

1 P2Y2 purinergic receptor is induced following human cytomegalovirus infection  
2 and its activity is required for efficient viral replication

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17 **Running Title:** Role of purinergic receptors in HCMV infection

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## Abstract

25 Human cytomegalovirus (HCMV) manipulates many aspects of host cell biology to  
26 create an intracellular milieu optimally supportive of its replication and spread. The current study  
27 reveals a role for purinergic signaling in HCMV infection. The levels of several components of  
28 the purinergic signaling system, including the P2Y2 receptor, were altered in HCMV-infected  
29 fibroblasts. P2Y2 receptor RNA and protein are strongly induced following infection.  
30 Pharmacological inhibition of receptor activity or knockdown of receptor expression markedly  
31 reduced the production of infectious HCMV progeny. When P2Y2 activity was inhibited, the  
32 accumulation of most viral RNAs tested and viral DNA was reduced. In addition, the level of  
33 cytosolic calcium within infected cells was reduced when P2Y2 signaling was blocked. The  
34 HCMV-coded UL37x1 protein was previously shown to induce calcium flux from the smooth  
35 endoplasmic reticulum to the cytosol, and the present study demonstrates that P2Y2 function is  
36 required for this mobilization. We conclude that P2Y2 supports the production of HCMV  
37 progeny, possibly at multiple points within the viral replication cycle that interface with  
38 signaling pathways induced by the purinergic receptor.

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## Importance

48 HCMV infection is ubiquitous and can cause life-threatening disease in  
49 immunocompromised patients, debilitating birth defects in newborns, and has been increasingly  
50 associated with a wide range of chronic conditions. Such broad clinical implications result from  
51 the modulation of multiple host cell processes. This study documents that cellular purinergic  
52 signaling is usurped in HCMV-infected cells and that the function of this signaling axis is critical  
53 for efficient HCMV infection. Therefore, we speculate that blocking P2Y2 receptor activity has  
54 the potential to become an attractive novel treatment option for HCMV infection.

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## Introduction

71 Human cytomegalovirus (HCMV) is a beta herpesvirus that infects a large percentage of  
72 the adult population worldwide. Infection in immunocompetent people is typically  
73 asymptomatic. In contrast, HCMV is a leading opportunistic pathogen in immunosuppressed  
74 individuals [1-4] and is a major infectious cause of birth defects [5, 6].

75 HCMV causes broad cellular effects that likely contribute to the diverse pathologies  
76 associated with infection. One mechanism utilized by the virus to change the biology of infected  
77 cells is via the regulation of expression levels and activities of cell surface proteins [7, 8]. Here,  
78 we describe the role of purinergic receptors during HCMV infection. Purinergic receptors are  
79 ubiquitous cell surface receptors that are activated by extracellular adenosine di- (ADP) and tri-  
80 (ATP) phosphates (P2 receptors) or adenosine (P1 receptors). P2 purinergic receptors are further  
81 divided into ionotropic P2X and metabotropic P2Y families. P2X receptors are ATP-gated ion  
82 channels and P2Y receptors are G protein-coupled receptors that are activated by adenine and  
83 uridine nucleotides or nucleotide sugars. Seven subtypes of P2X receptors (P2X1-7) and eight of  
84 P2Y receptors (P2Y1, P2Y2, P2Y4, P2Y6, P2Y11, P2Y12, P2Y13, and P2Y14) have been  
85 identified [9, 10].

86 Signaling via the P2Y receptors triggers the activation of a heterotrimeric G protein,  
87 which leads to the activation of effector protein phospholipase C (PLC), and generation of  
88 diacylglycerol (DAG) and inositol 1,4,5-triphosphate ( $IP_3$ )[11]. DAG stimulates protein  
89 kinase C (PKC)[12]. The activation of PKC has been shown to increase the expression of early  
90 growth response protein 1 (Egr-1) [13], a transcription factor critical for DNA synthesis,  
91 proliferation, and migration of fibroblasts and other cells [14].  $IP_3$  mobilizes cytosolic  $Ca^{2+}$  from  
92 the smooth endoplasmic reticulum (SER). It has been observed that in lung fibroblasts, P2Y2 is

93 the only purinergic receptor subtype that, when activated, causes the mobilization of intracellular  
94  $\text{Ca}^{2+}$  [15]. P2Y2-mediated intracellular  $\text{Ca}^{2+}$  increases have been implicated in promoting the  
95 proliferation and migration of hepatocellular carcinoma cells in mice [16]. Moreover, P2Y2  
96 signaling was found to stimulate HIV-1 viral fusion through the activation of proline-rich  
97 tyrosine kinase 2 (Pyk2) [17].

98 P2X receptors differ notably from their P2Y counterparts in ligand selectivity. While  
99 P2Y receptors recognize a wide range of agonists, P2X receptors are activated only by ATP.  
100 Moreover, P2X receptors are assembled as trimeric proteins. Specifically, P2X5 mainly  
101 functions as heterotrimers involving P2X1, P2X2, or P2X4 [18]. The expression of P2X5  
102 receptors is normally restricted to the trigeminal mesencephalic nucleus of the brainstem,  
103 sensory neurons, cervical spinal cord, and some blood vessels [19]. P2X5 receptor expression in  
104 atypical locations has been linked to cancer [20-22]. Activation of the P2X5 receptor causes an  
105 influx of cations ( $\text{Na}^+$ ,  $\text{K}^+$ , and  $\text{Ca}^{2+}$ ) across the plasma membrane [23].

106 Knowing that purinergic receptors regulate cellular calcium levels and that an increase in  
107 calcium levels has been observed following HCMV infection [24], it is intriguing to speculate  
108 that members of the family of purinergic receptors could be important factors during HCMV  
109 infection. It has been reported previously that a few members of the family of purinergic  
110 receptors exhibited higher expression in HCMV-infected cells, however their molecular role  
111 during infection has not been found [25, 26]. The regulation of  $\text{Ca}^{2+}$  release is critical for viral  
112 DNA synthesis and the production of infectious progeny [27]. It has been found that the HCMV  
113 immediate early protein pUL37x1 induces the mobilization of  $\text{Ca}^{2+}$  from SER to the cytosol [28].  
114 Altering the release of  $\text{Ca}^{2+}$  from SER affects the activity of  $\text{Ca}^{2+}$ -dependent ER chaperones,  
115 resulting in the accumulation of unfolded proteins and contributing to the unfolded protein

116 response [29]. In addition, the  $\text{Ca}^{2+}$ -dependent protein kinase,  $\text{PKC}\alpha$ , is activated following  
117 infection, leading to the production of large (1-5  $\mu\text{m}$  diameter) cytoplasmic vesicles at late times  
118 during infection [30]. The presence of these vesicles correlates with the efficient accumulation of  
119 enveloped virions. The release of  $\text{Ca}^{2+}$  from SER during infection is also expected to influence  
120  $\text{Ca}^{2+}$ -dependent processes that occur in the mitochondria. For instance,  $\text{Ca}^{2+}$  uptake can stimulate  
121 aerobic metabolism and enhance ATP production [31, 32].  $\text{Ca}^{2+}$  can also induce the activity of  
122  $\text{Ca}^{2+}$ /calmodulin-dependent protein kinase kinase (CaMKK), which activates 5' AMP-activated  
123 protein kinase (AMPK). AMPK activity has been shown to support HCMV-induced changes to  
124 the infected cell metabolome, as well as to be necessary for HCMV DNA synthesis and the  
125 expression of viral late genes [33, 34]. Interestingly, blocking viral DNA synthesis by inhibiting  
126 viral DNA polymerase, pUL54, can serve as an effective therapeutic measure for HCMV  
127 infection [35]. Additionally, cellular phosphoinositide 3-kinase (PI3-K) and p38 kinase activities  
128 were found to be required for viral DNA replication and the production of infectious progeny  
129 [36, 37]. As both PI3-K and p38 are downstream factors of the purinergic receptor signaling axis  
130 [38, 39], it is conceivable that purinergic receptors can have an effect on viral DNA synthesis.  
131 Finally, since  $\text{Ca}^{2+}$  plays a central role in mediating apoptosis, regulating the concentration of  
132 intracellular  $\text{Ca}^{2+}$  may affect the infected cell's sensitivity to apoptotic stimuli [40, 41].

133 Although the viral pUL37x1 protein has already been implicated in mediating  $\text{Ca}^{2+}$   
134 release during HCMV infection, there may also be other pathways that affect intracellular  $\text{Ca}^{2+}$   
135 levels. Therefore, we speculated that the purinergic receptor-mediated signaling may work  
136 alongside pUL37x1 in regulating  $\text{Ca}^{2+}$  release into the cytosol, influencing the efficiency of  
137 HCMV infection. In fact, one study using mouse fibroblasts showed that extracellular ATP and  
138 UTP could stimulate the mobilization of  $\text{Ca}^{2+}$  from intracellular stores by an  $\text{IP}_3$ -mediated

139 pathway downstream of P2Y2 signaling that is independent of PKC activation [42]. To test this  
140 notion, we used pharmacological agents and siRNA technology to study the consequences of  
141 inhibiting purinergic receptors during HCMV infection. We provide evidence that P2Y2  
142 inhibition interferes with the expression of viral genes and the release of infectious progeny,  
143 whereas P2X5 inhibition enhances HCMV yield. P2Y2-mediated signaling affects HCMV  
144 infection at the stage of viral DNA synthesis and significantly contributes to the regulation of  
145 intracellular  $\text{Ca}^{2+}$  homeostasis.

146

## 147 **Methods and Materials**

148 **Cells, viruses and drugs.** Human foreskin fibroblasts (HFF) and human lung fibroblasts  
149 (MRC5) were cultured in Dulbecco's Modified Eagle Medium (DMEM; Sigma-Aldrich)  
150 supplemented with 10% fetal bovine serum (FBS). Penicillin and streptomycin were added to the  
151 media for all experiments except those involving siRNA transfection.

152 HFF cells stably expressing the viral protein, pUL37x1, were made using the pLVX-  
153 EF1 $\alpha$  promotor lentivirus packaging plasmid (Clontech). To insert AD169 UL37x1 between  
154 EcoRI-BamHI sites of the pLVX-EF1 plasmid, the sequence was amplified by PCR from  
155 BADwt, a BAC containing the AD169 strain [43]. Lentivirus particles were generated using  
156 sequence-confirmed plasmids containing UL37x1 or GFP as a control. Following lentiviral  
157 treatment, cells expressing pUL37x1 or GFP were selected using 2  $\mu\text{g}/\text{ml}$  puromycin, and protein  
158 expression was evaluated by Western blot using mouse monoclonal antibody 4B6-B [28].

159 Previously described HCMV strains TB40/E, TB40-GFP, AD $\omega$ t and ADsubUL37x1  
160 HCMV strains were used in these studies [43, 44]. For some experiments, TB40/E virus was  
161 inactivated by irradiation (50 J/m<sup>2</sup>) in a Stratalinker (Stratagene) [45]. A pUL37x1-deficient

162 derivative of AD<sup>wt</sup>, AD<sub>subUL37x1</sub> [43], lacks the AD169 genomic sequence from 169,144 to  
163 169,631 and includes kanamycin resistance and *LacZ* markers [46]. All viral stocks were purified  
164 by centrifugation through a 20% D-sorbitol cushion containing 50mM Tris·HCl, 1mM MgCl<sub>2</sub>,  
165 pH 7.2, and resuspended in DMEM. Infections were performed by treating cells with viral  
166 inoculum for 2 h, followed by removal of inoculum and washing with phosphate-buffered saline  
167 (PBS) before applying with fresh media.

