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1 **RESEARCH ARTICLE**

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3 # Limited replication of human cytomegalovirus in a trophoblast cell line

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ABSTRACT

Several viruses, including human cytomegalovirus (HCMV), are thought to replicate in the placenta. However, there is little understanding of the molecular mechanisms involved in HCMV replication in this tissue. We investigated replication of HCMV in the extravillous trophoblast cell line SGHPL-4, a commonly used model of HCMV replication in the placenta. We found limited HCMV protein expression and virus replication in SGHPL-4 cells. This was associated with a lack of trophoblast progenitor cell protein markers in SGHPL-4 cells, suggesting a relationship between trophoblast differentiation and limited HCMV replication. We proposed that limited HCMV replication in trophoblast cells is advantageous to vertical transmission of HCMV as there is a greater opportunity for vertical transmission when the placenta is intact and functional. Furthermore, when we investigated the replication of other vertically transmitted viruses in SGHPL-4 cells we found some limitation to replication of Zika virus, but not herpes simplex virus. Thus, limited replication of some, but not all, vertically transmitted viruses may be a feature of trophoblast cells.

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1 **INTRODUCTION**

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3 TORCH (Toxoplasma, Other [including Zika virus], Rubella, 4 Cytomegalovirus, Herpes [herpes simplex 1 and herpes simplex 2]) pathogens 5 and viruses such as human immunodeficiency virus, parvovirus B19, hepatitis E 6 virus and varicella-zoster virus are widely studied as vertical transmission is an 7 important route of their dissemination in human populations and infection *in utero* 8 can have significant consequences for the health of the mother, fetus and/or child 9 (1-5).

10 TORCH pathogens and the viruses mentioned above can reach the 11 placenta via ascending or descending infections. Transvaginal ascending 12 infections can reach the fetus via infection of the amniotic membranes (1), 13 whereas in descending infection interaction of pathogens with trophoblast cells at 14 the fetal-maternal interface can be key to vertical transmission (1). In the 15 placenta the key features of the maternal-fetal interface are the branch-like 16 villous protrusions from the placenta that invade the maternal decidual tissue, 17 anchoring the placenta to the decidual tissue, and the floating villous protrusions 18 that are in direct contact with maternal blood (1). Covering these protrusions are 19 a layer of syncytiotrophoblasts and a layer of cytotrophoblasts. At the tip of the 20 protrusions are extravillious trophoblasts (EVTs). Maternal to fetal transmission 21 of pathogens across the placenta can occur via several routes including infection 22 of invasive EVTs, paracellular or transcellular transport, transfer of infected cells 23 across the maternal-fetal interface and infection of the amniotic membranes (1,

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2). TORCH pathogens are not restricted to using a single method to cross the maternal to fetal interface (1).

Virus infection of the placenta and subsequent infection of the fetus can cause a number of pathologies, including stillbirth and congenital disease (1-3). Human cytomegalovirus (HCMV) infection can cause placental dysfunction that leads to preterm birth, fetal growth restriction and direct injury to the fetus leading to miscarriage (1-3). Notably, HCMV is the most common congenital disease and is a major cause of blindness, deafness, cognitive impairment/neuronal disability (for example, cerebral palsy) and neonatal mortality (2, 3). HCMV seroprevalence in women of reproductive age can reach 100% in some populations and in most populations HCMV infection of pregnant women results in congenital HCMV infection in up to 2% of all live births (6-9). As multiple strains of HCMV exist, convalescent immunity from previous HCMV infection is not likely sufficient to be sufficient protect against future HCMV infections (10). Thus, seropositive mothers remain at risk of acquiring and vertically transmitting HCMV (10). Congenital infection can arise from primary infection of the mother or from reactivation of latent HCMV during pregnancy (1, 3). The impact of acquiring HCMV disease *in utero* can have long term consequences, especially as HCMV can establish a lifelong latent infection in an infected host that can be reactivated to cause further disease throughout their lifetime (11). However, it is interesting and important to note that acquisition of HCMV *post-partum*, for example via breast milk, may not lead to severe HCMV related disease (12).

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1 There are different mechanisms by which HCMV can cross the maternal-
2 fetal interface in the placenta and ultimately cause congenital disease. Maternal
3 IgG antibodies can facilitate immunoglobulin meditated transcytosis of HCMV in
4 EVT cells by binding HCMV and the neonatal Fc receptor for IgG (13).
5 Alternatively, HCMV can infect EVT cells (13). Presently, infection of Guinea
6 pigs with Guinea pig cytomegalovirus (GPCMV) is the only robust model of
7 cytomegalovirus vertical transmission, as GPCMV can infect Guinea pig
8 trophoblasts and the amniotic sac, plus infection of Guinea pigs can recapitulate
9 hearing loss found in congenital infection of humans (2, 14-17). New models of
10 congenital rhesus cytomegalovirus infection are developing rapidly (18) and it will
11 soon be possible to understand how useful they will be in understanding vertical
12 transmission of HCMV.
13 To understand vertical transmission of HCMV across the placenta it is
14 largely necessary to rely upon studies of HCMV infection of human explant tissue
15 and human EVT cell lines. A common EVT cell line used to study HCMV
16 replication in the placenta are SGHPL-4 cells. Studies using these cells have
17 suggested many pathogenic mechanisms in which HCMV could perturb EVT
18 function and cause placental and fetal damage *in utero* (3). These include
19 inhibiting invasion and migration of EVT and destruction of EVT via apoptosis (3).
20 However, there is very little understanding of the molecular basis of HCMV
21 replication in SGHPL-4 cells. Therefore, we set out to study HCMV replication in
22 SGHPL-4 cells. We also asked if HCMV infection of SGHPL-4 cells was similar to
23 infection by other viruses that can be acquired via ascending or descending

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1 infection, such as herpes simplex virus (HSV) and Zika virus (ZIKV). Like HCMV,

2 these viruses are a major cause of complications in pregnancy and fetal infection

3 by these viruses results in serious congenital disease (1).

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1 **METHODS**

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3 **Cells.** Human foreskin fibroblast (HFF) cells (CRL-1684, clone Hs29) were
4 obtained from the American Type Culture Collection (ATCC, USA)). Vero cells
5 were a kind gift from Donald Coen (Harvard Medical School). Human
6 adenocarcinomic alveolar basal epithelial cell line A549 and A549-N^{pro}
7 (expressing the bovine diarrhea virus N-terminal protease (N^{pro})) cells were kindly
8 provided by Steve Goodbourn (St George's, University of London). The
9 aforementioned cells were maintained in Dulbeccos Modified Eagles Medium
10 (DMEM) (Gibco) containing 10% fetal bovine serum (FBS) (Gibco), plus 1%
11 penicillin-streptomycin (Invitrogen). SGHPL-4 and SGHPL-5 (19) cells were kind
12 gifts from Guy Whitley (St George's, University of London). All trophoblast cell
13 lines were maintained in Rosslyn Park Memorial Institute (RPMI) media (Gibco)
14 or HAM F-12 media (Gibco) containing 10% fetal bovine serum (FBS) (Gibco),
15 plus 1% penicillin-streptomycin (Invitrogen). HepG2 cells were generously
16 provided by Joe Grove (UCL) and incubated in DMEM containing 10% FBS, plus
17 1% penicillin-streptomycin.

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19 **Human cytomegalovirus strains and mutants.** HCMV strain
20 Merlin(RCMV1111), which contains deletions in open reading frames encoding
21 RL13 and UL128, has been reported elsewhere (20). Merlin(R1111)UL36GFP, a
22 virus that expresses a fusion peptide of HCMV UL36 and green florescent protein
23 (GFP) separated by a self-cleaving P2A protein sequence has been described

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elsewhere (21). All virus stocks were generated by low multiplicity infections of HFF cells. Viral titre was determined by virus titration on HFF cells. HCMV strains TB40/E (generated from a bacterial artificial chromosome encoding the TB40/E genome (22)) and AD169 were generously provided by Matthew Reeves (UCL) and Donald Coen (Harvard Medical School), respectively.

FACS analysis. Uninfected or infected cells were infected as described in the figure legends. At the time points indicated in the text, cells were trypsinized, washed once in phosphate buffered saline (PBS), and then resuspended in PBS. Green florescent protein expression in cells was analyzed using florescence-activated cell sorting (FACS). In each case, 10,000 cells were acquired using BD FACSCalibur cytometer. Data were analyzed using FlowJo V10.

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Herpes simplex virus strains. HSV-1 strain KOS was a kind gift from Donald Coen (Harvard Medical School), while HSV-1 strains 17+, MG1 and SG16 were all gifts from Stacey Efstathiou and Mike Nicholl (NIBSC). All HSV-2 strains were generously donated by David Knipe (Harvard Medical School). All virus stocks were generated by low multiplicity infections of Vero cells. Viral titre was determined by virus titration on Vero cells.

Zika virus strains. Zika virus (ZIKV) *ZIKV/H. sapiens/Brazil/PE243/2015* (abbreviated to PE243, isolated from an infected human patient, Brazil, 2015), has been previously described (23). Strains VR-84 (strain MR766, isolated from

experimental forest sentinel rhesus monkey, Uganda, 1947) and VR-1845 (strain P6-740, isolated from *Aedes aegypti*, Malaysia, 1966) were obtained from American Type Culture Collection (ATCC, USA). All virus stocks were generated by low multiplicity infections of A549-N^{pro} cells. Viral titre was determined by virus titration on Vero cells.

Determination of viral titer by virus titration. Titers were determined by serial dilution of viral supernatant onto HFF (for HCMV) or Vero (for HSV and ZIKV) cell monolayers, which were then overlaid with DMEM containing 5% FBS (Gibco), 0.6% (w/v) methylcellulose and 1% penicillin-streptomycin (Invitrogen). After incubation for 3 days (HSV), 5 days (ZIKA) or 14 days (HCMV), cells were stained with crystal violet and plaques in the infected cell monolayers were counted to determine plaque forming units (p.f.u./ml)

MTT cytotoxicity assays. In this colorimetric assay the ability of cellular NAD(P)H-dependent cellular oxidoreductase enzymes to reduce the tetrazolium dye 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to formazan was measured. HFF cells were seeded at $1x10^4$ per well into 96-well plates. After overnight incubation to allow cell attachment, cells were treated as indicated in the figure legend and text. At 72 hours post infection MTT assays were then performed according to the manufacturer's instructions (GE Healthcare).

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Western blotting. Lysate of uninfected or infected HFF cells (see text and figure legends) were prepared for western blotting by washing cells once in PBS, the suspending cells directly in 2x Laemmli buffer containing 5% β-mercaptoethanol before incubating at 95°C for 5 mins. Western blotting of proteins separated on 8% or 10% polyacrylamide gels was carried out as described elsewhere (24), using antibodies recognizing IE1/2, UL44, pp28, (all Virusys, 1:1000 dilution), β-actin (SIGMA, 1:5000 dilution), PDGFRa (ABCAM ab203491, 1:1000 dilution) Hand1 (ABCAM, 1:1000 dilution), GATA-3, GATA-4 and HMGA2 (all Cell Signaling Technology, all 1:1000 dilution). All primary antibodies were incubated overnight at 4°C and detected using anti-mouse- or anti-rabbit-horseradish peroxidase (HRP) conjugated antibodies (Cell Signaling Technology). Chemiluminescence solution (GE Healthcare) was used to detect secondary antibodies on film. Where necessary blots were stripped and re-probed. Where indicated, relative band intensity (band intensity relative to β-actin signal in the same lane) was analyzed using ImageJ software, obtained from the NIH (USA).

