Inhibition of Human Cytomegalovirus Replication by Interferon Alpha can involve multiple anti-viral factors

Shabab Chowdhury[a], Katie A Latham[a], Andy C Tran[a], Christopher J Carroll[b], Richard J Stanton[c], Michael P Weekes[d], Stuart J D Neil[e], Chad M Swanson[e] & Blair L Strang[a #]

Institute of Infection & Immunity[a], Institute of Molecular & Cellular Sciences[b], St George's, University of London, London, UK; Division of Infection and Immunity, Cardiff University School of Medicine, Cardiff, UK[c]; Cambridge Institute for Medical Research, School of Clinical Medicine, University of Cambridge, Cambridge, UK[d]; Department of Infectious Diseases, School of Immunology & Microbial Sciences, King's College London, London, UK[e].

Running Title: IFNα inhibits HCMV replication

#Address correspondence to Blair L Strang, bstrang@sgul.ac.uk

ABSTRACT

The shortcomings of current direct acting anti-viral therapy against human cytomegalovirus (HCMV) has led to interest in host directed therapy. Here we re-examine the use of interferon proteins to inhibit HCMV replication utilizing both high and low passage strains of HCMV. Pre-treatment of cells with interferon alpha (IFNα) was required for robust and prolonged inhibition of both low and high passage HCMV strains, with no obvious toxicity, and was associated with an increased anti-viral state in HCMV infected cells. Pre-treatment of cells with IFNα led to poor expression of HCMV immediate-early proteins from both high and low passage strains, which was associated with presence of the anti-viral factor SUMO-PML. Inhibition of HCMV replication in the presence of IFNα involving ZAP proteins was HCMV strain-dependent, wherein a high passage HCMV strain was obviously restricted by ZAP and a low passage strain was not. This suggested that strain specific combinations of antiviral factors were involved in inhibition of HCMV replication in the presence of IFNα. Overall, this work further supports the development of strategies involving IFNα that may be useful to inhibit HCMV replication and highlights the complexity of the antiviral response to HCMV in the presence of IFNα.

INTRODUCTION

The betaherpesvirus human cytomegalovirus (HCMV) remains a notable cause of human morbidity and mortality worldwide (1). While many different vaccine candidates are in development, there is no widely available vaccine against HCMV (1). Those direct acting anti-HCMV drugs currently in clinical use have many shortcomings, including drug resistance and toxicity (1-3).

Recently developed approaches to inhibiting HCMV replication have focused on host directed therapy, which positively or negatively influences cellular factors involved in HCMV replication (3). Prominent examples of these strategies include the use of artemisinin compounds and kinase inhibitors that inhibit cellular factors required for HCMV replication (3). However, other areas of host directed therapy remain largely unexplored and/or require re-examination. This includes exploring use of different interferon proteins to inhibit HCMV replication, as there may be multiple interferon proteins capable of inhibiting HCMV replication. Plus, there are aspects of interferon action on HCMV replication which have not been recently revisited, such as the possible reversible inhibition of HCMV replication in the presence and absence of interferon (4).

Exposure of cells to interferon proteins results in an intracellular anti-viral state, which comes about by interaction of interferon proteins with cell surface receptors, leading to an intracellular signaling cascade, which promotes expression of multiple anti-viral proteins (5). Arguably, the type I interferon

response, stimulated by interferon α or β proteins, is the most widely studied interferon response against HCMV.

Inhibition of HCMV replication by type I interferon proteins has a long history, with early reports suggesting that interferon could not inhibit HCMV replication (6). However, this may have been the result of low concentrations of type I interferon proteins used in the study (6). More recent studies have strongly indicated that the type I interferon response is inhibitory to HCMV replication. This data includes a report where HCMV secretion was inhibited upon administration of IFNα to patients (7), several reports indicating that pre-treatment of cells before infection *in vitro* with type I interferon proteins restricted HCMV replication (4, 8-11), and the observation that inhibition of type I interferon signaling allows greater replication of HCMV *in vitro* (12). Additionally, numerous reports of HCMV encoded mechanisms to antagonize interferon production, antagonize interferon signaling or antagonize the function of anti-viral proteins expressed in response to interferon emphasize that the type I interferon response is a barrier to HCMV replication (13-15).

However, it is not well understood how the presence of type I interferon proteins affects the molecular mechanisms of HCMV replication, in particular the ability of different HCMV strains to evade inhibition by the type I interferon response. We have recently demonstrated that a low passage strain of HCMV whose genome content is similar HCMV clinical isolates (strain Merlin) was able to efficiently inhibit the production of the anti-viral factor zinc finger antiviral protein (ZAP) in response to type I interferon, whereas a high passage laboratory

strain commonly used in laboratory experiments and containing a genomic deletion plus several mutations (strain AD169) could not (16). Comparison of how these different HCMV strains might replicate in the presence of type I interferon proteins and how this might relate to the action of anti-viral factors such as ZAP has not, to our knowledge, been performed.

HCMV interaction with interferon proteins beyond the type I response also requires further exploration. For example, the interaction of HCMV with the type III interferon response. Although type I (IFNα/β) and III (IFNλ) interferon proteins utilize different receptor complexes to activate intracellular signaling, the repertoires of anti-viral proteins produced by each response is broadly similar (5). These repertoires include proteins such ZAP (17), that we and others have demonstrated are anti-HCMV proteins (16, 18, 19). This suggests that, like IFNα/β, IFNλ proteins could inhibit HCMV replication. This is supported by reports that certain gene alleles associated with expression of IFNλ proteins can positively or negatively affect control of HCMV replication *in vivo* (20-22) and a report that treatment of intestinal cells with IFN$\lambda 3$ could limit HCMV protein production (23). HCMV replication in the presence of IFN$\lambda 3$ was not assessed in the aforementioned study (23).

Additionally, there may be long-standing observations about the interaction of HCMV and interferons that may need to be revisited to understand how those proteins can be used in a therapeutic setting.. A previous study examining treatment of HCMV infected human fibroblast cell cultures from foreskin biopsies with type I interferon indicated that inhibition of HCMV

replication was reversible (4). Similar observations have been made upon infection of murine cells, including fibroblastic murine cells, with a betaherpesvirus related to HCMV, murine cytomegalovirus (MCMV) (24-26). In those experiments the presence of type I interferon could promote MCMV latency (24-26). Together, these data suggest reversible inhibition of virus replication by type I interferon is a common feature of betaherpesvirus replication. To our knowledge, the reversible effects of type I interferon on HCMV replication has not been revisited since (4).

As we have previously suggested that there may be differences in the ability of HCMV strains to inhibit responses to IFNα (16), we set out to test the ability of a high passage laboratory strain (AD169) and a low passage strain Merlin(R1111), similar to wild type HCMV, to replicate in the presence of a IFNα or IFNλ3. While IFNλ3 had very little effect on HCMV replication, we observed robust and prolonged inhibition of HCMV replication upon pre-treatment of cells with IFNα before HCMV infection, with no obvious toxicity. This inhibition of HCMV replication was associated with loss of immediate early protein expression and the presence of the known anti-viral protein PML-SUMO. Consistent with our previous observations (16), the role of ZAP in inhibiting HCMV replication was strain dependent; wherein ZAP inhibited replication of AD169, but Merlin considerably less so. Our observations emphasize that multiple anti-viral proteins, including ZAP, are likely responsible for inhibition of HCMV replication in the presence of IFNα.

MATERIALS & METHODS

Cells and viruses

Human foreskin fibroblast (HFF) cells (clone Hs27) were obtained from American Type Culture Collection, no. CRL-1634 (ATCC, Manassas, VA). Bulk populations of HFF cells containing CRISPR inhibiting expression of either Luciferase or all ZAP isoforms have been previously described (16). All cells were maintained in complete media: Dulbecco's Modified Eagle's Medium (DMEM) (Gibco) containing 10% (v/v) fetal bovine serum (FBS) (Gibco), plus 1% mixture of penicillin and streptomycin.

HCMV strain AD169 was a gift from Donald Coen (Harvard Medical School). The generation of HCMV strain Merlin(R1111), which contains mutations in genes RL13 and UL128 to allow release of cell-free virus, from a bacmid has been reported previously (27). AD169 virus expressing green fluorescent protein (GFP) from an ectopic site in the AD169 genome under control of the HCMV major immediate early (IE) promoter (AD169-GFP) was generated from a bacmid as described in (28). Merlin(R2582), a virus derived from Merlin(R1111) that expresses a fusion peptide of the HCMV IE protein UL36 and GFP separated by a self-cleaving P2A protein sequence, has also been previously described (29). In all cases, titers were determined by serial dilution of viral supernatant onto HFF monolayers, which were then covered in DMEM containing 5% (v/v) FBS, antibiotics and 0.6% (w/v) methylcellulose. After

incubation for 14 days, cells were stained with crystal violet and plaques in the infected cell monolayers were counted. Titre was expressed as plaque-forming units (p.f.u.)/ml.

Viral yield reduction assays

HFF cells were plated at 5×10^4 cells per well in 24-well plates. After overnight incubation, cells were either infected with 5×10^4 plaque forming units (p.f.u.) of HCMV or incubated for a further 24 hours in the presence or absence of interferon or drug without infection. After this further 24 hour period cells incubated in the presence or absence of interferon or drug without infection were infected with 5×10^4 p.f.u. of HCMV. HCMV viruses used in each experiment are indicated in the text and figure legends. In each case, after virus adsorption for 1 hour at 37°C, cells were washed and incubated with 0.5 ml of media in the presence or absence of interferons or drug throughout virus replication. Infected cells were incubated for 96 hours at 37°C before supernatant was removed from cells for analysis of virus titre by plaque counting, as described in the previous section. Data showing virus production over time is shown in Figure S1A.

Interferons and Ruxolitinib

Interferon-α (INTRON A (interferon alfa-2b), Merck) was a kind gift from Steve Goodbourn (St George's, University of London). Interferon-λ3 was purchased from Bio-Techne. Both interferon proteins were resuspended in complete cell culture media. Ruxolitinib was purchased from Cambridge Bioscience and resuspended in dimethyl sulfoxide (DMSO). In all experiments cells were treated with 1000U/ml of Interferon-α (or the equivalent volume of complete cell culture), 100 ng/ml Interferon-λ3 (or the equivalent volume of complete cell culture) or 10 μM Ruxolitinib (or the equivalent volume of DMSO). The final concentration of DMSO in all experiments was maintained at <1% (v/v). In all experiments, cells were treated with the concentrations of interferons mentioned above as these were the concentrations at which maximum inhibition of HCMV replication was observed. Use of higher concentrations of interferons did not yield greater inhibition of HCMV replication (Fig. S1B and S1C).

Western blotting

Conditions under which HFF or HFF CRISPR cells were infected are detailed in the text and figure legends. Lysate of uninfected or infected cells were prepared for western blotting by washing the cells once in phosphate-buffered

196 saline (PBS, SIGMA), suspending the cells directly in 2 x Laemmli buffer
197 containing 5% β–mercaptoethanol, and incubating at 95°C for 5 min.

198 Proteins were separated on 8% or 10% (v/v) polyacrylamide gels and
199 transferred to a Hybond-ECL membrane (Amersham Biosciences) using a semi-
200 dry protein transfer apparatus. The membranes were blocked at room
201 temperature for at least 90 min using TBS containing 0.1% Tween-20 and 5%
202 dried powdered milk (TBSTM) and then incubated overnight at 4°C in TBSTM
203 plus primary antibodies: antibodies recognizing HCMV IE1/2, UL57, or pp28, (all
204 Virusys, 1:1000 dilution), β-actin (SIGMA, 1:5000 dilution), ZAP (Abcam,
205 ab154680, 1:5000 dilution, recognizing all ZAP isoforms), MxA, STAT1-Tyr701p
206 (both Cell Signaling, #43575, 1:1000 dilution), PML (Bethyl, A301-167A, 1:1000
207 dilution), MRPS39 (PTCD3) (ProteinTech, 25158-1-AP, 1:1000 dilution) and
208 TOM20 (ProteinTech, 11802-1-AP, 1:1000 dilution).

209 After incubation in TBSTM with primary antibodies, the membrane was
210 washed extensively with TBST and incubated for 60 min at room temperature
211 with TBSTM containing anti-mouse- or anti-rabbit-horseradish peroxidase (HRP)
212 conjugated antibodies (Millipore and Cell Signaling Technologies, respectively),
213 to detect primary antibodies. After further washing with TBST and TBS,
214 chemiluminescence solution (GE Healthcare) were used to detect secondary
215 antibodies on x-ray film (GE Healthcare).

216 Where indicated in the text relative band intensity (band intensity relative
217 to β-actin signal in the same lane) was analyzed using ImageJ software, obtained
218 from the NIH (USA).

