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Monkeypox virus Replication and Host Response in Vaginal and Ectocervical Epithelial Cells

Davide Mariotti[a], Ludovica Picarone[b], Alessandra D'Auria[c], Luigi Rosa[a], Valentina Valeriani[a], Daniele Pietrucci[b], Silvia Meschi[a], Fabrizio Carletti[a], Valentina Mazzotta[d], Guido Antonelli[c], Enrico Girardi[e], Carolina Scagnolari[c], Giovanni Chillemi[f,g], Andrea Antinori[d], Fabrizio Maggi[a], Giulia Matusali*[a,h]

[a] *Laboratory of Virology, National Institute for Infectious Diseases "Lazzaro Spallanzani"-IRCCS, Rome, Italy*
[b] *Department for Innovation in Biological, Agro-food and Forest systems, University of Tuscia, Viterbo, Italy*
[c] *Department of Molecular Medicine, Lab. of Virology, Sapienza University of Rome, affiliated to Istituto Pasteur Italia-Fondazione Cenci Bolognetti, Bolognetti, Rome, Italy*
[d] *Clinical and Research Department, National Institute for Infectious Diseases "Lazzaro Spallanzani"- IRCSS, Rome, Italy*
[e] *Scientific Direction, National Institute for Infectious Diseases "Lazzaro Spallanzani"- IRCSS, Rome, Italy*
[f] *Department of Experimental Medicine, University of Rome Tor Vergata, Rome, Italy*
[g] *Bioinformatics Research Unit, National Institute for Infectious Diseases "Lazzaro Spallanzani"-IRCCS, Rome, Italy*
[h] *Department of Biology and Biotechnology "Charles Darwin" Sapienza University of Rome, Rome, Italy*

*****Correspondence to giulia.matusali@uniroma1.it

Monkeypox virus Replication and Host Response in Vaginal and Ectocervical Epithelial Cells

Abstract

Recent mpox outbreaks have shown a predominant transmission through sexual contact. Replication-competent virus has been detected in seminal fluid, while in female patients, vaginal lesions, vertical transmission, and miscarriage risk have been reported. This study explored the susceptibility of the lower female genital tract (LFGT) to monkeypox virus (MPXV) infection, the role of sex-hormones in modulating viral replication, and host-virus molecular interactions. Human vaginal (VK2/E6E7) and ectocervical (Ect1/E6E7) epithelial cells were exposed to MPXV clade IIb, and viral replication was assessed. The influence of sex-hormones was evaluated after pretreatment with physiological concentrations of 17-β-estradiol or progesterone. Cellular genes' expression was determined by RT-qPCR and RNAseq, and ELISA was used for protein release analysis.

Both cell lines supported productive MPXV infection. 17-β -estradiol and progesterone slightly reduced viral replication in Ect1/E6E7. At 48 hours post-infection, compared to uninfected control, 216 differentially expressed genes (DEGs) were identified in MPXV infected VK2/E6E7 and 11 in Ect1/E6E7, with nine shared DEGs involved in protein folding (HSPA6), chemotaxis (CXCL3, ARC), inflammation and lymphoproliferation (IL11, IL1RL1, MMP-1), and tissue remodelling (IGFN1, MMP-1). MPXV infection significantly increased MMP-1 release in both cell lines, and MMP-1 inhibitors reduced infectious virus production. IFN-β and IFN-λ1 were induced earlier and more pronouncedly in Ect1/E6E7 which also showed slower viral replication than VK2/E6E7. Our analysis demonstrated the MPXV-mediated modulation of common and tissue-specific cellular pathways in the LFGT. The perturbation of tissue remodelling and inflammation in this district has the potential to affect reproductive health and susceptibility to sexually-transmitted-infections.

Keywords: Monkeypox virus; female genital tract; sexually transmitted viruses; emerging viruses; antiviral response

Introduction

Monkeypox virus (MPXV) is a double-stranded DNA virus belonging to the Orthopoxvirus genus within the Poxviridae family, causing mpox, a viral zoonosis. Transmission can occur through exposure to infected animals or by close contact with bodily fluids or lesions of an infected person, or via fomites (e.g. household contacts) [1]. Vertical transmission [2] and occupational infections in health care professionals have also been described [3].

Based on phylogenetic analyses, MPXV is divided into two major clades: clade I and clade II. Each clade is further subdivided into two subclades (Ia and Ib; IIa and IIb). Clade Ia circulates in several Central African countries and is predominantly associated with zoonotic spillover events from animal reservoirs. In contrast, clade Ib emerged as the cause of a major outbreak in 2023 in the Democratic Republic of the Congo (DRC) and has since been characterized by sustained human-to-human transmission across multiple countries. Clade IIa has been detected mainly in animal species. Clade IIb, first identified in Nigeria, has circulated continuously in human populations since 2016 and was responsible for the large, ongoing global outbreak that began in 2022. From a geographical perspective, mpox cases reported outside Africa are predominantly—but not exclusively—associated with clade IIb. Within Africa, the epidemiological situation is more complex, characterized by co-circulation of clades I and II in Central and Southern Africa, while clade II predominates in West Africa. However, diagnostic capacity and genomic surveillance efforts vary substantially between countries, leading to underreporting of cases and incomplete clade assignment in some regions [4].

The ongoing global outbreak of clade IIb mpox has caused over 100,000 cases [5] , 97% of which were in countries with no previous reports of local spread [6]. Although this global mpox epidemic was predominantly associated with sexual contact among men who have sex with men (MSM) [6], cases in women and cases acquired through heterosexual contact were documented [7–10]. In 2022–2023, the percentage of infections reported in female patients varied from less than 1% to 8% [11], while in previous outbreaks the infected women represented over 30% of the cases (linked to either clade I or II) [12,13].

On 14 August 2024, the WHO declared the public health emergency of international concern (PHEIC) due to the increase in mpox cases in the DRC and its expansion to neighbouring countries with no previously reported cases. In some areas of the country, a new offshoot of clade I, clade Ib, has been spreading from person to person.

Data from the DRC collected during the last year revealed 51.9% of infections in female patients, with 29% of sex workers, suggesting again a role for sexual transmission [14]. During the clade IIb

mpox 2022-2023 epidemics, some gender specific differences have been found in symptom prevalence, with genital rash more common among men (>50% of cases) than women (about 30%), where nevertheless sexual transmission remained the primary route of viral spread [11,15] and vaginal lesions correlated to vaginal sexual intercourse [7].

The occurrence of vulvar and vaginal rashes as well as the presence of the virus in vaginal fluids, indicate viral replication in the lower female genital tract (LFGT) [11,16,17].

The genital mucosa represents the first line of entry for sexually-transmitted viruses, the vagina and the cervix are the principal gateways, primarily but not exclusively, when the infection is driven by the presence of the virus in the seminal fluid [18]. MPXV DNA was repeatedly detected in semen, and the presence of infectious viral particles was described in this genital fluid [19]. For MPXV, studies carried out on clade IIb cases suggest that sexual transmission depend mainly on the contact with genital sores where much higher viral load and positivity rate have been measured compared to genital fluids [8,20], nevertheless the effect of semen on spreading the virus remains unclear.

To understand if the enhanced human-to-human transmission observed during the 2022 outbreak sustained by MPXV clade IIb was dependent on the route of infection, African rodents *Mastomys natalensis* were subjected to intraperitoneal, rectal, vaginal, aerosol, or transdermal inoculation and viral replication and shedding compared [21]. Results showed high susceptibility of the anal and genital mucosae and increased shedding upon ano-genital inoculation. Vaginal exposure resulted in high viral levels in a wide range of tested tissues (e.g. blood, bladder, brain, colon, kidney, liver, lung, and spleen) with evidence of vaginal and uterine MPXV lesions and mixed inflammatory leukocyte infiltrates [21].

The infection of the LFGT is of concern since vertical transmission may result from ascending infection, and perinatal infections may occur at the time of birth. Acquiring MPXV during pregnancy can have consequences for both the mother and the foetus, and can lead to congenital mpox [22,23].

The influence of sex-hormones on innate antiviral immunity and inflammation has been described with differential effects on viral infections outcomes reported [24–26]. The physiological fluctuation of sex hormones during the menstrual cycle, pregnancy and with aging has an impact on response to microbes [24]. Estradiol is considered to be generally protective against sexually transmitted viral infections, while progesterone and progestin-based hormonal contraceptives have been shown to increase susceptibility. These effects have been mainly described for HIV and HSV-2 [27,28], while in the case of Zika virus, a hormonal stage-dependent role for IFN-λ in controlling vaginal infection was demonstrated in the mouse model [29]. The role of sex hormones in modulating the susceptibility to MPXV is currently unknown. Moreover, few studies analysed the interplay of MPXV with the

host cellular machinery. Pattern recognition receptors (PRRs) are functionally expressed in the tissues of the female reproductive tract, where they detect viral and microbial components and induce interferon (IFN) responses [30,31].

In particular, both type I and III IFN (IFN-I and IFN-III) are produced by epithelial cells in the female genital tract. They limit the replication of sexually transmitted viruses such as HIV and HSV, recruit immune cells, and maintain mucosal integrity [32,33].

More recently, IFN-ε, which is constitutively expressed in the female reproductive tract, has been shown to play a unique role in controlling Zika virus spread and modulating inflammation [34]. No studies are available on the expression of IFN-I/III and related IFN pathways, such as PRRs involved in the recognition of MPXV [35,36], and ISGs in MPXV-infected vaginal and ectocervical epithelial cells.

This study aimed to investigate the susceptibility of cells from the LFGT to MPXV infection, the influence of sex hormones on viral replication, and the host response at the vaginal and cervical levels.