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1 **RESULTS**

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3 **HCMV protein expression in SGHPL-4 cells.** SGHPL-4 cells are EVT
4 trophoblast cell lines taken from placental tissue of a first trimester pregnancy
5 termination (19). SGHPL-4 cells had been previously used in several studies of
6 placental trophoblast function upon infection of HCMV (3, 25-32) and were
7 known to support replication of an HCMV-green fluorescent protein (GFP)
8 reporter mutant (26). However, there was no comparison made to how well
9 HCMV replicates in other cells compared to replication in SGHPL-4 (26). Indeed,
10 to our knowledge, there had been no rigorous examination of the ability of HCMV
11 to replicate in SGHPL-4 cells and no examination of HCMV protein expression in
12 SGHPL-4 cells.

13 We noted that SGHPL-4 cells expressed the simian virus 40 (SV40)
14 middle T antigen, whose presence is required to maintain SGHPL-4 cell viability
15 (19). As this protein promotes progression through the cell cycle, we
16 hypothesized that HCMV replication in SGHPL-4 cells would be inefficient. It has
17 been observed elsewhere that serum starvation of cells can arrest the cell cycle
18 at a stage (G0/G1) which is advantageous for efficient HCMV immediate early
19 gene expression (33). Therefore, we incubated HFF and SGHPL-4 cells in a high
20 concentration of serum (10% volume/volume of the tissue culture media (10%
21 (v/v)), then either continued incubation under these conditions and infected cells
22 with HCMV in 10% (v/v) media, or incubated cells in a low concentration of
23 serum (0.5% volume/volume of the tissue culture media (0.5% (v/v)) for 24 hours

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1 then infected cells in 0.5% (v/v) media (Fig. 1A). All infections were carried out
2 using the HCMV virus Merlin(R1111) (20, 31). The genome of this virus is similar
3 to wild type HCMV genomes, but does not express HCMV proteins RL13 (which
4 promotes cell to cell spread of HCMV) or UL128 (part of the viral glycoprotein
5 gH/gL/UL128-UL131 pentamer complex which mediates virus entry into certain
6 cell types) (20). We prepared cell lysate for western blotting at 24-hour intervals
7 post infection (Fig. 1A) and used western blotting to assay the production of the
8 immediate early proteins IE1 and IE2 over time in infected cells (Fig. 1B(i)). We
9 observed robust expression of IE1 and IE2 proteins in HFF cells over time in the
10 presence of both high and low concentrations of serum (Fig. 1B(i), lanes 2-4 and
11 10-12, respectively). Compared to protein expression in HFF cells, we found
12 limited IE1 and IE2 protein expression in SGHPL-4 cells incubated in 10% (v/v)
13 media (Fig. 1B(i), lanes 6-8). However, compared to protein expression in HFF
14 cells, we observed only a modest defect in IE1 and IE2 protein expression in
15 SGHPL-4 cells incubated in 0.5% (v/v) media (Fig. 1B(i), lanes 14-16). Although
16 IE1 and IE2 protein expression was similar in HFF and SGHPL-4 cells at 24 h.p.i.
17 (Fig. 1B(i), compare lanes 10 and 14), we observed that while protein expression
18 increased in HFF over time (Fig. 1B(i), lanes 10-12), protein expression in
19 SGHPL-4 cells did not (Fig. 1B(i), lanes 13-16). Furthermore, with increased
20 passage of cells the aforementioned phenotype became more prominent
21 (compare panels in Fig. 1B(i) (early passage) with panels in Figs. 1B(ii) and
22 1B(iii) (later passages of the same cells)), to the extent that IE1 or IE2 protein
23 expression after 24 hours post infection could not be observed in SGHPL-4 cells

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1 (Fig. 1B(iii)). Therefore, immediate-early protein expression in SGHPL-4 cells
2 was possible under serum starvation conditions. However, this protein
3 expression in SGHPL-4 cells was not comparable with protein expression in HFF
4 cells either over the time of infection or with increasing passage of cells.

5 Comparable expression of IE1 or IE2 in HCMV infected HFF and SGHPL-
6 4 cells at 24 h.p.i. in cells of low and high passage incubated in 0.5% (v/v) media
7 (Fig. 1B(i)-(iii)) suggested that there was no obvious barrier to HCMV entry in
8 SGHPL-4 cells compared to HCMV entry in HFF cells. To confirm this we
9 incubated low and high passage cells in 0.5% (v/v) media and infected cells with
10 a derivative of Merlin(R1111), Merlin(R1111)UL36GFP, which expresses GFP
11 early in HCMV infection (21). After 24 hours, uninfected and infected cells were
12 analyzed by FACS (Fig. S1). We found no obvious difference in the number of
13 low or high passage HFF and SGHPL-4 cells expressing GFP, suggesting that
14 there was no obvious barrier to HCMV entry in SGHPL-4 cells and the HCMV
15 penatmer glycoprotein complex was not required for viral entry.

16 As we had seen a decrease in IE1 and IE2 protein expression over time in
17 early passage SGHPL-4 cells compared to HFF cells, we then investigated if this
18 defect in immediate early protein expression was associated with defects in early
19 and late HCMV protein expression. Therefore, we prepared cell lysates for
20 western blotting from early passage HFF and SGHPL-4 cells, both of which were
21 incubated in 0.5% (v/v) media for 24 hours before infection. Western blotting was
22 carried out using antibodies recognizing immediate-early (IE1 and IE2), early
23 (UL44) and late (pp28) viral proteins (Fig. 1C). We observed similar IE1 and IE2

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protein expression in HFF and SGHPL-4 cells to that seen in Figure 1B (Fig. 1C lanes 2-4 and 6-8, respectively). Compared to protein expression in HFF cells (Fig. 1C, lanes 2-4), we found limited UL44 and pp28 protein expression in SGHPL-4 cells (Fig. 1C, lanes 6-8). Therefore, the defect in immediate-early protein expression in SGHPL-4 cells was associated with limited early and late HCMV protein expression in those cells.

Replication of HCMV in SGHPL-4 cells. As we had observed limited HCMV protein expression in SGHPL-4 cells incubated in low serum concentration media (Fig. 1), we hypothesized that limited protein expression would be associated with limited production of HCMV virus. Therefore, we tested the ability of HCMV to replicate in HFF and SGHPL-4 cells incubated in 10% and 0.5% (v/v) media in both low and high passage cells. The experimental plan is shown in Figure 2A. Robust HCMV replication was found in low passage HFF cells incubated in both 10% and 0.5% (v/v) media (Fig. 2B). Compared to HCMV replication in HFF cells, limited replication of HCMV was found in low passage SGHPL-4 cells incubated in 10% (v/v) media, but replication of HCMV was greater in low passage SGHPL-4 cells incubated in 0.5% (v/v) media (Fig. 2B). Continued passage of cells had no obvious effect on HCMV replication in HFF cells (Fig. 2C). Similarly, compared to HCMV replication in HFF cells, limited replication of HCMV was found in high passage SGHPL-4 cells incubated in either 10% or 5% (v/v) media (Fig. 2C). Therefore, HCMV replication was limited in SGHPL-4 cells compared to HFF cells and differed with passage of cells.

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1 Although we did not find any data suggesting a barrier to HCMV
2 Merlin(R1111) entry into SGHPL-4 cells (Figs. 1 and S1), we speculated that
3 different HCMV strains may have different abilities to replicate in SGHPL-4 cells.
4 We incubated low passage HFF and SGHPL-4 cells in 0.5% (v/v) media and
5 infected them with Merlin(R1111), AD169 (an HCMV strain that does not express
6 the HCMV glycoprotein pentamer and a large number of proteins that influence
7 HCMV pathogenesis (31)) or TB40/E (and HCMV strain similar to wild type
8 Merlin that does express the HCMV glycoprotein pentamer (31)) (Fig. S2A). We
9 found no obvious difference in the ability of any HCMV strain to replicate in
10 SGHPL-4 cells. Similar data was observed using high passage SGHPL-4 cells
11 (data not shown). Using western blotting, we also examined expression of the
12 HCMV receptor platelet derived growth factor receptor alpha (PDGFRα) that is
13 required for entry of HCMV virus lacking the glycoprotein pentamer (Fig. S2B).
14 Consistent with data described elsewhere (28), SGHPL-4 cells expressed
15 PDGFRα, though not to the same extent as human fibroblast cells. Interestingly,
16 we observed that serum starvation of HFF and SGHPL-4 cells increased
17 PDGFRα expression in both cell lines, suggesting that serum starvation may aid
18 virus entry into both cell lines.

19 We also considered that HCMV replication in SGHPL-4 may be delayed
20 compared to HFF. Therefore, we analyzed HCMV Merlin (R1111) replication
21 over a time course of infection in low passage HFF and SGHPL-4 cells incubated
22 in 0.5% (v/v) media (Fig. S2C). HCMV replication in HFF cells increased over
23 time, until after 5 days post infection when cell death was evident in the infected

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HFF cell monolayer and no more time points were examined. HCMV replication in SGHPL-4 cells was limited compared to HCMV replication in HFF cells, peaking at 5 days post infection and then declining after that time point. Therefore, a delay in HCMV replication in SGHPL-4 cells was unlikely to reflect the limited HCMV replication we observed.

Cell viability of uninfected and infected trophoblast cell lines. We observed poor protein production and virus replication in SGHPL-4 cells infected with HCMV (Figs. 1 and 2). It was speculated that this may have been due to poor cell viability in the presence or absence of high concentrations of serum and/or HCMV. Therefore, we assayed cell viability by measuring the ability of cellular mitochondrial enzymes lost during apoptosis to reduce the compound MTT (Fig. 3). Low or high passage HFF and SGHPL-4 cells were incubated in either 10% or 0.5% (v/v) media and in the presence or absence of HCMV, then exposed to MTT. When we analyzed MTT reduction under the aforementioned conditions, we found that there was no obvious difference in the viability of HFF cells in high or low concentration serum media, nor was cell viability affected by the presence of virus or the passage of the cells (compare Figs. 3B and 3D). However, there was a modest difference in the viability of high passage SGHPL-4 cells in both the presence of 0.5%(v/v) media and/or HCMV (Compare Figs. 3E). Therefore, a defect in cell viability may have contributed to the limited HCMV protein expression and replication seen in high passage SGHPL-4 cells (Figs. 1 and 2). However, this reduction in cell viability was modest and there were likely

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1 other factors that contributed to limited HCMV protein expression and replication
2 in SGHPL-4 cells.
3
4 **Analysis of TBPC proteins in trophoblast cell lines.** We gave further
5 consideration as to why HCMV replication in SGHPL-4 cells was limited. It had
6 been reported that robust replication of HCMV was possible in first trimester
7 trophoblast progenitor cells (TBPC) (34) and that HCMV infection could alter the
8 expression of TBPC regulatory proteins (Hand1, GATA3, GATA4 and HMGA2)
9 involved in self renewal and differentiation that define TBPCs (35). Other reports
10 had indicated that HCMV replication in cells differentiated into cytotrophoblasts or
11 in trophoblasts from full term placental tissue was limited (36-39). As all
12 trophoblasts differentiate from progenitor cells, this suggested that HCMV
13 replication in trophoblast cells that had differentiated from progenitor cells may
14 have been inefficient. We hypothesized that SGHPL-4 cells may have moved
15 beyond the trophoblast progenitor stage of their differentiation and this may have
16 been associated with the limited HCMV replication we had observed.
17 To investigate this, we prepared cell lysate from uninfected and HCMV
18 infected low passage serum starved HFF and SGHPL-4 cells. Antibodies
19 recognizing the aforementioned TBPC regulatory proteins were used in western
20 blotting of cell lysates (Fig. 4). We found that uninfected SGHPL-4 cells did not
21 express all four TBPC regulatory proteins at levels detectable in our assay (Figs.
22 4A-4D), indicating that SGHPL-4 cells were not TBPCs. Hand1 and HGMA2
23 were detected in the presence or absence of HCMV, but to different extents, in

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both cell lines (Figs. 4A and 4D). GATA3 and GATA4 expression was detected in control cell lines, SGHPL-5 and HepG2 cells (Figs. 4B and 4C, respectively), but not in either HFF or SGHPL-4 cells (Figs. 4B and 4C).