Cell number and viability (MTT) assays

To count cells, HFF cells were seeded at high or low concentrations cells per well into 24-well plates. High numbers of cells (5×10^4 cells per well) were to assess cell viability, whereas low numbers of cells (5×10^3 cells per well) were to assess both cell viability and cell proliferation. After overnight incubation to allow cell attachment, cells were treated for 96 hours with IFNα or left untreated. Cells were removed from wells using trypsin, resuspended in complete cell media and counted using a Countess Automated Cell Counter (Invitrogen) using the manufacturers instructions.

In MTT assays, HFF were seeded at high (5×10^3 cells per well) or low (5×10^2 cells per well) numbers cells per well into 96 well plates. After overnight incubation to allow cell attachment, cells were treated for 96 hours with IFNα or left untreated. MTT assays were carried out on cells in the wells of 96 well plates according to the manufacturer's instructions (GE Healthcare). 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 in colorimetric assay, read on a FLUOstar Omega Microplate Reader.

Flow cytometry analysis

Cells were infected with GFP expressing viruses as described in the text and figure legends. At 24 hours post infection, uninfected and infected cells were trypsinized, washed once in phosphate buffered saline (PBS), and then resuspended in PBS. GFP expression in cells was analyzed using flow cytometry. In each case, 10,000 cells were acquired using a Beckman Coulter CytoFLEX S cytometer. Data were analyzed using FlowJo V10 to determine number of GFP positive cells and mean fluorescent intensity of GFP positive cells.

RESULTS

Pre-treatment of HFF cells with IFNα robustly inhibited replication of HCMV

We compared inhibition of AD169 and Merlin(R1111) replication when IFNα was added to human foreskin fibroblast (HFF) cells at the time of infection or when HFF cells were pre-treated with IFNα before infection (Figs. 1A(i) and 1A(iv), respectively). Addition of IFNα at the time of infection had a modest effect on AD169 replication, but no obvious impact on Merlin(R1111) replication (Figs 1A(ii) and (iii)). However, pre-treatment of HFF cells with IFNα before infection resulted in robust inhibition of both AD169 and Merlin(R1111) replication (Figs. 1A (v) and (vi)), indicating that setting an anti-viral state within HFF cells before infection was required for robust inhibition of replication of both HCMV strains.

To confirm that inhibition of type I interferon signaling before infection could influence HCMV replication, in the absence of exogenous IFNα HFF cells were also pre-treated with the Janus-kinase inhibitor Ruxolitinib (Fig. 1B(i)), which inhibits intracellular signaling from the type 1 interferon receptor. Treatment of HFF cells with Ruxolitinib resulted in increased replication of both AD169 and Merlin(R1111) (Fig. 1B(ii) and 1B(iii)), confirming that type 1 interferon signaling could inhibit replication of both HCMV strains.

We also analyzed how pre-treatment of HFF cells with IFNα before infection stimulated an anti-viral state. We analyzed expression of MxA, a canonical protein expressed in response to type 1 interferon proteins, in the

presence and absence of IFNα (Fig. 2). The presence of IFNα stimulated MxA expression in uninfected HFF cells. In AD169 and Merlin(R1111) infected HFF cells pre-treated with IFNα, MxA expression was greater than in HFF cells not pre-treated with IFNα. This increased anti-viral state in infected HFF cells pre-treated with IFNα largely persisted over the course of infection in HFF cells infected with both HCMV strains, which could explain why robust inhibition of both AD169 and Merlin(R1111) was observed in Figure 1.

Treatment with IFNα did not cause overt cellular cytotoxicity

As shown by many small molecule inhibitors of HCMV replication (3), anti-viral activity can be due to cellular cytotoxicity of compounds, not an anti-viral effect. Therefore, to ensure that inhibition of HCMV replication by AD169 and Merlin(R1111) seen in Figure 1 was not due to cellular cytotoxicity caused by exposure of cells to IFNα, we analyzed cell division (Fig. S2A) and cellular cytotoxicity (Fig. S2B) in uninfected cells exposed to IFNα. These assays were carried out using a high number of HFF cells (similar to that used in Figs. 1 and 2) to examine cell viability and a low number of HFF cells to examine both cell viability and cell proliferation of IFNα treated cells.

Treatment of HFF cells with IFNα did not result in any reduction in cell numbers using high or low numbers of cells (Fig. S2A). However, when cells were analyzed with a cytotoxicity assay that measures mitochondrial activity

298 (MTT assay), treatment of HFF cells with IFNα resulted in a modest deficit in cell
299 viability at low numbers of cells (Fig. S2B). When HFF cells were analyzed for
300 expression of mitochondrial proteins (mitochondrial ribosome protein MRPS39
301 and mitochondrial membrane protein TOM20), no difference in the expression of
302 mitochondrial proteins was found in the presence and absence of IFNα (Fig.
303 S1C). Therefore, the deficit in mitochondrial activity in the presence of IFNα at
304 low cell concentrations was not due to a deficit in number of mitochondria in
305 those conditions. Overall, these data indicated that exposure of cells to IFNα did
306 not cause obvious cytotoxicity and, therefore, indicated that the inhibition of
307 HCMV replication seen in Figure 1 was unlikely to be due to cytotoxic effects of
308 IFNα on cells. However, IFNα could have very modest effect on HFF cell viability
309 under certain conditions, which may have had a minor influence on the ability of
310 HFF cell cultures to support HCMV replication.
311
312 **Robust inhibition of HCMV replication by IFNα, but not IFNλ3**
313
314 Before examining the action of IFNα in greater detail, we also compared
315 the ability of IFNα and IFNλ3 to inhibit HCMV replication. As in Figure 1, pre-
316 treatment of HFF cells with IFNα resulted in robust inhibition of AD169 and
317 Merlin(R1111) replication (Figs. S3A(i) and S3A(ii)). Pre-treatment of HFF cells
318 with IFNλ3 resulted in a modest, but not statistically significant, inhibition of either
319 HCMV strain (Fig. S3A(i) and S3A(iii)). Consistent with these observations, we
320 observed obvious differences in the ability of IFNα and IFNλ3 to stimulate

phosphorylation of STAT1 (STAT-Tyr701p, which occurs on activation of both the type I and type III interferon receptors) and the expression of MxA (Fig. S3B). Thus, the differences in the ability of the different interferon proteins to inhibit HCMV replication likely reflected their abilities to simulate an anti-viral state in HFF cells. In this experimental setting only IFNα could robustly inhibit HCMV replication.

Inhibition of HCMV replication was associated with inhibition of HCMV entry and protein expression

To understand how IFNα inhibited HCMV replication, we examined expression of proteins produced from each class of HCMV transcription (Fig. 3A). Pre-treatment of HFF cells with IFNα resulted in poor protein expression from each class (IE1/2, UL57 and pp28) in both AD169 and Merlin(R1111) infected HFF cells (Fig. 3B). Poor of UL57 and pp28 expression was likely the result of poor immediate-early protein (IE1/IE2) expression. IFNα inhibited IE protein production at all times points tested during AD169 and Merlin(R1111) replication (Fig. S1C).

We then sought to understand if poor of IE1/2 expression in the presence of IFNα was due to a defect in HCMV entry into the cell or due to lack of protein expression in infected cells. Therefore, we assayed the ability of HCMV viruses expressing green fluorescent protein (GFP) (AD169-GFP and Merlin(R2582) (28, 29) to infect cells. We reasoned that if the number of cells expressing GFP in the

presence and absence of IFNα was similar, IFNα did not inhibit HCMV entry into the cell. Furthermore, we reasoned that if the number of cells expressing GFP was similar in the presence and absence of IFNα, but the fluorescence intensity of GFP expressing cells decreased in the presence of IFNα, then pre-treatment of cells with IFNα did not inhibit HCMV entry into the cell, but did inhibit protein expression from the HCMV genome.

We observed that pre-treatment of HFF cells with IFNα resulted in a decrease in both the number of cells expressing GFP and the fluorescence intensity of GFP in infected cells (Figs. 3C and 3D), albeit the effect of IFNα on Merlin(R2582) was very modest in one experimental replicate, affecting the overall statistical significance of the data (Fig. 3D). Overall, however, we interpreted the data in Figure 3D as pre-treatment of HFF cells with IFNα possibly led to both inhibition of HCMV entry into the cell and inhibition of HCMV protein expression in the infected cell.

Inhibition of HCMV replication was associated with the presence of PML-SUMO proteins

We reasoned that inhibition of HCMV replication was caused by anti-viral proteins expressed in the presence of IFNα. A range of anti-viral proteins expressed in response to type I interferon proteins have anti-HCMV activity (18). To our knowledge, none of these proteins are known to inhibit HCMV entry into

366 the cell. Therefore, we focused on examining anti-viral proteins expressed in the
367 presence of IFNα that could inhibit IE protein production.

368 The first anti-viral barrier to replication that the HCMV genome likely
369 encounters in the nucleus are PML bodies. Proteins in PML bodies, including
370 PML, come into contact with incoming HCMV genomes and form structures that
371 can contribute to inhibition of HCMV transcription (30), including transcriptional
372 inhibition of the alternatively spliced HCMV mRNA that encodes IE1 and IE2.
373 Key to the anti-HCMV function of PML is the post-translational addition of SUMO
374 proteins to PML (PML-SUMO) (31). Antagonism of PML SUMOylation by HCMV
375 IE1 antagonizes the anti-viral effects of PML bodies (31). It has been
376 demonstrated that transcription of mRNA encoding PML and other anti-viral
377 proteins in PML bodies is stimulated by the presence of IFNβ during HCMV
378 infection (32).

379 As in Figure 3, pre-treatment of HFF cells with IFNα led to loss of IE1 and
380 IE2 expression. Expression of PML isoforms can vary between different cell
381 lines, as can the molecular weights of those isoforms (31, 33, 34). Here we
382 observed expression of PML forms in our HFF cell line similar to those previously
383 reported for another HFF cell line (34). In the absence of virus or IFNα a version
384 of PML (isoform I (PML-I)) was detectable at approximately 120kDa, as could at
385 least one high molecular weight SUMOylated form of PML-I, but smaller
386 molecular weight isoforms of PML could not be detected (Fig. 4). In the presence
387 of virus without IFNα treatment, PML-I expression increased, but expression of
388 SUMOylated PML-I proteins increased less so (Fig. 4), consistent with

antagonism of PML SUMOylation by IE1 (31). However, upon treatment with IFNα high molecular weight forms of SUMOylated PML-I could be observed in HCMV infected cells (Fig. 4). Therefore, a combination of both IFNα treatment and HCMV replication led to an anti-viral state associated with the presence of PML-SUMO proteins in both AD169 and Merlin(R1111) in infected HFF cells. As PML-SUMO was present in uninfected HFF cells treated with IFNα, it is possible that expression of IE1 from incoming HCMV genomes was not sufficient to overcome the block to HCMV replication by PML-SUMO proteins already present in the cell. Therefore, the presence of PML-SUMO in infected HFF cells treated with IFNα was associated with poor immediate-early protein expression.

Inhibition of HCMV replication in the presence of IFNα by ZAP was strain-dependent

Anti-viral proteins other than PML expressed in the presence of IFNα (IDO1, RIPK2) can inhibit HCMV replication (35, 36) and may have been involved in inhibition of HCMV replication that we observed here. Additionally, we and others have recently observed that ZAP proteins can inhibit HCMV replication, including the ZAP isoform whose expression is stimulated by IFNα, ZAP-S (16, 18, 19). Additionally, we found that inhibition of HCMV replication by ZAP was strain-dependent, as AD169 replication was restricted by ZAP but Merlin(R1111) replication was not (16). These phenotypes were associated with downregulation of ZAP-S in Merlin(R1111) infected cells, but not AD169 infected

412 cells (16). As we did not see strain-dependent differences in resistance to
413 IFNα in IFNα pre-treated cells, we investigated ZAP expression in cells pre-
414 treated with IFNα.

415 We analyzed ZAP isoform expression in the presence and absence of
416 IFNα. Pre-treatment of HFF cells with IFNα resulted in expression of ZAP-S in
417 uninfected cells (Fig. 5A). Upon infection, pre-treatment of HFF cells with IFNα
418 resulted in greater expression of both ZAP-L and ZAP-S in both AD169 and
419 Merlin(R1111) infected HFF cells (Fig. 5). However, consistent with our previous
420 observations (16), we observed an obvious decrease in ZAP-S expression over
421 time in Merlin(R1111), but not AD169, infected HFF cells in the presence and
422 absence of IFNα (Fig. 5). However, similar to expression of MxA (Fig. 2), we
423 found that expression of both ZAP isoforms was greater in the presence of IFNα,
424 suggesting that although ZAP-S expression obviously decreased in
425 Merlin(R1111) infected cells, there was sufficient ZAP-S to inhibit Merlin(R1111)
426 replication in HFF cells pre-treated IFNα.

427 We then assayed HCMV replication in the presence and absence of ZAP
428 isoforms. Using bulk populations of HFF cells containing CRISPR that inhibited
429 the expression of both ZAP isoforms (ZAP-L and ZAP-S) (16), we confirmed that
430 the loss of ZAP expression did not compromise the ability of IFNα to set an anti-
431 viral state (37), as similar MxA expression was seen in the presence and
432 absence of ZAP proteins in IFNα treated cells (Fig. 6A). Additionally, this data
433 confirmed the loss of ZAP isoform expression in HFF cells.