Materials and Methods

Cells and virus

Human Vaginal Epithelial Cells VK2/E6E7 and Human Ectocervical Epithelial Cells Ect1/E6E7 (CRL-2616 and CRL-2614 obtained from ATCC, Manassas, Virginia) were cultured in Keratinocyte Serum-Free Medium (KSFM), containing 0.1 ng/mL Human Recombinant Epithelial Growth Factor (EGF) and 0.05 mg/mL Bovine Pituitary Extract (BPE) (Thermo Fisher Scientific, Waltham, Massachusetts). Vero E6 cells (CRL-1586 obtained from ATCC, Manassas, Virginia) were cultured in Eagle's Minimum Essential medium (E-MEM) containing L-glutamine (Sigma-Aldrich, St. Louis, Missouri) and 10% Foetal Bovine serum (Corning, New York). All media were supplemented with 50 U/mL penicillin and 50 µg/mL streptomycin; cells were cultured in a humidified atmosphere (5% CO_2) at 37°C.

MPXV stock (*hMpxV/Italy/un-INMI-Pt2/2022, clade/lineage IIb* B.1,) was produced and titrated on Vero E6 cells, the stock was Mycoplasma free and the sequence information can be found at GISAID: EPI_ISL_13251120 or GenBank: ON745215.1, (see also https://www.european-virus-archive.com/virus/mpxv-monkeypox-virus-human-2022-italy-strain-hmpxvitalyun-inmi-pt22022-cladelineage-iib-b1).

The metalloproteinase 1 (MMP-1) inhibitors used were Batimastat (HY-13564), CTS-1027 (HY-10398), and CP-471474 (HY-W011085) (MedChemExpress, New Jersey). The lactate dehydrogenase release assay (LDH-Glo Cytotoxicity Assay) and ATP content assay (CellTiter-Glo

Luminescent Cell Viability Assay) (both from Promega, Madison, Wisconsin) were used to estimate the viability and cytotoxicity following DMSO or MMP-1 inhibitors treatment.

Viral infection

VK2/E6E7 or Ect1/E6E7 cells were seeded in 6-well plates at 3.5×10^5 cells per well. The day after, cell monolayers were infected with low passages MPXV (passage 2) in KSFM at a multiplicity of infection (M.O.I.) of 0.1. Viral inoculum or medium only [for not infected cell controls (ni)] was applied to the cells for 2 hours at 37°C and 5% CO_2. Viral inoculum was then removed, cells rinsed twice with DPBS 1X (Corning, New York), and fresh KSFM added. At different time points [2,8,24,48,72 hours post infection (hpi)], cell culture supernatants and cell lysates were collected and stored at -80°C.

Assessment of MPXV replication by PCR and viral titration

DNA was extracted from cell lysates with the Quick-DNA™ Microprep Kit (Zymo Research, Irvine, California) and from cell culture supernatants with the QIAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. For the assessment of the viral replication, a home-made system was set up using an MPXV West African specific (G2R_WA) PCR assay with the protocol published by Li et al. [37] on the RotorGeneQ platform. The target viral gene of the PCR assay is the G2R; Primer sequences are the following: Forward primer (5′-CACACCGTCTCTTCCACAGA); Reverse primer (5′-GATACAGGTTAATTTCCACATCG). The RNAse P gene amplification was inserted as a human sample integrity/extraction control (Red channel - TXR615).

To estimate the infectivity of MPXV released by VK2/E6E7 and Ect1/E6E7 cells, serial dilutions of cell culture supernatants were put in contact with sub-confluent Vero E6 cells seeded in 96-well plates in E-MEM containing 2% FBS. Cells were observed daily for cytopathic effect (CPE) and viral titre (50% Tissue Culture Infective Dose, $TCID_{50}$/mL) estimated by the Reed-Muench method at day 6.

Sex-steroid hormones treatment

17-β-estradiol or progesterone (Sigma-Aldrich, Saint Louis, Missouri, USA) were used to perform a 7-day treatment on VK2/E6E7 and Ect1/E6E7 cells prior to MPXV infection. The hormone concentrations used reflected the physiological levels of these hormones at different stages of the menstrual cycle and pregnancy: follicular phase and menopause (0.1 nM 17-β-estradiol or 1 nM progesterone), ovulation/luteal phase and first trimester of pregnancy (1 nM 17-β-estradiol or 10

nM progesterone), second trimester of pregnancy (10 nM 17-β-estradiol or 100 nM progesterone) [38,39]. Hormone treatment was maintained during and after the infection.

RNA extraction and sequencing

Total RNA was extracted from MPXV-infected and not infected cells at 48 hpi from the VK2/E6E7 and Ect1/E6E7 using Quick-RNA MicroPrep kit (Zymo Research Corporation, Irvine, CA, USA) and eluted in 15 μl of Elution buffer. RNA concentration was measured by NanoDrop™ 2000/2000c Spectrophotometers (Thermo Scientific, Italy). RNA samples were processed to remove ribosomal RNA. Stranded Total RNA libraries were then prepared and sequenced on the Illumina NovaSeq 6000 platform using paired-end 2x100 cycles. The average number of reads per sample was 14,831,857, ranging from a minimum of 9,235,664 to a maximum of 18,246,167.

Data processing and functional annotation analyses

Raw FASTQ files were quality-checked using FastQC v0.11.9 [40]. Adapter sequences and low-quality reads were trimmed using Trimmomatic v0.39 [41]. The resulting high-quality reads were aligned to the human reference genome (GRCh38/hg38) using STAR v2.7.9a [42] with default parameters. Gene-level quantification was performed using featureCounts v2.0.8 [43]. Read counts were then imported into R v4.2.2, and differential gene expression analysis was performed using the DESeq2 package v1.38.3 [44]. Genes were considered differentially expressed if they exhibited an absolute fold change ≥ 2 and an adjusted p-value ≤ 0.05 when comparing MPXV-infected cells to uninfected controls at 48 hpi. To explore the biological significance of differentially expressed genes, Gene Ontology (GO) enrichment analysis was performed using the clusterProfiler R package v4.6.2 [45].

Cellular gene expression by RT-qPCR

RNA was extracted from cell lysates using the Quick-RNA™ Microprep Kit (Zymo Research, Irvine, California), according to the manufacturer's instructions. MPXV infected cells and uninfected cell controls were collected at different time points [2, 8, 24, 48 and 72 hpi] and mRNA levels of selected genes of the IFN pathway [Toll like receptor 9 (TLR9), Cyclic GMP-AMP Synthase (cGAS), Stimulator Of Interferon Response CGAMP Interactor (STING), type I Interferons (IFN-α, IFN-β and IFN-ε), type III IFN (IFN-λ1 and IFN-λ2/3) and their receptor subunit Interleukin-28 Receptor (IL28R), and Interferon Stimulated gene 15 (ISG15) and Protein Kinase R (PKR)] were measured by RT Real-Time PCR using the LightCycler-480II instrument (Roche, Basel, Switzerland) as previously reported [46]. The housekeeping gene β-actin was used as

an internal control. All Real-Time PCR reactions were performed in duplicate. The following primers and probes were purchased from Integrated DNA Technologies (Coralville, IA, USA) and added to Probes Master Mix (Roche, Basel, Switzerland) at 500 and 250 nM, respectively, in a final volume of 20 µl: ACTB (Hs.PT.39a.22214847), TLR9 (Hs.PT.58.40576968), cGAS (MB21D1, Hs.PT.58.20682405), STING (TMEM173, Hs.PT.58.20781952), IFNA2, IFNB1, IFNE, IFNL1, IFNL2/3, PKR [46,47]. Primer pair and probes for IL28R [47], were added to the Probes Master Mix at 600 and 400 nM, respectively, in a final volume of 20 µl.

Anti-MPXV activity of IFNs

VK2/E6E7 and Ect1/E6E7 cell lines were seeded in 96-well plates (18,000 cells/well) in KSFM, containing 0.1 ng/mL EGF and 0.05 mg/mL Bovine BPE (Thermo Fisher Scientific, Waltham, Massachusetts). Cells were treated with 1000 IU/mL, 100 IU/mL and 10 IU/mL of IFN-β (Avonex, Biogen, Cambridge, MA, USA) and IFN-λ1 (Interferon Lambda 1, human cell expressed, pbl assay science, Piscataway, USA) preparations for 24 hours. After 24 hours, the plate was washed with DPBS and each well was inoculated with MPXV in KSFM at a M.O.I. of 0.1. Viral inoculum was applied to the cells for 2 hours at 37°C and 5% CO_2. Viral inoculum was then removed, cells rinsed twice with DPBS 1X (Corning, New York), and fresh KSFM added. Cultivation was continued for 72 hours and supernatants were collected from IFN-treated and untreated cells. Each experiment was performed in triplicate. Titration of the virus was performed on Vero E6 cells as previously described. The reduction in yield of each virus obtained at different concentrations of IFNs was calculated by subtracting the viral titre obtained in untreated cells from that obtained in IFN-treated cells.

ELISA

The levels in the cell culture supernatants of the soluble Human Matrix metalloproteinase 1 (MMP-1) were assessed with the Human Matrix metalloproteinase 1 (MMP-1) ELISA kit (Cusabio, Houston, Texas, USA) according to the manufacturer's instructions.

Statistical analysis

Wilcoxon test was used to estimate the replicative dynamics of MPXV in VK2/E6E7 and Ect1/E6E7. Friedman test followed by Dunn's test was used to compare MPXV DNA levels and viral titres upon sex-hormones treatment and for MMP-1 release analysis. One-sample t-test was applied to estimate the effect of MMP-1 inhibitors on viral replication.

One-way ANOVA with Bonferroni correction was used to compare the expression levels of IFN-related genes between MPXV-infected VK2/E6E7 and Ect1/E6E7 cells and uninfected control cells, as well as between MPXV-infected cells treated with IFN-β or IFN-λ1 and untreated MPXV-infected cells. A p-value of <0.05 was considered statistically significant. All statistical analyses and graphical representations were performed using GraphPad Prism version 8.0.2.