We also noted that HCMV infection of SGHPL-4 cells did not result in differences in TBPC regulatory protein expression that had been previously reported in HCMV infection of TBPC cells (35). For example, we did not observe loss of HMGA2 expression in SGHPL-4 cells (Fig. 4D), which had been reported in HCMV infected TBPC cells (35). This data further indicated that SGHPL-4 cells did not have TBPC-like properties.

Therefore, the inability to detect all TBPC regulatory proteins in uninfected or HCMV infected SGHPL-4 cells was associated with limited HCMV protein expression and replication in those cells (Figs. 1 and 2). This suggested that once trophoblast cells had left their progenitor state, HCMV replication became limited.

HSV and ZIKV replication in trophoblast cell lines. We then investigated replication of other TORCH pathogen viruses in SGHPL-4 cells to understand if, like HCMV, replication of those viruses was limited in those cells. Therefore, we tested the ability of viruses related and unrelated to HCMV (the herpesvirus HSV and the flavivirus ZIKA, respectively) to replicate in SGHPL-4 cells.

It has been reported that there are differences in the genome content of laboratory and wild type strains of HSV-1 and HSV-2, although how these

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changes reflect virus replication and pathogenesis is largely unclear or unknown (40-47). It was, therefore, unknown if there would be differences in the ability of HSV-1 and HSV-2 to replicate in SGHPL-4 cells. Thus, we tested the ability of both laboratory and wild type HSV-1 and HSV-2 strains to replicate in HFF and SGHPL-4 cells.

We found no obvious difference in the ability of HSV-1 laboratory strain virus 17+ to replicate in low passage cells incubated in either high or low concentrations of serum (10% and 0.5% (v/v) media, respectively) (Figs. 5A and 5B), indicating that the serum concentration of the media had no obvious effect on HSV replication. When we tested the ability of laboratory and wild type HSV-1 and HSV-2 strains to replicate in HFF and low passage SGHPL-4 cells incubated in 10% (v/v) media we found that there was similar replication of each strain in HFF and SGHPL-4 cells (Fig 5C). However, we observed a trend wherein replication of virus in SGHPL-4 cells was moderately limited compared to HSV replication in HFF cells, but not to any degree of statistical relevance. We also tested replication of each strain in high passage HFF and SGHPL-4 cells. We found that there was no statistical difference in the ability of any HSV strain to replicate in either high passage HFF and SGHPL-4 cells compared to virus replication at in early passage cells (Figs. 5C and 5D).

Therefore, HSV could replicate in both trophoblast cell lines, regardless of cell culture conditions. There was no statistical difference in the ability of HSV-1 or HSV-2 laboratory or wild type strains to replicate in trophoblast cell lines.

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When we tested the ability of ZIKV to replicate in SGHPL-4. In the first instance we wished to compare ZIKV replication in SGHPL-4 cells to replication in HFF cells. ZIKV has been reported in human dermal fibroblast cells (48). However, in preliminary experiments we found very poor replication of ZIKV in HFF cells, regardless of multiplicity of infection used or time allowed for ZIKV to replicate (data not shown). It was unknown if the differences between our observations and those made elsewhere were due to the cell line or strain of ZIKV used or another experimental difference. Therefore, we decided to test ZIKV replication in SGHPL-4 cells compared to replication in Vero cells, a cell line known to allow robust replication of ZIKV. We did not know if there would be differences in the ability of different ZIKV strains to replicate in trophoblast cell lines. Therefore, we tested the ability of ZIKV laboratory strains VR-84 and VR-1845 to replicate in SGHPL-4 cells, as well as ZIKV strain PE243, a recently described strain that has not been extensively passaged in culture (23). There was no obvious difference in the ability of ZIKV strain VR-1845 to replicate in Vero or SGHPL-4 cells at high or low serum concentrations (10% and 0.5% (v/v) media, respectively) (Figs. 6A and 6B). We tested the ability of all ZIKV strains to replicate in Vero or low passage SGHPL-4 cells incubated in 10% (v/v) media (Fig. 6C). We found that all ZIKV showed robust replication in Vero cells, but considerably less replication in SGHPL-4 cells, regardless of what strain was used (Fig. 6C). We observed similar results when the experiments were repeated using high passage cells (Fig. 6D).

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We considered that ZIKV replication in Vero cells may be more efficient than replication in SGHPL-4 cells as Vero cells do not produce type I interferon (49, 50). Therefore, we compared the ability of ZIKV to replicate in either Vero cells, human A549 cells or human A549-N^{pro} cells, which do not have a functional type I interferon signaling system due to the presence of the N^{pro} protein that promotes proteasomal degradation of interferon regulatory factor 3 (IRF3) (51) (Fig. S3). Consistent with observations made elsewhere (52), we found that replication of ZIKV was similar in all three cell types. This was likely due to the ability of ZIKV to inhibit the type I interferon response to infection (23). This indicates that robust replication of ZIKV in Vero was not due to the absence of a type I interferon response to the virus.

Therefore, replication of Zika virus strains in SGHPL-4 cells was poor compared to replication in Vero cells. There was no obvious difference in replication of different Zika virus strains in any cell line tested. However, like HCMV, Zika virus replication in our trophoblast cell lines was limited compared to the control cell line used in our experiments.

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DISCUSSION

As yet, we have little understanding of the molecular basis of HCMV replication in SGHPL-4 cells. We found that HCMV protein expression and HCMV replication in SGHPL-4 cells was limited. Limited viral protein expression may have been, in part, due to the presence of the SV40 T antigen in both cell lines. However, circumventing that issue, we found limitation of HCMV protein expression may have been a feature of HCMV replication in EVT cells. We argue that limited viral protein expression and replication may be of advantage to HCMV during vertical transmission.

Limiting virus replication in placental cells would allow HCMV to generate sufficient progeny for the virus to be transmitted from mother to fetus, but not cause pathologies associated with HCMV infection that would damage the placenta and inhibit vertical transmission. It is also interesting to note that transmission of HCMV from mother to fetus in the first trimester of pregnancy may not be as efficient as transmission in the third trimester (53, 54). This may tally with our observations that there is limited HCMV replication in SGHPL-4 cell lines, which derived from placentas taken from pregnancies in the first trimester (19).

The molecular basis of the limited HCMV protein expression that we have observed is unknown. It remains unknown why IE protein expression in SGHPL-4 cells decreases over time after 24 hours post infection and why this should be associated with passage of cells in culture. To our knowledge, this has not been

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observed before in any other setting of HCMV infection. Microscopy experiments examining SGHPL-4 cells infected with HCMV expressing UL36GFP suggest that all infected cells loose GFP expression over time (data not shown). This suggested a model where loss of protein expression occurs in all infected SGHPL-4 cells over time, rather than a model where only a subset of infected SGHPL-4 cells are permissive for HCMV replication. We speculated that the reduction in IE protein expression was associated either with repression of transcription from the HCMV major immediate-early promoter or was associated with proteolysis of IE protein in infected cells. However, treatment of HCMV infected SGHPL-4 cells with either histone deacetylase inhibitors or the ubiquitin mediated proteasome inhibitor MG132 had no obvious effect on IE protein expression in SGHPL-4 cells under any condition tested so far (data not shown). Therefore, IE transcriptional repression or protein proteolysis was unlikely to be associated with the HCMV IE protein expression phenotype we observed in SGHPL-4 cells. Rather, there may be an issue with IE RNA metabolism and/or protein translation in HCMV infected SGHPL-4 cells that resulted in the loss of IE protein expression. Our data were consistent with a previous report of poor HCMV-GFP reporter virus replication in SGHPL-4 cells (26). However, it was not possible to directly compare our data with this previous report as it was not stated what cell culture conditions were used in the aforementioned study (26).

We also considered if the presence of the SV40 T antigen was limiting HCMV replication in SGHPL-4 cells. Expression of the SV40 T antigen in fibroblasts is a barrier to HCMV entry into cells, via the loss of the viral receptor

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1 platelet-derived growth factor receptor α (PDGFRα), and decreased expression
2 of IE2, but not IE1, over the course of HCMV replication (55). As we observe no
3 obvious barrier to HCMV entry into SGHPL-4 cells and decreases in both IE1
4 and IE2 expression over time in HCMV infected SGHPL-4 cells, it is unlikely that
5 the presence of the SV40 T antigen was directly responsible for the limited
6 HCMV replication we observed in those cells.

7 We argue that the difference in the ability of HCMV to replicate in SGHPL-
8 4 cells compared to HFF cells was not due to the ability of HCMV to enter the
9 SGHPL-4 cells. The Merlin(R1111) virus that we used throughout our studies
10 does not express the HCMV pentamer glycoprotein complex that allows virus
11 entry into a range of cell types other than fibroblasts. It has been demonstrated
12 that pentamer expression on Merlin virions only results in a very modest increase
13 on the ability of Merlin to enter SGHPL-4 cells (less than 2-fold compared to
14 Merlin virions not expressing pentamer) (28). However, the pentamer
15 glycoprotein complex was essential for Merlin to enter another human
16 trophoblast cell line, HTR (28). This is likely due to the presence of PDGFRα on
17 SGHPL-4 cells, but not HTR cells (28). HCMV can regulate the amount of
18 glycoprotein pentamer complexes that are displayed on HCMV virions (56).
19 Therefore, HCMV entry into different trophoblast cells may require involve viral
20 glycoprotein complexes and assessing where PDGFRα is expressed in placental
21 tissue may be of importance to understanding HCMV replication. It is possible
22 that the glycoprotein pentamer is not displayed on all HCMV virions and that the
23 presence of glycoprotein pentamer on HCMV virions is not essential for HCMV

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1 replication in the placenta. However, the pentamer complexes of Guinea pig
2 cytomegalovirus and rhesus cytomegaloviruses must be present for those
3 viruses to be efficiently vertically transmitted and cause congenital infection (16,
4 18). Therefore, the presence of the HCMV pentamer may also be important in
5 vertical transmission and development of congenital disease in humans.

6 Further investigation is required to understand what limitations there are
7 on HCMV replication in primary trophoblasts. We observed that differences in the
8 ability of HCMV to replicate in different EVT cell lines was associated with the
9 differentiation state of the cell line. In future experiments it will be important to
10 understand the differentiation state of primary trophoblast cells or trophoblast cell
11 lines used and how that impacts upon the ability of HCMV to replicate in those
12 cells. To our knowledge, it is not yet possible to alter the differentiation of
13 SGHPL-4 cells to address the relationship between progenitor cell status and
14 ability of HCMV to replicate in those cells.

15 SGHPL-4 cells are commonly used to study HCMV replication (3, 25-30).
16 Based on the data we present here future studies using SGHPL-4 cells to
17 investigate HCMV replication may need to consider the cell culture conditions
18 used and how that might influence HCMV protein expression.