Upon examination HFF cells infected with AD169 or Merlin(R1111) that did or did not express ZAP isoforms, we found that pre-treatment of HFF cells with IFNα before infection resulted in a decrease in HCMV replication in all conditions tested (Figs. 6B(i) and 6B(ii)). However, in agreement with our previous observations (16), we found that in the presence and absence of IFNα loss of ZAP proteins could increase AD169, but not Merlin(R1111), replication (Figs. 6b(ii) and (iii)). As in our previous work, we interpret this strain-dependent inhibition of HCMV replication as the ability of Merlin(R1111), but not AD169, to control ZAP expression, in particular ZAP-S (16). Thus, although ZAP isoform expression was greater in the presence of IFNα (Fig. 5A), the decrease in ZAP-S expression observed (Fig. 5A) may have been sufficient to prevent inhibition of Merlin(R1111) replication by ZAP.

In summary, IFNα could inhibit replication of both AD169 and Merlin(R1111), but in the presence of IFNα ZAP was an inhibitor of AD169, but not Merlin(R1111). Therefore, there may be strain-dependent combinations of anti-viral proteins inhibiting replication of either AD169 or Merlin(R1111). The combinations of proteins inhibiting either AD169 or Merlin(R1111) replication can include known anti-HCMV proteins expressed in the presence of type I interferon proteins, such as ZAP, PML-SUMO, RIPK2 and IDO (16, 18, 19, 31, 35, 36).

IFNα had prolonged repressive effects on HCMV replication

It has previously been reported that inhibition of HCMV replication in human fibroblast cultures derived from foreskin biopsies by IFNα was reversible (4), similar to observations made upon infection of several murine cell types with MCMV, including fibroblastic murine cells (24-26). Reversible inhibition of HCMV replication would not be advantageous to any therapeutic strategy. Therefore, we investigated if the action of IFNα on HCMV replication was reversible, as previously reported (4).

As above (Fig. 1), pre-treatment of HFF cells with IFNα with continuous treatment of infected HFF cells with IFNα led to robust inhibition of AD169 and Merlin(R1111) replication, which was associated with loss of IE1 and IE2 expression (Figs. 7A(i), 7A(ii) and 7B). However, near identical results were found when infected HFF cells were pre-treated with IFNα, but IFNα was not maintained in the cell culture from the time of infection (Figs. 7A(i), 7A(iii) and 7B). Therefore, the anti-viral state set by pre-treatment of HFF cells with IFNα could not be overcome by either HCMV strain when IFNα was absent after infection, demonstrating that in these experimental conditions IFNα had prolonged repressive effects on HCMV replication. We attempted to maintain HCMV infected HFF cells infected with high and low MOIs and treated with IFNα in culture past 7-10 days. However, this resulted in destruction of cell monolayers and no infectious HCMV virus could be detected (data not shown). This suggested that in our hands inhibition of HCMV replication by IFNα may have been irreversible. Consistent with our analysis in the previous sections, these

prolonged or irreversible effects could have been caused by a combination of anti-viral factors acting on HCMV.

DISCUSSION

When IFNα was added to HFF cells at the time of infection replication of the high passage laboratory strain AD169 was modestly inhibited, whereas replication of the low passage HCMV strain Merlin was not. This was consistent with our previous observations that compared to Merlin, AD169 did not effectively control the anti-viral state cause by type I interferons at the time of infection (16, 38), likely due to downregulation of type I interferon signaling proteins in Merlin infected cells (38). However, when cells were exposed to IFNα before infection, replication of both HCMV strains was robustly inhibited. Therefore, there were strain-dependent differences in the ability of HCMV to evade the type I interferon response, but this was dependent upon when HCMV encountered the intracellular anti-viral state stimulated by IFNα. This emphasized that if IFNα is to be used in a therapeutic setting it would be advantageous to ensure that it is administered early in infection so that HCMV encounters the intracellular anti-viral state when the virus enters the cell. Additionally, our data suggests that high concentrations of IFNα may be required to efficiently inhibit HCMV replication. Both of these points would make therapeutic use of IFNα challenging and may help explain contradictory findings about the inhibitory effects of outlined in the Introduction (6, 7). However, the apparent prolonged effects of IFNα on HCMV replication may indicate that continual treatment with IFNα is not required.

In this study we examined the ability of IFNα to inhibit replication of HCMV strains that infect cells as cell-free virus. It has been previously demonstrated

that replication of cell-associated HCMV Merlin was not obviously inhibited in cells pre-treated with type I interferons (11). Thus, treatment of HCMV patients using exogenous IFNα may inhibit replication of cell-free virus during HCMV transmission and have less effect on virus dissemination *in vivo*.

In contrast to the robust inhibition of HCMV replication by IFNα, we found a modest, but not statistically significant, inhibition of HCMV replication by IFNλ3. This was likely due to the inability of IFNλ3 to set a robust anti-viral state within cells. This may have been due to weak interactions between IFNλ3 and its cognate cell surface receptor (39) or differences in expression of type I and type III receptors on the cell line used here. To our knowledge, there are no reports testing the ability of IFNλ proteins other than IFNλ3 (IFNλ1, IFNλ2 and IFNλ4) to inhibit HCMV replication. Our data suggests that further examination of interaction between HCMV and IFNλ3 may be of interest in attempts to inhibit HCMV replication. These experiments will likely require development of novel infectious models of HCMV replication involving cell lines that are highly susceptible to activation of an anti-viral state via IFNλ proteins, such as hepatocyte or intestinal cells (39). Such models do not appear to be available at present. For example, IFNλ3 has been shown to inhibit IE protein expression in a transformed intestinal cell line (23), but transformed cells such as those do not support HCMV replication.

The intrinsic ability of PML bodies to inhibit transcription from the HCMV genome, and their antagonism by the HCMV protein IE1, has been widely studied (40, 41). The anti-viral role of PML bodies in response to type I interferon

proteins has been previously investigated, showing that an increase in mRNA encoding the HCMV inhibitory proteins, including PML, in response to IFNβ produced from cells upon HCMV infection (32). Here, we expand on those observations (32) by analyzing protein expression, showing that while PML protein is expressed in response to IFNα, expression of both PML and PML-SUMO is greatest in HCMV infected cells treated with IFNα. This overexpression of PML and PML-SUMO that HCMV encounters when it entered the cell and attempted to replicate was most likely a major cause of inhibition of immediate-early protein expression and, therefore, HCMV replication.

However, other factors were likely to be involved in inhibition of HCMV replication in cells treated with IFNα. For example, screens of proteins produced in response to type I interferon signaling indicate that anti-viral factors such as RIPK2 and IDO are likely to inhibit HCMV replication in the conditions we use here (18, 35, 36). We also investigated the role of ZAP in inhibition of HCMV replication (16, 18, 19). Consistent with our previous observations (16), we found differences in the ability of AD169 and Merlin(R1111) to evade ZAP function. However, we conclude that the ability of IFNα to inhibit replication of HCMV was likely due to a combination of anti-viral proteins expressed in response to IFNα, including ZAP and SUMO-PML, and there may be strain-dependent differences in anti-viral factors required to inhibit replication of different HCMV strains.

Contrary to a previous report (4), we found that IFNα caused prolonged, or perhaps irreversible, inhibition of HCMV replication under the conditions we used here. In the aforementioned study (4), HCMV replication was undetectable for up

to 16 days in the presence of IFNα and then could be detected when IFNα was withdrawn from the culture of fibroblasts derived from foreskin biopsies at 16 days post infection. It is possible that the differences in our studies are the result of differences in experimental procedure. We speculate that the HFF cell line used in our studies and the primary cell cultures taken from foreskin biopsies used previously (4) may be able to support HCMV replication in different ways. We further speculate that primary cell cultures from foreskin biopsies used previously (4) may have contained as yet unrecognized fibroblastic or monocytic cells capable of supporting IFNα-dependent HCMV latency and reactivation. However, while different conditions for this experiment could be explored, we demonstrate conditions under which inhibition of HCMV replication is irreversible, which will be useful for considering future therapeutic strategies involving IFNα.

Additionally, our data contrasts with that seen during MCMV infection and suggests that reversible inhibition of virus replication by type I interferons may not be a feature conserved across betaherpesvirus replication. In further contrast, previous reports link the presence of IFNα to the establishment of MCMV latency and the withdrawal of IFNα from MCMV infected murine cell cultures was required for reactivation of MCMV from latency (24-26). Whereas proteins expressed in response to type I interferon proteins are down regulated during HCMV latency in human monocytes (42), indicating that the type I interferon response is detrimental to the establishment of HCMV latency and/or HCMV reactivation from latency. Thus, the two betaherpesvirues have different relationships with the type I interferon response during virus latency. That said,

our observations made in this study and aforementioned work on HCMV latency (42) suggest that treatment with IFNα will have inhibitory effects on both productive HCMV replication and HCMV latency/reactivation.

Any future strategy targeting HCMV replication or HCMV latency/reactivation may benefit from utilizing newly developed IFNα mutants that can stimulate an antiviral response without potently stimulating immunomodulatory or antiproliferative effects that may be harmful *in vivo* (43-46).

ACKNOWLEDGMENTS

We would like to express our thanks to Don Coen and Steve Goodbourn for providing reagents. We also thank Connor Bamford, Steeve Boulant and Megan Stanifer for helpful conversations.

FUNDING INFORMATION

This work was supported by a St George's, University of London, Institute of Infection & Immunity Research Support Grant (BLS), Medical Research Council LID Studentship MR/W006677/1 (KAL), Medical Research Council grants MR/S000844/1, MR/W018519/1 (SJDN, CMS), MC_UU_12014/3 (AJD), MR/W025647/1 (MPW) and MR/S00971X/1 (RJS), plus a Wellcome Trust Senior Research Fellowship (WT098049AIA) (SJDN). The funders had no role in data collection, interpretation or the decision to submit the work for publication. For the purpose of open access, the author has applied a Creative Commons Attribution (CC-BY) license to any Author Accepted Manuscript version arising from this submission.

AUTHOR CONTRIBUTIONS

SC: Investigation, Methodology, Formal Analysis. KAL: SC: Investigation, Methodology, Formal Analysis, Funding. ACT: Investigation, Methodology,

Formal Analysis. CJC: Methodology, Resources. RJS: Formal Analysis, Writing-Original Draft Preparation, Writing-Review and Editing, Funding. MPW: Formal Analysis, Writing-Original Draft Preparation, Writing-Review and Editing, Funding. SJDN: Conceptualization, Formal Analysis, Writing-Original Draft Preparation, Writing-Review and Editing, Funding. CMS: Conceptualization, Formal Analysis, Writing-Original Draft Preparation, Writing-Review and Editing, Funding. BLS: Conceptualization, Investigation, Methodology, Formal Analysis, Data Curation, Resources, Writing-Original Draft Preparation, Writing-Review and Editing, Supervision, Project Administration, Funding.

CONFLICT OF INTERESTS

The authors declare no conflicts of interest.