Results

MPXV replication in human epithelial vaginal and ectocervical cells

To determine whether MPXV could replicate in epithelial cells derived from the LFGT, VK2/E6E7 and Ect1/E6E7 cells were exposed to a clade IIb viral stock (M.O.I. 0.1) derived from the 2022 outbreak. We assessed viral replication by measuring viral DNA (vDNA) copies in cells and supernatants and infectious particles shedding in the supernatants of infected cells. A significant increase in cell-associated viral DNA content was observed at 24 hpi compared to 2 hpi in both VK2/E6E7 (274-fold increase; p=0.0002; Figure 1A) and Ect1/E6E7 cells (199-fold increase; p=0.0039; Figure 1B). Further increases in the vDNA cellular content were observed at 48 hpi and 72 hpi (Fig. 1A-B). A similar trend was observed when analysing the release of vDNA (Fig. 1C-D) in both cell lines. Viral particle shedding in VK2/E6E7 cells significantly increased at 48 hpi (19-fold compared to 2 hpi, p=0.0001) and 72 hpi (65-fold compared to 2 hpi, p=0.0001) (Figure 1E).
In Ect1/E6E7 cells, compared to 2 hpi, the release of viable MPXV showed a 5-fold increase at 48 hpi (p=0.0039) and reached levels comparable to those observed in the vaginal cells at 72 hpi (59-fold increase, p=0.0039) (Figure 1F). Viral release was accompanied by a Cytopathic effect which became evident starting 48 hpi (Supplementary Figure 1).

Sex-steroid hormones' treatment slightly decreases MPXV replication in ectocervical cells

The influence of 17-β-estradiol or progesterone pretreatment at levels measured during the follicular, ovulation/luteal phases or pregnancy on MPXV replication was investigated. VK2/E6E7 and Ect1/E6E7 cells were pretreated for one week with either 17-β-estradiol (0.1, 1, 10 nM; corresponding to 27, 270, and 2700 pg/mL, respectively) or progesterone (1, 10, 100 nM; corresponding to 0.3, 3, and 31 ng/mL, respectively). When comparing untreated (nt) and treated samples at each time point tested (2, 24, 48, 72 hpi), no marked differences in the titres of infectious MPXV nor an impact on vDNA in the supernatant were observed in VK2/E6E7 cells (Supplementary Figure 2A and Figure 2A). In Ect1/E6E7 cells at 72 hpi, the highest dose of 17-β-estradiol determined a 3.4-fold decrease (SEM ±0.72) in the release of vDNA (p=0.0089) and a 3.2-

fold (SEM ±1.4) decrease (not statistically significant p=0.54) in the titre of infectious MPXV (Figure 2B and Supplementary Figure 2B). The progesterone treatment resulted in a similar trend, at 72 hpi, the incubation with 10 nM and 100 nM progesterone determined 5.8 (SEM ±3.9) and 5-fold (SEM ±2.5) decrease of vDNA copies/mL of the supernatant, respectively. The decrease in the infectious titre of MPXV (10nM: 2.4-fold, SEM ±1.28; 100nM: 3.1-fold, SEM ±1.22) was nevertheless not statistically significant (Figure 2B and Supplementary Figure 2B).

MPXV replication differentially alters the transcriptome of vaginal and ectocervical cells

To further explore how female genital cells respond to MPXV infection, we analysed the effect of infection on the transcriptome of VK2/E6E7 and Ect1/E6E7 epithelial cell lines. RNAseq analysis of MPXV-infected cells vs uninfected control was performed at 48 hpi. After MPXV infection, a total of 216 genes were differentially expressed (DEGs; Log_2 fold change>2; adjusted p value <0.05) in VK2/E6E7 cells, 41 genes were down-regulated and 175 were up-regulated (Figure 3A). Among the down-modulated transcripts, we observed those involved in the metabolism of steroids and fatty acids (BBOX, PSAPL1, HSD17B3, PPARGC1A, WNT4, PLA2G4F), mitochondrial function and homeostasis (TNSF10, PPARGC1A, DCN), nucleotide metabolic process (GMPR), autophagy (TRIM22, DCN), and tissue development (WNT6, WNT10A, WNT4). The expression levels of Dual-specificity phosphatases transcripts (DUSP 1, 2, 5, 6, 8), involved in the regulation of MAPKs activity, were up-regulated. The expression of genes involved in inflammation and the regulation of leukocyte recruitment (CXCL2, CXCL3, CXCL8, CCL20, TNFSF14, SLIT2, ABL2, PTGS2, MIR221, MPO, IL-11, IL1RL1) were also significantly increased. The Gene Ontology (GO) enrichment analysis in VK2/E6E7 indicated that the molecular functions of DEGs were associated with MAPK phosphatase, cytokine/chemokine, and DNA binding transcription activities (Figure 3C). The GO Biological Processes analysis revealed enrichment for the response to unfolded protein and stress signalling, regulation of MAPK cascade, cell migration, chemotaxis, and myeloid cell differentiation (Figure 3D).

MPXV-infected Ect1/E6E7 cells showed 11 DEGs compared to controls; the OAS2 gene was down-modulated, and the other 10 markedly up-modulated (HSPA6, CXCL3, ARC, IL11, IL1RL1, MMP-1, IGFN1, FTCD, TRPV3, LOC101928841/ADPRHL1 ADP-ribosylhydrolase like 1) (Figure 3B). The OAS2 gene has a crucial role in antiviral response, and the FTCD encoded protein is associated with histidine metabolism. The other nine DEGs are in common with those observed in VK2/E6E7 cells and involved in protein folding (HSPA6), cell chemotaxis and migration (CXCL3, ARC), inflammation and lymphoproliferation (IL11, IL1RL1, MMP-1), cell adhesion and tissue remodelling (IGFN1, MMP-1) (Figure 3E).

Matrix metallo-protease inhibition decreases MPXV replication in vaginal and ectocervical cells

We then analysed more in depth the modulation of one of the factors which resulted to be upregulated upon MPXV infection in both the cell lines, the MMP-1. MMP-1 is a collagenase which can be released from cells and participate in the degradation of the extracellular matrix [48]. The shedding of MMP-1 might increase tissue damage and facilitate viral dissemination from the epithelium to the underlying connective tissues.

The transcriptional up-modulation of the MMP-1 corresponded to a 3-fold increase in the release of this protein into the supernatant of MPXV infected VK2/E6E7 at 48 hpi (ni= 2.04 ± 0.72, MPXV= 5.44 ± 0.79; p=0.0399) and 72 hpi (ni= 2.44 ± 0.95, MPXV= 7.33 ± 1.44; p=0.0202) (Figure 4A). In Ect1/E6E7, at 72 hpi, a 4-fold increased release (p=0.0141) was observed in MPXV-infected (ni= 2.06 ± 1.22 MPXV= 8.24 ± 0.97) cells (Figure 4B).

We then treated both the cell lines with three different compounds with inhibitory activity against MMP-1: Batimastat, CTS-1027, and CP-471474. The cells were treated soon after the removal of the viral inoculum, DMSO was used at the corresponding concentrations as a control and not infected samples were used for comparison of cell viability.

Based on preliminary viability assays (Supplementary figure 3) and the IC50 for MMP-1 inhibition, three concentrations of Batimastat (1.2 μM; 0.12 μM, 0.012 μM), CTS-1027 (2.5 μM; 0.63 μM, 0.16 μM), and CP-471474 (5 μM; 1.25 μM, 0.31 μM) were used. The release of infectious particles, measured at 72 hpi, was inhibited in a dose-dependent manner in VK2/E6E7 cells by the three compounds. The infectious titres measured in the supernatant significantly decreased when the cells were treated with the highest concentrations of Batimastat (0.26, p=0.0052); CTS-1027 (0.28, p=0.0319), and CP-471474 (0.16; p=0.0088) compared to controls (DMSO=1) (Figure 4C). The vDNA content in cell lysates decreased in presence of CP-471474 (5 μM; p=0.0109) compared to controls (DMSO=1) (Supplementary Figure 4). A decrease in infectious particles release was also observed in Ect1/E6E7 cells when treated with CTS-1027 (2.5 μM: 0.23, p=0.0182; 0.63μM: 0.58, p=0.1632; 0.16μM: 0.21 p=0.0052), and CP-471474 (5μM: 0.06; p=0.0007; 1.25 μM: 0.68, p=0.1753; 0.31μM: 0.37, p=0.0101) compared to controls (DMSO=1) (Figure 4D). The vDNA content in cell lysates decreased in presence of CTS-1027 (2.5 μM, p=0.0181) and CP-471474 (5 μM; p=0.0290) compared to controls (DMSO=1) (Supplementary Figure 4). To further exclude the possibility that the reduction in viral production was caused by compound-associated cytotoxicity, we performed a cytotoxicity assay and quantified LDH release in the supernatants of treated and untreated control cells (Supplementary Figure 3). Treatment with CTS-1027 and CP-471474

resulted in LDH levels comparable to those observed in the DMSO control, indicating no measurable cytotoxic effects at the tested concentrations. In contrast, Batimastat exhibited a modest impact on cell viability: in VK2/E6E7 cells, one biological replicate showed reduced viability at 1.2, 0.12, and 0.012 μM, in Ect1/E6E7 higher LDH release was observed only at 1.2 μM.