168 Virus titers were determined based on viral IE1 expression on a reporter plate containing  
169 fibroblasts [34, 47]. At 24 hpi, cells were assayed by indirect immunofluorescence using a  
170 primary antibody against the viral IE1 protein [48] and secondary goat anti-mouse antibody  
171 conjugated to Alexa Fluor 488 (Molecular Probes). Nuclei were stained using Hoescht dye  
172 (Thermo Fisher Scientific). IE1-positive nuclei were quantified using the Operetta High-Content  
173 Imaging System (PerkinElmer). In some cases, titers were determined by TCID<sub>50</sub> analysis, and  
174 calculations were performed according to the Reed and Muench formula [49]. All virus stocks  
175 were titered by the TCID<sub>50</sub> method.

176 Kaempferol (Sigma-Aldrich) was dissolved in DMSO and stored at 4°C until use.  
177 PPADS tetrasodium salt (Torcisor) was dissolved in dimethyl water and stored at -20°C until use.  
178 Cytotoxicity assays for drugs were performed at 96 h post treatment using the CellTiter 96®  
179 AQueous One Solution Cell Proliferation Assay (Promega, Madison, WI), according to the  
180 manufacturer's protocol. Cell viability was measured based on absorbance at 490 nm using a  
181 SpectraMax Plus spectrometer (Molecular Devices).

182  
183 **siRNA knockdown experiments.** siRNAs targeting P2Y2 (siP2Y2), P2X5 (siP2X5), ENPP4  
184 (siENPP4) or ENTPD2 (siENTPD2) were designed and purchased from Life Technologies.  
185 HFFs were grown to ~80% confluence in DMEM supplemented with 10% FBS and then

186 transfected with siRNA using Lipofectamine<sup>®</sup> RNAiMAX Reagent (Life Technologies). Cells  
187 transfected with non-specific, scrambled siRNA (siSc) (Life Technologies) served as a negative  
188 control. Following a 24 h-incubation, cells were washed with PBS and either infected with  
189 TB40/E-GFP virus or mock infected.

190  
191 **Assay for viral entry.** For drug assays, kaempferol, PPADS, or solvent was applied to 95-100%  
192 confluent HFFs for 1 h, and for siRNA analyses, cells were transfected with siP2Y2, siP2X5 or  
193 siSc for 24 h. Cells were then washed with PBS and infected at a multiplicity of 1 TCID<sub>50</sub>/cell or  
194 mock infected for 1 h at 4°C. Virus that had not penetrated cells was removed with a low-pH  
195 citrate buffer (40 mM citric acid, 10 mM KCl, 135 mM NaCl, pH 3.0) [50]. Cells were then  
196 incubated at 37°C in DMEM containing 10% FBS. Cells were either fixed in cold MeOH and  
197 immunostained for viral IE1 protein at 24 hpi, as described above, or viral DNA was isolated 1 h  
198 after the reaction temperature was raised to 37°C, using the QIAamp DNA Mini kit (Qiagen) and  
199 quantified by quantitative polymerase chain reaction (qPCR) performed in duplicate using SYBR  
200 Green master mix (Applied Biosystems) using the QuantStudio™ 6 Flex Real-Time PCR System  
201 (Applied Biosystems). Primers for the viral UL123 locus were used (Table 1). Fold change was  
202 calculated using the ΔΔC<sub>t</sub> method and GAPDH was used as an internal control [51].

203  
204 **Nucleic acid and protein analyses.** For RNA-Seq analysis of protein-coding RNAs, MRC-5  
205 human lung fibroblasts were infected with the AD169 strain of HCMV. RNA from mock- and  
206 HCMV-infected cells was collected at 48 hpi and isolated using the miRNeasy kit (Qiagen.).  
207 RNA quality was analyzed using the Bioanalyzer 2100 (Agilent Technologies). TruSeq Stranded  
208 Total RNA Library Prep Kit with Ribo-Zero (Illumina) was used to generate cDNA libraries as  
209 per the manufacturer's instructions.. Briefly, cytosolic ribosomal RNA (rRNA) was depleted

210 from total RNA using biotinylated probes that selectively bind rRNA species. The resulting  
211 rRNA-depleted RNA underwent fragmentation, reverse transcription, end repair, 3'-end  
212 adenylation, adaptor ligation and subsequent PCR amplification and SPRI bead purification  
213 (Beckman Coulter). The unique barcode sequences were incorporated in the adaptors for  
214 multiplexed high-throughput sequencing. The final product was assessed for its size distribution  
215 and concentration using BioAnalyzer High Sensitivity DNA Kit (Agilent) and Kapa Library  
216 Quantification Kit (Kapa Biosystems). The libraries were pooled, denatured and loaded onto a  
217 HiSeq Rapid Paired-Read flow cell (Illumina), which was subjected to 2X100 cycles of  
218 sequencing by an Illumina HiSeq 2500 (Illumina). Illumina CASAVA pipeline Version 1.8 was  
219 used to extract de-multiplexed sequencing reads. Sequencing reads were normalized using the  
220 trimmed mean of M-values method (TMM), mapped to the human reference genome and  
221 transcripts annotated to which reads have been mapped. Mapped reads were counted and the  
222 differential gene expression between triplicate samples from mock- and HCMV-infected cells  
223 was computed based on counts per million (CPM). Ingenuity Pathway Analysis cloud software  
224 (Qiagen) was used to group the genes identified as significantly up- or down-regulated into gene  
225 ontologies and to determine which cellular networks were the most significantly regulated in  
226 HCMV-infected cells.

227 For quantitative reverse transcription PCR (qRT-PCR) analysis of RNA, RNA was  
228 extracted from samples collected in QIAzol lysis reagent using the miRNeasy kit (Qiagen).  
229 Complimentary DNA (cDNA) was made from 1 µg of total RNA with oligo dT and MultiScribe  
230 reverse transcriptase (Applied Biosystems), according to the manufacturer's protocol. Reverse  
231 transcription reactions were run at 25°C for 10 min, 48°C for 30 min, and 90°C for 5 min.  
232 Primers used in the study are listed in Table 1. qPCR was performed in duplicates on equal

233 volumes of cDNA using SYBR Green master mix (Applied Biosystems) on the QuantStudio™ 6  
234 Flex Real-Time PCR System (Applied Biosystems). Transcript levels were analyzed using the  
235  $\Delta\Delta Ct$  method and GAPDH was used as an internal control [51]. Error ranges are reported as  
236 standard error of the mean (SEM).

237 For quantification of viral DNA, total DNA was isolated from HCMV-infected cells or  
238 media at 96 hpi, and viral DNA was quantified by qPCR using primers specific for UL123 gene  
239 and a standard curve created by serially diluting BADwt DNA. To calculate the particle-to-  
240 infectious unit ratio, media collected at 96 hpi was titered and viral DNA was isolated from  
241 DNase I-treated (10U/mL; 30 min. at 37C) virions to calculate viral DNA copy number/mL as a  
242 measure of virus particles.

243 For protein analysis, fibroblasts were harvested using lysis buffer (50 mM Tris-HCl, pH  
244 7.5, 5 mM EDTA, 100 mM NaCl, 1% Triton X-100, 0.1% SDS, and 10% glycerol). Samples  
245 were mixed with 6x SDS sample buffer (325 mM Tris pH 6.8, 6% SDS, 48% glycerol, 0.03%  
246 bromophenol blue containing 9% 2-mercaptoethanol). Equal protein amounts of the different  
247 samples were separated by electrophoresis (SDS-PAGE) and transferred to ImmunoBlot  
248 polyvinylidene difluoride (PVDF) membranes (BioRad Laboratories). Western blot analyses  
249 were performed using primary antibodies recognizing P2Y2 (H-70; Santa Cruz Biotechnology),  
250 P2X5 (Sigma-Aldrich),  $\beta$ -actin-HRP (Abcam), or pUL37x1-specific mouse monoclonal antibody  
251 4B6-B [28]. Goat anti-mouse and donkey anti-rabbit (GE Healthcare Biosciences) conjugated  
252 with horseradish peroxidase (HRP) were used as secondary antibodies. Western blots were  
253 developed using WesternSure ECL Detection Reagents (Licor).

254  
255 **Intracellular calcium assay.** HFFs were cultured to 95% confluence, drug-treated and infected  
256 at a multiplicity of ~1 with ADwt, AdsubUL37x1 viruses or mock infected. At 20 hpi, cells were

washed and incubated in media containing 2  $\mu$ M Fluo-4 AM dye (Life Technologies) for 30 min. at 37°C. After washing with PBS, images were captured using the Operetta High-Content Imaging System (PerkinElmer) and the fluorescent signal was measured based on 10 cells per experimental arm using ImageJ software [52]. Background fluorescence was subtracted and fold change was determined relative to control samples.

262

## Results

## 264 HCMV infection elevates the steady-state levels of several purinergic receptors

To investigate the differential expression of purinergic receptors during infection, we performed whole RNA sequencing of MRC-5 fibroblasts infected with the AD169 strain of HCMV. RNA from mock and infected cells was analyzed in three independent experiments where samples were collected at 48 hpi, allowing us to analyze RNAs during the late phase of the viral replication cycle. Several components involved in purinergic receptor signaling are expressed at significantly higher levels in the infected cells, including P2Y2, P2X5, ENPP4 and ENTPD2, and several others were reduced (Table 2).

To confirm the results obtained from RNA-Seq and extend the findings to a different fibroblast cell population and virus strain, we used qRT-PCR to investigate the expression levels of the RNAs identified in our RNA-seq analysis. HFFs were infected with the TB40/E-GFP strain of HCMV at a multiplicity of 3 TCID<sub>50</sub>/cell. At 48 hpi, HCMV-infected cells expressed 76.41 ± 20.10 times more P2Y2, 2.74 ± 0.54 times more P2X5, 9.98 ± 3.22 times more ENPP4 and 45.55 ± 16.54 times more ENTPD2 RNA than mock-infected controls (Fig. 1).

We also examined the kinetics of P2Y2 and P2X5 receptor expression throughout the viral replication cycle. HFFs were infected at a multiplicity of 3 TCID<sub>50</sub>/cell, and samples were

280 collected after various time intervals. P2Y2 and P2X5 RNAs were assayed by qRT-PCR and  
281 receptor proteins were assayed by Western blot. P2Y2 RNA was elevated at 24 hpi and increased  
282 as the infection progressed (Fig. 2A). P2Y2 protein became highly abundant at 48 hpi and its  
283 levels stayed elevated through the last time point assayed at 120 hpi (Fig. 2C). These results also  
284 qualitatively match the mass spectroscopy-based analysis of cell surface proteins, which  
285 determined that P2Y2 protein is 3-, 4- and 5-fold more abundant at 24, 48, and 72 hpi,  
286 respectively, compared to uninfected cells [8]. In the case of P2X5, its transcript levels increased  
287 up to 48 hpi and then decreased later in infection (Fig. 2B). This corresponded with increasing  
288 levels of P2X5 protein between 24 and 72 hpi, which then markedly decreased later in infection  
289 (Fig. 2C).