REFERENCES

1. Griffiths P, Reeves M. Pathogenesis of human cytomegalovirus in the immunocompromised host. Nat Rev Microbiol. 2021;19(12):759-73. doi: 10.1038/s41579-021-00582-z

2. Coen DM, Schaffer PA. Antiherpesvirus drugs: a promising spectrum of new drugs and drug targets. Nat Rev Drug Discov. 2003;2(4):278-88. doi: 10.1038/nrd1065

3. Strang BL. Toward inhibition of human cytomegalovirus replication with compounds targeting cellular proteins. J Gen Virol. 2022;103(10). doi: 10.1099/jgv.0.001795

4. Rodriguez JE, Loepfe TR, Stinski MF. Human cytomegalovirus persists in cells treated with interferon. Brief report. Arch Virol. 1983;77(2-4):277-81. doi: 10.1007/BF01309276

5. Mesev EV, LeDesma RA, Ploss A. Decoding type I and III interferon signalling during viral infection. Nat Microbiol. 2019;4(6):914-24. doi: 10.1038/s41564-019-0421-x

6. Glasgow LA, Hanshaw JB, Merigan TC, Petralli JK. Interferon and cytomegalovirus in vivo and in vitro. Proc Soc Exp Biol Med. 1967;125(3):843-9. doi: 10.3181/00379727-125-32220

7. Emodi G, O'Reilly R, Muller A, Everson LK, Binswanger U, Just M. Effect of human exogenous leukocyte interferon in cytomegalovirus infections. J Infect Dis. 1976;133 Suppl:A199-204. doi: 10.1093/infdis/133.supplement_2.a199

8. Nakamura K, Eizuru Y, Minamishima Y. Effect of natural human interferon-beta on the replication of human cytomegalovirus. J Med Virol. 1988;26(4):363-73. doi: 10.1002/jmv.1890260404

9. Sainz B, Jr., LaMarca HL, Garry RF, Morris CA. Synergistic inhibition of human cytomegalovirus replication by interferon-alpha/beta and interferon-gamma. Virol J. 2005;2:14. doi: 10.1186/1743-422X-2-14

10. Delannoy AS, Hober D, Bouzidi A, Wattre P. Role of interferon alpha (IFN-alpha) and interferon gamma (IFN-gamma) in the control of the infection of monocyte-like cells with human cytomegalovirus (HCMV). Microbiol Immunol. 1999;43(12):1087-96. doi: 10.1111/j.1348-0421.1999.tb03365.x

11. Murrell I, Bedford C, Ladell K, Miners KL, Price DA, Tomasec P, et al. The pentameric complex drives immunologically covert cell-cell transmission of wild-type human cytomegalovirus. Proc Natl Acad Sci U S A. 2017;114(23):6104-9. doi: 10.1073/pnas.1704809114

12. McSharry BP, Forbes SK, Avdic S, Randall RE, Wilkinson GW, Abendroth A, et al. Abrogation of the interferon response promotes more efficient human cytomegalovirus replication. J Virol. 2015;89(2):1479-83. doi: 10.1128/JVI.02988-14

13. Dell'Oste V, Biolatti M, Galitska G, Griffante G, Gugliesi F, Pasquero S, et al. Tuning the Orchestra: HCMV vs. Innate Immunity. Front Microbiol. 2020;11:661. doi: 10.3389/fmicb.2020.00661

662 14. Becker T, Le-Trilling VTK, Trilling M. Cellular Cullin RING Ubiquitin
663 Ligases: Druggable Host Dependency Factors of Cytomegaloviruses. Int J Mol
664 Sci. 2019;20(7). doi: 10.3390/ijms20071636
665 15. Goodwin CM, Ciesla JH, Munger J. Who's Driving? Human
666 Cytomegalovirus, Interferon, and NFkappaB Signaling. Viruses. 2018;10(9). doi:
667 10.3390/v10090447
668 16. Lista MJ, Witney AA, Nichols J, Davison AJ, Wilson H, Latham KA, et al.
669 Strain-Dependent Restriction of Human Cytomegalovirus by Zinc Finger Antiviral
670 Proteins. J Virol. 2023:e0184622. doi: 10.1128/jvi.01846-22
671 17. Nchioua R, Kmiec D, Muller JA, Conzelmann C, Gross R, Swanson CM,
672 et al. SARS-CoV-2 Is Restricted by Zinc Finger Antiviral Protein despite
673 Preadaptation to the Low-CpG Environment in Humans. mBio. 2020;11(5). doi:
674 10.1128/mBio.01930-20
675 18. Lin YT, Chiweshe S, McCormick D, Raper A, Wickenhagen A, DeFillipis V,
676 et al. Human cytomegalovirus evades ZAP detection by suppressing CpG
677 dinucleotides in the major immediate early 1 gene. PLoS Pathog.
678 2020;16(9):e1008844. doi: 10.1371/journal.ppat.1008844
679 19. Gonzalez-Perez AC, Stempel M, Wyler E, Urban C, Piras A, Hennig T, et
680 al. The Zinc Finger Antiviral Protein ZAP Restricts Human Cytomegalovirus and
681 Selectively Binds and Destabilizes Viral UL4/UL5 Transcripts. mBio.
682 2021;12(3):e02683-20. doi: 10.1128/mBio.02683-20
683 20. Bravo D, Solano C, Gimenez E, Remigia MJ, Corrales I, Amat P, et al.
684 Effect of the IL28B Rs12979860 C/T polymorphism on the incidence and features

685 of active cytomegalovirus infection in allogeneic stem cell transplant patients. J
686 Med Virol. 2014;86(5):838-44. doi: 10.1002/jmv.23865
687 21. Egli A, Levin A, Santer DM, Joyce M, O'Shea D, Thomas BS, et al.
688 Immunomodulatory Function of Interleukin 28B during primary infection with
689 cytomegalovirus. J Infect Dis. 2014;210(5):717-27. doi: 10.1093/infdis/jiu144
690 22. Manuel O, Wojtowicz A, Bibert S, Mueller NJ, van Delden C, Hirsch HH, et
691 al. Influence of IFNL3/4 polymorphisms on the incidence of cytomegalovirus
692 infection after solid-organ transplantation. J Infect Dis. 2015;211(6):906-14. doi:
693 10.1093/infdis/jiu557
694 23. Brand S, Beigel F, Olszak T, Zitzmann K, Eichhorst ST, Otte JM, et al. IL-
695 28A and IL-29 mediate antiproliferative and antiviral signals in intestinal epithelial
696 cells and murine CMV infection increases colonic IL-28A expression. Am J
697 Physiol Gastrointest Liver Physiol. 2005;289(5):G960-8. doi:
698 10.1152/ajpgi.00126.2005
699 24. Dag F, Dolken L, Holzki J, Drabig A, Weingartner A, Schwerk J, et al.
700 Reversible silencing of cytomegalovirus genomes by type I interferon governs
701 virus latency. PLoS Pathog. 2014;10(2):e1003962. doi:
702 10.1371/journal.ppat.1003962
703 25. Holzki JK, Dag F, Dekhtiarenko I, Rand U, Casalegno-Garduno R, Trittel
704 S, et al. Type I Interferon Released by Myeloid Dendritic Cells Reversibly Impairs
705 Cytomegalovirus Replication by Inhibiting Immediate Early Gene Expression. J
706 Virol. 2015;89(19):9886-95. doi: 10.1128/JVI.01459-15

26. Sitnik KM, Krstanovic F, Godecke N, Rand U, Kubsch T, Maass H, et al. Fibroblasts are a site of murine cytomegalovirus lytic replication and Stat1-dependent latent persistence in vivo. Nat Commun. 2023;14(1):3087. doi: 10.1038/s41467-023-38449-x

27. Stanton RJ, Baluchova K, Dargan DJ, Cunningham C, Sheehy O, Seirafian S, et al. Reconstruction of the complete human cytomegalovirus 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

28. Strang BL, Bender BJ, Sharma M, Pesola JM, Sanders RL, Spector DH, et al. A mutation deleting sequences encoding the amino terminus of human cytomegalovirus UL84 impairs interaction with UL44 and capsid localization. J Virol. 2012;86(20):11066-77. doi: 10.1128/JVI.01379-12

29. 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

30. Scherer M, Read C, Neusser G, Kranz C, Kuderna AK, Muller R, et al. Dual signaling via interferon and DNA damage response elicits entrapment by giant PML nuclear bodies. Elife. 2022;11. doi: 10.7554/eLife.73006

31. Schilling EM, Scherer M, Reuter N, Schweininger J, Muller YA, Stamminger T. The Human Cytomegalovirus IE1 Protein Antagonizes PML Nuclear Body-Mediated Intrinsic Immunity via the Inhibition of PML De Novo SUMOylation. J Virol. 2017;91(4). doi: 10.1128/JVI.02049-16

730 32. Ashley CL, Glass MS, Abendroth A, McSharry BP, Slobedman B. Nuclear
731 domain 10 components upregulated via interferon during human cytomegalovirus
732 infection potently regulate viral infection. J Gen Virol. 2017;98(7):1795-805. doi:
733 10.1099/jgv.0.000858
734 33. Wagenknecht N, Reuter N, Scherer M, Reichel A, Muller R, Stamminger
735 T. Contribution of the Major ND10 Proteins PML, hDaxx and Sp100 to the
736 Regulation of Human Cytomegalovirus Latency and Lytic Replication in the
737 Monocytic Cell Line THP-1. Viruses. 2015;7(6):2884-907. doi: 10.3390/v7062751
738 34. Seitz S, Heusel AT, Stamminger T, Scherer M. Promyelocytic Leukemia
739 Protein Potently Restricts Human Cytomegalovirus Infection in Endothelial Cells.
740 Int J Mol Sci. 2022;23(19). doi: 10.3390/ijms231911931
741 35. Kapoor A, Forman M, Arav-Boger R. Activation of nucleotide
742 oligomerization domain 2 (NOD2) by human cytomegalovirus initiates innate
743 immune responses and restricts virus replication. PLoS One. 2014;9(3):e92704.
744 doi: 10.1371/journal.pone.0092704
745 36. Zimmermann A, Hauka S, Maywald M, Le VTK, Schmidt SK, Daubener W,
746 et al. Checks and balances between human cytomegalovirus replication and
747 indoleamine-2,3-dioxygenase. J Gen Virol. 2014;95(Pt 3):659-70. doi:
748 10.1099/vir.0.061994-0
749 37. Shaw AE, Rihn SJ, Mollentze N, Wickenhagen A, Stewart DG, Orton RJ,
750 et al. The antiviral state has shaped the CpG composition of the vertebrate
751 interferome to avoid self-targeting. PLoS Biol. 2021;19(9):e3001352. doi:
752 10.1371/journal.pbio.3001352

753 38. Weekes MP, Tomasec P, Huttlin EL, Fielding CA, Nusinow D, Stanton RJ,
754 et al. Quantitative temporal viromics: an approach to investigate host-pathogen
755 interaction. Cell. 2014;157(6):1460-72. doi: 10.1016/j.cell.2014.04.028
756 39. Guo C, Reuss D, Coey JD, Sukumar S, Lang B, McLauchlan J, et al.
757 Conserved Induction of Distinct Antiviral Signalling Kinetics by Primate Interferon
758 Lambda 4 Proteins. Front Immunol. 2021;12:772588. doi:
759 10.3389/fimmu.2021.772588
760 40. Scherer M, Schilling EM, Stamminger T. The Human CMV IE1 Protein: An
761 Offender of PML Nuclear Bodies. Adv Anat Embryol Cell Biol. 2017;223:77-94.
762 doi: 10.1007/978-3-319-53168-7_4
763 41. Scherer M, Stamminger T. Emerging Role of PML Nuclear Bodies in
764 Innate Immune Signaling. J Virol. 2016;90(13):5850-4. doi: 10.1128/JVI.01979-
765 15
766 42. Elder EG, Krishna BA, Williamson J, Lim EY, Poole E, Sedikides GX, et al.
767 Interferon-Responsive Genes Are Targeted during the Establishment of Human
768 Cytomegalovirus Latency. mBio. 2019;10(6). doi: 10.1128/mBio.02574-19
769 43. Levin D, Schneider WM, Hoffmann HH, Yarden G, Busetto AG, Manor O,
770 et al. Multifaceted activities of type I interferon are revealed by a receptor
771 antagonist. Sci Signal. 2014;7(327):ra50. doi: 10.1126/scisignal.2004998
772 44. Urin V, Levin D, Sharma N, Harari D, Schreiber G. Fine Tuning of a Type
773 1 Interferon Antagonist. PLoS One. 2015;10(7):e0130797. doi:
774 10.1371/journal.pone.0130797

45. Nganou-Makamdop K, Billingsley JM, Yaffe Z, O'Connor G, Tharp GK, Ransier A, et al. Type I IFN signaling blockade by a PASylated antagonist during chronic SIV infection suppresses specific inflammatory pathways but does not alter T cell activation or virus replication. PLoS Pathog. 2018;14(8):e1007246. doi: 10.1371/journal.ppat.1007246

46. Viox EG, Hoang TN, Upadhyay AA, Nchioua R, Hirschenberger M, Strongin Z, et al. Modulation of type I interferon responses potently inhibits SARS-CoV-2 replication and inflammation in rhesus macaques. Sci Immunol. 2023;8(85):eadg0033. doi: 10.1126/sciimmunol.adg0033

785 **FIGURE LEGENDS**

786

787 **Figure 1 HCMV replication in HFF cells treated with IFNα or Ruxolitinib.** (A)
788 (i) and (iv) Diagram of experiments: HFF cells were treated with IFNα at the time
789 of infection (or left untreated) or pre-treated for 24 hours with IFNα (or left
790 untreated) and then infected in the presence and absence of IFNα. Treatment of
791 cells continued throughout infection with either AD169 or Merlin(R1111) for 96
792 hours. (ii) and (v) Titre in plaque forming units/ml (p.f.u./ml) of each experiment.
793 (iii) and (iv) Fold decrease in HCMV titre in the presence of IFNα compared to
794 HCMV titre from infected untreated cells. (B) (i) Diagram of experiments: HFF
795 cells were treated with Ruxolitinib (Ruxo) or the equivalent volume of DMSO at
796 the time of infection or pre-treated with Ruxo or the equivalent volume of DMSO
797 then infected in the presence of either Ruxo or DMSO. Treatment of cells
798 continued throughout infection with either AD169 or Merlin(R1111). (ii) Titre in
799 plaque forming units/ml (p.f.u./ml) of each experiment. (iii) Fold increase in
800 HCMV titre in the presence of Ruxo compared to HCMV titre from infected cells
801 treated with DMSO. In each figure data is representative of three independent
802 experiments (black data points) and presented as average (block) and standard
803 deviation (error bars) of the data. Statistical relevance was examined used a
804 student t test. ns = not significant (ns), p=<0.05 (*,**).