MPXV infection differentially triggers the antiviral response in vaginal and ectocervical cells

As emerging evidence has highlighted the key role of IFN in controlling sexually transmitted viral infections in the female genital tract, and having observed a dysregulation in the IFN-induced OAS expression in Ect1/E6E7 cells through RNA-seq, we therefore carried out a comprehensive analysis of the gene expression of multiple IFN-related genes involved in the recognition and control of MPXV in female genital cells using RT-real time PCR assays. These genes include pattern recognition receptors (PRRs), such as Toll-like receptor 9 (TLR9) [35], cyclic GMP-AMP synthase (cGAS) [36] and adaptor stimulator of interferon response CGAMP interactor (STING) [36], type I IFNs (IFN-α, IFN-β and IFN-ε) [46,47,49], type III IFNs (IFN-λ1 and IFN-λ2/3) [50,51], their receptor subunit interleukin-28 receptor (IL28R) [50], and downstream ISGs (ISG15 and protein kinase R (PKR)) [46,52–54]. The expression of PRR mRNAs did not significantly increase as a result of MPXV infection in either the VK2/E6E7 or Ect1/E6E7 cell lines (Figure 5A and 5B). The only exception was a slightly increased cGAS expression of 2.28-fold at 72 hpi in VK2/E6E7 cells ($p=0.002$; Figure 5A). IFN-I and IFN-III exhibited distinct expression kinetics between the two cell types analysed (Figure 5C-F). In MPXV-infected VK2/E6E7 cells, IFN-β increased from 2- to 4.5-fold at 24, 48, and 72 hpi ($p<0.0001$ at 24 and 48h; $p=0.0041$ at 72h; Figure 5C). In contrast, IFN-β was more highly induced with an earlier peak in Ect1/E6E7 than in VK2/E6E7 cells, with a ~6.3-fold increase at 24 hpi ($p=0.0218$ at 2h; $p=0.0024$ at 8h; $p<0.001$ at 24h; Figure 5D). MPXV also induced delayed expression of IFN-α in both cell lines, with moderate increases observed at 48 (~3-fold) and 72 (~3-fold) hpi in VK2/E6E7 and Ect1/E6E7 cells, respectively ($p=0.0001$ for both time points; Figure 5C and 5D). Furthermore, MPXV did not induce alteration in the IFN-ε expression in both the cell lines (Figure 5C and 5D).

Regarding changes in IFN-λs after MPXV infection, both IFN-λ1 and IFN-λ2/3 increased slightly at 24 hpi (3.18-fold, $p<0.0001$) and between 24 and 48 hpi (2.24-fold at 24h, $p=0.0421$; 3.18-fold at 48h, $p<0.0001$, respectively) in VK2/E6E7 cells (Figure 5E). Conversely, IFN-λ1 was rapidly increased, reaching a 15-fold increase at 8 hpi, with expression levels maintained between 7- and 4-fold at 24 and 48 hpi, respectively ($p<0.0001$ for all the time points, Figure 5F) in Ect1/E6E7 cells. Furthermore, IFN-λ2/3 was induced by ~3-fold between 48 ($p=0.0038$) and 72 hpi ($p<0.0001$) in

Ect1/E6E7 cells (Figure 5F). No significant changes in the transcript expression of the ISGs, PKR and ISG15, were observed in either cell line (Figure 5G and 5H).

As we observed a slight induction of IFN-β and IFN-III in MPXV-infected VK2/E6E7 and Ect1/E6E7 cells, we also evaluated the anti-MPXV activity of IFN-β and IFN-λ1 at concentrations of 1000 IU/mL, 100 IU/mL, and 10 IU/mL in both cell lines.

MPXV exhibited resistance to both IFN-β and IFN-λ1 preparations, which were ineffective in blocking MPXV replication in the two cell lines studied, with the only exception of a slight reduction in MPXV titre after treatment with 1000 IU/mL of IFN-β on VK2/E6E7 (log_{10} reduction value of 0.32; Supplementary table 1).

Discussion

Following the global mpox outbreak, which reached its epidemiological peak in the spring-summer of 2022, MPXV continues to circulate, and the current scenario is characterized by the co-circulation of different clades in African countries. Meanwhile, the majority of MPXV cases outside Africa are still attributed to clade IIb [55]. The sexual transmission of MPXV is thought to be primarily caused by contact with skin lesions in the genital area; nevertheless, infectious virus is released in semen [56,57] and semen driven infection cannot be excluded. Little is known about the interaction of MPXV with the LFGT, apart from the clinical observations of genital lesions and the detection of viral DNA in cervico-vaginal swabs [7,58,59]. Here we investigated the replication of MPXV clade IIb in human epithelial cells derived from the LFGT, specifically vaginal and ectocervical cells, and demonstrated the virus' ability to infect these cells productively. The release of infectious MPXV particles was observed in both cell lines, with the ectocervical cell line exhibiting a slower release rate. We then examined the modulation of viral infection by sex-steroid hormones. The levels of these hormones physiologically vary during the menstrual cycle, during pregnancy, and with age. A complex interaction between the innate and adaptive immune systems, as well as sex hormones, has been described, also shaping the response to viral infections [31]. In our settings, the pretreatment of vaginal and ectocervical cells with different doses of 17-β-estradiol or progesterone showed a low or negligible effect on viral replication, suggesting the absence of a direct role of sex-hormones on the susceptibility of these epithelial cells to MPXV. In ectocervical cells, higher doses of 17-β-estradiol and progesterone showed a low down-modulation of vDNA release, accompanied by a decrease in viral particle release that was not statistically significant. The differences in sex hormone responses observed between vaginal and ectocervical cells, although modest in terms of modulation of viral replication, are not unexpected, given their distinct cellular backgrounds and the differential hormone responsiveness previously described for these tissues

[31,60–62]. Further investigations are needed to determine if these hormones may affect MPXV replication in a more physiological context, such as a 3D system incorporating a multilayered epithelium, stromal cells, and immune cells. Indeed, estradiol and progesterone have been shown to impact the thickness of the epithelial barrier and differentially modulate innate and adaptive immune cells and humoral products [31,63], factors to be evaluated in human-based complex models.

The impact of viral infection on the host metabolic machinery was investigated through RNAseq performed at 48 hpi, when the productive infection was evident in both the cell lines, but the cytopathic effect had not advanced as it did at later time points. The transcriptomic analysis revealed a profound dysregulation of biological processes and molecular functions in vaginal cells with over 200 DEGs compared to non-infected controls. The modulated genes were involved in a wide range of functions, including the metabolism of lipids and nucleotides, mitochondrial homeostasis, autophagy, tissue development, MAPKs activity, inflammation and leukocyte recruitment. A recent report describes a dynamic phosphorylation of both host and viral proteins in MPXV IIb-infected human foreskin fibroblasts, suggesting a key role in this process for MAPKs [64]. The comparison of the impact on the cellular transcriptome of different MPXV clades was assessed in a colon organoid model, in which a more extensive alteration of stress-responsive genes was observed upon clade I vs clade II [65].

We observed a less pronounced transcriptomic modulation upon infection of ectocervical cells, further highlighting the differential impact of MPXV on epithelial cells originating from distinct districts within the lower female genital tract. The more limited impact is consistent with the slower viral release kinetics observed for these cells. Nevertheless, MPXV infected ectocervical and vaginal cells shared nine common DEGs including transcripts encoding for the MMP-1. MMP-1 is a collagenase which cleaves a broad range of substrates including extracellular matrix (ECM) proteins, chemokines and cytokines (CCL-2, -7, -13; CXCL5,6,11,12, pro-IL-1β, IL-1β, IL-8, IGFBP-2, -3, -5; TNF-α) [48,66]. Thus, MMP-1 is involved in ECM and tissue remodelling and inflammation, including during embryo development and reproduction [66], and its action could facilitate viral spread in infected tissues. We observed an increase in the release of MMP-1 upon MPXV infection and a decrease in viral replication in the presence of its inhibitors, suggesting a role for this protein in modulating viral infection. With the exception of a slight effect of Batimastat on cell viability, the observed decrease in viral replication was not due to compound toxicity. The inhibitors used were not exclusively specific for the MMP-1, also acting on other MMPs; nevertheless, the inhibition of viral replication was evident at concentrations within the range of the IC50 described for MMP-1. The role of other MMPs cannot be ruled out; indeed, MMPs are

dynamically interconnected in a complex network which dysregulation may lead to unbalanced homeostasis and pathological outcomes [48].

Of note, the dysregulation of MMP-1 and other MMPs (e.g. MMP-2 and MMP-9), as well as increased levels of IL11 and CXCL3 (two other common DEGs), have been involved in cervical and uterine tissue changes in cases of adverse birth outcomes [67–72]. Whether these pathways could be involved in the foetal loss or premature birth observed in MPXV-infected women warrant further studies [22,23].

The transcriptomic analysis, carried out at 48 h post-infection (hpi), did not show a significant modulation of transcripts involved in IFN-mediated antiviral response. However, in ectocervical cells, a down-modulation of OAS2 was observed. To investigate more in-depth the interplay of MPXV with host antiviral response pathways, we performed kinetics experiments, which revealed common and differential patterns in the two LFGT cell lines studied. In particular, IFN-β and IFN-λ1 were induced earlier and more pronouncedly in ectocervical cells than in vaginal cells. These results are consistent with the slower viral release and less extensive impact of infection on the host transcriptome observed in Ect1/E6E7 cells. However, both IFN-β and IFN-λ1 appeared ineffective in inhibiting MPXV replication in the two LFGT cell lines studied. Previous studies have demonstrated that IFN-β significantly reduced viral replication in MPXV-infected CAST/EiJ mice [36] and exhibited anti-MPXV activity in vitro [73]. However, the extent to which MPXV replication was inhibited by IFN-β in vitro varied greatly, depending on the target cells used, with primary cells showing stronger suppression [73]Therefore, the resistance phenotype of MPXV to IFN-β and IFN-λ1, which was observed in both the VK2/E6E7 and Ect1/E6E7 cell lines, may be due to the influence of specific cell types on IFN antiviral activity, reflecting variability in IFN receptor expression, JAK–STAT signalling and basal or inducible levels of ISGs. Our results also confirm previous findings that MPXV has a low capacity to stimulate variations in the expression of innate immune sensors and antiviral effector genes in vitro [46,74]. Indeed, no significant changes were observed after MPXV infection for the ISGs analysed, nor for the PRRs involved in MPXV recognition. Remarkably, MPXV also did not cause any changes in the expression of IFN- ε in both vaginal and ectocervical cell lines. Similarly, the expression of IFN-ε was not altered by infection with herpes simplex virus 2 (HSV-2) [75], nor by stimulation in vitro with PRR ligands [75]. This lack of IFN-ε gene regulation by PRR pathways is consistent with the absence of response elements for IRFs, NF-κB and STAT pathways, as observed with other IFN genes [75].

