other studies have shown how artificially citrullinated Epstein–Barr virus (EBV) proteins could be specifically recognized by RA sera. Importantly, a recent in silico analysis of transcriptome datasets from lung biopsies of SARS-CoV-2-infected patients has proposed a putative role of PADs in COVID-19.

In this study, we unveil a signature of HCMV infection based on PAD-mediated citrullination of multiple cellular proteins to disrupt host-defense mechanisms.

**Results**

**HCMV infection induces protein citrullination.** To begin to characterize protein citrullination during HCMV infection, we performed immunoblot analysis of protein lysates obtained from human foreskin fibroblasts (HFFs) infected with the HCMV strain AD169 at different time points, using the citrulline-specific probe rhodamine–phenylglyoxal (Rh–PG). We observed an increase in total protein citrullination in lysates from HCMV-infected HFFs at 48 h and, albeit to a lesser extent, at 96 h post-infection (hpi) compared to uninfected control cells (mock) (Fig. 1a).

We next sought to determine whether PAds played a role in enhanced citrullination by HCMV. RT-qPCR analysis revealed that PAD2, 4, and 6 genes were all expressed at significantly higher levels in HCMV-infected HFFs at 24 hpi compared to mock-infected controls (i.e., ~4.8–, ~2.7, and ~1.7-fold, respectively) (Fig. 1b). In particular, both PAD2 and 4 genes were upregulated at 8 hpi, reaching a peak 24 hpi and decreasing afterward (Fig. 1c). In contrast to their mRNA expression levels, PAD2 and 4 protein levels were already increased at 24 hpi and remained elevated for up to 72 hpi (Fig. 1d), suggesting that transcription and translation of PAds are differentially regulated upon HCMV infection. By contrast, the other PAd isoforms were expressed at very low (PAD3) or undetectable levels (PAD1 and 6) and did not vary following HCMV infection (Fig. 1b and Supplementary Fig. 1a), indicating that PAD2 and 4 are the only PAd enzymes involved in HCMV–induced citrullination.

**PAD protein levels are induced by HCMV immediate early 1 (IE1) protein.** To gain more insight into the mechanisms responsible for PAD2 and 4 transcriptional upregulation, we assessed the promoter activity of both PAD2 and 4 genes taking advantage of a dual-luciferase assay system. To this end, HFFs were transiently transfected with luciferase reporter plasmids carrying the wild-type promoter region of either PAD2 or PAD4. Twenty-four hours after electroporation, cells were infected with wild-type or UV-inactivated HCMV. UV-inactivation of HCMV was confirmed by assessing its ability to produce viral immediate early antigen (IEA) compared to the intact virus (Supplementary Fig. 1b). As shown in Supplementary Fig. 1c, HCMV infection led to a robust induction of the luciferase activity driven by either the PAD2 or PAD4 promoter (~7– and 3.5-fold, respectively), whereas UV-HCMV infection failed to induce a similar response, indicating that the synthesis of one or more viral proteins during the initial stage of infection is critical for transcriptional activation of PAD2 and 4 genes.

We next proceeded to determine which viral gene product was responsible for the upregulation of PAD2, the most potently induced PAd member following HCMV infection (Fig. 1b, c). Treatment of HCMV-infected HFFs with the protein synthesis inhibitor cycloheximide (CHX) completely shut down HCMV–induced PAD2 expression (Fig. 1e). Fittingly, UV-inactivated HCMV failed to induce PAD2 expression (Fig. 1e). In contrast, treatment with the viral DNA synthesis inhibitor phosphonoformic acid (PFA) did not seem to affect PAD2 upregulation (Fig. 1e), indicating that true late viral proteins are not involved in PAD regulation.

These results, together with the observation that the early kinetics of PAD induction (Fig. 1c) paralleled that of viral IE1 or IE2 gene expression, raised the hypothesis that IE gene products may play a functional role in PAD induction. Indeed, adenoviral-mediated overexpression of IE1 (AdVIE1) but not IE2 (AdVIE2) led to a substantial upregulation of both PAD2 protein expression (Fig. 1f), indicating that IE1 is at least one of the HCMV products regulating PAD gene expression. Consistently, HFFs infected with a recombinant HCMV strain lacking the IE1 protein (AD169ΔIE1) displayed much lower levels of PAD2 protein expression at 48 hpi compared to cells transduced with the wild-type control (AD169), an effect that was reversed by AdVIE1 coinfection (Fig. 1g). In line with previous results, induction of citrullination by the AD169 strain was also confirmed using the citrulline-specific probe Rh–PG (Supplementary Fig. 1d).