290 Taken together, data from both RNA-Seq and qRT-PCR analyses confirm that HCMV  
291 infection in fibroblasts causes an increase in the expression of the cellular purinergic receptors  
292 P2Y2 and P2X5, as well as the cell surface ectonucleotidases ENPP4 and ENTPD2.

293

#### 294 **Viral gene expression is necessary for the normal modulation of P2Y2 and P2X5 expression**

295 Since the overexpression of these receptors was seen during the early and late phases of  
296 infection, we wished to determine whether the binding of the virion to the fibroblast cell surface  
297 was sufficient to induce their upregulation. To do so, we used UV-irradiated TB40/E virus that  
298 can bind to and enter cells but cannot express its genes. HFFs were infected with the untreated  
299 virus or UV-irradiated virus at a multiplicity of 3 TCID<sub>50</sub>/cell, samples were collected at 24 and  
300 48 hpi and receptor transcript levels were quantified by qRT-PCR. Compared to cells infected  
301 with untreated TB40/E virus, those infected with the UV-irradiated virus exhibited 90% lower  
302 expression of P2Y2 RNA (Fig. 3A) but 40% higher expression of P2X5 (Fig. 3B). This finding

303 suggests that viral binding and entry is not sufficient to produce the increased levels of P2Y2  
304 transcript normally seen during infection. However, viral gene expression appears not to be  
305 required for the upregulation of P2X5 expression.

306

307 **P2Y2 and P2X5 receptors have opposite effects on HCMV yield**

308 To determine whether the upregulated expression of these purinergic receptors and  
309 ectonucleotidases is related to HCMV pathogenesis, we assessed the effects of inhibiting their  
310 expression or activity on the production of infectious progeny.

311 First, we employed siRNAs to knock down their expression. Specific siRNAs designed to  
312 target the P2Y2 (siP2Y2), P2X5 (siP2X5), ENPP4 (siENPP4) and ENTPD2 (siENTPD2)  
313 transcripts were used to inhibit the expression of these four genes. We verified that the siRNAs  
314 reduced expression of their targeted transcripts by using qRT-PCR (Fig. 4A). HFFs were  
315 transfected with the selected siRNAs for 24 h before HCMV infection at a multiplicity of 3  
316 TCID<sub>50</sub>/ml. At 120 hpi, the media was collected and viral titer assayed. Knockdown of the P2Y2  
317 (~15% of its normal level) or P2X5 receptor (~10% of its normal level) reduced the viral yield  
318 by ~95% or increased the yield by almost 400%, respectively (Fig. 4B), compared to viral titer of  
319 the media collected from scrambled siRNA (siSc)-treated cells. In contrast, siRNA-mediated  
320 knockdown of ENPP4 or ENTPD2 had minimal effects on the amount of infectious progeny  
321 released by the infected cells (Fig. 4B).

322 Seeing the significant effect of P2Y2 and P2X5 knockdown on HCMV yield, we focused  
323 further study on the roles of these two receptors. To investigate the kinetics of viral release  
324 during infection, HFFs were transfected with selected siRNAs for 24 h before HCMV infection  
325 at a multiplicity of 3 TCID<sub>50</sub>/cell. Differences in the amount of virus released from cells

326 transfected with siP2Y2 (reduced virus yield) or siP2X5 (increased virus yield) relative to  
327 control cells transfected with siSc were first observed at 72 hpi and gradually increased up to 120  
328 hpi (Fig. 4C).

329 We next employed pharmacological perturbations to confirm the roles of the receptors  
330 during HCMV infection. Kaempferol is a selective P2Y2 receptor antagonist [17, 53]. PPADS  
331 has ~10-fold higher affinity for blocking P2X5 than other P2X family members [23, 54]. We  
332 used kaempferol and PPADS at 50  $\mu$ M in our studies, because the drugs are commonly used to  
333 treat fibroblasts at concentrations of 20-100  $\mu$ M [55-58] and we detected little toxicity when  
334 uninfected fibroblasts were treated at doses ranging from 0-400  $\mu$ M (Fig. 5A, B). To test the  
335 effects of inhibiting P2Y2 and P2X5 receptor activity on the release of viral progeny, HFFs were  
336 pretreated with drug for 1 h prior to HCMV infection at a multiplicity of 3 TCID<sub>50</sub>/cell. After  
337 allowing 2 h for viral entry into cells, cells were washed and supplemented with media  
338 containing either kaempferol or PPADS. Media with drug was replaced every 24 h until media  
339 samples were collected at 96 hpi and assayed for infectious virus. Kaempferol decreased the viral  
340 yield by more than 99%, whereas PPADS nearly tripled the yield (Fig. 5C). Therefore, we  
341 conclude that elevated P2Y2 receptor activity normally facilitates infection whereas the P2X5  
342 receptor plays an antiviral role.

343

344 **Inhibiting P2Y2 and P2X5 purinergic receptors does not affect the efficiency of viral entry  
345 into fibroblasts.**

346 We next aimed to elucidate the point during infection at which the two purinergic  
347 receptors play a role. A possible explanation for the observed changes in viral yield upon  
348 inhibiting the activity or expression of P2Y2 and P2X5 is that the cell surface purinergic

349 receptors may function in the initial entry of the virus into fibroblasts. Although this notion did  
350 not fit well with the kinetics of receptor expression following HCMV infection (Fig. 2),  
351 purinergic signaling was shown to be involved in the entry step for HIV-1 infection [17, 59].  
352 Therefore, we tested this possibility using both pharmacological antagonists and siRNA-  
353 mediated expression knockdown. First, we assessed the efficiency of viral entry into cells that  
354 have been pre-treated with kaempferol or PPADS compared to solvent controls. Cells were  
355 treated with drugs and after 1 h, cells were washed and HCMV-infected at a multiplicity of 1  
356 TCID<sub>50</sub>/cell for 1 h at 4°C to allow virus attachment to the cell surface. Then, the viral particles  
357 that did not attach were removed and cells were incubated at 37°C, allowing the attached virus to  
358 penetrate the cells. After 24 h, the cells were fixed and immunostained for IE1 protein. Pre-  
359 treatment with either drug did not significantly alter the percentage of cells expressing IE1  
360 protein compared to solvent controls (Fig. 6A). To confirm these results, we assessed the  
361 efficiency of viral entry in cells transfected with siP2Y2, siP2X5 or siSc as a control. Cells were  
362 transfected with the appropriate siRNA for 24 h before HCMV infection at a multiplicity of 1  
363 TCID<sub>50</sub>/cell. After 1 h at 4°C, unattached virions were removed and cultures were shifted to  
364 37°C. One hour later, samples were collected and viral DNA copy number was quantified by  
365 qPCR. Again, results showed no significant differences in fold change of viral DNA copies  
366 between the siP2Y2 or siP2X5 transfected cells and the siSc controls (Fig. 6B). We conclude that  
367 inhibiting P2Y2 and P2X5 activity or expression does not affect viral entry into HFFs.  
368

### 369 **Inhibiting P2Y2 reduces the accumulation of viral transcripts**

370 To probe when and how the cellular P2Y2 receptor might affect viral gene expression  
371 subsequent to virus entry, we analyzed viral gene expression in siSc- and siP2Y2-treated cells at

372 various times during the viral replication cycle. qRT-PCR was used to quantify transcript levels  
373 for representatives of the three main classes of viral genes. The data show that infection in cells  
374 transfected with siP2Y2 resulted in a reduction of immediate early (UL123, UL122), early  
375 (UL26, UL54), and late (UL69, UL82, UL99) viral transcripts compared to siSc controls (Fig.  
376 7A-B, D-H). Viral RNAs were most dependent on P2Y2 during the late phase of infection,  
377 consistent with the timing of P2Y2 RNA accumulation (Fig. 2). Intriguingly, one viral transcript,  
378 immediate early UL37x1, was expressed at higher levels in cells transfected with siP2Y2 than in  
379 control cells (Fig. 7C). UL37x1 has been implicated in the mobilization of intracellular  $\text{Ca}^{2+}$   
380 during infection [28, 30].

381

382 **P2Y2 receptor affects the efficiency of viral DNA synthesis**

383 One of the strongest inhibitory effects of P2Y2 knockdown was seen on the accumulation  
384 of RNA coding for the viral DNA polymerase, UL54 (Fig. 7E). Given its direct role in viral  
385 DNA replication [60], we tested whether P2Y2 could have an impact on viral DNA (vDNA)  
386 accumulation. Total DNA was isolated from HCMV-infected control and P2Y2-deficient cells  
387 and vDNA copy numbers were measured by qPCR using primers specific to UL123. There were  
388 about 5-fold fewer copies of vDNA in P2Y2-deficient cells than in control cells (Fig. 8A). This  
389 supports the hypothesis that P2Y2 inhibition prevents viral DNA accumulation by blocking viral  
390 DNA synthesis. To confirm this result, we also compared the vDNA copy numbers in released  
391 viral particles between siP2Y2-treated cells and siSc-treated controls. Similarly, when viral  
392 genomic DNA was isolated from viral particles and quantified, there were also nearly 5 times  
393 fewer copies of vDNA released from P2Y2-deficient cells compared to control cells (Fig. 8B).  
394 To confirm that lower viral DNA synthesis is the predominant explanation for the decrease in

395 infectious particles found in the media collected from P2Y2-deficient cells (Fig. 4B, C and 5C),  
396 we decided to test the alternative hypothesis that progeny released from P2Y2 knockdown cells  
397 are less infectious by investigating if there are any effects of P2Y2 inhibition on the infectivity of  
398 virions. Accordingly, the ratios of vDNA copy number to infectious unit were calculated, and  
399 there was no significant difference between the siP2Y2-treated cells and siSc controls (Fig. 8C).  
400 Therefore, the reduction in HCMV yield observed in the P2Y2 knockdown condition is caused  
401 by reduced viral DNA synthesis and release of viral particles, and not by reduced infectivity of  
402 the virions released from P2Y2-deficient cells. Taken together, it appears that P2Y2 is critical for  
403 viral DNA synthesis. Inhibiting the P2Y2 receptor reduces viral DNA synthesis and causes a  
404 drastic reduction in viral titer.