This study has some limitations, mainly related to the use of immortalized epithelial cell lines and the inclusion of only one MPXV clade. Although immortalized cells do not fully replicate the

complexity of primary tissues, they remain the most accessible and reproducible models for studying vaginal and ectocervical biology, as primary cells from the lower female genital tract are difficult to obtain. The VK2/E6E7 and Ect1/E6E7 cell lines used here have been extensively characterized, shown to closely resemble the morphology and functional properties of their tissues of origin, to respond to sex hormones, and to be widely adopted in infectious disease research [27,61,62,76–79]. Nevertheless, future work will benefit from more physiological systems—such as organotypic epithelial models, tissue explants, or organ-on-chip devices—to better capture the multilayered structure, immune components, and hormonal environment of the female genital mucosa.

A second limitation is that we focused exclusively on MPXV clade IIb. While clade IIb has been less frequently associated with infections in women, it remains the globally dominant lineage and thus provides relevant insight into the interaction between circulating MPXV strains and the lower female genital tract. At the same time, recent epidemiological evidence suggests a stronger association between clade Ib and mpox acquisition through heterosexual contact [80], indicating that additional MPXV lineages may exhibit distinct tissue tropism or transmission dynamics in women. Comparative studies involving multiple clades will therefore be essential to determine shared and divergent determinants of pathogenicity, transmissibility, and host response in the female genital tract.Overall, our results indicate a dysregulation of tissue remodelling, development, and inflammation by MPXV, which could have a significant impact on the homeostasis of the lower reproductive tract. Whether these processes are involved in reproductive health, including fertility, the acquisition of STIs, or adverse birth outcomes should be investigated.

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Author's contribution: DM, AD, LR, VV, SM performed experiments and analysed data; LP, DP performed bioinformatic analyses; FC provided material and contributed to methodology; CS, GC analysed data, supervised experimental procedures, and contributed to manuscript draft; EG, FM, CS, GA, VM, AA supervised the project and contributed to study funding; GM conceived the study, supervised experimental procedures, contributed funding, analysed data, and wrote the manuscript. All authors provided critical feedback and contributed to shaping the research, analysis, and manuscript.

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Requests for further information and resources should be directed to and will be fulfilled by the corresponding author.

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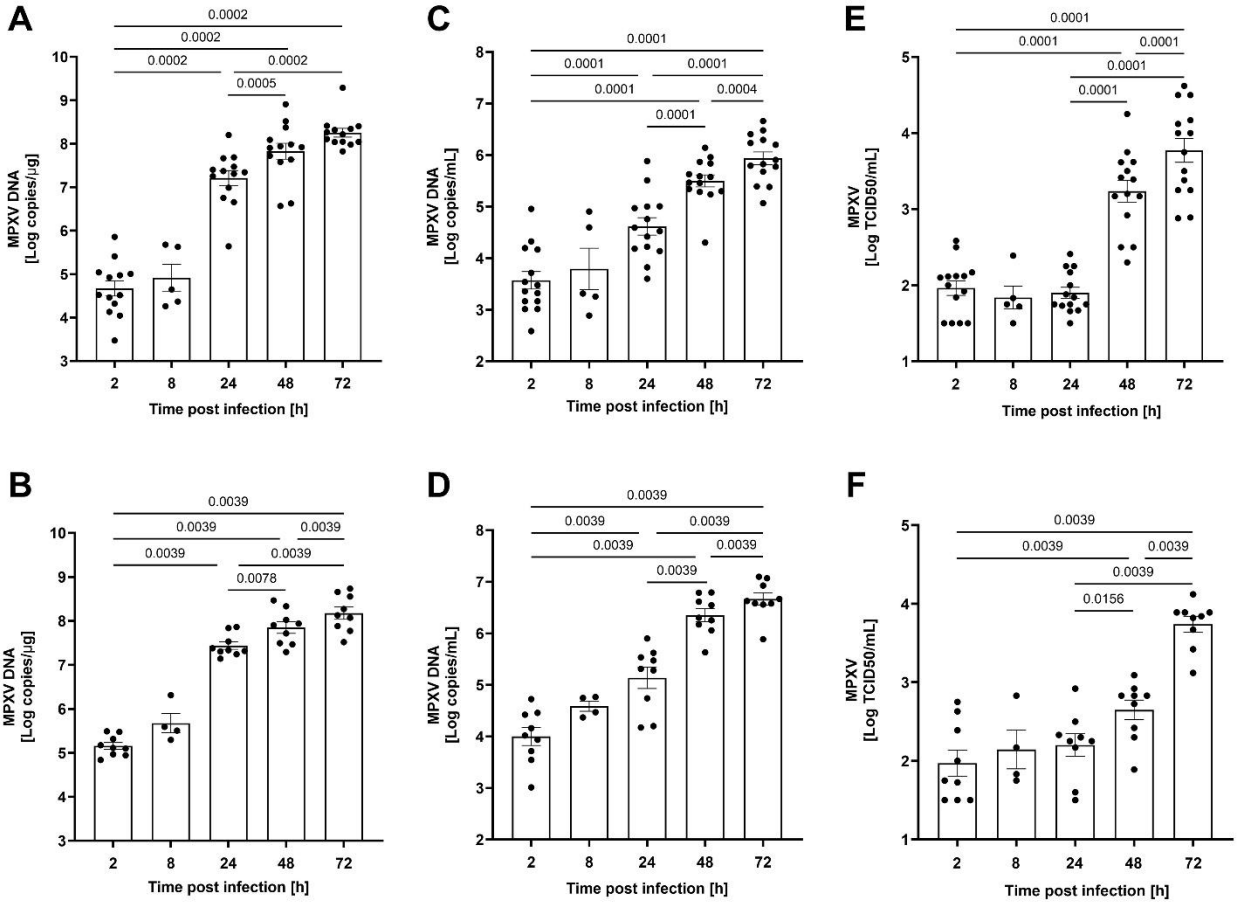