**PAD inhibition blocks HCMV replication.** Next, we asked whether citrullination induced by HCMV infection would affect viral replication. To answer this question, we assessed viral plaque formation in HCMV-infected HFFs (MOI 0.1) treated for 1 h prior to infection with increasing concentrations of CI-amidine (CI–A) (25–200 µM), a cell-permeable pan-PAD inhibitor. After 7 days of continuous exposure to CI–A, we observed a dose-dependent downregulation in the number of viral particles in HCMV-infected HFFs, with a complete suppression at 100 µM. Assessment of cell cytotoxicity by means of the 3-(4,5-dime-thylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay in HFFs treated under the same conditions ruled out the possibility that the antiviral activity was related to the cytotoxic effects
of the drug (Fig. 2a). The half-maximal inhibitory concentration (IC50) of CI-A was found to be 36 µM (Fig. 2a). To further corroborate these data, we also measured viral DNA synthesis by quantitative real-time PCR (qPCR) in similarly treated cells. Remarkably, at the low concentration of 25 µM, CI-A treatment reduced the rate of HCMV DNA replication by approximately threefold compared to untreated cells (Fig. 2b). In line with the plaque-forming activity data, treatment with 100 µM CI-A completely shut down HCMV DNA replication (Fig. 2b). Next, HFFs were treated for 1 h prior to HCMV infection with 100 µM CI-A, infected with increasing MOI (0.01–1) of HCMV, and incubated for an additional 144 h in the presence of the inhibitor. At 144 hpi, the number of plaque-forming units (PFUs) was completely suppressed at MOIs ranging from 0.01 to 0.1 (Fig. 2c). Interestingly, CI-A maintained its antiviral effect even at higher HCMV MOIs (0.5–1). Consistently, this remarkable reduction in the number of viral particles was observed also in HCMV-infected HFFs treated with BB-Cl-amidine (BB-Cl-A)14, a second-generation pan-PAD inhibitor, at nontoxic concentrations much lower than CI-A of 1 and 0.5 µM, with a complete suppression at 1 µM (Supplementary Fig. 2a).

To determine whether the antiviral activity of the compound was limited to HCMV or could be extended to other viruses, we assessed the effect of increasing CI-A concentrations on the replication of: (i) herpes simplex virus 1 (HSV-1) and 2 (HSV-2), two members of the Herpesviridae family; (ii) a clinical isolate of adenovirus as a prototype of a non-enveloped DNA virus; and (iii) the RNA virus human immunodeficiency virus 1 strain IIIb (HIV-1IIIb). Interestingly, only HSV-1 and -2 displayed impaired viral growth in CI-A-treated cells (IC50 ∼ 66 and 21 µM) (Supplementary Fig. 2b), whereas the replication rates of adenovirus and HIV-1IIIb were only marginally affected by the
PAD inhibitor (Supplementary Fig. 2c). Of note, the slight reduction in adenosine replication observed in HFFs cells treated with higher doses of Cl-A was probably due to the cytotoxic effect of the compound, as judged by the MTT assay.

To further demonstrate that Cl-A reduces HCMV-driven citrullination in HFFs, we assessed total protein citrullination levels using an anti-peptidylcitrulline antibody (clone F95). In line with the results obtained with the Rh–PG probe (Fig. 1a), protein citrullination peaked at 48 hpi and decreased at 96 hpi (Fig. 2d). As expected, in the presence of Cl-A, protein citrullination was efficiently suppressed at either time points (Fig. 2d). Next, PAD enzymatic activity was measured by means of an in vitro antibody-based assay using histone 3 as a substrate (Fig. 2e). The reliability of the assay was first assessed using increasing amounts of human recombinant PAD2 in the presence or absence of Cl-A (Supplementary Fig. 1e). Consistent with the kinetics of PAD2 and PAD4 protein expression and with the citrullination profile, PAD catalytic activity was enhanced at 24 hpi, peaked at 48 hpi, and decreased at 72 hpi. Importantly, HCMV-induced PAD activity was significantly inhibited by Cl-A (100 μM) at 48 and 72 hpi (Fig. 2e). Finally, to assess the extent of HCMV replication, total protein extracts from HCMV-infected HFFs treated with or without Cl-A at various time points post infection were subjected to immunoblotting using antibodies against the corresponding IEA, early (UL44), and late (pp28) proteins. While Cl-A treatment only marginally affected IEA expression, it inhibited the expression of viral early and late genes (Fig. 2f), indicating that PAD enzymes support the HCMV productive cycle by fostering the expression of early and late genes.

To confirm that the antiviral activity of Cl-A was due to PAD inhibition and not to an off-target effect of the compound, we generated PAD knockout (KO) HFFs using clustered regularly interspaced short palindromic repeat (CRISPR)/Cas9 technology. Primary cell lines carrying mutations in genes encoding PAD1 (PAD1 KO), PAD2 (PAD2 KO), PAD3 (PAD3 KO), PAD6 (PAD6 KO), or PAD4 (PAD4 KO) were generated using five different guide RNAs (gRNAs). Tracking of indels by decomposition (TIDE) analysis showed an overall knockdown efficiency ranging from 35 to 45% for each of the PAD KO cell lines (Supplementary Fig. 3). Consistently, immunoblot analysis revealed a reduction in PAD2, 4, and 3 protein expression signals between 50 and 60% (Fig. 3a and Supplementary Fig. 4a). With regard to PAD1 and 6, we could only rely on the aforementioned TIDE analysis (Supplementary Fig. 3) as the expression levels of these two PAD isoforms were barely detectable in HFFs, even after HCMV infection (Supplementary Fig. 1a). Although we failed to achieve total suppression of PAD gene expression, the overall knockdown efficiency proved to be sufficient enough to allow us to perform subsequent functional experiments.