19 While our studies highlighted how HCMV replication in EVT cells was
20 limited, we also noted that there was limited replication of ZIKV in SGHPL-4 cells.
21 Again, we argue that limited viral replication may be advantageous during vertical
22 transmission of virus, as it would allow virus replication and maximize the
23 opportunity for virus transmission across a healthy maternal to fetal tissue

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1 interface. In future it will be important to understand the molecular basis of limited

2 ZIKV replication.

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AUTHOR CONTRIBUTIONS

KH: Investigation. NS: Investigation. NB: Methodology, Investigation. ACT: Investigation CLD: Methodology, Resources, Writing-Review and Editing. AK: Methodology, Resources Writing-Review and Editing, Funding RJS: Methodology, Resources, Writing-Review and Editing, Funding BLS: Conceptualization, Methodology, Investigation, Data Curation, Writing-Original Draft Preparation, Writing-Review and Editing, Supervision, Project Administration, Funding.

CONFILCTS OF INTEREST

The authors declare there are no conflicts of interest.

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1 **FIGURE LEGENDS**

2

3 **Figure 1 HCMV protein expression in SGHPL-4 cells.** (A) Timeline of cell
4 preparation, cell infection and sample collection. (B) Lysate from uninfected or
5 infected HFF and SGHPL-4 cells (HCMV strain Merlin(R1111), multiplicity of
6 infection of 1) were prepared for western blotting at the time points (hours post
7 infection (h.p.i.)) indicated above the figure (also, see panel (A)). The passage
8 number (p) of the cells used is indicated to the far left of the figure. Figures (i)-(iii)
9 are increasing passage of the same HFF or SGHPL-4 cells. (C) Lysates from
10 uninfected or infected HFF and SGHPL-4 cells (p8 and p14, respectively) (HCMV
11 strain Merlin(R1111), multiplicity of infection of 1) incubated in 0.5% (v/v) media
12 24 hours prior to infection and during infection were prepared for western blotting
13 at the time points indicated above the figure. In both (B) and (C) uninfected cells
14 harvested at the time of infection are shown as 0 h.p.i.. Proteins recognized by
15 the antibodies used in the experiment are indicated to the right of each western
16 blot panel. In each experiment the presence of β-actin was assayed to assess
17 the amount of cell lysate assayed in each lane. The positions of molecular weight
18 markers (kDa) are indicated to the left of each the figure.

19

20 **Figure 2 HCMV replication in SGHPL-4 cells.** (A) Timeline of cell preparation,
21 cell infection and sample collection. (B) Low and (C) high passage HFF or
22 SGHPL-4 cells (passages 6-10 and 13-15, respectively) were treated as shown
23 in panel (A) and infected with HCMV strain Merlin(R1111) at a multiplicity of

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1 infection of 1. Viruses were harvested at 96 hours post infection and viral titre
2 (plaque forming units (p.f.u.)/ml) was determined by titration of viral supernatant
3 on HFF cells. Data from three independent experiments was presented in each
4 figure. The bar chart and error bars represent the mean and standard deviation
5 of that data, respectively. The statistical difference between the indicated
6 conditions was measured using an unpaired t test (two-tailed) and is indicated
7 above each figure. A statistically relevant difference was where p=<0.05.
8
9 **Figure 3 Cell viability in the presence and absence of HCMV and serum.** (A)
10 Timeline of cell preparation, cell infection and sample collection. (B-E) Low and
11 high passage HFF and SGHPL cells (HFF passage 6-9 and 15-18, SGHPL-4
12 passage 13-15 and 19-21) were treated as shown in (A) and infected with HCMV
13 strain Merlin(R1111) at a multiplicity of infection of 1. At 72 h.p.i. cell viability was
14 determined using an MTT assay (Arbitrary units (AU)). Each data point
15 represents the data from eight biological replicates in each condition. The bar
16 and error bars represent the mean and standard deviation of that data,
17 respectively. The data in this figure is representative of two independent
18 experiments measured at 72 h.p.i.. The statistical difference between the
19 indicated conditions was measured using an unpaired t test (two-tailed) and is
20 indicated above each figure. A statistically relevant difference was where
21 p=<0.05. Not significant (ns). Uninfected (un.). Infected (in).
22

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1 **Figure 4 Trophoblast protein expression in uninfected and HCMV infected**
2 **cells.** Lysates from uninfected or infected HFF or SGHPL-4 cells (HCMV strain
3 Merlin(R1111), multiplicity of infection of 1) (HFF passage 7, SGHPL-4 passage
4 14) incubated in 0.5% (v/v) media 24 hours prior to infection and during infection
5 were prepared for western blotting at the time points (h.p.i.) indicated above the
6 figure. Uninfected cells harvested at the time of infection are shown as 0 h.p.i..
7 Where indicated, lysate from the control cell lines SGHPL-5 and HepG2 were
8 analyzed. Proteins recognized by the antibodies used in the experiment are
9 indicated to the right of each western blot panel. In each experiment the
10 presence of β-actin was assayed to assess the amount of cell lysate analyzed in
11 each lane. The positions of molecular weight markers (kDa) are indicated to the
12 left of the figure. In figure C, each panel shows data from the same exposure of
13 film.
14
15 **Figure 5 HSV replication in HFF and SGHPL-4 cell lines.** (A) Timeline of cell
16 preparation, cell infection and sample collection. (B) Low passage HFF and
17 SGHPL-4 cells (HFF passage 6-9, SGHPL-4 passage 13-15) were incubated in
18 either 10% or 5% (v/v) media and infected with the HSV-1 strain 17+. (C) Low
19 passage or (D) high passage HFF and SGHPL-4 cells (HFF passage 6-9 and 15-
20 18, SGHPL-4 passage 13-15 and 19-21) were incubated in 10% (v/v) media and
21 infected with the HSV-1 and HSV-2 strains indicated at the top of the figures
22 (multiplicity of infection 1). In all experiments viruses were harvested at 48 hours
23 post infection and viral titre (p.f.u./ml) was determined by titration of viral

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supernatant on Vero cells. The data from three independent experiments was presented in each figure. The bar chart and error bars represent the mean and standard deviation of that data, respectively. The statistical difference between the indicated conditions was measured using an unpaired t test (two-tailed) and is indicated above each figure. A statistically relevant difference was where $p=<0.05$. Not significant (ns).

Figure 6 ZIKV replication in HFF and SGHPL-4 cell lines. (A) Timeline of cell preparation, cell infection and sample collection. (B) Low passage HFF and SGHPL-4 cells (HFF passage 7-9, SGHPL-4 passage 14-16) were incubated in either 10% or 5% (v/v) media and infected with the Zika strain VR-1845. (C) Low passage or (D) high passage HFF and SGHPL-4 cells (HFF passage 6-9 and 15-18, SGHPL-4 passage 13-15 and 19-21) were incubated in 10% (v/v) media and infected with the ZIKV strains indicated at the top of the figures (multiplicity of infection 0.1). In all experiments viruses were harvested at 48 h.p.i and viral titre (p.f.u./ml) was determined by titration of viral supernatant on Vero cells. The data from three independent experiments was presented in each figure. The bar chart and error bars represent the mean and standard deviation of that data, respectively. The statistical difference between the indicated conditions was measured using an unpaired t test (two-tailed) and is indicated above each figure. A statistically relevant difference was where $p=<0.05$.

CONFIDENTIAL

REFERENCES

1. Coyne CB, Lazear HM. Zika virus - reigniting the TORCH. Nat Rev Microbiol. 2016;14(11):707-15. doi: 10.1038/nrmicro.2016.125
2. Britt WJ. Maternal Immunity and the Natural History of Congenital Human Cytomegalovirus Infection. Viruses. 2018;10(8). doi: 10.3390/v10080405
3. Njue A, Coyne C, Margulis AV, Wang D, Marks MA, Russell K, et al. The Role of Congenital Cytomegalovirus Infection in Adverse Birth Outcomes: A Review of the Potential Mechanisms. Viruses. 2020;13(1). doi: 10.3390/v13010020
4. Pereira L. Congenital Viral Infection: Traversing the Uterine-Placental Interface. Annu Rev Virol. 2018;5(1):273-99. doi: 10.1146/annurev-virology-092917-043236
5. Arora N, Sadovsky Y, Dermody TS, Coyne CB. Microbial Vertical Transmission during Human Pregnancy. Cell Host Microbe. 2017;21(5):561-7. doi: 10.1016/j.chom.2017.04.007
6. Cannon MJ, Schmid DS, Hyde TB. Review of cytomegalovirus seroprevalence and demographic characteristics associated with infection. Rev Med Virol. 2010;20(4):202-13. doi: 10.1002/rmv.655
7. Kenneson A, Cannon MJ. Review and meta-analysis of the epidemiology of congenital cytomegalovirus (CMV) infection. Rev Med Virol. 2007;17(4):253-76. doi: 10.1002/rmv.535

CONFIDENTIAL

8. Dollard SC, Grosse SD, Ross DS. New estimates of the prevalence of neurological and sensory sequelae and mortality associated with congenital cytomegalovirus infection. Rev Med Virol. 2007;17(5):355-63. doi: 10.1002/rmv.544

9. Fowler KB, Stagno S, Pass RF. Maternal immunity and prevention of congenital cytomegalovirus infection. JAMA. 2003;289(8):1008-11. doi: 10.1001/jama.289.8.1008

10. Britt WJ. Human Cytomegalovirus Infection in Women With Preexisting Immunity: Sources of Infection and Mechanisms of Infection in the Presence of Antiviral Immunity. J Infect Dis. 2020;221(Suppl 1):S1-S8. doi: 10.1093/infdis/jiz464

11. Dupont L, Reeves MB. Cytomegalovirus latency and reactivation: recent insights into an age old problem. Rev Med Virol. 2016;26(2):75-89. doi: 10.1002/rmv.1862

12. Bardanzellu F, Fanos V, Reali A. Human Breast Milk-acquired Cytomegalovirus Infection: Certainties, Doubts and Perspectives. Curr Pediatr Rev. 2019;15(1):30-41. doi: 10.2174/1573396315666181126105812

13. Maidji E, McDonagh S, Genbacev O, Tabata T, Pereira L. Maternal antibodies enhance or prevent cytomegalovirus infection in the placenta by neonatal Fc receptor-mediated transcytosis. Am J Pathol. 2006;168(4):1210-26. doi: 10.2353/ajpath.2006.050482

CONFIDENTIAL

1 14. Weisblum Y, Panet A, Haimov-Kochman R, Wolf DG. Models of vertical
2 cytomegalovirus (CMV) transmission and pathogenesis. Semin Immunopathol.
3 2014;36(6):615-25. doi: 10.1007/s00281-014-0449-1
4 15. Woolf NK, Ochi JW, Silva EJ, Sharp PA, Harris JP, Richman DD.
5 Ganciclovir prophylaxis for cochlear pathophysiology during experimental guinea
6 pig cytomegalovirus labyrinthitis. Antimicrob Agents Chemother. 1988;32(6):865-
7 72. doi: 10.1128/AAC.32.6.865
8 16. Coleman S, Choi KY, Root M, McGregor A. A Homolog Pentameric
9 Complex Dictates Viral Epithelial Tropism, Pathogenicity and Congenital Infection
10 Rate in Guinea Pig Cytomegalovirus. PLoS Pathog. 2016;12(7):e1005755. doi:
11 10.1371/journal.ppat.1005755
12 17. Choi KY, El-Hamdi NS, McGregor A. Requirements for guinea pig
13 cytomegalovirus tropism and antibody neutralization on placental amniotic sac
14 cells. J Gen Virol. 2020;101(4):426-39. doi: 10.1099/jgv.0.001394
15 18. Taher H, Mahyari E, Kreklywich C, Uebelhoer LS, McArdle MR, Mostrom
16 MJ, et al. In vitro and in vivo characterization of a recombinant rhesus
17 cytomegalovirus containing a complete genome. PLoS Pathog.
18 2020;16(11):e1008666. doi: 10.1371/journal.ppat.1008666
19 19. Choy MY, St Whitley G, Manyonda IT. Efficient, rapid and reliable
20 establishment of human trophoblast cell lines using poly-L-ornithine. Early
21 Pregnancy. 2000;4(2):124-43. doi:
22 20. Stanton RJ, Baluchova K, Dargan DJ, Cunningham C, Sheehy O,
23 Seirafian S, et al. Reconstruction of the complete human cytomegalovirus