Figure 1

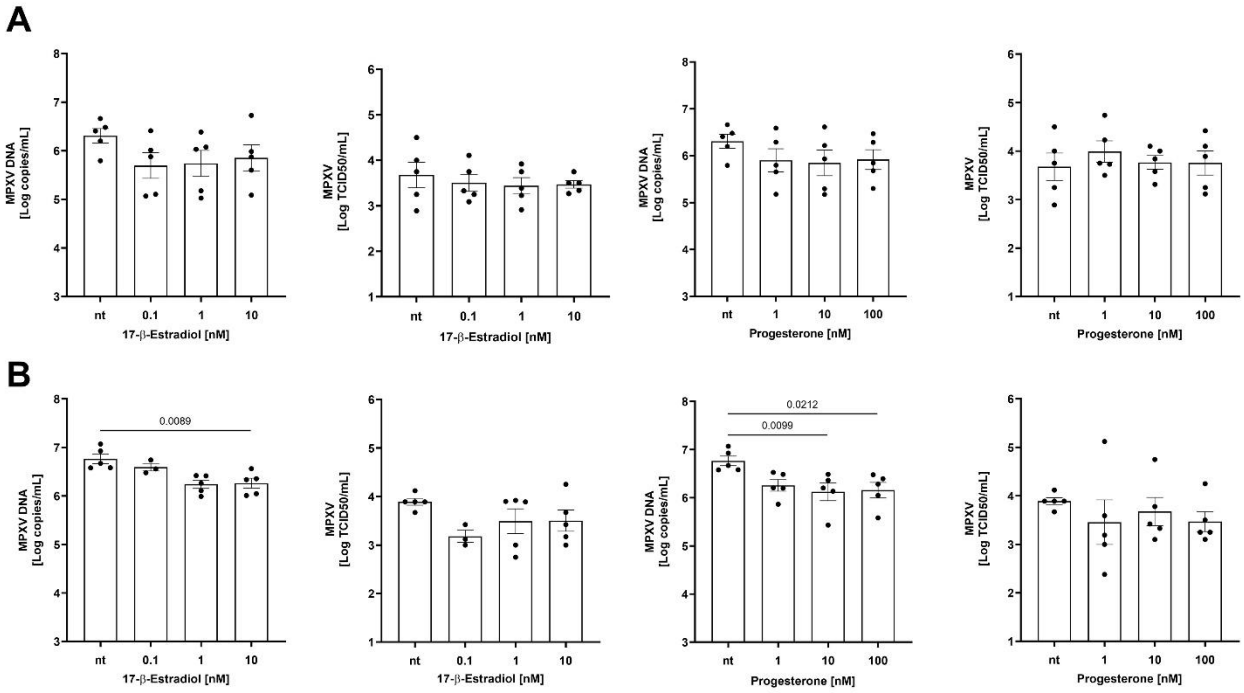


Figure 2

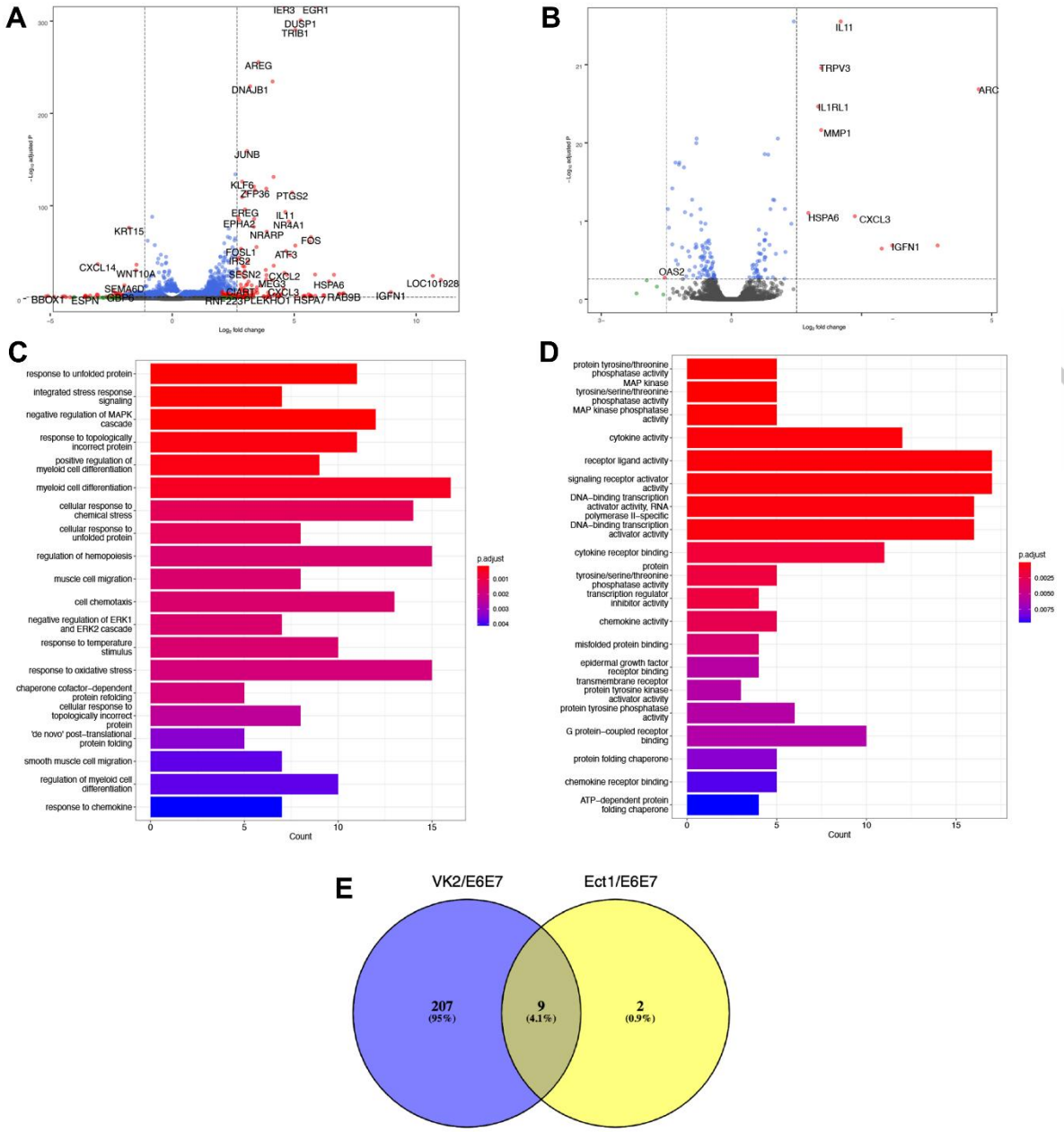

A
IER3
EGR1
DUSP1
TRIB1
AREG
DNAJB1
JUNB
KLF6
ZFP36
PTGS2
EREG
IL11
EPHA2
NR4A1
NRARP
FOS
KRT15
FOSL1
ATF3
IRS2
CXCL14
WNT10A
SESN2
CXCL2
MEG3
HSPA6
LOC101928
SEMA6D
CYP1B1
CXCL3
RAB9B
IGFN1
BBOX1
ESPN
GBP6
RNF223
PLEKHO1
HSPA7
B
IL11
TRPV3
ARC
IL1RL1
MMP1
HSPA6
CXCL3
IGFN1
OAS2
C
response to unfolded protein
integrated stress response signaling
negative regulation of MAPK cascade
response to topologically incorrect protein
positive regulation of myeloid cell differentiation
myeloid cell differentiation
cellular response to chemical stress
cellular response to unfolded protein
regulation of hemopoiesis
muscle cell migration
cell chemotaxis
negative regulation of ERK1 and ERK2 cascade
response to temperature stimulus
response to oxidative stress
chaperone cofactor-dependent protein refolding
cellular response to topologically incorrect protein
'de novo' post-translational protein folding
smooth muscle cell migration
regulation of myeloid cell differentiation
response to chemokine
Count
p.adjust
0.001
0.002
0.003
0.004
D
protein tyrosine/threonine phosphatase activity
MAP kinase tyrosine/serine/threonine phosphatase activity
MAP kinase phosphatase activity
cytokine activity
receptor ligand activity
signaling receptor activator activity
DNA-binding transcription activator activity, RNA polymerase II-specific
DNA-binding transcription activator activity
cytokine receptor binding
protein tyrosine/serine/threonine phosphatase activity
transcription regulator inhibitor activity
chemokine activity
misfolded protein binding
epidermal growth factor receptor binding
transmembrane receptor protein tyrosine kinase activator activity
protein tyrosine phosphatase activity
G protein-coupled receptor binding
protein folding chaperone
chemokine receptor binding
ATP-dependent protein folding chaperone
Count
p.adjust
0.0025
0.0050
0.0075
E
VK2/E6E7
Ect1/E6E7
207
(95%)
9
(4.1%)
2
(0.9%)