To assess whether PADS are required for HCMV replication, we performed a standard plaque assay and qPCR as described above. As shown in Fig. 3b, c, PAD2 and PAD4 KO cells, respectively, displayed 0.5 and 1 logs of viral load reduction, and ~60 and 70% decrease in viral replication, at an MOI of 0.1. In line with these results, a consistent decrease in UL44 protein expression levels was also detected in infected PAD2, and PAD4 KO cell lines at 48 hpi (Fig. 3a). As expected, depletion of PAD1 (PAD1 KO), 3 (PAD3 KO), or 6 (PAD6 KO) in HFFs did not affect the HCMV replication rates compared to wild-type control cells (Supplementary Fig. 4b, c). Thus, PAD2 and 4 but not PAD1, 3, and 6 sustain the viral cell cycle of HCMV. These results were further confirmed in HFFs transfected with siRNA against PAD2 and PAD4, where a protein knockdown efficiency of ~100 and 70%, respectively (Supplementary Fig. 4d), resulted in an even higher viral reduction of 1 and 2 logs, respectively (Supplementary Fig. 4e). In addition, while siRNA control-transfected HFFs treated with 100 μM Cl-A showed a 1-log reduction in the viral load, no significant differences were observed in PAD2- or PAD4-depleted cells (Supplementary Fig. 4e), indicating no predominant role of one isoform over the other in our working model.

IFN-inducible proteins are the main targets of PAD-mediated citrullination. To identify which proteins (i.e., cellular and/or viral) were deinamitized during HCMV infection, we performed citrullinome analysis on HCMV-infected cells harvested at 48 and 96 hpi. Consistent with our earlier findings, we observed a massive increase in overall protein citrullination in HCMV-infected cells at both 48 and 96 hpi compared to uninfected control cells, even though citrullination levels were slightly diminished at 96 hpi compared to 48 hpi (Fig. 4a and Supplementary Data 1, 2, and 3), confirming our previous results (Fig. 1a). In addition to detecting numerous citrullinated viral proteins—35 at 48 hpi and 40 proteins at 96 hpi—we noticed an even higher number of...
citrullinated host cell proteins—177 at 48 hpi and 122 at 96 hpi. Using PANTHER software, we were able to identify a wide range of citrullinated host proteins falling into various functional classes, among which cytoskeletal proteins, chaperones, oxidoreductase, hydrolase, and nucleic acid binding proteins were more frequently found at both time points of infection (Supplementary Fig. 5a). Of particular interest was the significant citrullination at 48 hpi of several members of the interferon (IFN)-induced protein with tetratricopeptide repeat (IFIT) family, such as IFIT1, IFIT2 and IFIT3, and of the IFN-inducible myxovirus resistance 1 (Mx1) gene product (Fig. 4a, left panel)—at 96 hpi, citrullination of these proteins was no longer detectable (Fig. 4a, right panel).

To validate these findings, total proteins from mock or infected HFFs at 48 hpi were immunoprecipitated with the anti-citrulline F95 antibody and subjected to immunoblotting using antibodies against Mx1, IFIT1, IEA, UL44, and pp65. As shown in Supplementary Fig. 5b, all five proteins were robustly deiminated following infection with HCMV.

To corroborate our finding that IE1 is crucial for PAD induction and subsequent citrullination, we performed an additional citrullinome analysis on uninfected HFFs, and wild-type AD169, or mutant AD169ΔIE1-infected HFFs at 48 hpi (Fig. 4b and Supplementary Data 4 and 5). Consistent with previous results, we detected a significant overall protein citrullination in AD169-infected cells at 48 hpi, with IFIT1, IFIT2, and IFIT3, and Mx1 being among the most highly deiminated cellular proteins compared to uninfected control cells (Fig. 4b, left panel). In contrast, upon infection with AD169ΔIE1, we observed a 6.3-, 3.5-, and 2.2-fold decrease in IFIT1, Mx1, and IFIT3 enrichment, respectively (Fig. 4b, right panel), which further supports the crucial role played by the IE1 protein in PAD-mediated citrullination during HCMV infection.
The IFN-inducible proteins IFIT1 and Mx1 exert antiviral activity against HCMV. IFITs are a family of antiviral RNA-binding proteins highly expressed during antiviral immune responses. In this regard, IFIT family members, known primarily for their antiviral activity against RNA viruses, have only recently been implicated in the innate immune response against DNA viruses. Specifically, Li and Swaminathan have shown that human IFIT1, IFIT2, and IFIT3 proteins can suppress lytic replication of the Kaposi’s sarcoma-associated herpesvirus (KSHV). Furthermore, Zhang et al. demonstrated that IFIT1 overexpression significantly impairs HCMV replication in astrocytes, whereas IFIT1 knockdown sustains the viral cycle of HCMV.

The other IFN-inducible target of HCMV-induced deimination, Mx1, is a member of the dynamin-like large GTPase family involved in protection against negative-stranded RNA virus infection and several DNA viruses. Interestingly, MX2 has been recently shown to display antiviral activity against herpesviruses.

To gain further insight into the role of these genes during HCMV infection, we measured virus production in HCMV-infected HFFs after siRNA-mediated depletion of Mx1 or IFIT1—the latter having been shown to exert antiviral activity against HCMV only in fetal astrocytes upon lentiviral overexpression. Following transfection with specific siRNAs against IFIT1 or Mx1 (siIFIT1, siMx1) and subsequent infection with HCMV for 48 h, we achieved a ~60% reduction in IFIT1 protein expression and near to complete silencing of Mx1 protein (Supplementary Fig. 6a). Importantly, in both cases, gene silencing resulted in 1-log higher levels of virus production compared to siCTRL-transfected cells (Fig. 5a).