405

#### 406 **P2Y2 and UL37x1 cooperate to regulate intracellular Ca<sup>2+</sup> during infection**

407 One of the most striking findings in our analysis of the role of purinergic receptors on  
408 viral gene expression during HCMV infection is the fact that of all the viral genes studied, only  
409 one was upregulated in the siP2Y2-treated cells compared to siSc-treated control cells (Fig. 7C).  
410 This was the transcript of the viral immediate early UL37x1 gene. Previously, UL37x1 has been  
411 shown to be involved in the release of Ca<sup>2+</sup> from SER stores to increase cytosolic Ca<sup>2+</sup>  
412 concentrations during infection [28, 30]. Interestingly, P2Y2 is a G-protein coupled receptor that,  
413 when activated, leads to the downstream hydrolysis of PIP<sub>2</sub> into IP<sub>3</sub>, which is also known to  
414 cause the release of Ca<sup>2+</sup> ions from SER stores [11, 61]. Because of the potential involvement of  
415 P2Y2 in Ca<sup>2+</sup> homeostasis following infection, we investigated the effects of inhibiting P2Y2  
416 expression on intracellular Ca<sup>2+</sup> levels. To do so, fibroblasts were infected with either wild-type  
417 AD<sup>wt</sup> virus or AD<sup>subUL37x1</sup> virus lacking the UL37x1 gene at a multiplicity of 1 TCID<sub>50</sub>/cell

418 or mock infected, and cultures were treated 2 h later with kaempferol or solvent control. Using a  
419 multiplicity of 1 TCID<sub>50</sub>/cell allowed us also to measure Ca<sup>2+</sup> levels in infected and uninfected  
420 cells in the same experimental well. At 20 hpi, we used the fluorescence-based fluo-4 AM  
421 calcium assay to compare the changes in intracellular Ca<sup>2+</sup> concentrations. We determined that  
422 wild type virus caused ~2.5-fold increase in Ca<sup>2+</sup> levels compared to uninfected cells (Fig. 9A).  
423 We postulated that if P2Y2 contributes to elevated levels of Ca<sup>2+</sup> in infected cells, then we should  
424 see a partial decrease of Ca<sup>2+</sup> levels in cells either infected with AD<sup>wt</sup> and treated with  
425 kaempferol or infected with AD<sup>subUL37x1</sup>. Instead, we saw that in both instances, Ca<sup>2+</sup> was  
426 reduced almost to the level found in uninfected cells (Fig. 9A). Interestingly, Ca<sup>2+</sup> levels did not  
427 decrease further in cells infected with AD<sup>subUL37x1</sup> and treated with kaempferol. Therefore,  
428 these data imply that P2Y2 and pUL37x1 work together to elevate Ca<sup>2+</sup> levels during HCMV  
429 infection and that the lack of either component prevents cells from increasing intracellular  
430 calcium levels during infection.

431 To further test this hypothesis, we investigated the effect of UL37x1 expression on Ca<sup>2+</sup>  
432 homeostasis in HFF cells without the background of infection. For this purpose, a UL37x1-  
433 expressing lentiviral pLVX-EF1 plasmid was created. HFF cells were transduced with the vector,  
434 and subjected to puromycin selection. Two cellular clones were obtained [pLVX-UL37x1(1) and  
435 pLVX-UL37x1(2)] that exhibited stable expression of pUL37x1. The cells were treated with  
436 kaempferol or solvent control for 1 h. Then, Ca<sup>2+</sup> levels were measured using the fluo-4 AM  
437 assay and compared to levels in wild type HFF cells. No significant changes in Ca<sup>2+</sup> levels were  
438 observed between samples (Fig. 9B), which supports our conclusion that increased expression of  
439 pUL37x1 in the absence of P2Y2 overexpression is not sufficient to stimulate the robust increase  
440 in calcium seen in HCMV-infected cells.

441 As a control, cells transduced with pLVX-UL37x1 or pLVX-GFP plasmids were  
442 analyzed by Western blot assay for the level of pUL37x1 expression. The assay confirmed the  
443 pUL37x1 overexpression only in pLVX-UL37x1 plasmid-transduced HFF cells (Fig. 9C).  
444 Because inhibiting the P2Y2 receptor activity decreases the extent to which intracellular  $\text{Ca}^{2+}$  is  
445 mobilized during infection, we propose that P2Y2 signaling plays a critical role in controlling  
446 intracellular  $\text{Ca}^{2+}$  during HCMV infection.

447 Since previous studies as well as our current study have shown that the viral pUL37x1  
448 protein is involved in the mobilization of intracellular  $\text{Ca}^{2+}$  from calcium stores in the SER and  
449 knowing that P2Y2 expression was upregulated early in infection, we aimed to further elucidate  
450 the relationship between the viral UL37x1 and cellular P2Y2. Specifically, we assessed whether  
451 the upregulation of P2Y2 expression during viral infection may be a downstream effect of the  
452 immediate early kinetics of UL37x1 expression [62]. Since the upregulation of P2Y2 transcript is  
453 most evident around 48hpi, it is conceivable that the viral pUL37x1 protein increases the  
454 expression of P2Y2 and that increased P2Y2 activity further increases intracellular  $\text{Ca}^{2+}$  levels  
455 for the remainder of the infection. In order to test this idea, HFFs were infected with either the  
456 AD<sub>sub</sub>UL37x1 virus or the wild type AD<sub>wt</sub> virus. Samples were collected at 24 hpi. P2Y2,  
457 UL54, UL123 and UL37x1 transcript levels were quantified by qRT-PCR. If the hypothesis that  
458 P2Y2 upregulation is dependent on pUL37x1 expression were true, then P2Y2 transcripts would  
459 be significantly lower in cells infected with AD<sub>sub</sub>UL37x1 than AD<sub>wt</sub> virus. However, although  
460 P2Y2 expression was appeared somewhat lower in cells infected with AD<sub>sub</sub>UL37x1 compared  
461 to those infected with AD<sub>wt</sub>, the difference was not significant (Fig. 10A). Interestingly, it was  
462 also noted that unlike siP2Y2-treated cells, AD<sub>sub</sub>UL37x1-infected cells did not exhibit lower  
463 UL54 RNA levels compared to AD<sub>wt</sub>-infected cells (Fig. 10B). The same expression levels of

464 UL123 in cells infected with either AD<sup>wt</sup> or AD<sup>subUL37x1</sup> confirm that the cells received an  
465 equal amount of virus (Fig. 10C). As expected, UL37x1 expression was only evident in AD<sup>wt</sup>-  
466 and not in AD<sup>subUL37x1</sup>-infected cells (Fig. 10D). Both clonal populations of cells expressing  
467 pUL37x1 were characterized by a robust expression of pUL37x1, but there was only a minimally  
468 higher level of P2Y2 protein in these cells compared to controls expressing GFP (Fig. 9C).  
469 Therefore, these data suggest that viral pUL37x1 is likely not a significant upstream activator of  
470 P2Y2 expression.

471 Taken together, these findings suggest that both cellular P2Y2 and viral pUL37x1  
472 cooperate to modulate intracellular Ca<sup>2+</sup> within HCMV-infected cells.

473

474

## Discussion

475 The purpose of this study was to investigate the role of purinergic signaling during  
476 HCMV infection in human fibroblasts. RNA-Seq analysis identified several members of the  
477 purinergic receptor network whose expression was modulated upon infection (Table 2). qRT-  
478 PCR analysis confirmed that infection increased the cellular P2Y2, P2X5, ENPP4 and ENTPD2  
479 transcripts. Elevated expression of purinergic receptors during HCMV infection has been  
480 reported previously. In myeloid cells, HCMV infection increases the expression of the P2Y5  
481 receptor [63]. In endothelial cells, HCMV infection has been associated with higher levels of the  
482 P2Y2, P2Y1, and P2X7 receptors, as well as ecto-5' nucleotidase CD73 [25, 26]. Yet, the impact  
483 of these altered expression patterns on the efficiency of HCMV infection has not been  
484 investigated. We now report that siRNA targeting ENPP4 and ENTPD2 transcripts did not affect  
485 viral yield (Fig. 4B). In contrast, inhibiting the activity or expression of P2Y2 and P2X5  
486 receptors had a robust effect on the amount of infectious progeny released from infected cells

487 (Fig. 4B, C). These findings suggest that, at least in fibroblasts, purinergic signaling through the  
488 P2Y2 and P2X5 receptors is important in HCMV infection independent from the activity of  
489 ectonucleotidases.

490 Inhibiting P2Y2 and P2X5 by using pharmacological compounds or siRNA technology  
491 drastically altered HCMV yield. It is noteworthy that, even though both receptors are activated  
492 by ATP, they had completely opposite effects on the production of HCMV progeny. Compared  
493 to control treated cells, infection in P2Y2-deficient cells led to a drastic reduction (~95% lower)  
494 in the number of infectious virions released (Fig. 4B, C), which demonstrated that P2Y2 supports  
495 HCMV replication. On the other hand, infection in P2X5-deficient cells resulted in enhanced (4x  
496 higher) viral release compared to control treated cells (Fig. 4B, C), suggesting that P2X5  
497 antagonizes HCMV replication.

498 Our analysis of P2Y2 and P2X5 expression kinetics supported the view that these  
499 receptors are independently regulated and have different functions during HCMV infection.  
500 While active viral gene expression is necessary for the upregulation of P2Y2 expression (Fig.  
501 3A), it is not for the increased expression of P2X5 (Fig. 3B). Moreover, P2Y2 gradually  
502 increased in expression from 2 to 96 hpi, as seen at both RNA and protein levels (Fig. 2A, C),  
503 P2X5 expression increased up to 48 hpi (RNA) or 72 hpi (protein) and then decreased  
504 immediately afterwards (Fig. 2B, C). The gradual increase in P2Y2 expression is consistent with  
505 a supportive role for the receptor throughout the viral replication cycle. Conversely, the initial  
506 spike in the levels of the inhibitory P2X5 receptor might be antagonized by a virus-mediated  
507 mechanism that suppresses its expression later after infection.

508 How do the P2Y2 and P2X5 receptors modulate HCMV replication? The P2X5  
509 purinergic receptor is a non-selective cation channel that is gated by extracellular ATP. It is

510 typically found on cells of the skin, gut, bladder, thymus, skeletal muscle, and spinal cord and is  
511 thought to play important roles in cell differentiation [64]. For instance, it has been shown to be  
512 involved in the differentiation of skin keratinocytes and mucosal epithelial cells during turnover  
513 in the gut and bladder [19, 65]. P2X5 receptors have also been observed to play various roles in  
514 regulating osteoblastic differentiation and proliferation, triggering differentiation of skeletal  
515 muscle satellite cells, and the differentiation of human fetal epidermis [66, 67]. Although the  
516 expression of P2X5 receptors have been associated with differentiating and proliferating cell  
517 types, the exact mechanism by which its signaling affects these cellular processes is still unclear.  
518 Thus it is difficult to speculate on how a block to its activity might influence HCMV replication.

519 P2Y2 is a G-protein coupled receptor that can be activated by extracellular ATP and  
520 UTP. HCMV infection of P2Y2 knockdown cells exhibited reduced expression of most viral  
521 genes tested, including UL54, the viral DNA polymerase catalytic subunit (Fig. 7E). Reduced  
522 pUL54 levels would be expected to reduce key downstream events in the viral replication cycle.  
523 Indeed, P2Y2-deficient cells exhibited reduced accumulation of viral DNA (Fig. 8A) and late  
524 viral RNAs (Fig. 7F-H). Although it is likely that the receptor has additional effects on HCMV  
525 replication, its effect on viral DNA accumulation, followed by inhibition of late RNAs that  
526 depend on active DNA replication for their synthesis, can explain the low viral yield from P2Y2-  
527 deficient cells.