CONFIDENTIAL

genome in a BAC reveals RL13 to be a potent inhibitor of replication. J Clin Invest. 2010;120(9):3191-208. doi: 10.1172/JCI42955

21. Nightingale K, Lin KM, Ravenhill BJ, Davies C, Nobre L, Fielding CA, et al. High-Definition Analysis of Host Protein Stability during Human Cytomegalovirus Infection Reveals Antiviral Factors and Viral Evasion Mechanisms. Cell Host Microbe. 2018;24(3):447-60 e11. doi: 10.1016/j.chom.2018.07.011

22. Sinzger C, Hahn G, Digel M, Katona R, Sampaio KL, Messerle M, et al. Cloning and sequencing of a highly productive, endotheliotropic virus strain derived from human cytomegalovirus TB40/E. J Gen Virol. 2008;89(Pt 2):359-68. doi: 10.1099/vir.0.83286-0

23. Donald CL, Brennan B, Cumberworth SL, Rezelj VV, Clark JJ, Cordeiro MT, et al. Full Genome Sequence and sfRNA Interferon Antagonist Activity of Zika Virus from Recife, Brazil. PLoS Negl Trop Dis. 2016;10(10):e0005048. doi: 10.1371/journal.pntd.0005048

24. Strang BL, Stow ND. Circularization of the herpes simplex virus type 1 genome upon lytic infection. J Virol. 2005;79(19):12487-94. doi: 10.1128/JVI.79.19.12487-12494.2005

25. LaMarca HL, Nelson AB, Scandurro AB, Whitley GS, Morris CA. Human cytomegalovirus-induced inhibition of cytotrophoblast invasion in a first trimester extravillous cytotrophoblast cell line. Placenta. 2006;27(2-3):137-47. doi: 10.1016/j.placenta.2005.03.003

CONFIDENTIAL

26. LaMarca HL, Sainz B, Jr., Morris CA. Permissive human cytomegalovirus infection of a first trimester extravillous cytotrophoblast cell line. Virol J. 2004;1:8. doi: 10.1186/1743-422X-1-8

27. van Zuylen WJ, Ford CE, Wong DD, Rawlinson WD. Human Cytomegalovirus Modulates Expression of Noncanonical Wnt Receptor ROR2 To Alter Trophoblast Migration. J Virol. 2016;90(2):1108-15. doi: 10.1128/JVI.02588-15

28. Naing Z, Hamilton ST, van Zuylen WJ, Scott GM, Rawlinson WD. Differential Expression of PDGF Receptor-alpha in Human Placental Trophoblasts Leads to Different Entry Pathways by Human Cytomegalovirus Strains. Scientific reports. 2020;10(1):1082. doi: 10.1038/s41598-020-57471-3

29. Angelova M, Zwezdaryk K, Ferris M, Shan B, Morris CA, Sullivan DE. Human cytomegalovirus infection dysregulates the canonical Wnt/beta-catenin signaling pathway. PLoS Pathog. 2012;8(10):e1002959. doi: 10.1371/journal.ppat.1002959

30. Huynh KT, van Zuylen WJ, Ford CE, Rawlinson WD. Selective modulation of Wnt-binding receptor tyrosine kinase ROR2 expression by human cytomegalovirus regulates trophoblast migration. J Gen Virol. 2019;100(1):99-104. doi: 10.1099/jgv.0.001179

31. Wilkinson GW, Davison AJ, Tomasec P, Fielding CA, Aicheler R, Murrell I, et al. Human cytomegalovirus: taking the strain. Med Microbiol Immunol. 2015;204(3):273-84. doi: 10.1007/s00430-015-0411-4

CONFIDENTIAL

32. Isomura H, Stinski MF. The human cytomegalovirus major immediate-early enhancer determines the efficiency of immediate-early gene transcription and viral replication in permissive cells at low multiplicity of infection. J Virol. 2003;77(6):3602-14. doi: 10.1128/jvi.77.6.3602-3614.2003

33. Spector DH. Human cytomegalovirus riding the cell cycle. Med Microbiol Immunol. 2015;204(3):409-19. doi: 10.1007/s00430-015-0396-z

34. Zydek M, Petitt M, Fang-Hoover J, Adler B, Kauvar LM, Pereira L, et al. HCMV infection of human trophoblast progenitor cells of the placenta is neutralized by a human monoclonal antibody to glycoprotein B and not by antibodies to the pentamer complex. Viruses. 2014;6(3):1346-64. doi: 10.3390/v6031346

35. Tabata T, Petitt M, Zydek M, Fang-Hoover J, Larocque N, Tsuge M, et al. Human cytomegalovirus infection interferes with the maintenance and differentiation of trophoblast progenitor cells of the human placenta. J Virol. 2015;89(9):5134-47. doi: 10.1128/JVI.03674-14

36. Delorme-Axford E, Donker RB, Mouillet JF, Chu T, Bayer A, Ouyang Y, et al. Human placental trophoblasts confer viral resistance to recipient cells. Proc Natl Acad Sci U S A. 2013;110(29):12048-53. doi: 10.1073/pnas.1304718110

37. Fisher S, Genbacev O, Maidji E, Pereira L. Human cytomegalovirus infection of placental cytotrophoblasts in vitro and in utero: implications for transmission and pathogenesis. J Virol. 2000;74(15):6808-20. doi: 10.1128/jvi.74.15.6808-6820.2000

CONFIDENTIAL

38. Halwachs-Baumann G, Wilders-Truschnig M, Desoye G, Hahn T, Kiesel L, Klingel K, et al. Human trophoblast cells are permissive to the complete replicative cycle of human cytomegalovirus. J Virol. 1998;72(9):7598-602. doi: 10.1128/JVI.72.9.7598-7602.1998

39. Hemmings DG, Kilani R, Nykiforuk C, Preiksaitis J, Guilbert LJ. Permissive cytomegalovirus infection of primary villous term and first trimester trophoblasts. J Virol. 1998;72(6):4970-9. doi: 10.1128/JVI.72.6.4970-4979.1998

40. Wang H, Davido DJ, Morrison LA. HSV-1 strain McKrae is more neuroinvasive than HSV-1 KOS after corneal or vaginal inoculation in mice. Virus Res. 2013;173(2):436-40. doi: 10.1016/j.virusres.2013.01.001

41. Macdonald SJ, Mostafa HH, Morrison LA, Davido DJ. Genome sequence of herpes simplex virus 1 strain McKrae. J Virol. 2012;86(17):9540-1. doi: 10.1128/JVI.01469-12

42. Macdonald SJ, Mostafa HH, Morrison LA, Davido DJ. Genome sequence of herpes simplex virus 1 strain KOS. J Virol. 2012;86(11):6371-2. doi: 10.1128/JVI.00646-12

43. Newman RM, Lamers SL, Weiner B, Ray SC, Colgrove RC, Diaz F, et al. Genome Sequencing and Analysis of Geographically Diverse Clinical Isolates of Herpes Simplex Virus 2. J Virol. 2015;89(16):8219-32. doi: 10.1128/JVI.01303-15

44. Lamers SL, Newman RM, Laeyendecker O, Tobian AA, Colgrove RC, Ray SC, et al. Global Diversity within and between Human Herpesvirus 1 and 2 Glycoproteins. J Virol. 2015;89(16):8206-18. doi: 10.1128/JVI.01302-15

CONFIDENTIAL

1 45. Colgrove R, Diaz F, Newman R, Saif S, Shea T, Young S, et al. Genomic
2 sequences of a low passage herpes simplex virus 2 clinical isolate and its
3 plaque-purified derivative strain. Virology. 2014;450-451:140-5. doi:
4 10.1016/j.virol.2013.12.014
5 46. Dudek TE, Torres-Lopez E, Crumpacker C, Knipe DM. Evidence for
6 differences in immunologic and pathogenesis properties of herpes simplex virus
7 2 strains from the United States and South Africa. J Infect Dis.
8 2011;203(10):1434-41. doi: 10.1093/infdis/jir047
9 47. Dolan A, Jamieson FE, Cunningham C, Barnett BC, McGeoch DJ. The
10 genome sequence of herpes simplex virus type 2. J Virol. 1998;72(3):2010-21.
11 doi: 10.1128/JVI.72.3.2010-2021.1998
12 48. Hamel R, Dejarnac O, Wichit S, Ekchariyawat P, Neyret A, Luplertlop N, et
13 al. Biology of Zika Virus Infection in Human Skin Cells. J Virol. 2015;89(17):8880-
14 96. doi: 10.1128/JVI.00354-15
15 49. Desmyter J, Melnick JL, Rawls WE. Defectiveness of interferon production
16 and of rubella virus interference in a line of African green monkey kidney cells
17 (Vero). J Virol. 1968;2(10):955-61. doi: 10.1128/JVI.2.10.955-961.1968
18 50. Mosca JD, Pitha PM. Transcriptional and posttranscriptional regulation of
19 exogenous human beta interferon gene in simian cells defective in interferon
20 synthesis. Mol Cell Biol. 1986;6(6):2279-83. doi: 10.1128/mcb.6.6.2279-
21 2283.1986
22 51. Hilton L, Moganeradj K, Zhang G, Chen YH, Randall RE, McCauley JW, et
23 al. The NPro product of bovine viral diarrhea virus inhibits DNA binding by

CONFIDENTIAL

interferon regulatory factor 3 and targets it for proteasomal degradation. J Virol. 2006;80(23):11723-32. doi: 10.1128/JVI.01145-06

52. Vicenti I, Boccuto A, Giannini A, Dragoni F, Saladini F, Zazzi M. Comparative analysis of different cell systems for Zika virus (ZIKV) propagation and evaluation of anti-ZIKV compounds in vitro. Virus Res. 2018;244:64-70. doi: 10.1016/j.virusres.2017.11.003

53. Enders G, Daiminger A, Bader U, Exler S, Enders M. Intrauterine transmission and clinical outcome of 248 pregnancies with primary cytomegalovirus infection in relation to gestational age. J Clin Virol. 2011;52(3):244-6. doi: 10.1016/j.jcv.2011.07.005

54. Picone O, Vauloup-Fellous C, Cordier AG, Guitton S, Senat MV, Fuchs F, et al. A series of 238 cytomegalovirus primary infections during pregnancy: description and outcome. Prenat Diagn. 2013;33(8):751-8. doi: 10.1002/pd.4118

55. Xu S, Schafer X, Munger J. Expression of Oncogenic Alleles Induces Multiple Blocks to Human Cytomegalovirus Infection. J Virol. 2016;90(9):4346-56. doi: 10.1128/JVI.00179-16

56. Li G, Nguyen CC, Ryckman BJ, Britt WJ, Kamil JP. A viral regulator of glycoprotein complexes contributes to human cytomegalovirus cell tropism. Proc Natl Acad Sci U S A. 2015;112(14):4471-6. doi: 10.1073/pnas.1419875112