Given the emerging evidence supporting an antiviral role of IFIT1 against HCMV infection, we asked whether ectopic expression of IFIT1 would curb viral spread in our system. To test this hypothesis, HFFs were transduced with AdVIFIT1 or LacZ as a control at a MOI of 10 and, 24 h post transduction (hpt), infected with HCMV at an MOI of 1 for 144 h. As expected, overexpression of IFIT1 (Supplementary Fig. 6b) reduced viral titer by ~2-log compared to AdVLacZ control (Fig. 5b), indicating that IFIT1 strongly impairs HCMV infection in HFFs.

In vitro citrullination of IFIT1 inhibits its binding to triphosphorylated RNA. In order to elucidate the effect of citrullination on IFIT1, we exploited its well-established ability to preferentially bind viral RNA harboring a triphosphate group at its C-terminus (5′-ppp-RNA), which has been shown to be detrimental for the growth of some RNA viruses containing 5′-ppp-RNA. Interestingly, the arginine at position 187 in a highly charged carboxy-terminal groove appears necessary for IFIT1 binding. In this context, we first attempted to determine whether this arginine 187 could be citrullinated by PAD2 after assessment of its in vitro citrullination using the Rh–PG (Supplementary Fig. 6c). LC-MS/MS peptide analysis of recombinant IFIT1 mock or treated with PAD2 for 3 h identified several arginine positions citrullinated in vitro by PAD2, including arginine 187 (Supplementary Data 6). We then conducted a gel shift assay to assess the ability of IFIT1 to bind to 5′-ppp-RNA according to its citrullination state. In vitro transcribed 5′-ppp-RNA was incubated with recombinant IFIT1, either mock or treated with PAD2, and then loaded on an agarose gel. As expected, mock IFIT1 induced a delay in 5′-ppp-RNA migration in comparison with control RNA, indicating the formation of an IFIT1-RNA complex. This effect was reduced when IFIT1 was previously treated with PAD2 for 3 h (Fig. 5c).

In vitro citrullination of UL44 affects long-chain DNA synthesis. To determine whether citrullination could also affect viral protein activity, we measured the ability of native vs. citrullinated forms of VPAP, also known as ICP36 or UL44, to cooperate with the viral DNA polymerase UL54 in promoting long-chain DNA synthesis, using poly (dA)-oligo (dT)12-18 as primer template (Fig. 5d, and Supplementary Fig. 6d). The choice of VPAP was due to its high deimination rate observed at both 48 and 96 hpi compared to mock control (Fig. 4a, and Supplementary Data 1 and 3). To perform this assay, we employed a truncated form of UL44 (UL44ΔC290) retaining all known biochemical activities of full-length UL44 for DNA polymerase stimulation of full-length UL44. As expected, in the presence of UL54 alone we could not detect long-chain DNA, whereas long-chain DNA synthesis was readily detected when both UL54 and UL44ΔC290 were added to the reaction (Fig. 5d and Supplementary 6d). Notably, PAD2- or PAD4-mediated citrullination of UL44ΔC290 reduced the...
synthesis of long-chain DNA substantially (Fig. 5d and Supplementary Fig. 6d). This inhibition was not due to carryover of PAD2 or PAD4 because addition of PAD2 and 4 to the reaction did not inhibit DNA synthesis (Fig. 5d and Supplementary Fig. 6d). Finally, in vitro citrullination of UL44ΔC290 was confirmed by reduced mobility on SDS-PAGE compared to untreated or mock-treated controls (Supplementary Fig. 6e), as shown previously for other citrullinated proteins.

Discussion

Citrullination is an irreversible PTM catalyzed by PADs enzymes. This modification results in the conversion of an arginine side chain into citrulline, with loss of one positive charge and consequent reduction in protein net charge. This PTM can in turn increase protein hydrophobicity and alter intra- and inter-molecular interactions, affecting protein conformation, stability, and binding of a wide range of proteins involved in numerous physiological processes, such as apoptosis, differentiation, and gene regulation. Eventually, these structural changes can lead to the gain or loss of protein functions. In this study, we have uncovered the role of citrullination in promoting viral persistence through the deimination of cellular proteins endowed with anti-viral activity. Among these, we have focused our attention on IFIT1 and Mx1, two IFN-stimulated genes (ISGs) with a well-established inhibitory activity against various DNA and RNA viruses. We show that silencing of either gene results in augmented virus production, whereas ectopic expression of IFIT1 inhibits viral growth, in good agreement with previous results obtained using KSHV-infected cells. Finally, we report that PAD-mediated in vitro deimination of IFIT1 severely affects its ability to bind to 5′-triphosphorylated RNA, thereby impairing the antiviral activity of this protein.

Taken together, our findings suggest that HCMV can trigger PAD-mediated deimination of arginine residues of cellular proteins in order to enhance viral growth, a possibility supported by previous work showing that the arginine at position 187 of the IFIT1 protein is necessary for its binding to 5′-triphosphorylated RNA. This residue is located within a highly charged carboxy-terminal groove that allows recognition and sequestration of viral nucleic acids. Thus, in future studies it will be crucial to characterize the yet unknown mechanisms of IFIT1- and Mx1-mediated inhibition of HCMV growth and to ascertain whether citrullination can hijack these defense pathways in vivo.