805

806 **Figure 2 MxA expression in the presence and absence of IFNα.** HFF cells
807 were pre-treated for 24 hours with IFNα (or left untreated) and then infected in

808 the presence and absence of IFNα. Treatment of cells continued throughout
809 infection with either AD169 or Merlin(R1111). HFF cell lysates were prepared for
810 western blotting at the time points indicated in the figure (hours post infection
811 (h.p.i.)). Uninfected HFF cell lysates were treated or untreated IFNα were also
812 prepared for western blotting at the time of infection (0 h.p.i.). (A) Western
813 blotting. Proteins recognized by the antibodies used in the experiment are
814 indicated to the right of the figure. The positions of molecular weight markers
815 (kDa) are indicated to the left of the figure. (B) Quantification of western blotting.
816 Relative band intensity (band intensity of MxA relative to β-actin signal in the
817 same lane, arbitrary units (A.U.)) was analyzed using ImageJ using data from
818 two independent experiments. The mean of each data point from those
819 experiments is shown. Hours post infection (h.p.i.).
820
821
822 **Figure 3 Western blotting and FACS analysis of HCMV infection in the**
823 **presence and absence of IFNα.** (A) Schematic of HCMV protein expression
824 with relevant proteins grouped into kinetic classes. (B) HFF cells were pre-
825 treated for 24 hours with IFNα (or left untreated) and then infected in the
826 presence and absence of IFNα. Treatment of cells continued throughout infection
827 with either AD169 or Merlin(R1111). Cell lysates were prepared for western
828 blotting at 96 hours post infection (h.p.i.). Uninfected cell lysates were treated or
829 untreated IFNα were also prepared for western blotting at the time of infection (0
830 h.p.i.). Proteins recognized by the antibodies used in the experiment are

831 indicated to the right of the figure and the positions of molecular weight markers
832 (kDa) are indicated to the left of the figure. (C and D) HFF cells were pre-treated
833 for 24 hours with IFNα (or left untreated) and then infected in the presence and
834 absence of IFNα. Treatment of cells continued throughout infection with AD169
835 or Merlin viruses expressing GFP. At 24 hours post infection cells were analyzed
836 using flow cytometry. (C) Flow cytometry data from a representative experiment.
837 (D) (i) number of cells expressing GFP (ii) mean fluorescent intensity of GFP
838 expressing cells. Data is representative of three independent experiments (black
839 data points) and presented as average (block) and standard deviation (error
840 bars) of the data.
841
842 **Figure 4 Western blotting of HCMV and PML proteins expressed in the**
843 **presence and absence of IFNα.** HFF cells were pre-treated for 24 hours with
844 IFNα (or left untreated) and then infected in the presence and absence of IFNα.
845 Treatment of cells continued throughout infection with either AD169 or
846 Merlin(R1111). Cell lysates were prepared for western blotting at the time points
847 indicated in the figure (hours post infection (h.p.i.)). Uninfected cell lysates were
848 treated or untreated IFNα were also prepared for western blotting at the time of
849 infection (0 h.p.i.). Proteins recognized by the antibodies used in the experiment
850 are indicated to the right of the figure and the positions of molecular weight
851 markers (kDa) are indicated to the left of the figure.
852

Figure 5 ZAP isoform expression in the presence and absence of IFNα. HFF cells were pre-treated for 24 hours with IFNα (or left untreated) and then infected in the presence and absence of IFNα. Treatment of cells continued throughout infection with either AD169 or Merlin(R1111). HFF cell lysates were prepared for western blotting at the time points indicated in the figure (hours post infection (h.p.i.)). Uninfected HFF cell lysates were treated or untreated IFNα were also prepared for western blotting at the time of infection (0 h.p.i.). (A) Western blotting. Proteins recognized by the antibodies used in the experiment are indicated to the right of the figure (large and small ZAP isoforms, ZAP-L and ZAP-S, respectively). The positions of molecular weight markers (kDa) are indicated to the left of the figure. (B) Quantification of western blotting. Relative band intensity (band intensity of each ZAP isoform relative to β-actin signal in the same lane, arbitrary units (A.U.)) was analyzed using ImageJ using data from two independent experiments. Hours post infection (h.p.i.). The mean of each data point from those experiments is shown.

Figure 6 HCMV replication in CRISPR containing cells in the presence and absence of IFNα. (A) Uninfected HFF CRISPR-Luc (Luciferase) or CRISPR-ZAP HFF cells were treated with IFNα or left untreated. Cell lysates were prepared for western blotting 24 hours post treatment. Proteins recognized by the antibodies used in the experiment are indicated to the right of the figure. The positions of molecular weight markers (kDa) are indicated to the left of the figure. (B) (i) HFF CRISPR-Luc or CRISPR-ZAP cells were pre-treated for 24 hours with IFNα (or

left untreated) and then infected in the presence and absence of IFNα. Treatment of cells continued throughout infection with either AD169 or Merlin(R1111) for 96 hours. Titre in plaque forming units/ml (p.f.u./ml) of each experiment was calculated. Data is representative of three independent experiments (black data points) and presented as average (block) and standard deviation (error bars) of the data. Statistical relevance was examined used a student t test. ns = not significant (ns), p=<0.05 (*,**). (ii) Fold decrease in HCMV titre in the presence of IFNα compared to HCMV titre from infected untreated cells. (iii) Fold increase in HCMV titre in CRISPR-ZAP cells compared to CRISPR-Luc cells.

Figure 7 HCMV replication in HFF cells in the continuous and discontinuous presence of IFNα. (A) (i) HFF cells were pre-treated for 24 hours with either IFNα or left untreated and then infected in the presence and absence of IFNα. Treatment of cells with IFNα continued throughout infection with either AD169 or Merlin(R1111) (+IFNα) or was discontinued at the time of infection (+/-IFNα). Titre in plaque forming units/ml (p.f.u./ml) of each experiment at 96 hours post infection was calculated. Data is representative of three independent experiments (black data points) and presented as average (block) and standard deviation (error bars) of the data. Statistical relevance was examined used a student t test. ns = not significant (ns), p=<0.05 (*,**). (ii) Fold decrease in HCMV titre in the continuous presence of IFNα (+IFNα) compared to HCMV titre from infected untreated cells. (iii) Fold decrease in HCMV titre in the discontinuous presence of IFNα (+/-IFNα) compared to HCMV titre from infected

899 untreated cells. (B) HFF cells were treated as in figure A and cell lysates were
900 prepared for western blotting at the time points indicated in the figure. Proteins
901 recognized by the antibodies used in the experiment are indicated to the right of
902 the figure. The positions of molecular weight markers (kDa) are indicated to the
903 left of the figure.
904
905 **Figure S1 Inhibition of HCMV strains in the presence of interferons (A)** HFF
906 cells were pre-treated for 24 hours with IFNα, IFNλ3 (or left untreated) and then
907 infected in the presence and absence of interferons. (A) Treatment of cells with
908 1000 U/ml IFNα continued throughout infection with either AD169 or
909 Merlin(R1111). Titre in plaque forming units/ml (p.f.u./ml) was determined from
910 virus supernatants collected at the time points indicated in the figure. (B and C).
911 Treatment of cells with interferons continued throughout infection with either
912 AD169 or Merlin(R1111) for 96 hours. Concentrations of interferons used are
913 indicated in each figure. Titre in plaque forming units/ml (p.f.u./ml) was
914 determined from virus supernatants collected at the 96 hours post infection. (C)
915 HFF cell lysates were prepared for western blotting at the time points indicated in
916 the figure (hours post infection (h.p.i.)). Uninfected HFF cell lysates were treated
917 or untreated IFNα were also prepared for western blotting at the time of infection
918 (0 h.p.i.). Proteins recognized by the antibodies used in the experiment are
919 indicated to the right of the figure. The positions of molecular weight markers
920 (kDa) are indicated to the left of the figure. AD169 (A), Merlin(R1111) (M).
921

Figure S2 Assessment of cell number and cytotoxicity in the presence and absence of IFNα. (A and B) Uninfected HFF cells plated at (i) high or (ii) low numbers were treated with IFNα or left untreated for 96 hours. Cell number or cell health in each condition was then investigated using cell counting or MTT assays. The percentage of data from IFNα treated cells compared to untreated cells was calculated. In each figure data is representative of three independent experiments (black data points) and presented as average (block) and standard deviation (error bars) of the data. (C) Uninfected HFF cells plated at high or low numbers were treated with IFNα or left untreated for 96 hours. At 96 hours post infection. time samples were prepared for western blotting. Proteins recognized by the antibodies used in the experiment are indicated to the right of the figure. The positions of molecular weight markers (kDa) are indicated to the left of the figure.

Figure S3 HCMV replication in HFF cells treated with IFNα or IFNλ3. (A) (i) HFF cells were pre-treated for 24 hours with either IFNα, IFNλ3 or left untreated and then infected in the presence and absence of IFNα or IFNλ3. Treatment of cells with interferon proteins continued throughout infection with either AD169 or Merlin(R1111) for 96 hours. Titre in plaque forming units/ml (p.f.u./ml) of each experiment was calculated. Data is representative of three independent experiments (black data points) and presented as average (block) and standard deviation (error bars) of the data. Statistical relevance was examined used a

student t test. ns = not significant (ns), p=<0.05 (*,**). (ii) and (iii) Fold decrease in HCMV titre in the presence of IFNα or IFNλ3, respectively, compared to HCMV titre from infected untreated cells. (B) Western blotting of uninfected HFF cells prepared at 24 hours post treatment with either IFNα or IFNλ3. Proteins recognized by the antibodies used in the experiment are indicated to the right of the figure. The positions of molecular weight markers (kDa) are indicated to the left of the figure.

Supplementary Data

Inhibition of Human Cytomegalovirus Replication by Interferon Alpha can involve multiple anti-viral factors

Shabab Chowdhury[a], Katie A Latham[a], Andy C Tran[a], Christopher J Carroll[b], Richard J Stanton[c], Michael P Weekes[d], Stuart J D Neil[e], Chad M Swanson[e] & Blair L Strang[a #]

Institute of Infection & Immunity[a], Institute of Molecular & Cellular Sciences[b], St George's, University of London, London, UK; Division of Infection and Immunity, Cardiff University School of Medicine, Cardiff, UK[c]; Cambridge Institute for Medical Research, School of Clinical Medicine, University of Cambridge, Cambridge, UK[d]; Department of Infectious Diseases, School of Immunology & Microbial Sciences, King's College London, London, UK[e].

#Address correspondence to Blair L Strang, bstrang@sgul.ac.uk

Figure S1 Inhibition of HCMV strains in the presence of interferons (A) HFF cells were pre-treated for 24 hours with IFNα, IFNλ3 (or left untreated) and then infected in the presence and absence of interferons. (A) Treatment of cells with 1000 U/ml IFNα continued throughout infection with either AD169 or Merlin(R1111). Titre in plaque forming units/ml (p.f.u./ml) was determined from virus supernatants collected at the time points indicated in the figure. (B and C). Treatment of cells with interferons continued throughout infection with either AD169 or Merlin(R1111) for 96 hours. Concentrations of interferons used are indicated in each figure. Titre in plaque forming units/ml (p.f.u./ml) was determined from virus supernatants collected at the 96 hours post infection. (C) HFF cell lysates were prepared for western blotting at the time points indicated in the figure (hours post infection (h.p.i.)). Uninfected HFF cell lysates were treated or untreated IFNα were also prepared for western blotting at the time of infection (0 h.p.i.). Proteins recognized by the antibodies used in the experiment are indicated to the right of the figure. The positions of molecular weight markers (kDa) are indicated to the left of the figure. AD169 (A), Merlin(R1111) (M).