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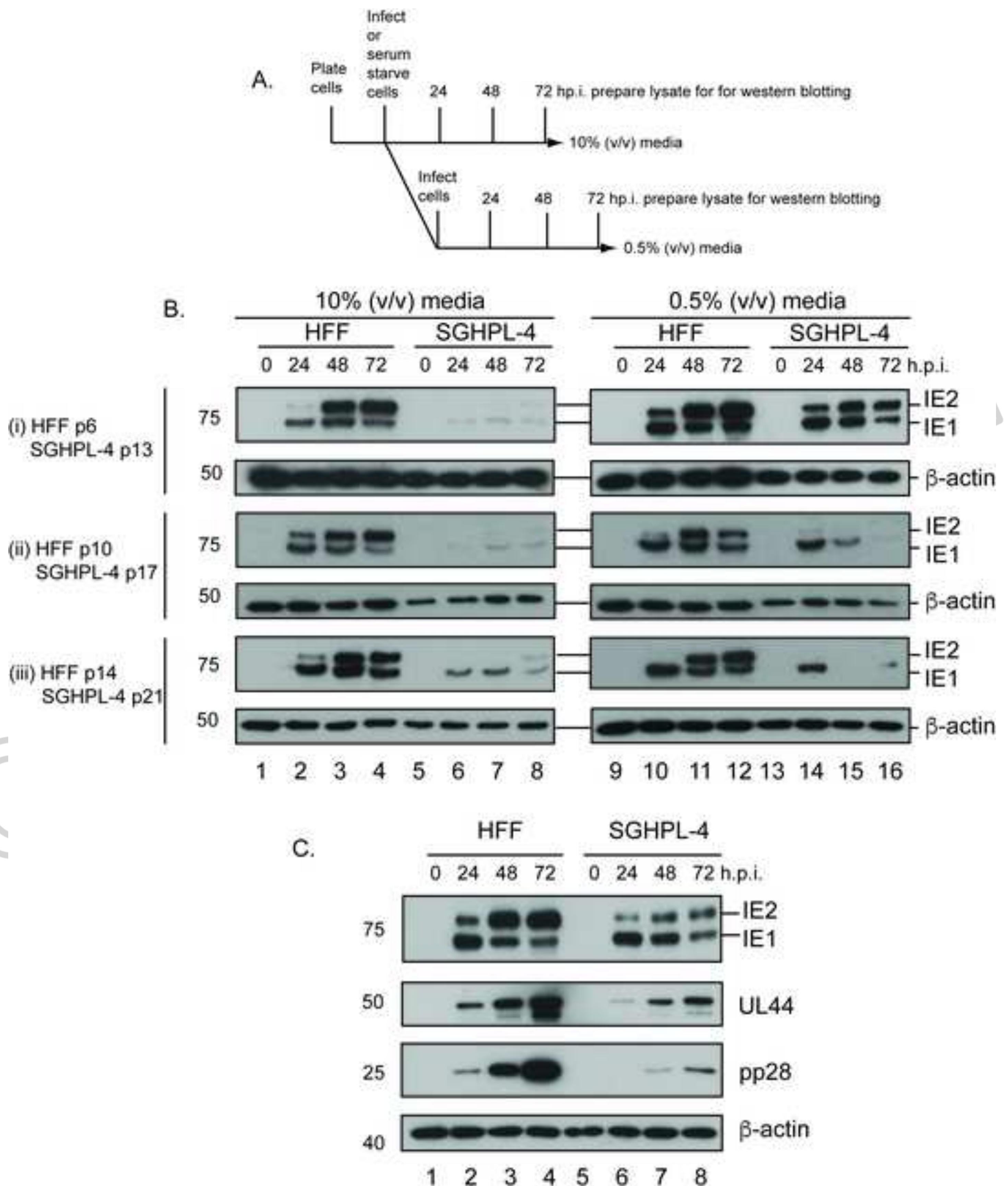

Figure 1

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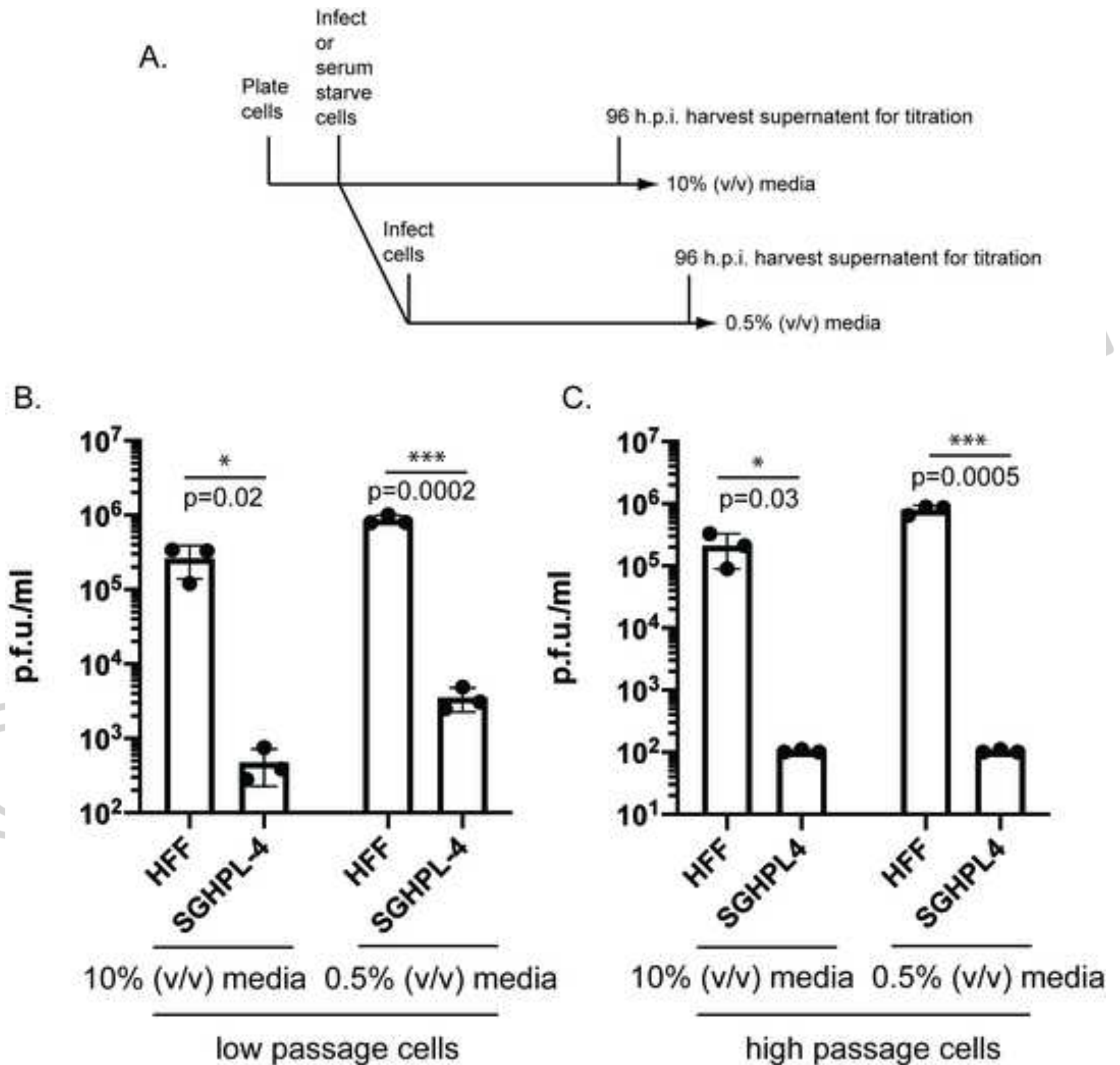

Figure 2

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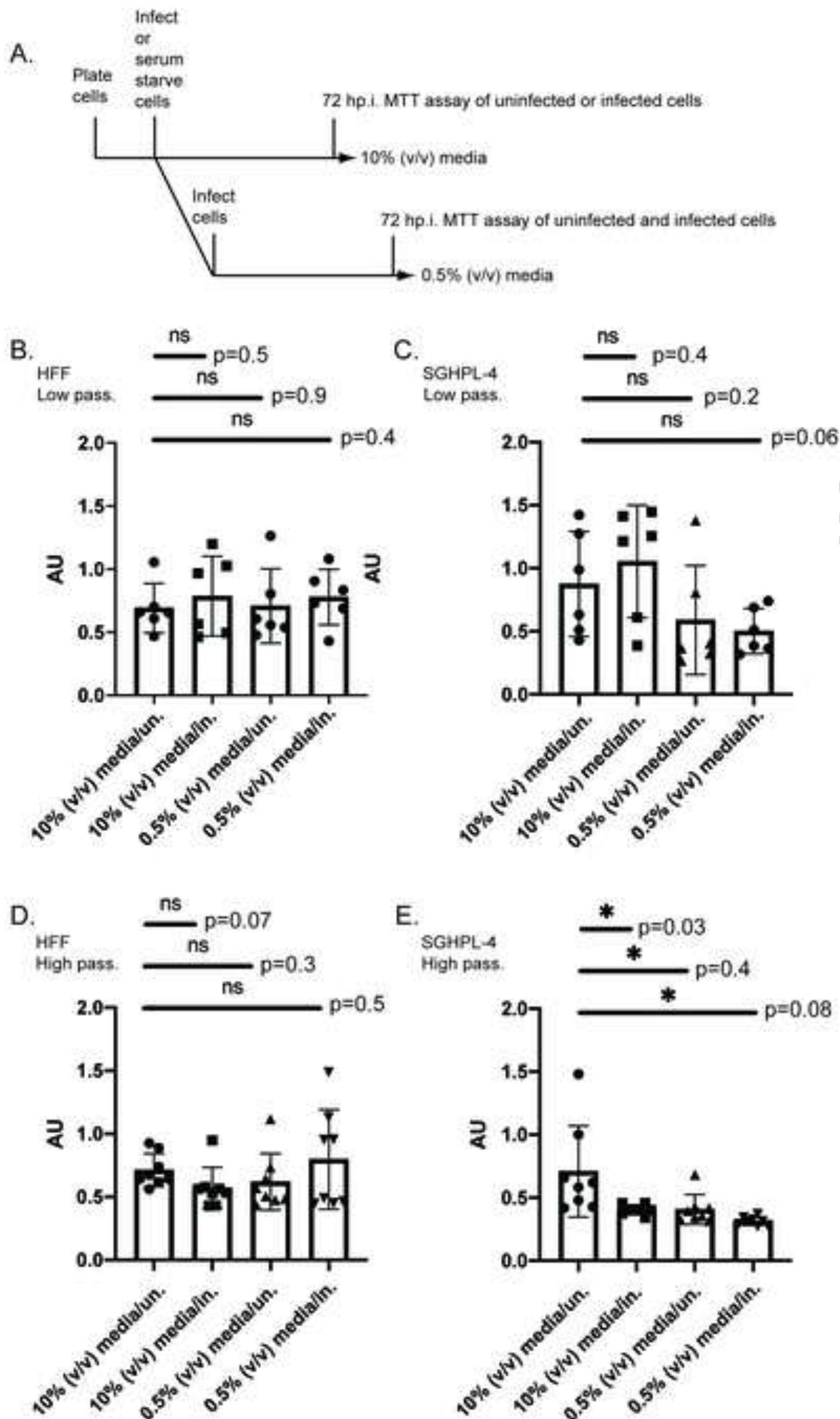