528 There are several plausible mechanisms by which purinergic signaling via the P2Y2  
529 receptor can affect viral DNA replication. It could affect DNA replication indirectly, through  
530 inhibition of an upstream viral function that we have not yet measured, or it could act directly at  
531 DNA replication. One potential mechanism is through p38 mitogen-activated protein kinases  
532 (MAPKs). Extracellular ATP and UTP can activate the MAPK kinase (MKK) 3/6-p38-MAPK

533 cascade via the P2Y2 receptor in glomerular mesangial cells [68]. p38 activation results from  
534 increased activity of MKK3/6, which are downstream effectors of P2Y2 signaling [69]. It has  
535 been reported that active p38 plays a critical role in HCMV viral DNA replication in infected  
536 human fibroblasts [69]. Thus, inhibiting P2Y2 could decrease MKK3/6 activity, which might  
537 then reduce p38 activation and viral DNA replication. This role for P2Y2 action within infected  
538 cells remains speculative, because the exact mechanism by which p38 regulates viral DNA  
539 replication via the UL54 polymerase has not yet been characterized.

540 In addition to its impact on p38 MAPK, the P2Y2 receptor has a well-established role in  
541 regulating cellular calcium levels [15, 16, 23]. Extracellular nucleotides act via the P2Y2  
542 receptor to induce intracellular  $\text{Ca}^{2+}$  mobilization from SER in a variety of cell types [70-72].  
543 The increase in intracellular  $\text{Ca}^{2+}$  can be blocked by PLC inhibitors and by low molecular weight  
544 heparin, indicating the involvement of IP<sub>3</sub>-sensitive intracellular  $\text{Ca}^{2+}$  stores, which is known to  
545 be downstream of P2Y2-mediated signaling [73]. As calcium homeostasis was found to be  
546 critical for an efficient HCMV infection [27, 28], it was intriguing to speculate that P2Y2  
547 inhibition may have important consequences for HCMV replication due to disruption of  
548 intracellular calcium regulation. Treating infected cells with drugs that disrupt SER  $\text{Ca}^{2+}$   
549 homeostasis inhibits the production of infectious progeny due to retarded accumulation of late  
550 gene products [27], patterns that were observed during HCMV infection in P2Y2 knockdown  
551 cells.

552 The first indication that P2Y2-dependent  $\text{Ca}^{2+}$  regulation may be important during  
553 HCMV infection came from analyzing differential expressions of viral genes in wild type cells  
554 and cells treated with siP2Y2. Among all tested viral transcripts only UL37x1 RNA levels were  
555 upregulated in P2Y2-deficient cells (Fig. 7C). It is known that the HCMV immediate early

556 protein pUL37x1 induces the mobilization of  $\text{Ca}^{2+}$  from the SER to the cytosol [28]. In addition,  
557 the  $\text{Ca}^{2+}$ -dependent protein kinase,  $\text{PKC}\alpha$ , is activated following infection, leading to the  
558 production of large (1-5  $\mu\text{m}$  diameter) cytoplasmic vesicles late after infection [30]. The  
559 presence of these vesicles correlated with the efficient accumulation of enveloped virions.  
560 Regulating intracellular  $\text{Ca}^{2+}$  levels may also influence viral DNA replication.  $\text{Ca}^{2+}$  can induce  
561 the activity of  $\text{Ca}^{2+}$ /calmodulin-dependent protein kinase kinase (CaMKK), which activates  
562 AMPK. AMPK activity has been shown to be necessary for HCMV DNA synthesis and the  
563 expression of viral late genes [33, 34]. In addition, it has been found that extracellular  
564 nucleotides stimulate the PI3-K/Akt pathway through P2Y2-mediated signaling involving  $\text{Ca}^{2+}$   
565 influx, CaM, and Src [74]. It was previously reported that treating infected fibroblasts with an  
566 inhibitor of PI3-K activity caused inhibition of viral DNA replication and a 4-log decrease in  
567 viral titers [36]. Although intriguing, it is more likely that P2Y2-mediated inhibition of viral  
568 UL54 is independent from its effects on intracellular  $\text{Ca}^{2+}$ . Cells infected with AD<sub>sub</sub>UL37x1,  
569 which lacks the UL37x1 gene, show reduced intracellular  $\text{Ca}^{2+}$  levels (Fig. 9A), but,  
570 nevertheless, do not show reduced UL54 RNA levels (Fig. 10B). Hence the effect P2Y2 on viral  
571 DNA accumulation may be distinct from its effects on intracellular  $\text{Ca}^{2+}$  concentrations.

572 To broaden our understanding on a potential role of P2Y2 in  $\text{Ca}^{2+}$  levels and the  
573 relationship between P2Y2 and UL37x1 during infection, we tested the effect of kaempferol, a  
574 P2Y2 antagonist, on intracellular  $\text{Ca}^{2+}$  in cells infected with wild-type HCMV (AD<sub>wt</sub>) versus the  
575 pUL37x1-deficient mutant virus (AD<sub>sub</sub>UL37x1). As shown previously [28], AD<sub>wt</sub>-infected  
576 cells exhibited increased free  $\text{Ca}^{2+}$  compared to uninfected cells, whereas AD<sub>sub</sub>UL37x1-  
577 infected cells had  $\text{Ca}^{2+}$  levels similar to those in uninfected cells (Fig. 9A). Interestingly,  
578 kaempferol treatment also lowered  $\text{Ca}^{2+}$  levels in AD<sub>wt</sub>-infected cells to its level in uninfected

579 cells and similar Ca<sup>2+</sup> levels were measured in kaempferol-treated, AD*sub*UL37x1-infected cells  
580 (Fig. 9A). Additionally, when we tested Ca<sup>2+</sup> levels in uninfected cells that overexpress UL37x1,  
581 no elevated levels of Ca<sup>2+</sup> were observed (Fig. 9B). While P2Y2-deficient cells were  
582 characterized by increased expression of UL37x1 (Fig. 7C), neither AD*sub*UL37x1-infected  
583 cells nor cells overexpressing UL37x1 affected P2Y2 RNA or protein levels (Fig.10A, 9C).  
584 Taken together, these results strongly suggest that P2Y2 has a critical role in regulating Ca<sup>2+</sup>  
585 levels following HCMV infection. Both P2Y2 and pUL37x1 are indispensable for maintaining  
586 favorable intracellular Ca<sup>2+</sup> levels during infection. It is not yet clear how the viral and cellular  
587 gene products cooperate in this process.

588 Aside from disrupting viral DNA replication and Ca<sup>2+</sup> homeostasis, a third mechanism by  
589 which P2Y2 activation may play a role in HCMV infection is through affecting the nuclear  
590 egress of the viral capsid. It has been reported that PKC, a downstream effector of P2Y2  
591 signaling, plays an important role in destabilizing the nuclear lamina by phosphorylating several  
592 types of nuclear lamin [75]. The phosphorylation and reorganization of the nuclear lamins is an  
593 essential part of viral nuclear egress [75]. It is conceivable, then, that a block to nuclear egress of  
594 HCMV capsids could contribute to the reduction in infectious virus produced following  
595 inhibition of P2Y2 (Fig. 4, 5).

596 It has been reported that during hypoxic conditions, fibroblasts release ATP, which  
597 activates P2Y2 to regulate cellular DNA synthesis via extracellular signal-regulated kinases 1  
598 and 2 (ERK<sub>1/2</sub>) induced Egr-1 activation [14]. Additionally, it has been found that in human  
599 cardiac fibroblasts, ATP-mediated activation of the P2Y2 receptor leads to cell cycle progression  
600 and cell proliferation [76]. Moreover, P2Y2 activation results in the activation of PKC, which  
601 acts in concert with ERK and PI3-K/PKC pathways to induce c-Fos protein expression and HeLa

602 cell proliferation [77]. Since P2Y2 is dramatically overexpressed in HCMV-infected cells, it  
603 would be interesting to understand how the expected upregulation of cellular DNA synthesis and  
604 cell cycle progression is avoided in HCMV-infected cells in order to maintain the cell cycle  
605 arrest characteristic of infection [78, 79].

606 Signaling via the P2Y2 receptor also may influence cell migration. P2Y2 activation  
607 increases MCF-7 breast cancer cell migration via the MEK-ERK<sub>1/2</sub> signaling pathway [80].  
608 Specifically, extracellular ATP can activate MAPKs through the P2Y2/PLC/PKC/ERK signaling  
609 pathway to induce the translocation of ERK<sub>1/2</sub> into the nucleus [13]. Also, fibroblasts appear to  
610 require PKC activation, which is downstream of P2Y2 signaling, in order to respond to  
611 hyaluronan stimulation with increased locomotion [81]. Taken together, it is conceivable that the  
612 overexpression of P2Y2 receptors during HCMV infection may lead to increased migration of  
613 the host cell. This not only has potential to facilitate dissemination of the virus within its infected  
614 host, it may also contribute to the likely oncomodulatory activity of HCMV [82].

615 In summary, our results show that purinergic signaling through the P2Y2 and P2X5  
616 receptors plays a critical role within infected cells and set the stage for additional investigations  
617 on the impact of this receptor family in HCMV biology. Further, our observation that a  
618 pharmacological block to P2Y2 dramatically reduces viral yield raises the possibility that P2Y2  
619 antagonists, if well tolerated, could prove to be attractive candidates for new HCMV therapies.

620

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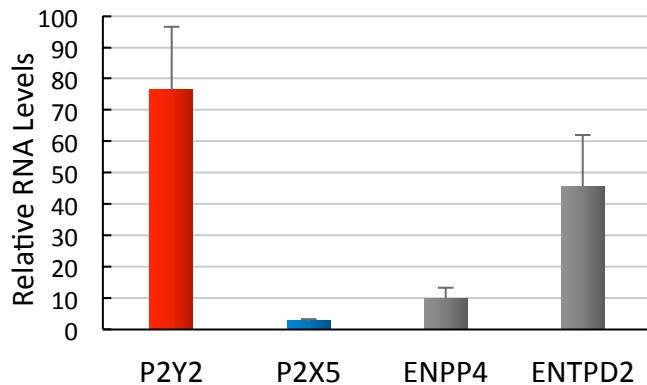
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<b>Gene</b>	<b>Forward Primer (5'→3')</b>	<b>Reverse Primer (5'→3')</b>
<b>UL123</b>	TGC TGT GCT GCT ATG TCT TAG AGG	TTG GTT ATC AGA GGC CGC TTGG
<b>UL122</b>	TGA CCG AGG ATT GCA ACGA	CGG CAT GAT TGA CAG CCTG
<b>UL37x1</b>	TCC CGC CTT GGT TAA GA	CGA GTT CTC ACC GTC AAT TA
<b>UL26</b>	CCA GCA GCT TCC AGT ATT C	ACC TGG ATC TGC CCT ATC
<b>UL54</b>	TGC TTT CGT CGG TGC TCT CTA AG	TGT GCG GCA GGT TAG ATT GACG
<b>UL69</b>	ACG AGT GTC AGA ACG AGA TGT GC	TGA AAC GAT AGG GTG CCA ACG C
<b>UL82</b>	AGA CGT CGA AGC GGT AAC AAC G	AGT CGT CAA GGC TCG CAA AGA C
<b>UL99</b>	ACG ACA ACA TCC CTC CGA CTTC	TCT GTT GCC GCT CCT CGT TATC
<b>P2Y2</b>	CCA CCT GCC TTC TCA CTA GC	TGG GAA ATC TCA AGG ACT GG
<b>P2X5</b>	CCT GCG GAG AGA GAA CTT GG	AGT TGA ATT TGG GGA AAC GGA TG
<b>ENPP4</b>	TTT GGT TGC CGA TGA AGG CT	CGT GGG CAG CTA GAA ATG GA
<b>ENPTD2</b>	TCA ATC CAG CTC CTT GAA CC	TCC CCA GTA CAG ACC CAG AC
<b>GAPDH</b>	CAA GAG CAC AAG AGG AAG AGA G	CTA CAT GGC AAC TGT GAG GAG