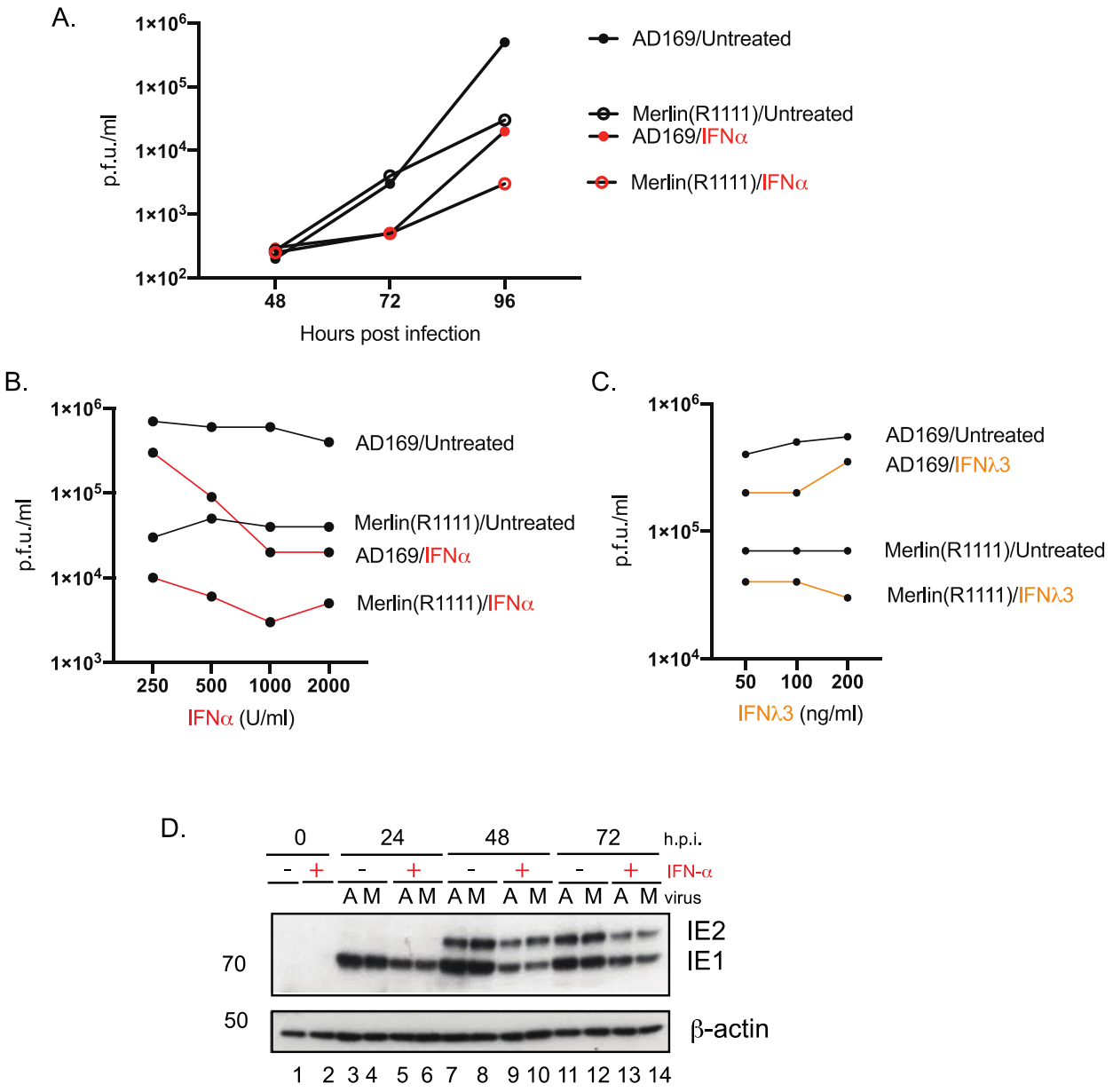

A.
1×10^6
1×10^5
1×10^4
1×10^3
1×10^2
p.f.u./ml
48
72
96
Hours post infection
AD169/Untreated
Merlin(R1111)/Untreated
AD169/IFNα
Merlin(R1111)/IFNα
B.
1×10^6
1×10^5
1×10^4
1×10^3
p.f.u./ml
250
500
1000
2000
IFNα (U/ml)
AD169/Untreated
Merlin(R1111)/Untreated
AD169/IFNα
Merlin(R1111)/IFNα
C.
1×10^6
1×10^5
1×10^4
p.f.u./ml
50
100
200
IFNλ3 (ng/ml)
AD169/Untreated
AD169/IFNλ3
Merlin(R1111)/Untreated
Merlin(R1111)/IFNλ3
D.
0
24
48
72
h.p.i.
- + - + - + - +
IFN-α
A M A M A M A M A M A M
virus
70
50
IE2
IE1
β-actin
1 2 3 4 5 6 7 8 9 10 11 12 13 14

Figure S1

Figure S2 Assessment of cell number and cytotoxicity in the presence and absence of IFNα. (A and B) Uninfected HFF cells plated at (i) high or (ii) low numbers were treated with IFNα or left untreated for 96 hours. Cell number or cell health in each condition was then investigated using cell counting or MTT assays. The percentage of data from IFNα treated cells compared to untreated cells was calculated. In each figure data is representative of three independent experiments (black data points) and presented as average (block) and standard deviation (error bars) of the data. (C) Uninfected HFF cells plated at high or low numbers were treated with IFNα or left untreated for 96 hours. At 96 hours post infection. time samples were prepared for western blotting. Proteins recognized by the antibodies used in the experiment are indicated to the right of the figure. The positions of molecular weight markers (kDa) are indicated to the left of the figure.

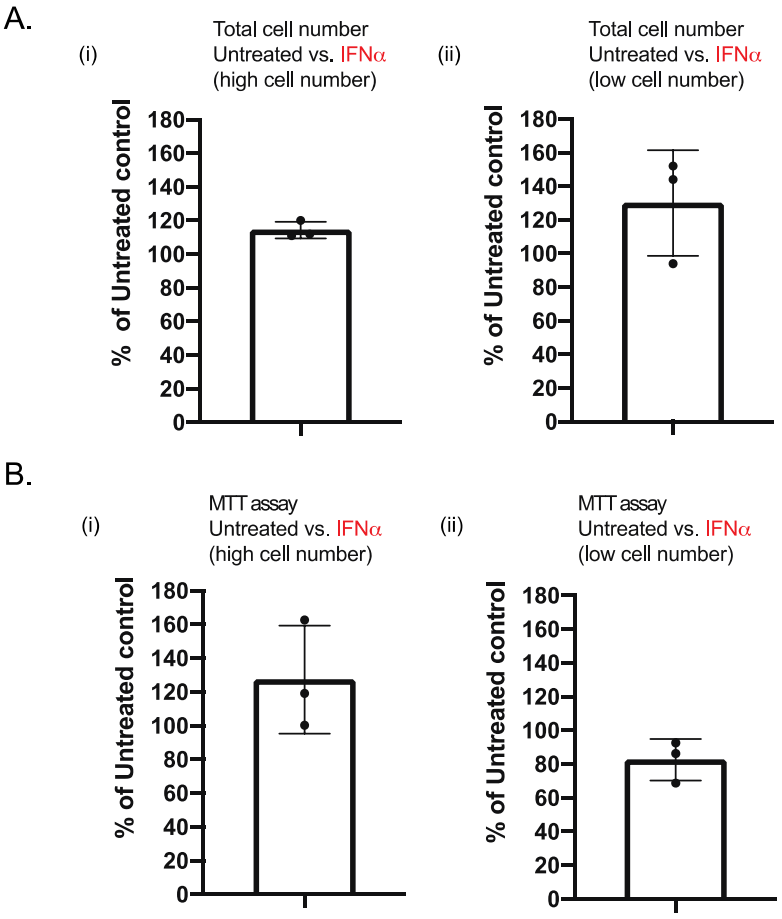
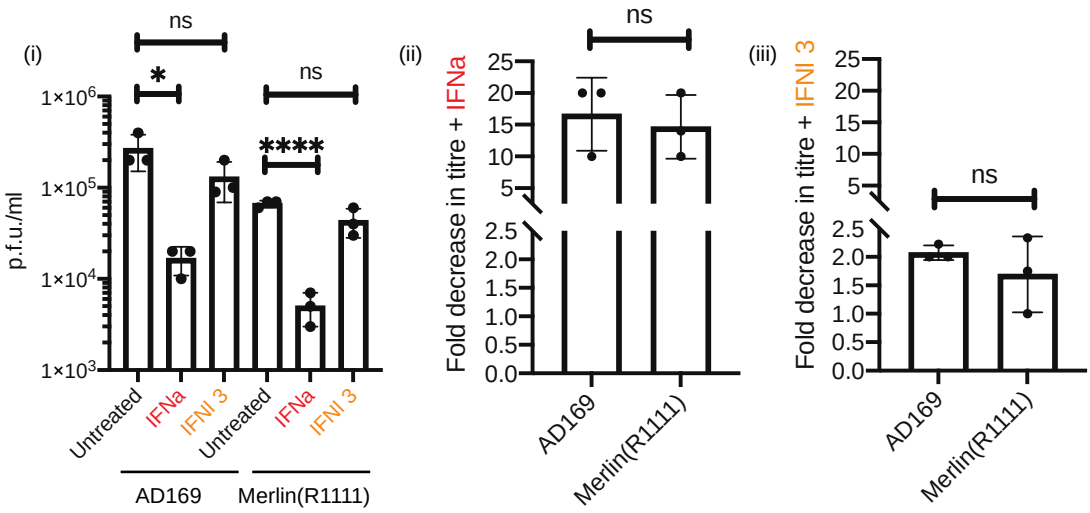

A.
(i)
Total cell number
Untreated vs. IFNα
(high cell number)
(ii)
Total cell number
Untreated vs. IFNα
(low cell number)
% of Untreated control
180
160
140
120
100
80
60
40
20
0
B.
(i)
MTT assay
Untreated vs. IFNα
(high cell number)
(ii)
MTT assay
Untreated vs. IFNα
(low cell number)
% of Untreated control
C.
High Conc
Low Conc
- + - + IFN-α
70
MRPS39
50
β-actin
70
MxA
15
TOM20
50
β-actin
1 2 3 4

Figure S2

Figure S3 HCMV replication in HFF cells treated with IFNα or IFNλ3. (A) (i) HFF cells were pre-treated for 24 hours with either IFNα**,** IFNλ3 or left untreated and then infected in the presence and absence of IFNα or IFNλ3. Treatment of cells with interferon proteins continued throughout infection with either AD169 or Merlin(R1111) for 96 hours. Titre in plaque forming units/ml (p.f.u./ml) of each experiment was calculated. Data is representative of three independent experiments (black data points) and presented as average (block) and standard deviation (error bars) of the data. Statistical relevance was examined used a student t test. ns = not significant (ns), p=<0.05 (*,**). (ii) and (iii) Fold decrease in HCMV titre in the presence of IFNα or IFNλ3, respectively, compared to HCMV titre from infected untreated cells. (B) Western blotting of uninfected HFF cells prepared at 24 hours post treatment with either IFNα or IFNλ3. Proteins recognized by the antibodies used in the experiment are indicated to the right of the figure. The positions of molecular weight markers (kDa) are indicated to the left of the figure.

A.


(i)
ns
*
ns
****
p.f.u./ml
1×10^6
1×10^5
1×10^4
1×10^3
Untreated
IFNa
IFNl 3
Untreated
IFNa
IFNl 3
AD169
Merlin(R1111)
(ii)
ns
Fold decrease in titre + IFNa
25
20
15
10
5
2.5
2.0
1.5
1.0
0.5
0.0
AD169
Merlin(R1111)
(iii)
Fold decrease in titre + IFNl 3
ns
AD169
Merlin(R1111)

B.