Figure 3

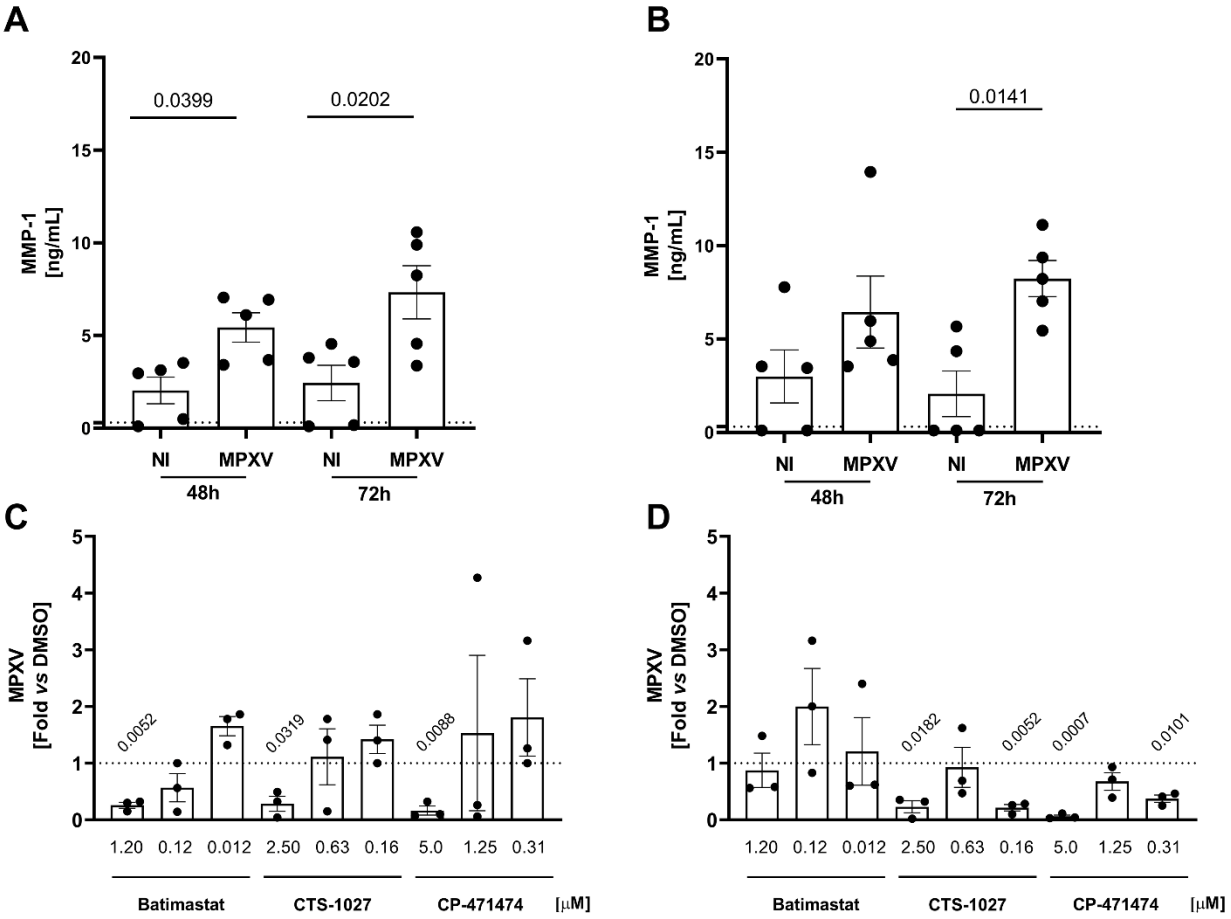


Figure 4

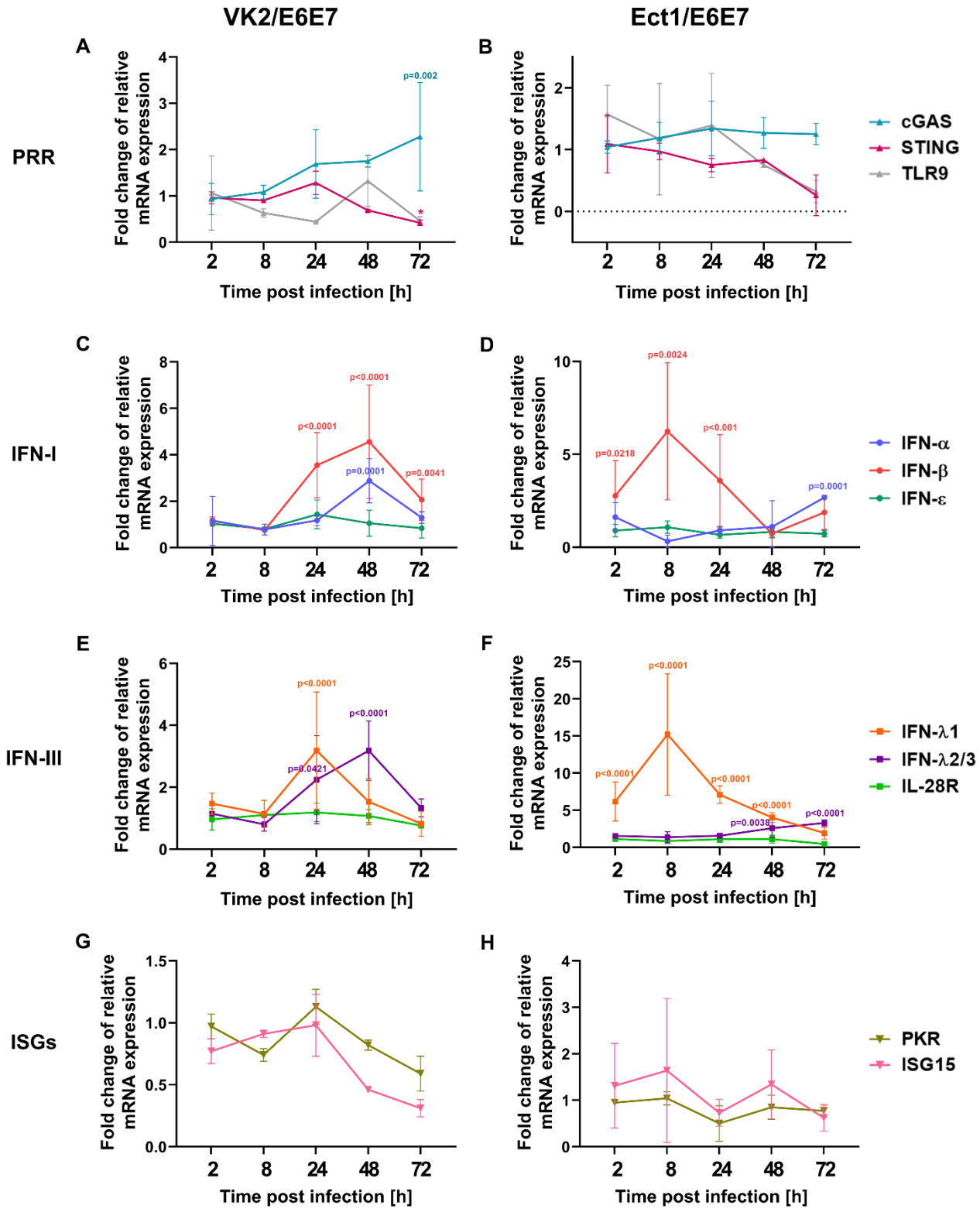

VK2/E6E7
Ect1/E6E7
A
B
PRR
Fold change of relative mRNA expression
Time post infection [h]
p=0.002
cGAS
STING
TLR9
C
D
IFN-I
p<0.0001
p=0.0001
p=0.0041
p=0.0024
p<0.001
p=0.0218
IFN-α
IFN-β
IFN-ε
E
F
IFN-III
p=0.0421
IFN-λ1
IFN-λ2/3
IL-28R
p=0.0038
G
H
ISGs
PKR
ISG15

Figure 5

Figure captions

Figure 1. MPXV replication in vaginal and ectocervical cells Replicative dynamics of MPXV in vaginal (VK2/E6E7) (A, C, E) and ectocervical (Ect1/E6E7) (B, D, F) cells in terms of released and cell-associated viral DNA and released viral particles infectious titre ($TCID_{50}$/ml) at 2, 8, 24, 48 and 72 hpi. Data are shown as mean ± SEM, and the Wilcoxon test addressed the statistical differences. *p*-values are indicated on the graphs. (A) VK2/E6E7 cell-associated viral DNA (2,24,48,72h n=13; 8h n=5). (B-D) Ect1/E6E7 cell-associated and released viral DNA (2,24,48,72h n=9; 8h n=4). (C-E) VK2/E6E7 released viral DNA and released viral particles infectious titre (2,24,48,72h n=13; 8h n=5). (F) Ect1/E6E7 released viral particles infectious titre (2,24,48,72h n=9; 8h n=4).

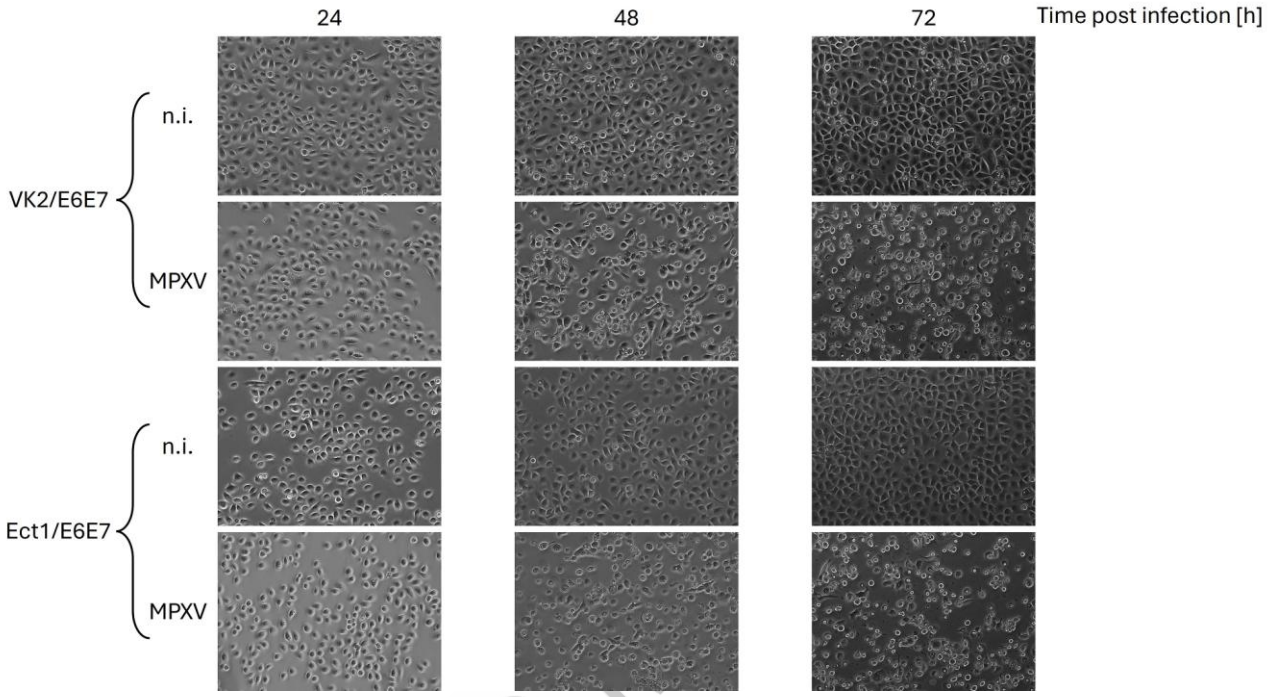
Figure 2. Effect of sex-hormones on MPXV replication. Comparison of MPXV levels and titre released from cells at 72 hpi in vaginal (VK2/E6E7) (A) and ectocervical (Ect1/E6E7) (B) cells with treatment with 17-β-estradiol or progesterone. Data are shown as mean ± SEM, and Friedman with Dunn's post-test addressed the statistical differences. p-values are indicated on the graphs. (A) n=5; (B) n=5 (0,1 nM 17-β-estradiol n=3).

Figure 3. Transcriptomic analysis of MPXV infected compared to not infected vaginal and ectocervical cells. Volcano plot of DEGs in vaginal (VK2/E6E7) (A) and ectocervical (Ect1/E6E7) (B) cells. Each point in the plot represents a single gene, with the x-axis indicating the Log_2 fold change in expression between MPXV-infected and uninfected cells, and the y-axis showing the $-log_{10}$ of the adjusted *p*-value. Genes with $|\ Log_2$ fold change $|\ > 2$ and adjusted p-value ≤ 0.05 are highlighted in red as significantly differentially expressed. Genes located to the right of the vertical threshold lines are upregulated, while those to the left are downregulated in infected cells. Panel C and D show the graphs relating to the analysis performed with Gene Ontology Biological Processes (C) and Molecular Functions (D) on the DEGs in vaginal cells. The pathways are classified using a colour gradation from red to blue, indicating the pathways most and least enriched in genes, respectively. Graphic representation using a Venn Diagram of the DEGs in the two cell lines (E) in which the genes located in the intersection between the two circles are differentially expressed in both cell lines, while those outside this area are exclusive to the respective cell line. (n=3)