Noteworthy, we demonstrate that HCMV IE1 protein is a pivotal factor for PAD induction and subsequent protein citrullination. In this regard, our citrullinome analysis reveals that a variety of cellular proteins, such as IFITs or Mx1, are significantly less citrullinated upon infection with the AD169ΔIE1 mutant compared to the wild-type virus, implying a central role of IE1 in mediating HCMV-induced citrullination events. The residual amount of citrullinated—IFN-inducible—proteins still detectable...
Fig. 4 Characterization of the citrullinated proteome (citrullinome) of HCMV-infected cells. 

**a** Volcano plot depicting the host (upper panel—circle) and viral (lower panel—triangle) citrullinated proteins of infected cells vs. mock-infected cells at 48 hpi (left panel) and 96 hpi (right panel). Cell lysates from uninfected (mock) or HCMV-infected HFFs (MOI 1) were exposed to a biotin-PG to isolate citrullinated proteins on streptavidin agarose. Bound proteins were then subjected to on-bead tryptic digestion and analyzed by LC-MS/MS—in the graph, every identified citrullinated protein corresponds to a dot. The x axis represents the ratio of citrullination between mock and infected cells at the indicated time points, while the y axis indicates the statistical significance. Both variables were plotted on a logarithmic scale ($n = 3$).

**b** Volcano plot depicting the host citrullinated proteins in HCMV AD169 (left panel) and AD169ΔIE1 (right panel) vs. mock-infected HFFs at 48 hpi. Cell lysates from uninfected (mock) or HCMV-infected (MOI 1) HFFs were exposed to a biotin-PG to isolate citrullinated proteins on streptavidin agarose. Bound proteins were then subjected to on-bead tryptic digestion and analyzed by LC-MS/MS—in the graph, every identified citrullinated protein corresponds to a dot. The x axis represents the ratio of citrullination between mock and infected cells at the indicated time points, while the y axis indicates the statistical significance. Both variables were plotted on a logarithmic scale ($n = 3$).
upon infection with AD169ΔIE1 suggests that other (viral) proteins may be implicated in PAD induction or that a small number of inactive PADs may be present inside the cell and still capable of citrullinating certain targets upon HCMV-mediated activation. Our findings are consistent with a recent study by Casanova et al. showing that HRV infection upregulates PAD activity and that citrullination of the human cathelicidin LL-37 suppresses its antiviral activity against HRV infection, corroborating the role of citrullination as a viral evasion mechanism employed by certain viruses. Noteworthy, citrullination of this host defense peptide has been shown to disrupt its antimicrobial role, both against bacterial and viral infections, including HRV, and increase its susceptibility to degradation41–46.

Another important observation of our study is that, upon cellular entry, several viral proteins are citrullinated as well, raising the hypothesis that citrullination may concomitantly

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**Fig. 5 The antiviral role of IFIT1 and Mx1 against HCMV.**

**a** HFFs were silenced for IFIT1 and Mx1 using specific siRNAs (siIFIT1, siMx1, respectively). As negative control cells were also similarly transfected with scrambled siRNA (siCTRL). At 24 hpt, cells were infected with HCMV at an MOI of 0.1 PFU/cell. Viral supernatants were collected at 144 hpi and analyzed by standard plaque assay. Values are expressed as means ± SEM of three independent experiments (siCTRL vs. siIFIT1 P = 0.0111, unpaired two-tailed t test; siCTRL vs. siMx1 P > 0.05, unpaired two-tailed t test). **b** HFFs were transduced with AdVIFIT1 or AdVLacZ at an MOI of 10 PFU/cell. Subsequently, cells were infected with HCMV at an MOI of 1. The extent of virus replication was measured at 144 hpi. Results are expressed as means ± SEM of three independent experiments (AdVLacZ vs. AdVIFIT1 P > 0.05, unpaired two-tailed t test). **c** Gel electrophoretic mobility shift assay of IFIT1 binding to PPP-7SKas RNA. 50 nM of in vitro transcribed 7SK 5'-ppp-RNA was incubated with only buffer (buffer), 5 µM of recombinant IFIT1, either untreated (UNT rIFIT1) or treated with PAD2 (PAD2 d rIFIT1) and loaded on a tris-glycine agarose gel. Data are representative of three experiments. **d** Long-chain DNA synthesis directed by UL54 in the presence or absence of purified UL44ΔC290 was assayed by measuring the incorporation of labeled [32P]TTP with a poly(dA)-oligo(dT) primer template. DNA products were resolved on an alkaline agarose gel that was exposed to film and, for quantification, to a phosphorescence screen followed by scanning with a Typhoon scanner. The image shows products directed by UL54 alone (lane 1), or by UL54 in the presence of untreated (UNT) UL44ΔC290 (lane 2), in the presence of UL44ΔC290 treated with PAD2 or PAD4 (PAD2 d, PAD4 d; lanes 3 and 4, respectively), in the presence of UNT UL44ΔC290 but with 270 pM PAD2 (lane 5) or 370 pM PAD4 (lane 6) added to the DNA synthesis reaction, or in the presence of UL44 incubated in PAD reaction buffer, but without PADs (mock-PAD, lane 7). One representative gel of three independent experiments is shown. Data are shown as the mean ± SEM, *P < 0.05, **P < 0.01, ***P < 0.001.
influence the activity of both viral and cellular proteins. Among viral proteins, we find that in vitro citrullination of UL44, an accessory protein that increases the DNA processivity of UL54,UL55, reduces its ability to drive long-chain DNA synthesis. Although the physiological role of UL44 citrullination during HCMV infection cannot be inferred from the results of this study, it is tempting to speculate that HCMV may exploit UL44 citrullination to fine-tune its DNA replication during specific phases of viral infection.