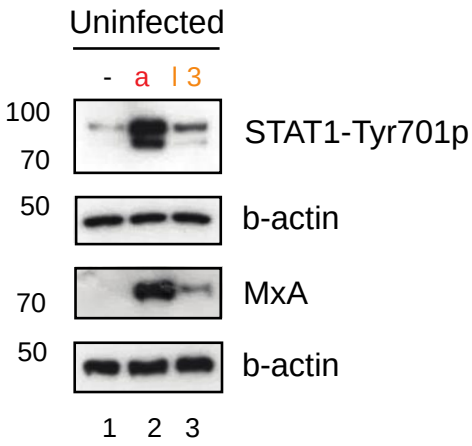

Uninfected
-
a
l 3
100
70
STAT1-Tyr701p
50
b-actin
70
MxA
50
b-actin
1
2
3

Figure S3

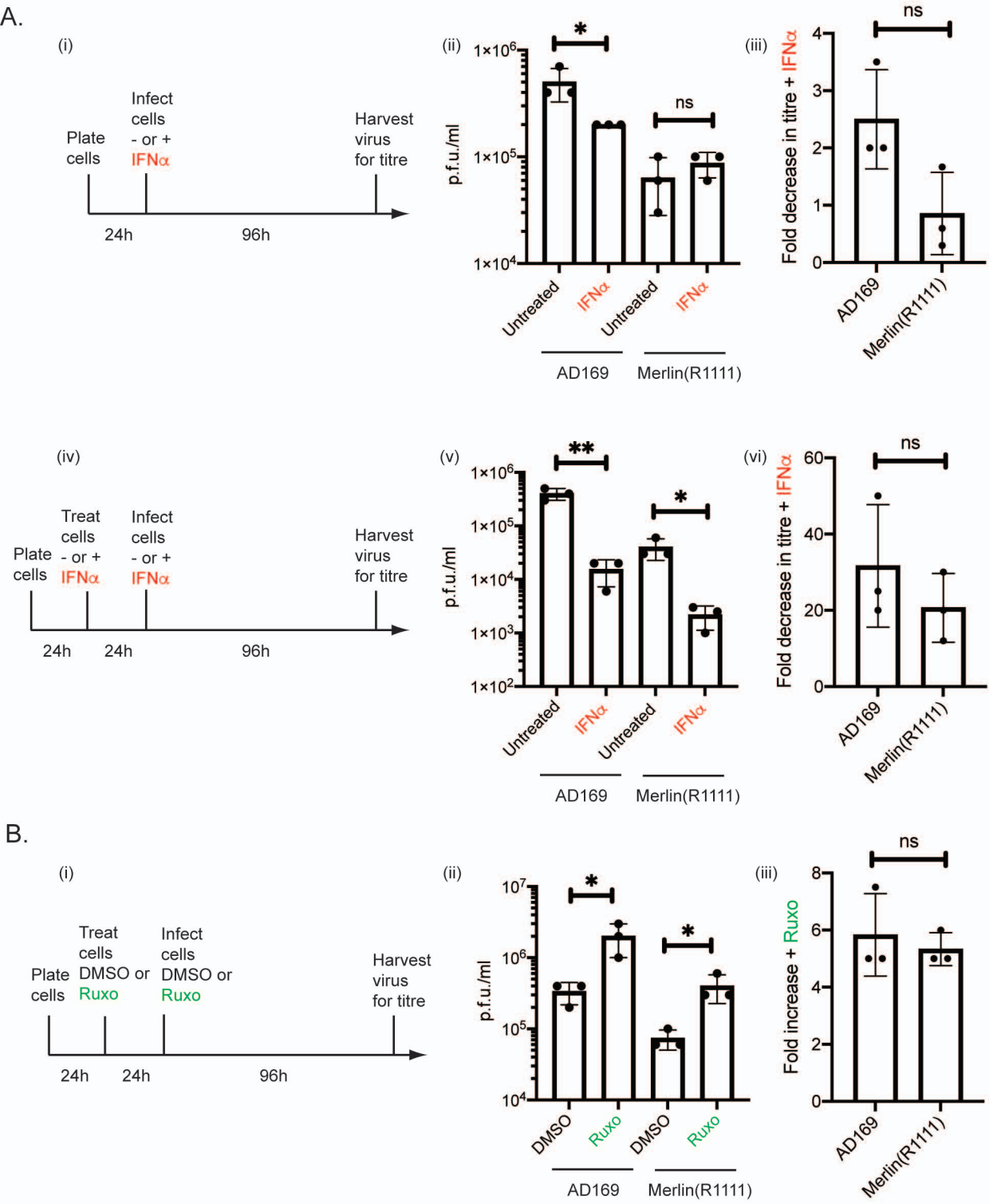


Figure 1

Figure 1

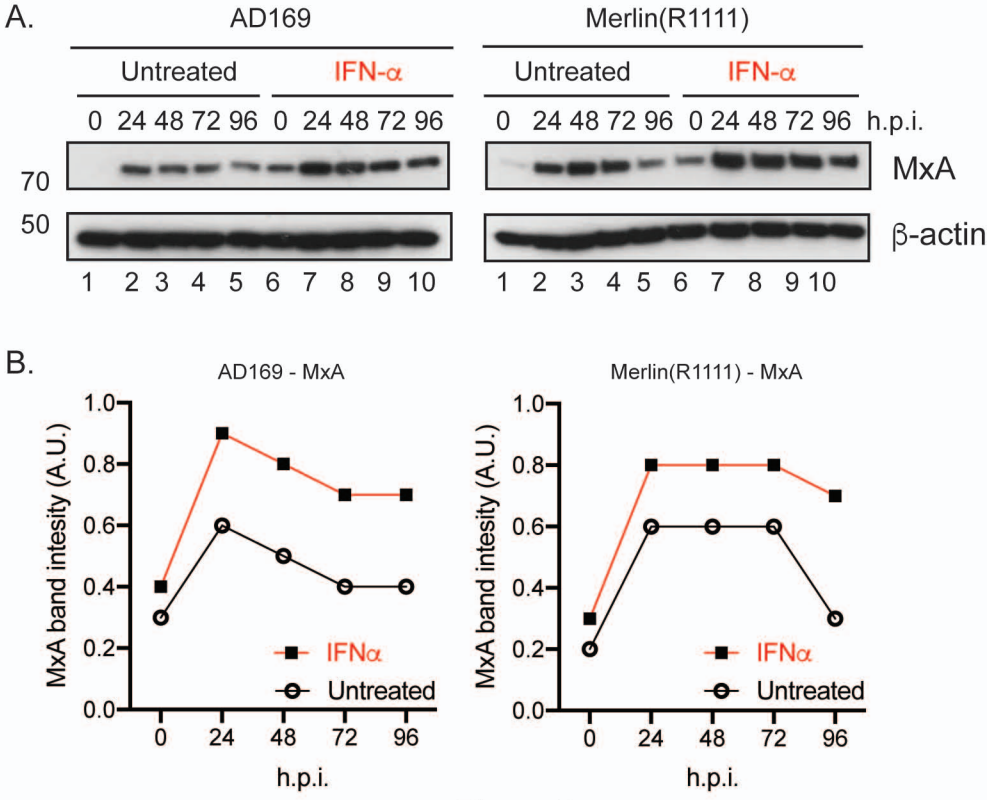

A.
AD169
Merlin(R1111)
Untreated
IFN-α
Untreated
IFN-α
0 24 48 72 96 0 24 48 72 96
0 24 48 72 96 0 24 48 72 96 h.p.i.
70
MxA
50
β-actin
1 2 3 4 5 6 7 8 9 10
1 2 3 4 5 6 7 8 9 10
B.
AD169 - MxA
Merlin(R1111) - MxA
MxA band intesity (A.U.)
1.0
0.8
0.6
0.4
0.2
0.0
IFNα
Untreated
0 24 48 72 96
h.p.i.

Figure 2

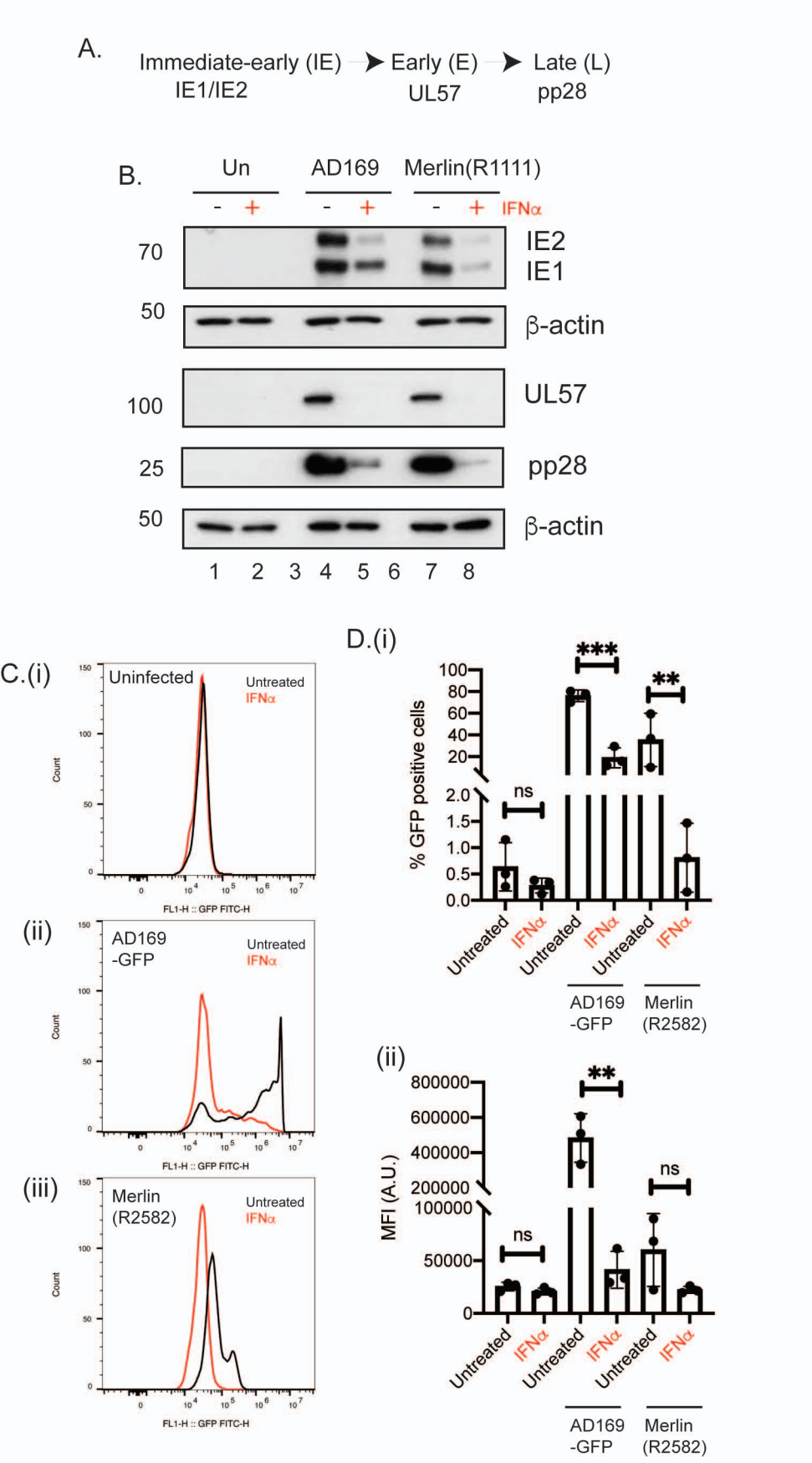

A.
Immediate-early (IE)
IE1/IE2
Early (E)
UL57
Late (L)
pp28
B.
Un
AD169
Merlin(R1111)
- + - + - + IFNα
70
IE2
IE1
50
β-actin
100
UL57
25
pp28
50
β-actin
1 2 3 4 5 6 7 8
C.(i)
Uninfected
Untreated
IFNα
Count
FL1-H :: GFP FITC-H
(ii)
AD169
-GFP
Untreated
IFNα
(iii)
Merlin
(R2582)
D.(i)
% GFP positive cells
***
**
ns
Untreated
IFNα
AD169
-GFP
Merlin
(R2582)
(ii)
MFI (A.U.)
**
ns

Figure 3

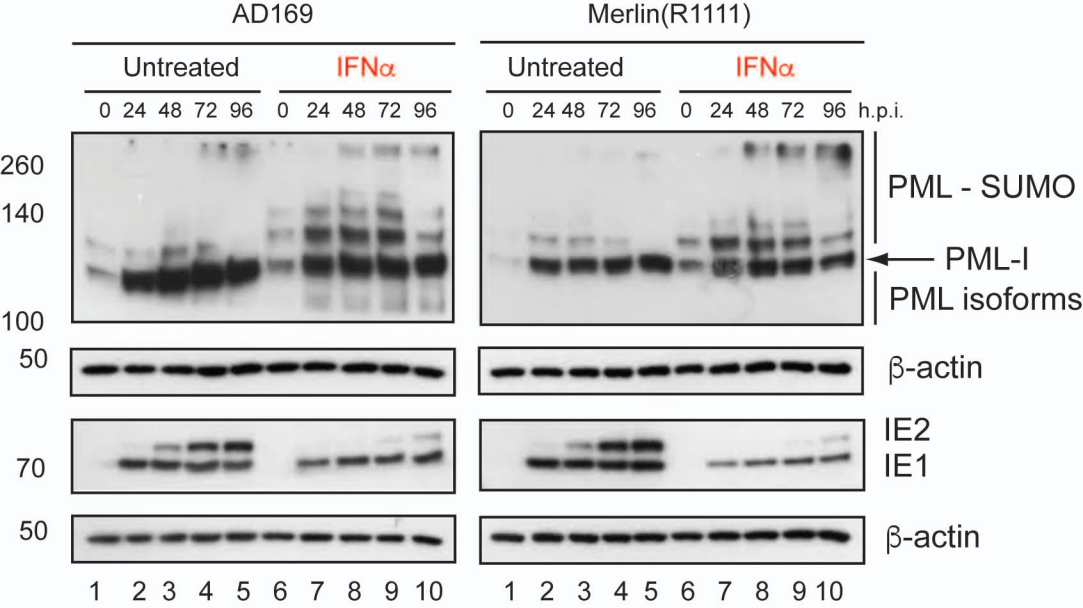

AD169
Merlin(R1111)
Untreated
IFNα
0 24 48 72 96 0 24 48 72 96
Untreated
IFNα
0 24 48 72 96 0 24 48 72 96 h.p.i.
260
140
100
PML - SUMO
PML-I
PML isoforms
50
β-actin
IE2
IE1
70
50
β-actin
1 2 3 4 5 6 7 8 9 10
1 2 3 4 5 6 7 8 9 10