Figure 3

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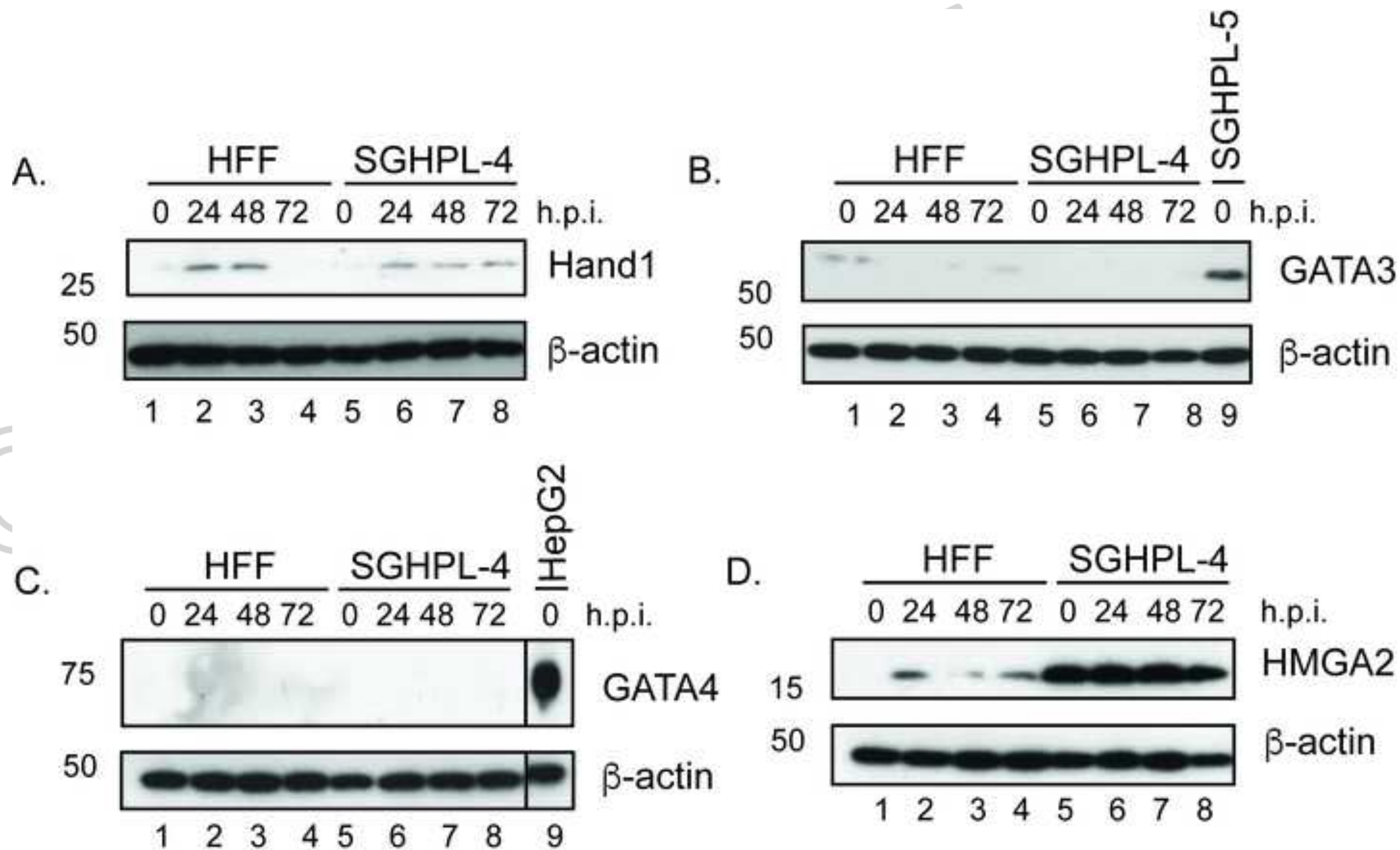

Figure 4

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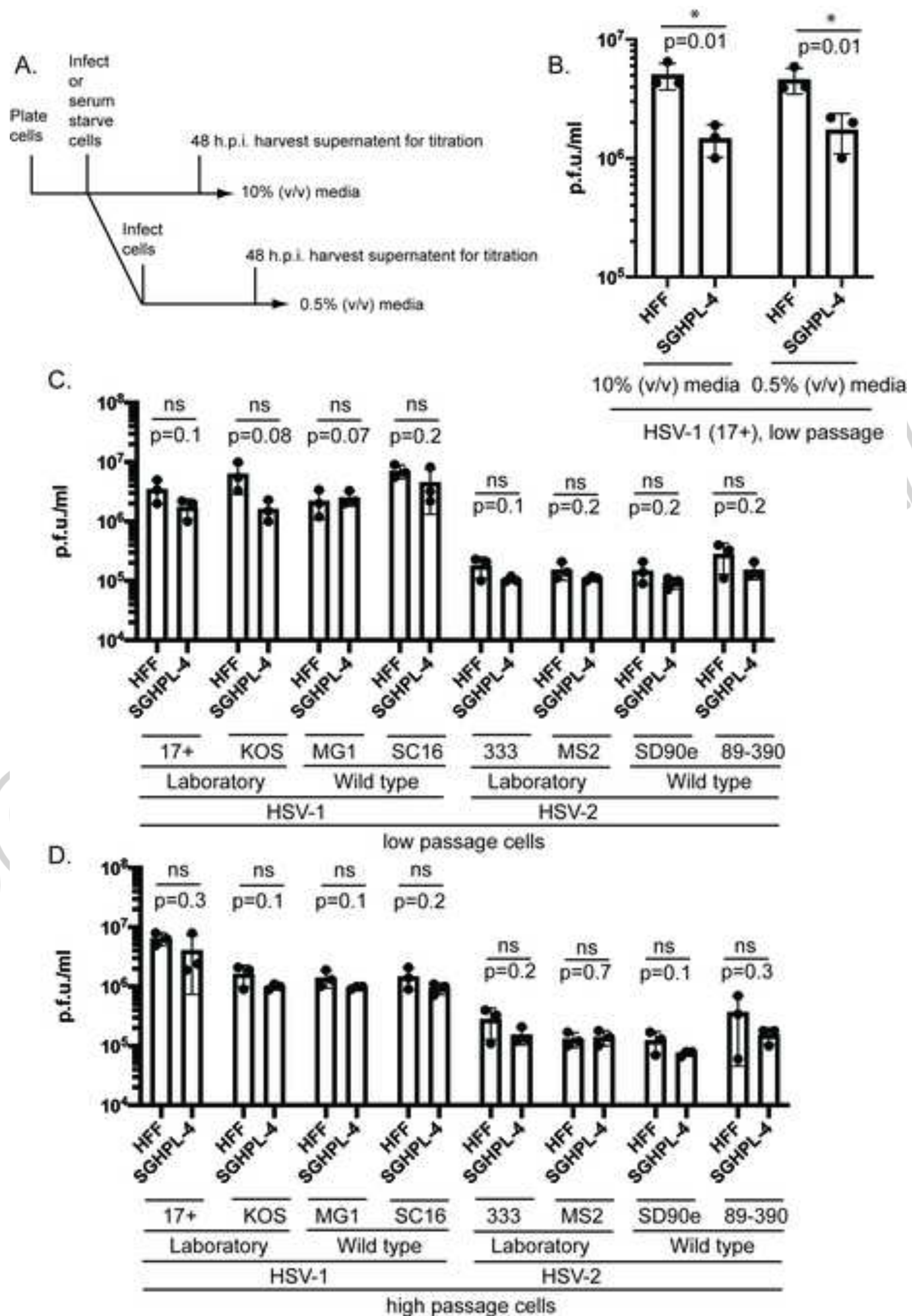

Figure 5

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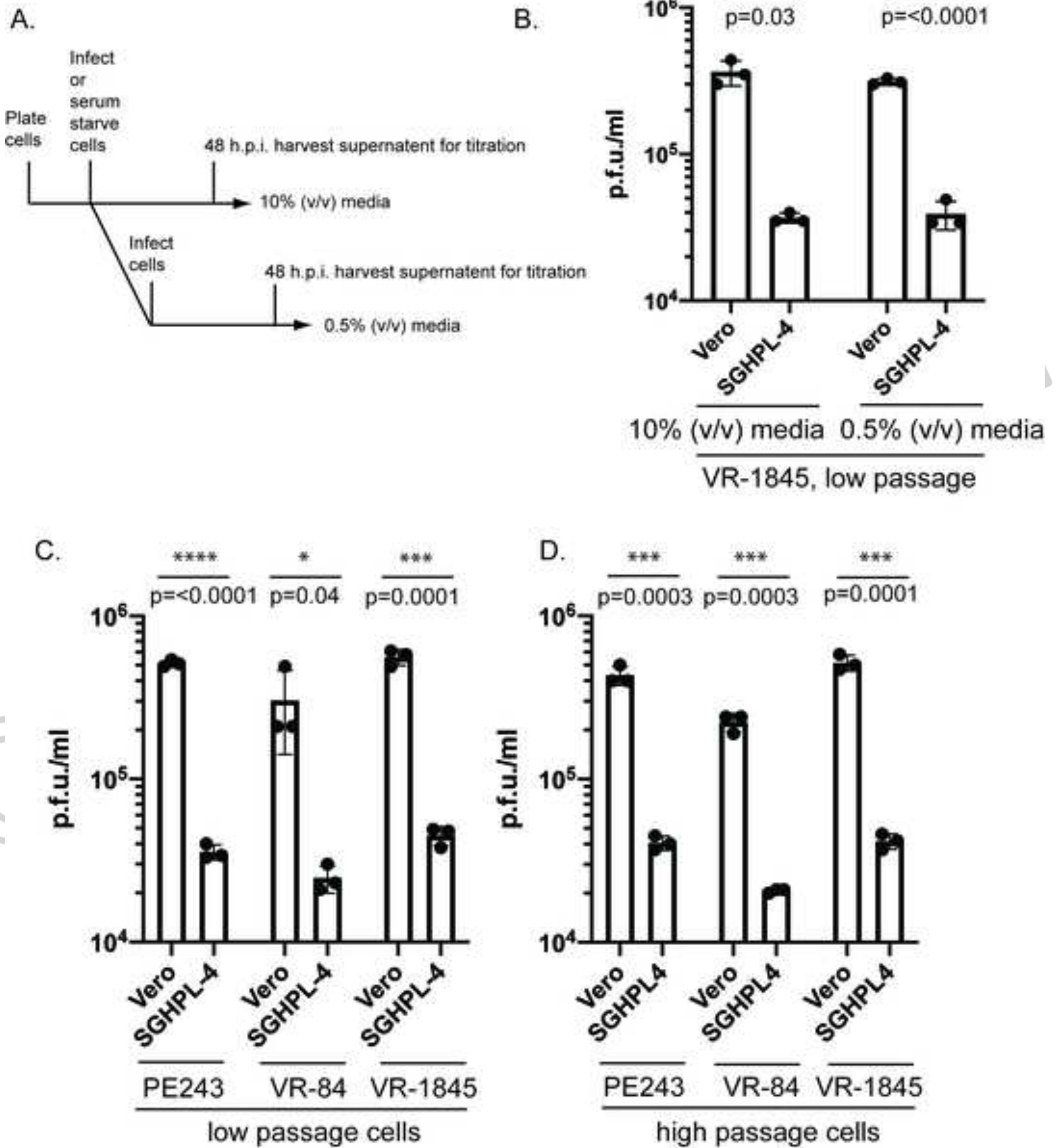

Figure 6

Click here to access/download;Supplementary Material;Supplementary Data.pdf

1 # Supplementary Data

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3 Limited replication of human cytomegalovirus in a trophoblast cell line

4

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7

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12 **Supplementary Figure 1 Analysis of fluorescent protein expression in HFF and**

13 **SGHPL-4 cells.** Low and high passage HFF and SGHPL cells (HFF passage 6, SGHPL-

14 4 passage 13) (A and C, B and D, respectively) were incubated in 0.5% (v/v) media for

15 24 hours before infection with an MOI of 0.5 with green fluorescent protein (GFP)

16 expressing virus Merlin(R1111)UL36GFP (green) or mock infected (grey). After 24 hours

17 uninfected and infected cells were analyzed for GFP expression using FACS. The

18 percentage of uninfected and infected cells detected in the FACS channel detecting GFP

19 in each condition is noted in each panel. The data presented in this figure is representative

20 of two independent experiments.