Overall, our observation that HCMV growth relies, in part, on PAD-mediated citrullination suggests the attractive prospect that deamination of host—and possibly—viral proteins may be exploited for the design of antiviral agents against HCMV and, more in general, herpesviruses. An alternative pharmacological strategy—perhaps less costly and time-consuming—could rely on repurposing existing PAD inhibitors, such as Cl-A, which we have shown to have a potent inhibitory effect against HCMV-induced citrullination and viral growth.

As HCMV has been implicated in various autoimmune, inflammatory, and cardiovascular diseases as well as cancer development, all characterized by a high degree of protein citrullination, it is possible that HCMV-mediated protein citrullination also constitutes a key event in the pathogenesis of such diseases.

Methods

Cells and viruses. Human foreskin fibroblasts (HFFs, ATCC SCRC-1041™), African green monkey kidney cells (Vero; Sigma-Aldrich 84113001), human HEK 293 from the human kidney (embryonic) (HEK 293, Sigma-Aldrich 85120602), HEK 293T (HEK 293T, ATCC CRL-3216™) were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal calf serum (FCS) (Sigma-Aldrich, Milan, Italy), CD4+ lymphoblastoid T (C8166, ECACC 88051601) were kindly provided by Thomas Stamminger (University of Ulm, Germany). UV-inactivated Merlin was prepared using a double pulse of UV-B light (1.2 J/cm²).

A clinical isolate of adenovirus was propagated in HEK 293 cells, whereas clinical isolates of HSV-1 and HSV-2 were grown in Vero cells and titrated by standard plaque assay,HSV-1Δ113 strain stock was prepared in C8166 cells, as previously describedCL-A was determined by the trypan blue exclusion technique at day 7 pi.

Reagents and proteins. Recombinant human PAD2, PAD4, and Cl-A were from Cayman Chemical (Ann Arbor, USA). Recombinant AdV stocks were kindly provided by R. J. Lebbink (UMC, Utrecht).

Cell viability assay. To determine cell viability, HFFs, HEK 293, and Vero were exposed to increasing concentrations of Cl-A. After 6 days of incubation, the number of viable cells was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method. Briefly, MTT solutions from the stock (final concentration 500 µg/ml) were added, and cells were incubated in a CO2 incubator in the dark for 2 h. Then, the medium was removed and the formazan crystals were dissolved using 100 µl of DMSO. Following the resulting color solution was transferred in a 96-well plate, and the absorbance was read at 570 nm using 630 nm as reference wavelength on a Victor X3 Multi-label Plate Reader (Perkin Elmer, Waltham, MA, USA; wallac 1420 work station software). The viability of the C8166 cells in presence of scalar concentrations of Cl-A was determined by the trypan blue exclusion technique at day 7 pi.

HIV-1 infection and antiviral assay. HIV-1Δ13 (5 ng/ml of HIV-1 gag p24) was pre-incubated for 1 h at 37 °C with the increasing amount of Cl-A (0, 10, 25, 75, 100, or 200 µM) and then added to C8166 cells (0.5 × 10⁶ cells/ml) for 2 h at 37 °C. After three washes in 1× phosphate-buffered saline (PBS), cells were seeded at 5 × 10⁵ cells/ml into a fresh medium plus the same drug concentration used in the pre-incubation step. The HIV-1 gag p24 level was determined in culture supernatants with the HIV-1 p24 antigen ELISA kit (Bionerieux, Marcy-Étoile, France). Mock-infected C8166 cells were used as a negative control. In parallel, HIV-1Δ13-infected C8166 cells were treated with 5 µM tenofovir (NIBSC, London, UK) with the same procedure used for the drug treatment.

RNA isolation and quantitative nucleic acid analysis. Total RNA was extracted using the NucleaseSpin RNA kit (Macherey-Nagel, Düren, Germany), and 1 µg was retrotranscribed using the Revert-Aid H-Minus First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Waltham, USA), according to the manufacturer’s instructions. Comparison of mRNA expression between samples (i.e., infected vs. untreated) was performed by a quantitative real-time PCR (qPCR) apparatus (Stratagene, San Diego, USA), using the primers reported in Supplementary Table 1. To determine the number of viral DNA genomes per nanogram of cellular reference DNA (GAPDH gene), viral DNA levels were measured by quantitative PCR on an Mx3000P apparatus (Stratagene, San Diego, USA). HCMV DNA copy numbers were normalized by the amount of human β-actin (ACTB) amplification reaction mixture. A standard curve of serially diluted genomic DNA mixed with an 1E1-encoding plasmid (from 10⁷ to 1 copy) was created in parallel with each analysis.

Detection of citrullination with rhodamine-phenylglyoxal (Rh-PG). Equal amounts of protein were diluted with 80% trichloroacetic acid and incubated with Rh-PG (final concentration 0.1 mM) for 30 min. The reaction was quenched with 100 mM i-citrate, then centrifuged at 21,100g for 10 min and washed with ice-cold acetone, and resuspended in 2× SDS loading dye for gel electrophoresis. Gels were imaged (excitation = 532 nm, emission = 580 nm) using a ChemiDoc MP Imaging System (Bio-Rad Laboratories Srl), and an enzymatic assay kit (Thermo Fisher Scientific Gmbh, Waltham, MA, USA). Scanning densitometry of the bands was performed using Image Lab (version 4.6.9; Bio-Rad Laboratories Srl, Segrate, Italy).