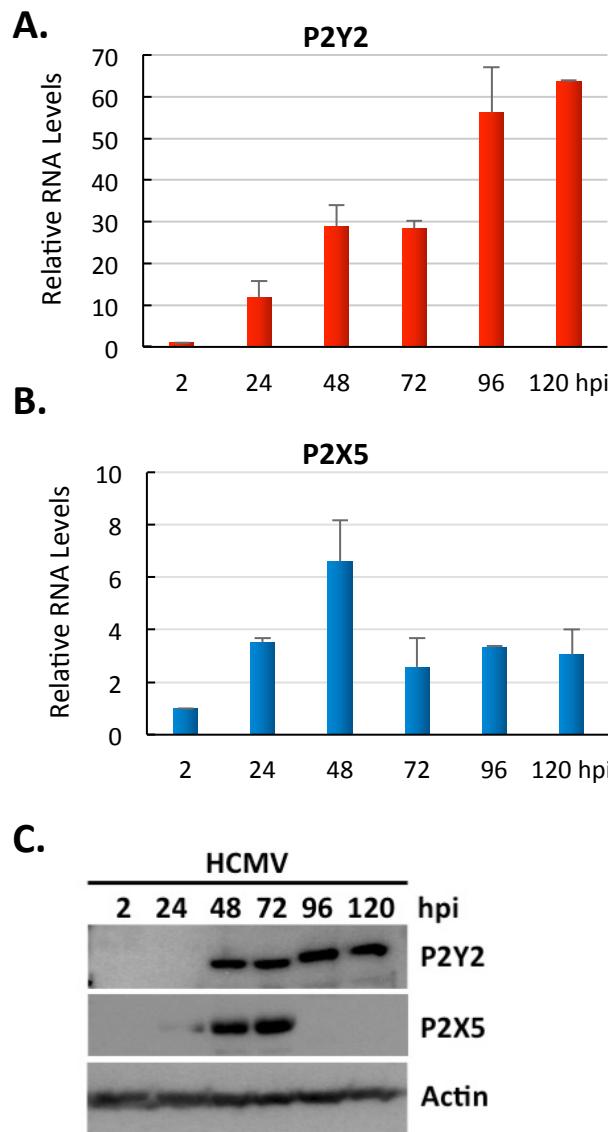
**TABLE 1.** Primers used in qPCR assays.

<b>Gene</b>	<b>Description</b>	<b>Log<sub>2</sub> (FC)</b>
<b>P2Y2</b>	Purinergic receptor, P2Y subtype 2	<b>7.49</b>
<b>ENPP4</b>	Ectonucleotide pyrophosphatase/phosphodiesterase 4	<b>4.20</b>
<b>P2X5</b>	Purinergic receptor, P2X subtype 5	<b>3.21</b>
<b>ENTPD2</b>	Ectonucleoside triphosphate diphosphohydrolase 2	<b>3.18</b>
<b>P2X1</b>	Purinergic receptor, P2X subtype 1	<b>2.78</b>
<b>ENTPD8</b>	Ectonucleoside triphosphate diphosphohydrolase 8	<b>2.65</b>
<b>PANX2</b>	Pannexin channel 2	<b>2.28</b>
<b>ENPP5</b>	Ectonucleotide pyrophosphatase/phosphodiesterase 5	<b>1.41</b>
<b>ENPP6</b>	Ectonucleotide pyrophosphatase/phosphodiesterase 6	<b>-3.66</b>
<b>ENPP7P10</b>	Ectonucleotide pyrophosphatase/phosphodiesterase 7 pseudogene 10	<b>-3.58</b>
<b>P2X6</b>	Purinergic receptor, P2X subtype 6	<b>-2.76</b>
<b>ENPP7P4</b>	Ectonucleotide pyrophosphatase/phosphodiesterase 7 pseudogene 4	<b>-2.17</b>
<b>P2X7</b>	Purinergic receptor, P2X subtype 7	<b>-1.97</b>
<b>NT5E</b>	Ecto-5'nucleotidase (CD73)	<b>-1.68</b>
<b>ENPP7P12</b>	Ectonucleotide pyrophosphatase/phosphodiesterase 7 pseudogene 12	<b>-1.55</b>

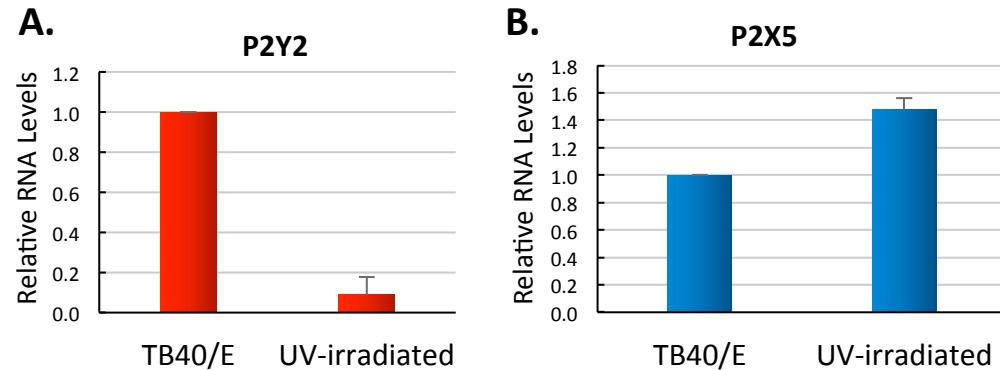
**TABLE 2.** Differential expression of members of the purinergic receptor network in HCMV-infected MRC-5 fibroblasts at 48 hpi compared to mock-infected controls. Log<sub>2</sub> fold change (FC) was calculated from sequence reads determined from triplicate biological samples.



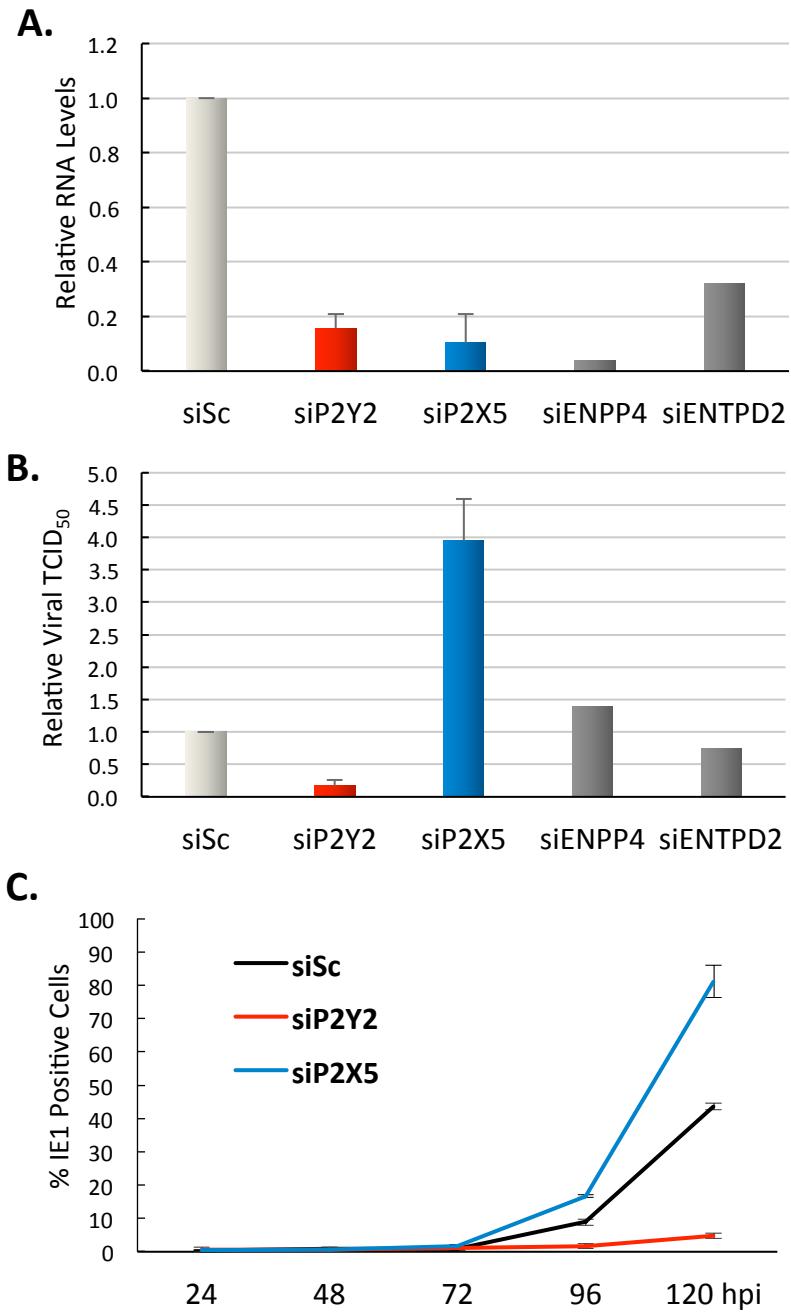
**FIG 1. HCMV infection increases the transcript levels of P2Y2, P2X5, ENPP4, and ENTPD2 at 48 hpi.** HFFs were infected with TB40/E-GFP virus (MOI=3) or mock infected for 2 h. Samples were collected at 48 hpi, transcripts were quantified by qRT-PCR and GAPDH was used as an internal control. Data are represented as fold change compared to mock-infected cells. Results were averaged between two biological replicates and error bars represent standard error of the mean (SEM).