21

22 **Supplementary Figure 2 Replication of different HCMV strains in HFF and SGHPL-**

23 **4 cells.** (A) Low passage HFF and SGHPL cells (HFF passage 6-10, SGHPL-4 passage

13-17) were incubated in 0.5% (v/v) media for 24 hours before infection with an MOI of 1 with the HCMV strains shown in the figure. After 96 hours post infection in 0.5% (v/v) media viral titre (p.f.u./ml) was determined by titration of viral supernatant on HFF cells. Each data point represents the data from three independent experiments. The bar chart and error bars represent the mean and standard deviation of that data, respectively. The statistical difference between the indicated conditions was measured using an unpaired t test (two-tailed) and is indicated above each figure. A statistically relevant difference was where p=<0.05. Not significant (ns). (B) Low and high passage HFF and SGHPL cells (HFF passage 6, SGHPL-4 passage 14) were prepared for western blotting or incubated in 0.5% (v/v) media for 24 hours before preparation for western blotting. Proteins recognized by the antibodies used in the experiment are indicated to the right of each western blot panel. The presence of β-actin was assayed to assess the amount of cell lysate assayed in each lane. The positions of molecular weight markers (kDa) are indicated to the left of the figure. (C) Cells were infected with Merlin(R1111) as in (A) and virus was harvested at the indicated time points. The data from three independent experiments was presented. The bar chart and error bars represent the mean and standard deviation of that data, respectively.

Supplementary Figure 3 Replication of ZIKV in different cell lines. The cell lines indicated in the figure were incubated in 10% (v/v) media and infected with the ZIKV strain PE243 (MOI 0.1). In all experiments viruses were harvested at 48 hours post infection and viral titre (p.f.u./ml) was determined by titration of viral supernatant on Vero cells. The data from three independent experiments was presented. The bar chart and error bars

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47 represent the mean and standard deviation of that data, respectively. The statistical
48 difference between the indicated conditions was measured using an unpaired t test (two-
49 tailed) and is indicated above each figure. A statistically relevant difference was where
50 $p=<0.05$.
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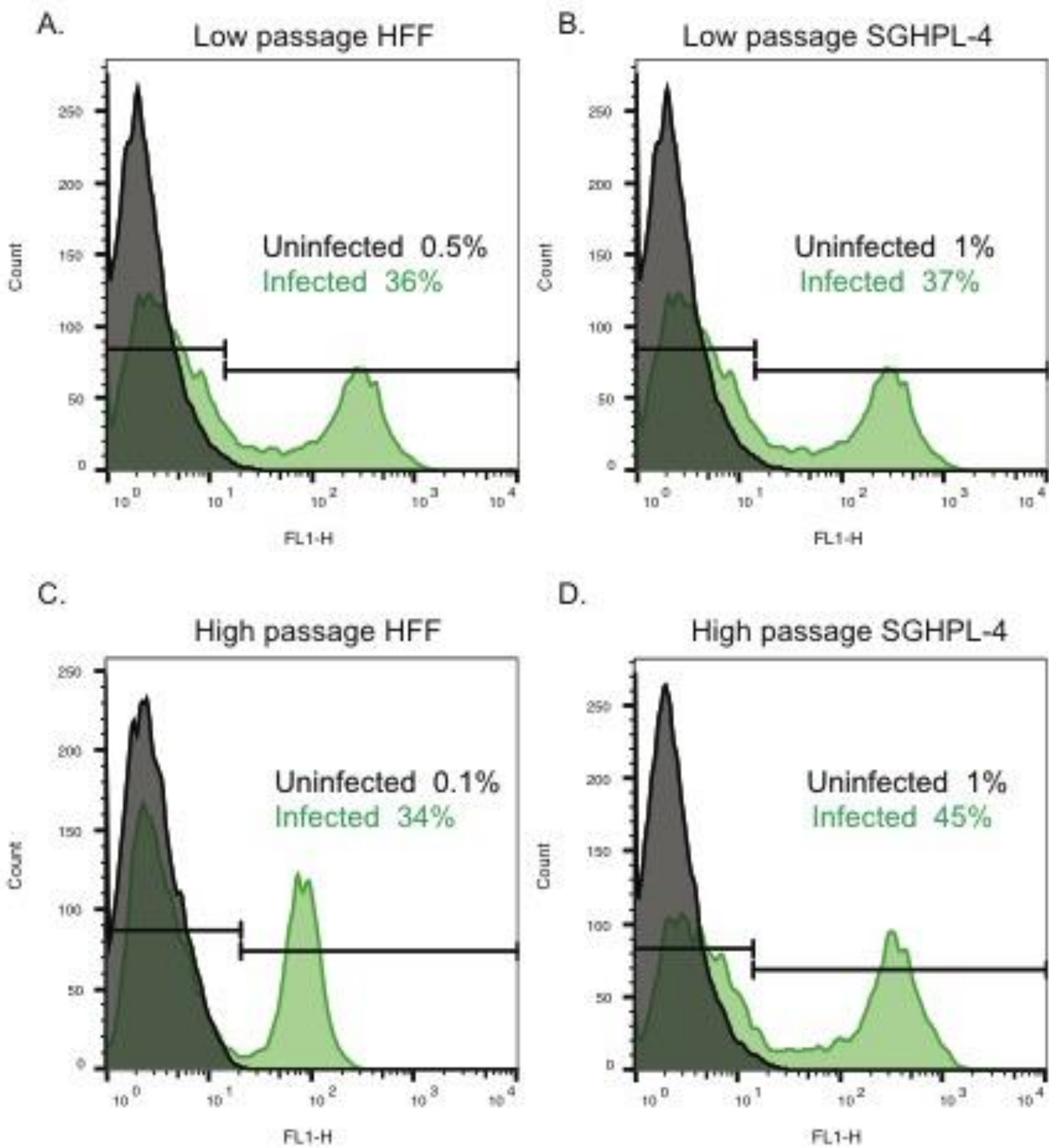

Supplementary Figure S1

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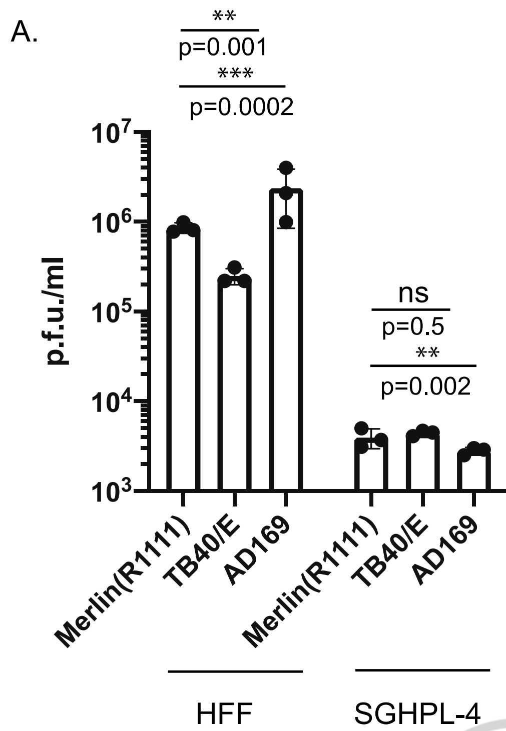

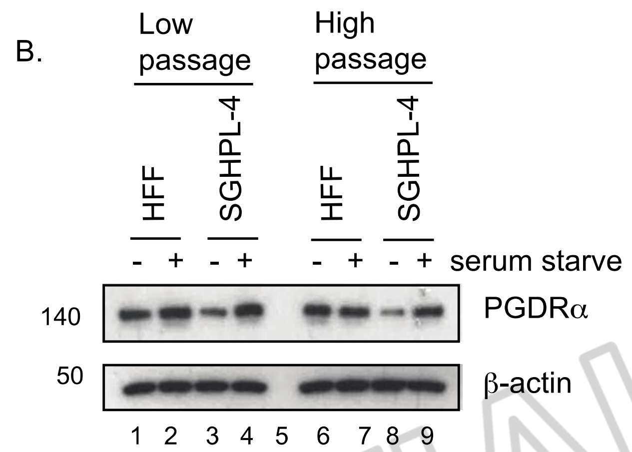

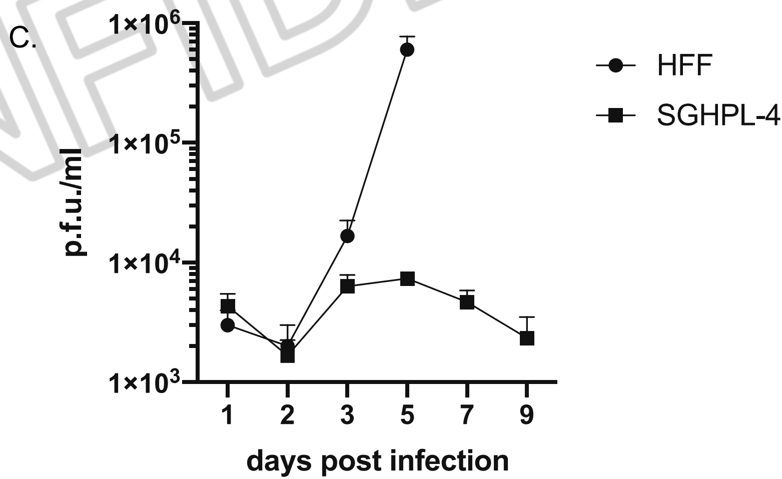

Supplementary Figure S2

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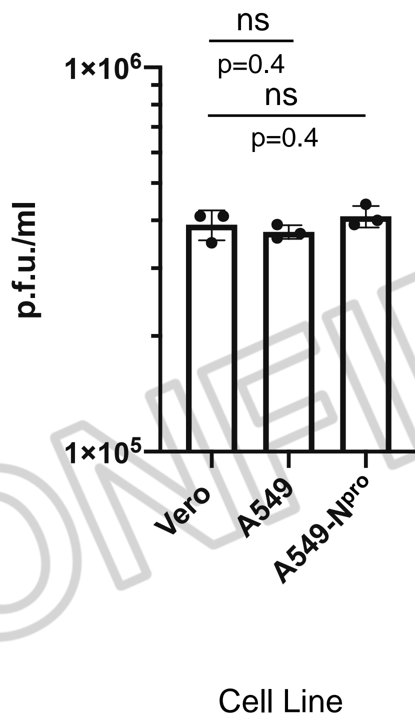

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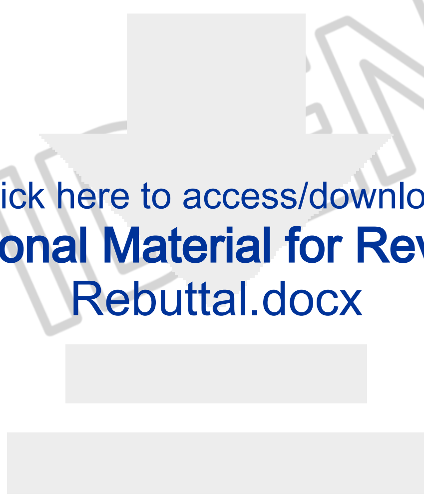

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