In vitro antiviral assay. HFFs, HEK 293, and Vero were incubated with increasing concentrations of Cl-A (0, 25, 50, 75, 100, or 200 µM) 1 h prior to being infected with HCMV, adenovirus or HSV-1 or HSV-2 at an MOI of 0.1. Following virus adsorption (2 h at 37 °C), cultures were maintained in medium containing the corresponding drug. HCMV cultures displayed a late cytopathology (7 days pi for HCMV, 6 days pi for adenovirus, and 48 hpi for HSV-1 and HSV-2). Thereafter, the cells and supernatants from the antiviral assay were harvested and disrupted by sonication. The extent of virus replication was then assessed by titrating the infectivity of supernatants by standard plaque assay on HFFs for HCMV, on HEK 293 for adenovirus, and on Vero cells for HSV-1/HSV-2.

Cell viability assay. To determine cell viability, HFFs, HEK 293, and Vero were exposed to increasing concentrations of Cl-A. After 6 days of incubation, the number of viable cells was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method. Briefly, MTT solutions from the stock (final concentration 500 µg/ml) were added, and cells were incubated in a CO2 incubator in the dark for 2 h. Then, the medium was removed and the formazan crystals were dissolved using 100 µl of DMSO. Following the resulting color solution was transferred in a 96-well plate, and the absorbance was read at 570 nm using 630 nm as reference wavelength on a Victor X3 Multi-label Plate Reader (Perkin Elmer, Waltham, MA, USA; wallac 1420 work station software). The viability of the C8166 cells in presence of scalar concentrations of Cl-A was determined by the trypan blue exclusion technique at day 7 pi.
antibody (1:2000, ab5103; Abcam, Cambridge, UK) and anti-rabbit IgG-Fc HRP-conjugated secondary antibody were used to detect citrullinated proteins (1:7000). Subsequently, 100 nM 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) (Sigma, Milan, Italy) was added to each well. The amplification of the signal by HRP was given by the blue color obtained after 30 min of incubation, which changed to yellow once stopped by the addition of 1 N HCl. The optical density (OD) was measured at 450-620 nm with a Victor X3 Multilabel Reader (Perkin Elmer, Waltham, MA, USA; Wallace 1240 work station software).

Construction of promoter-reporter plasmids. The 5'-flanking region of PAD2 was generated by PCR using Q5 High-Fidelity DNA polymerase (New England Biolabs, Ipswich, USA), pLightSwitch_Prom PAD2 (Active Motif, La Hulpe, Belgium), as the template, and the primers listed in Supplementary Table 3. The thermocycler (Biorad C1000 Touch Thermal Cycler) settings consisted of 30 s of incubation at 98 °C, followed by 35 cycles of 10 s at 98 °C, 10 s at the pre-designated annealing temperature and 30 s at 72 °C, and a final extension for 2 min at 72 °C. The 5'-flanking region of PAD4 was amplified by PCR using human genomic DNA from HFFs, as the template, and the primers listed in Supplementary Table 2. The PCR condition was an initial denaturation for 2 min at 95 °C, 35 cycles (95 °C for 30 s, 56 °C for 30 s, and 72 °C for 4 min) and a final extension at 72 °C for 8 min. The resulting amplification products were digested with XhoI (Thermo Fisher Scientific, Waltham, USA) and HindIII (Thermo Fisher Scientific, Waltham, USA) and cloned into the pGL2.0[uc2/Puro] Vector (Promega, Madison, USA), which encoded the Renilla luciferase gene driven by the SV40 promoter. After 24 h, cells were infected with HCMV, UV-HCMV, or mock (MOI of 1 PFU/ml). At 24 h, the cells were co-transfected with the pRL-SV40 vector (Promega, Madison, USA), which contained the Renilla luciferase gene driven by the SV40 promoter. Luciferase activity to the RLU measured for that of Renilla luciferase.

Luciferase assay. HFFs were electroporated using a Micro-Perator MP-100 (Thermo Fisher Scientific, Waltham, USA) according to the manufacturer’s instructions (a single 1300 V pulse, 30-ns pulse width). Briefly, 500 ng of each construct were used in every 2 × 10⁵ cells, which were plated in 24-well tissue culture plate System (Promega, Madison, USA) and verilux elements. All of the constructs were prepared using the PureYield Plasmid Mini-Prep System (Promega, Madison, USA) and verified by restriction mapping and complete sequencing. The resulting chromatograms were analyzed using Chromas software (2.6.6 (Technolium Ltd.).