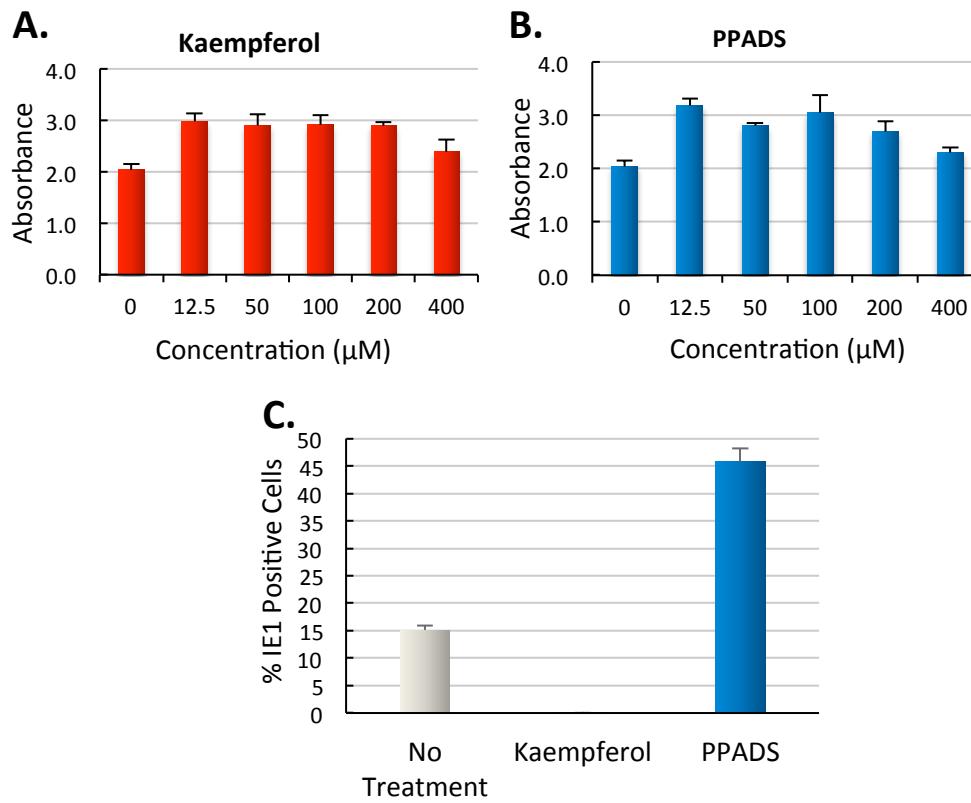
**FIG 2. Kinetics of P2Y2 and P2X5 expression in HCMV-infected HFFs. (A, B) P2Y2 and P2X5 RNA levels are modulated by infection.** HFFs were infected with TB40/E-GFP (MOI=3) or mock infected. RNA and protein samples were collected at various times after infection, transcripts were quantified by qRT-PCR and GAPDH was used as an internal control. Results are shown as fold change compared to mock-infected cells. Data were averaged for two biological replicates and error bars represent SEM. **(C) P2Y2 and P2X5 proteins are modulated by infection.** Proteins were subjected to Western blot analyses using antibodies specific for P2Y2 and P2X5. Actin was monitored as a loading control.



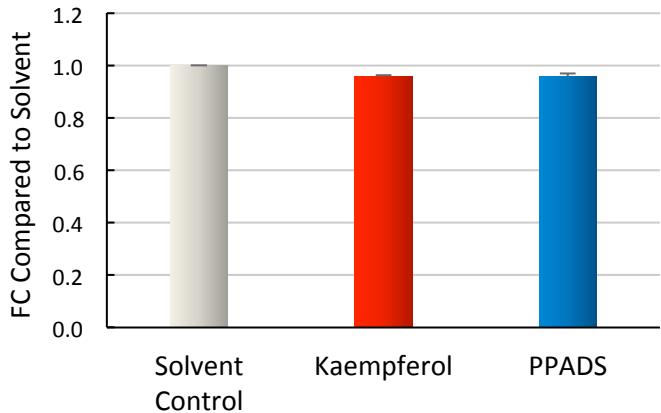
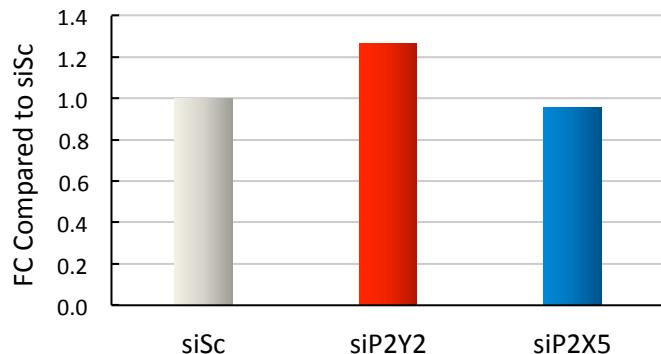
**FIG 3. UV-irradiated HCMV failed to increase P2Y2 but did increase P2X5 RNA levels.** Untreated or UV-irradiated TB40/E virus was applied to HFFs (MOI=3). Samples were collected at 48 hpi (P2Y2) or 24 hpi (P2X5), and transcripts were quantified by qRT-PCR. GAPDH was assayed as an internal control. Results are shown as fold change compared to untreated control virus condition. Data were averaged between two biological replicates and error bars represent SEM.



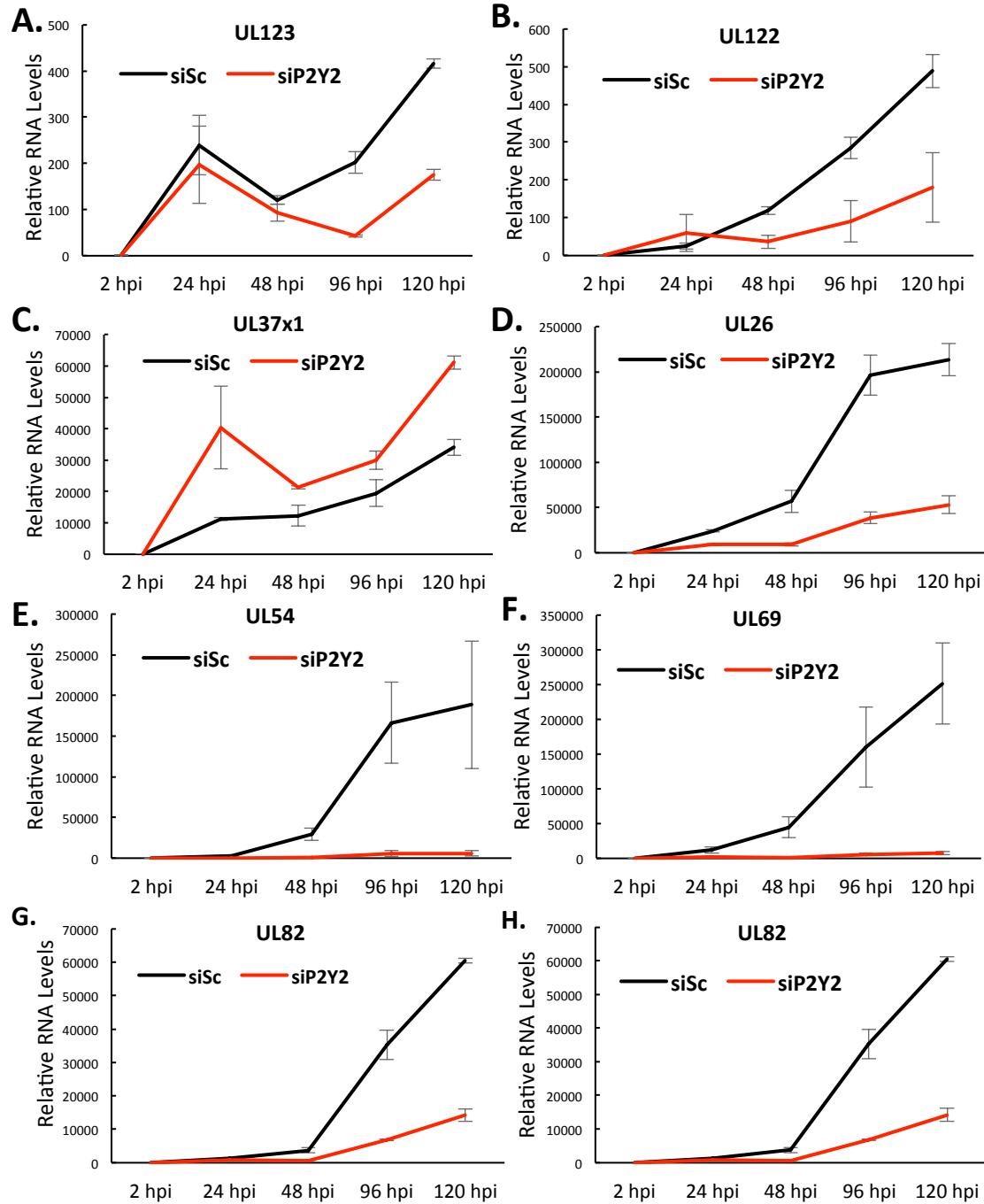
**FIG 4. siRNA-mediated knockdown of P2Y2 or P2X5 affects HCMV yield.** HFFs were transfected with siP2Y2, siP2X5, siENPP4, siENTPD2, or scrambled siRNA (siSc) as a control. After 24 h, cells were infected with TB40/E-GFP virus (MOI=3). **(A) siRNA validation.** Samples were collected at 48 hpi and qRT-PCR was used to measure levels of P2Y2, P2X5, ENPP4 and ENTPD2 transcripts. GAPDH was used as an internal control. The data are represented as fold change compared to siSc control. The mean fold change for three biological replicates are shown and error bars represent SEM. **(B) Effect of siRNA-mediated knockdowns on virus yield at 120 hpi.** TCID<sub>50</sub>/ml values were determined and fold change was calculated relative to siSc control. Results show the average fold change from two biological replicates and error bars represent SEM. **(C) Virus growth kinetics following siRNA-mediated knockdown.** Samples were collected after various time intervals. Viral titers were determined by applying the infectious media to a reporter plate of HFFs and immunostaining for IE1 protein 24 h later. The data are presented as percent of IE1-positive cells averaged from two biological replicates and error bars represent SEM.



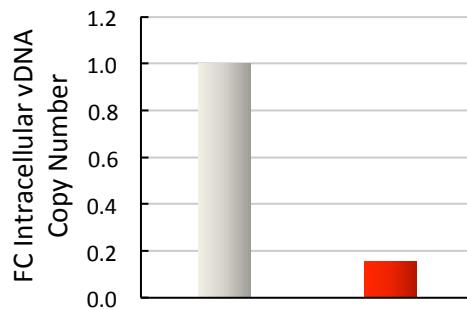
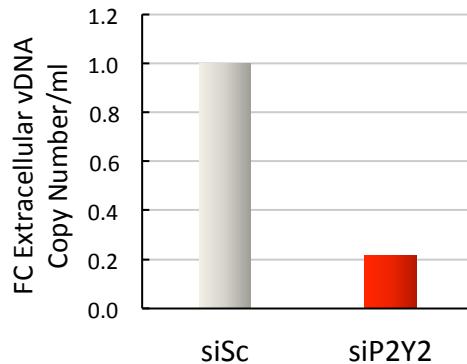
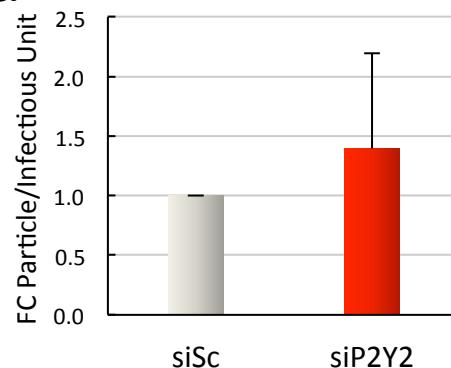
**FIG 5. Kaempferol and PPADS have opposite effects on HCMV yield. (A, B) Drug toxicity assays.** HFFs were treated with various concentrations of (A) kaempferol and (B) PPADS. The media with drugs was replenished every 24 h. At 96 h, cell viability was measured using the CellTiter 96<sup>®</sup> AQ<sub>ueous</sub> Assay. Results are shown as average absorbance from two biological replicates and error bars represent SEM. **(C) Drugs modulate the yield of HCMV.** HFFs were infected with TB40/E-GFP virus (MOI=3) or mock infected for 2 h and treated with either kaempferol (50  $\mu\text{M}$ ) or PPADS (50  $\mu\text{M}$ ). The drugs were replaced every 24 h until media samples were collected at 96 hpi. Viral yield was determined by monitoring expression of IE1 protein at 24 h after infection. Results were averaged between two biological replicates and error bars represent SEM.