Figure 4

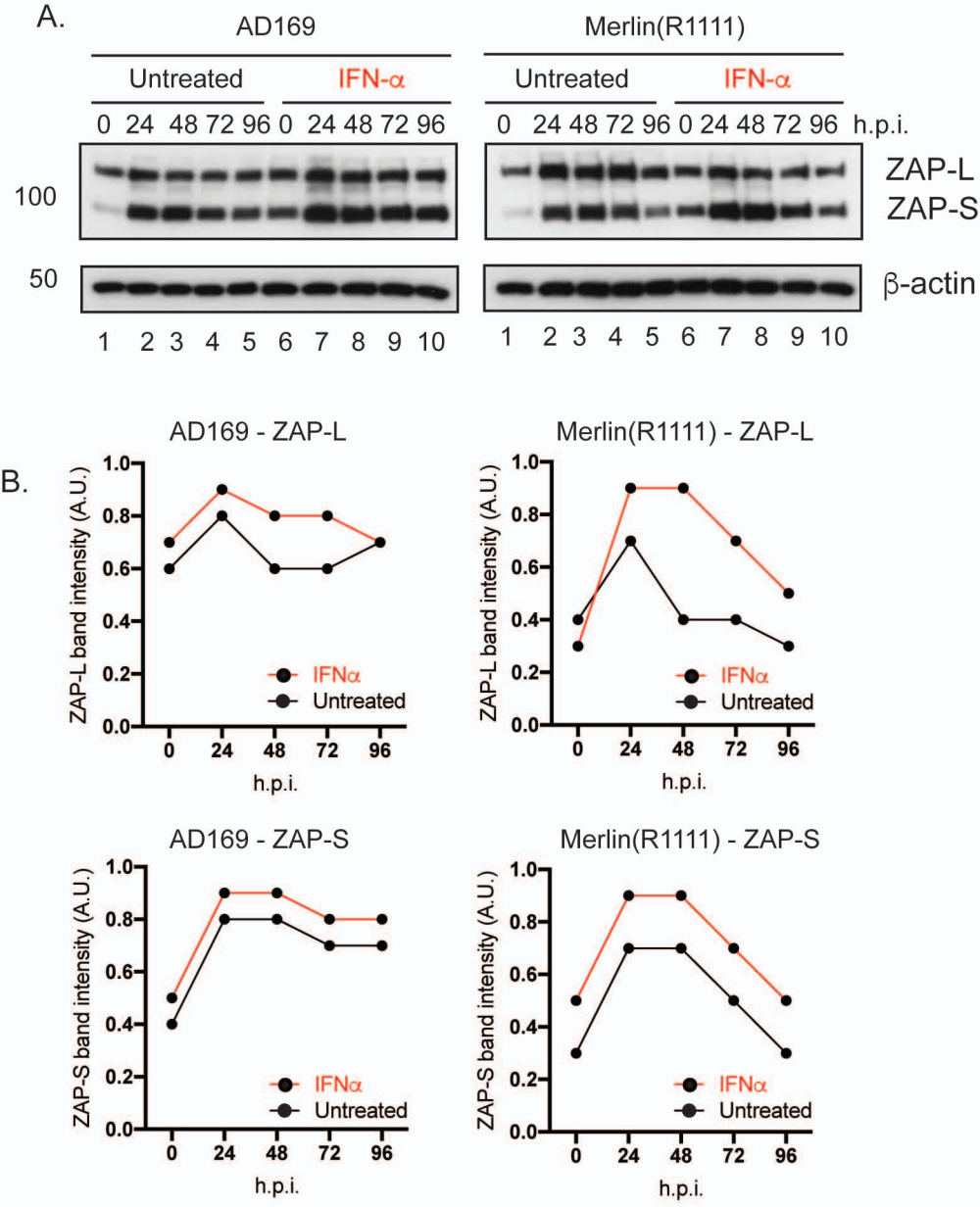

A.
AD169
Merlin(R1111)
Untreated
IFN-α
Untreated
IFN-α
0 24 48 72 96 0 24 48 72 96
0 24 48 72 96 0 24 48 72 96 h.p.i.
100
ZAP-L
ZAP-S
50
β-actin
1 2 3 4 5 6 7 8 9 10
1 2 3 4 5 6 7 8 9 10
B.
AD169 - ZAP-L
Merlin(R1111) - ZAP-L
ZAP-L band intensity (A.U.)
IFNα
Untreated
h.p.i.
AD169 - ZAP-S
Merlin(R1111) - ZAP-S
ZAP-S band intensity (A.U.)
0.0
0.2
0.4
0.6
0.8
1.0
0
24
48
72
96

Figure 5

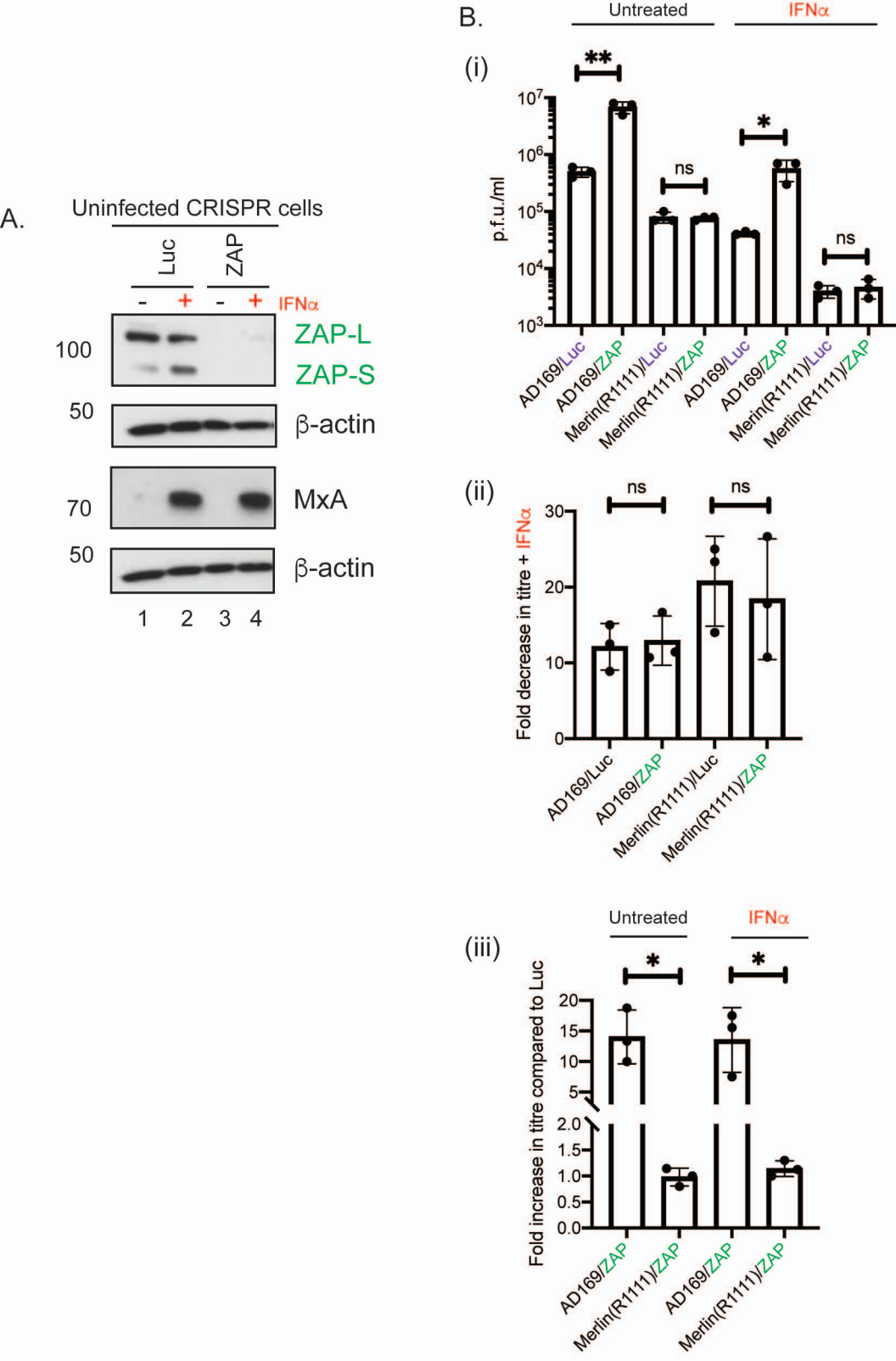

A.
Uninfected CRISPR cells
Luc
ZAP
- + - + IFNα
100
ZAP-L
ZAP-S
50
β-actin
70
MxA
50
β-actin
1 2 3 4
B.
Untreated
IFNα
(i)
p.f.u./ml
**
*
ns
ns
AD169/Luc
AD169/ZAP
Merlin(R1111)/Luc
Merlin(R1111)/ZAP
(ii)
Fold decrease in titre + IFNα
ns
ns
(iii)
Fold increase in titre compared to Luc
*
*

Figure 6

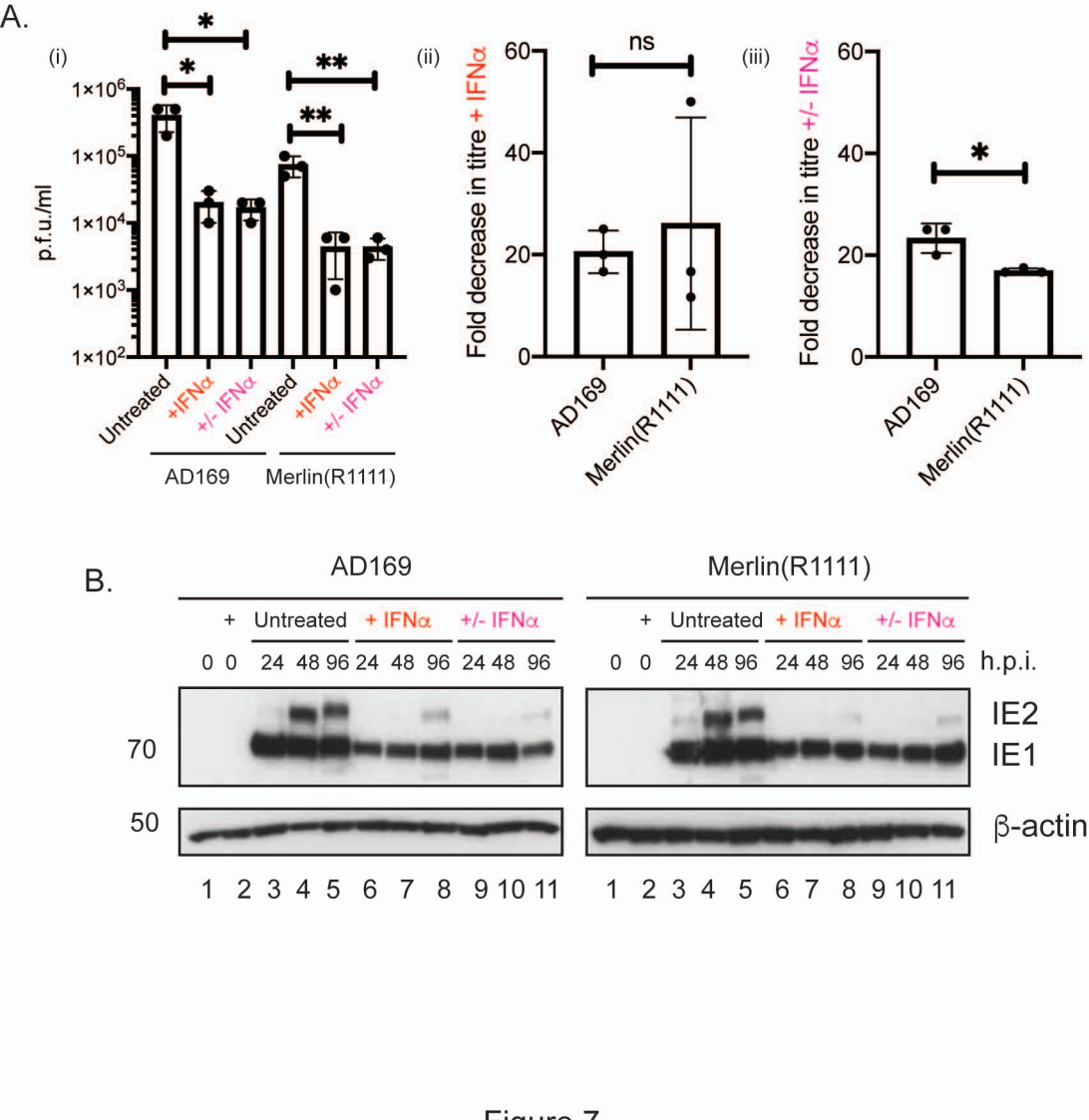

A.
(i)
p.f.u./ml
1×10^6
1×10^5
1×10^4
1×10^3
1×10^2
*
*
**
**
Untreated
+IFNα
+/- IFNα
Untreated
+IFNα
+/- IFNα
AD169
Merlin(R1111)
(ii)
Fold decrease in titre + IFNα
60
40
20
0
ns
AD169
Merlin(R1111)
(iii)
Fold decrease in titre +/- IFNα
60
40
20
0
*
AD169
Merlin(R1111)
B.
AD169
Merlin(R1111)
+
Untreated
+ IFNα
+/- IFNα
0 0 24 48 96 24 48 96 24 48 96
h.p.i.
70
50
IE2
IE1
β-actin
1 2 3 4 5 6 7 8 9 10 11

Figure 7