Citrulline analysis by mass spectrometry: sample preparation. Sample preparation in technical triplicates followed the procedure outlined in ref. 58. Equal amounts of cell lysates from each experimental group (300 μg) were diluted in buffer (100 mM HEPES pH 7.6) to a final concentration of 1 μg/μL and incubated with 20% trichloroacetic acid (TCA) and 0.5 mM biotin-PG59 for 30 min at 37 °C. Labelled proteins were precipitated on ice for 30 min. Samples were pelleted through tabletop centrifugation (21,100 x g, 15 min) at 4 °C. The supernatants were discarded, and the pellets were washed with cold acetone (300 μL). After drying for 5 min, the pellets were resuspended in 1.2% SDS in PBS by bath sonication and heating. Samples were then transferred to 15-mL screw-cap tubes and diluted in 1× PBS to a 0.2% SDS final concentration. Samples were incubated with streptavidin agarose slurry (Sigma-Aldrich, 170 μL) overnight at 4 °C and for an additional 3 h at 25 °C. After discarding the flow-through, the streptavidin beads were washed with 0.2% SDS in PBS (5 μL) for 10 min at 25 °C. The beads were then washed three times with 1X PBS (5 μL) and three times with water (5 μL) in order to remove any unbound proteins. Beads were then transferred to a microcentrifuge tube and heated in 1× PBS with 500 μL 6 M urea and 10 mM DTT (65 °C, 20 min). Proteins bound to the beads were then alkylated with iodoacetamide (20 mM, 37 °C for 30 min). The beads were successively pelleted by centrifugation (1400 x g for 3 min) and the supernatant was removed. The pellet was resuspended in a premixed solution of 2 μL urea, 1 mM CaCl₂, and 2 μg Trypsin Gold (Promega, Madison, USA) in PBS. These were shaken overnight at 37 °C. The supernatant was collected and the beads were washed twice with water (50 μL), each time collecting the supernatant. The fractions were combined, acidified with formic acid (5% final concentration), and stored at −20 °C until use.

Mass spectrometry. Liquid chromatography-mass spectrometry/mass spectrometry (LC-MS/MS) analysis was performed with an LTQ-Orbitrap Discovery mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA) coupled to an Easy-nLC HPLC (Thermo Fisher Scientific, Waltham, MA, USA). Samples were pressure-loaded onto a 250-μm fused-silica capillary hand-packed with 4 μm Agilent C18 reverse-phase resin (Phenomenex). Samples were separated on a hand-packed 250-μm fused-silica capillary column with a 5-μm tip Agilent C18 reverse-phase resin (Phenomenex). Peptides were eluted using a 10-h gradient of 0–100% Buffer B in Buffer A (Buffer A: 95% water, 5% acetonitrile, 0.1% formic acid; Buffer B: 20% water, 80% acetonitrile, 0.1% formic acid). The flow rate through the column was set to ~400 nL/min, and the spray voltage was set to 2.1 kV. Full MS scan (FTMS) and dependent MS2 scans (ITMS) of the 10 most abundant ions. The tandem MS data were searched by the SEQUEST algorithm using a concatenated target/decoy variant of the human and viral UniProt database. A static modification of +57.02146 on cysteine was specified to account for alkylation by iodoacetamide. SEQUEST output files were filtered using DTASelect 2.0.

Reductive dimethylation (ReDiMe) labeling and mass spectrometry for the citrulline analysis of wtAD169 and AD169ΔE1E1-infected cells. The trypsin digest was desalted using Pierce C18 spin column (catalog No 89870) according to the manufacturer’s protocol and was reconstituted in 200 μL of 100 mM triethylammonium bicarbonate, pH 8.5. 20% H₂O-CHO (4 μL, light formaldehyde), and D3-CDO (4 μL, medium formaldehyde), and D2-CDO (4 μL, heavy formaldehyde) were added to the mock, AD169ΔE1ΔE1, AD169ΔE1, and AD169ΔE1E1E1 infected samples, respectively. In total, 20 μL of 0.6 M sodium cyanoborohydride (NaBH₃CN) was then added to both the mock and AD169-infected samples, respectively, while 20 μL of 0.6 M sodium cyanoborodeuteride (NaBD₃CN) was added to the AD169ΔE1E1-infected samples. The samples were incubated at room temperature for 2 h. The samples were then cooled on ice and the reaction quenched with 4 μL of 20% ammonium hydroxide. Formic acid (8 μL) was then added to the samples. Heavy, medium, and light formaldehyde-labeled samples were mixed together, and the mixture was desalted and stored at −20 °C for proteomic analysis. Data was acquired using a NanoAcuity UPLC (Waters Corporation, Milford, MA) coupled to an Orbitrap Fusion Lumos Tribrid (Thermo Fisher Scientific, Waltham, MA) Mass spectrometer. Peptides were trapped and separated using an in-house 100 μm i.D. fused-silica pre-column (Kas1 frit) packed with 2 cm ProntoSil (Bischoff
The data that support the findings of this study are available within the paper and its supplementary information files. All other relevant data are available from the corresponding authors upon reasonable request. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE (1) partner project.

**Data availability**
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Author contributions

G.G., F.G., S.P., and S.L. conceived and designed the experiments. G.G., F.G., S.P., G.O., V.D.O., M.B., and V.G. performed the experiments and analyzed the data. A.I.S., P.R.T., E.W., S.M., and V.G. carried out the LC-MS/MS analyses. J.A.S. and R.J.L. supported the generation of the CRISPR/Cas9 KO cell lines. T.S. supported the design of the experiments with AD169AIE1. M.D.A., D.M.C., and A.P. critically revised the manuscript. All authors contributed to writing this manuscript.

Competing interests

The authors declare the following competing financial interest(s): P.R.T. founded Padlock Therapeutics and is entitled to payments from Bristol Myers Squibb if certain milestones are met. P.R.T. is a consultant for Celgene and Dharma Therapeutics. Italian Patent No. 102011596547852 issued in October 20, 2019 ("PAD2 PER USO NELLA PREVENZIONE E/O TRATTAMENTO O DIAGNOSI DI INFEZIONI DA VIRUS DELLA FAMIGLIA HERPESVIRIDAE").

Additional information

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