**A.****B.**

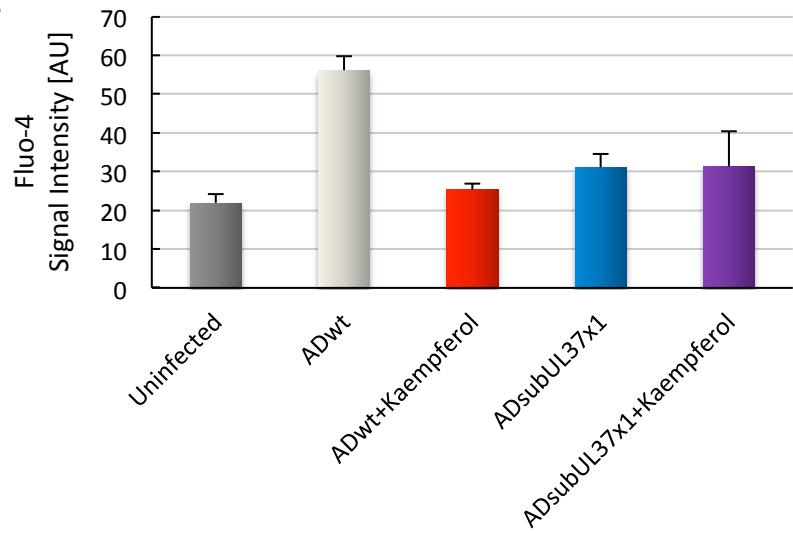
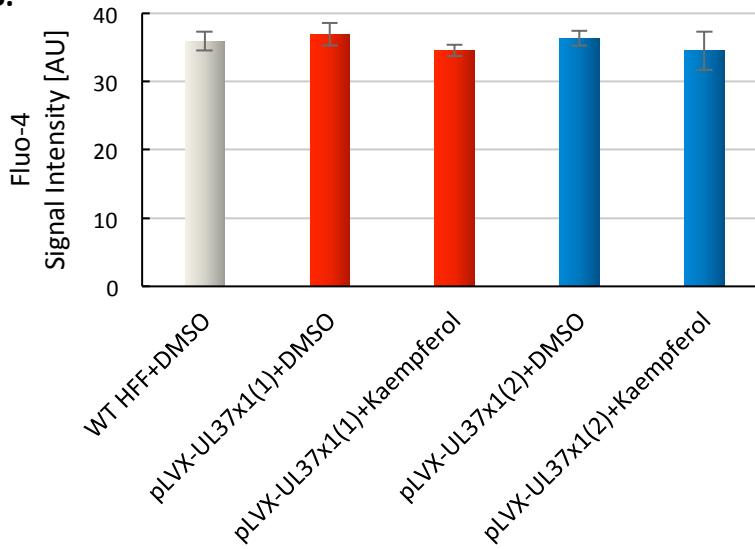
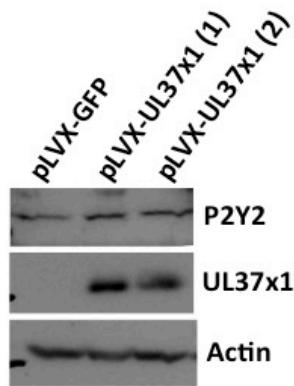
**FIG 6. P2Y2 and P2X5 receptors do not affect HCMV entry into HFFs.**  
HFFs were either (A) pre-treated with either kaempferol (50  $\mu$ M) or PPADS (50  $\mu$ M), or solvent control for 1 h, or (B) transfected with siP2Y2, siP2X5, or siSc as a control for 24 h. Then, they were infected with TB40/E-GFP virus (MOI=1) or mock-infected for 1 h at 4°C. Cells were washed with citrate buffer and incubated at 37°C. Viral entry was assayed by either (A) immunostaining for IE1 protein at 24 hpi or (B) qPCR quantification of intracellular viral DNA at 1 hpi. Results are reported as fold change (FC) compared to the solvent control or siSc control conditions. Data for two technical replicates were averaged and error bars represent SEM.



**FIG 7. P2Y2 receptor regulates levels of viral gene expression.** HFFs were transfected with siP2Y2 or siSc as a control. After 24 h, cells were infected with TB40/E-GFP virus (MOI=3) or mock infected, and RNA samples were collected after various time intervals. Viral transcript levels were determined by qRT-PCR with GAPDH serving as an internal control. The data are shown as fold change compared to transcript levels at 2 hpi, calculated from two technical replicates. Error bars represent SEM.

**A.****B.****C.**

**FIG 8. P2Y2 receptor affects viral DNA accumulation.** HFFs were transfected with siP2Y2 or siSc as a control. After 24 h, cells were infected with TB40/E-GFP virus (MOI=3) or mock infected. **(A) P2Y2 KD reduces intracellular vDNA.** At 96 hpi, total DNA was isolated from siSc- or siP2Y2-treated, HCMV-infected cells and the level of intracellular viral DNA was measured by qPCR. **(B) P2Y2 KD reduces extracellular vDNA.** At 96 hpi, total DNA was isolated from media of siSc- or siP2Y2-treated, HCMV-infected cells and the level of extracellular viral DNA was measured by qPCR. **(C) P2Y2 KD does not change the particle-to-infectious unit ratio.** The infectivity of virus in media collected at 96 hpi was titered and viral DNA was isolated from virions present in the media and quantified by qPCR to calculate a viral DNA copy number-to-infectious unit ratio. The results are depicted as fold change of this ratio. Data were averaged between two biological replicates and error bars represent SEM.

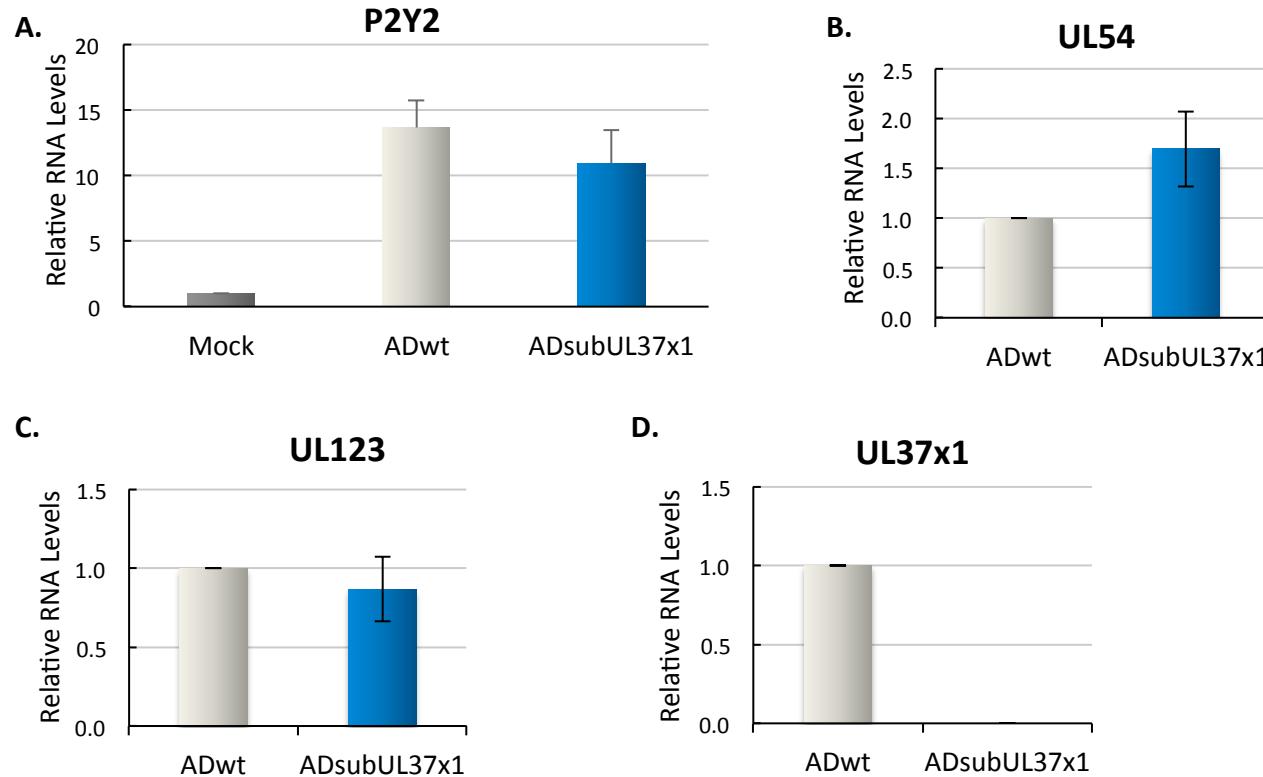
**A.****B.****C**

**FIG 9. P2Y2 and UL37x1 work cooperatively to regulate  $\text{Ca}^{2+}$  in HCMV-infected cells.**

**(A) Effects of kaempferol on  $\text{Ca}^{2+}$  levels in AD<sup>wt</sup> vs. AD<sub>UL37x1</sub> infected cells.** HFFs were infected with either AD<sup>wt</sup> or AD<sub>UL37x1</sub> (MOI=1) or mock infected for 2 h and treated with kaempferol (50  $\mu\text{M}$ ) or solvent control. Fluo-4 AM dye was applied at 20 hpi.

**(B) Effects of kaempferol on  $\text{Ca}^{2+}$  levels in wild type vs. UL37x1 overexpressing cells.** Wild type HFF (WT HFF) cells or HFF cells overexpressing UL37x1 [pLVX-UL37x1(1) and pLVX-UL37x1(2)] were treated with kaempferol (50  $\mu\text{M}$ ) or solvent control. Fluo-4 AM dye was applied 1 h later. Pictures of cells were taken using the Operetta High-Content Imaging System and the fluorescent signal was measured using ImageJ software based on 10 cells per experimental arm. Results are presented as the intensity of fluorescent signal in arbitrary units [AU].

**(C) P2Y2 and UL37x1 protein in UL37x1 overexpressing cells.** Protein samples were collected from two clones of MRC5 fibroblasts overexpressing UL37x1 [pLVX-UL37x1 (1) and pLVX-UL37x1 (2)] and control cells expressing GFP. Proteins were separated on SDS-PAGE and Western blot analysis was performed using antibodies recognizing P2Y2, UL37x1 and actin as a loading control.



**FIG 10. P2Y2 overexpression in HCMV-infected HFFs is not regulated by viral UL37x1.** HFFs were infected with either ADwt or ADsubUL37x1 virus (MOI=3) or mock-infected for 2 h. Samples were collected at 24 hpi. (A) P2Y2, (B) UL54, (C) UL123 and (D) UL37x1 transcript levels were determined by qRT-PCR with GAPDH serving as an internal control. Results are shown as fold change compared to mock-infected or Adwt-infected controls. Data are averaged across three biological replicates and error bars represent SEM.