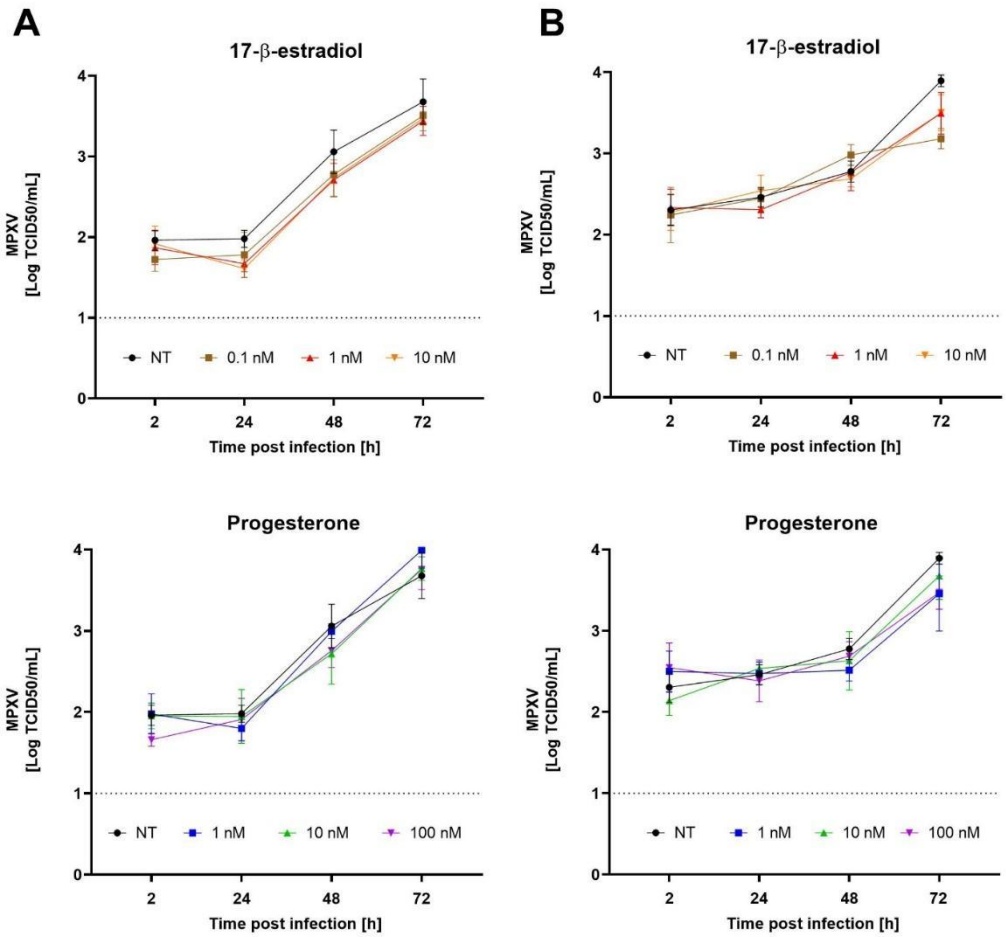
Figure 4. MMP-1 release upon MPXV infection of vaginal and ectocervical cells. MMP-1 levels in the supernatant of vaginal (VK2/E6E7) (A) and ectocervical (Ect1/E6E7) (B) cells infected or not with MPXV at 48 and 72 hpi (n=5) Friedman with Dunn's post-test addressed the statistical differences. MPXV titre released from vaginal (C) and ectocervical (D) cells concomitantly with treatment with MMP-1 inhibitors (n=3). Differences between DMSO and MMP-1 inhibitors treated samples were evaluated using One-sample t-test Data are shown as mean ± SEM. p-values are indicated on the graphs. C, D Dot line represents normalized viral release from DMSO treated cells. n.i.=not infected. (A,B) n=5; (C,D) n=3.

Figure 5. Antiviral IFN-I response upon MPXV infection of vaginal and ectocervical cells. In vitro expression of IFN-related genes in MPXV-infected immortalized human epithelial vaginal (VK2/E6E7) (panels A, C, E and G, n=5) and ectocervical (Ect1/E6E7) (panels B, D, F and H, n=4) cell lines. The genes are organized in each panel according to their function within the IFN pathway: panels A and B for pattern recognition receptors (TLR9, cGAS and STING); panels C and D for type I IFNs (IFN-α, IFN-β and IFN-ε); panels E and F for type III IFNs (IFN-λ1 and IFN-λ2/3); and panels G and H for IFN-stimulated genes (PKR and ISG15). Gene expression was measured at serial time points [2, 8, 24, 48 and 72 hpi] and calculated using the $2^{\wedge(-\Delta\Delta Ct)}$ method, normalized to β-actin. Data are expressed as the fold change (the normalized expression value of a

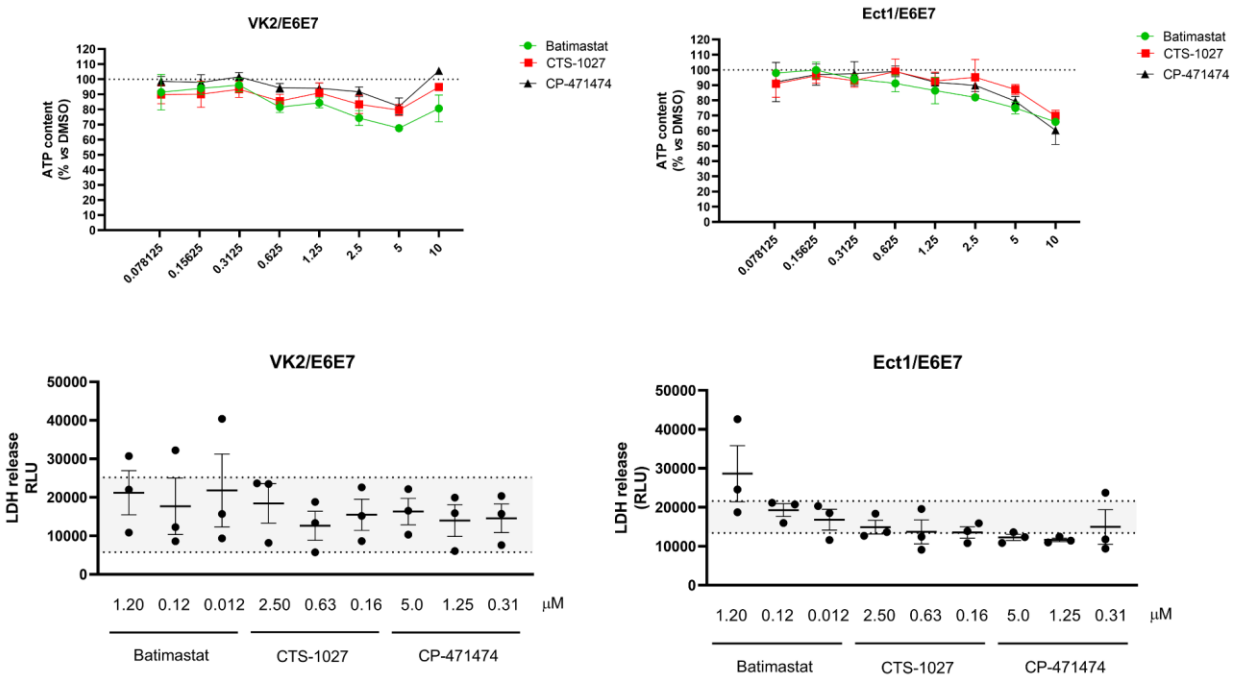
gene in MPXV-infected cells divided by the normalized expression value of the same gene in uninfected cells). Differences in mRNA levels between uninfected and MPXV-infected cells were evaluated using One-way ANOVA with Bonferroni correction.



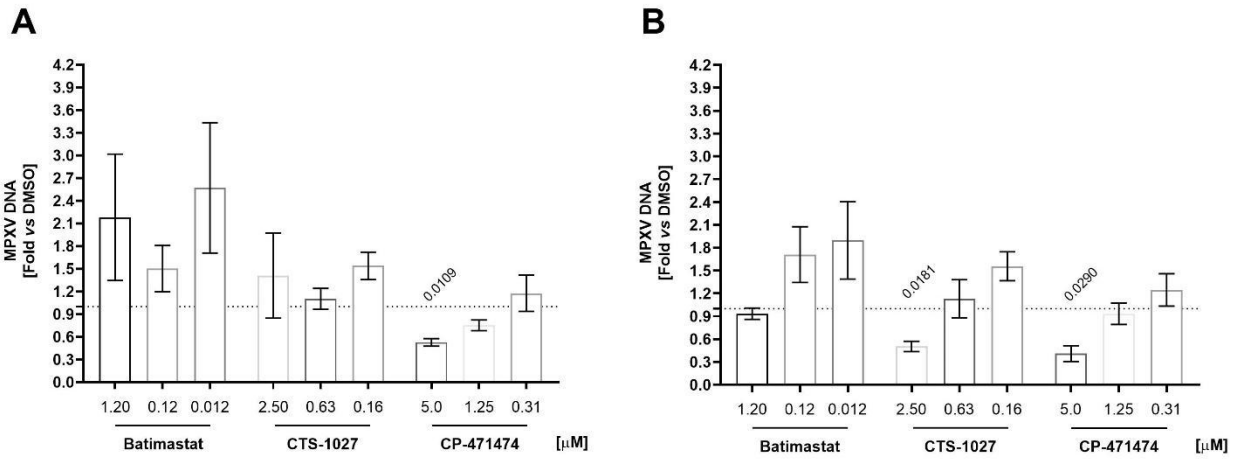
Supplementary Figure 1: Cytopathic effect upon MPXV infection of vaginal VK2/E6E7 and ectocervical Ect1/E6E7 cells. n.i.= not infected



Supplementary Figure 2: Time course of MPXV release from vaginal VK2/E6E7 (A) and ectocervical Ect1/E6E7 (B) cells after treatment with 17-β-estradiol or progesterone.



Supplementary Figure 3: Effect of MMP-1 inhibitors on cell viability. ATP content was measured as a preliminary test on VK2/E6E7 and Ect1/E6E7 cells treated with MMP-1 inhibitors for 72 hours at 10 to 0.078 µM (upper panel), dot lines represent 100% viability (DMSO treated sample). LDH release from vaginal VK2/E6E7 (n=3) and ectocervical Ect1/E6E7 (n=3) cells treated with MMP-1 inhibitors at the concentrations used in the infection experiment was measured at 72 hours post-treatment (lower panel), dot lines represent minimum and maximum levels of LDH released from DMSO treated cells. Data are shown as mean ± SEM.



Supplementary Figure 4: MPXV cell associated levels in vaginal VK2/E6E7 (A) and ectocervical Ect1/E6E7 (B) treated with different concentrations of MMP-1 inhibitors and measured at 72 hpi. Data are shown as mean ± SEM. p-values<0.05 are indicated on the graphs. Dot lines represent normalized cell associated MPXV DNA levels from DMSO treated cells. n=3.

Supplementary Table 1. Antiviral activity of IFN-β and IFN-λ1 against MPXV.

		VK2/E6E7		Ect1/E6E7	
	Concentration (IU/ml)	Virus Control (VC)	*Treated	Virus Control (VC)	*Treated
IFN-β	1000	3.53±0.41	3.21±0.26	2.77±0.54	2.66±0.43
	100	3.53±0.41	3.68±0.24	2.77±0.54	2.75±0.00
	10	3.53±0.41	3.50±0.00	2.77±0.54	2.75±0.42
IFN-λ1	1000	3.53±0.41	3.55±0.29	2.77±0.54	3.10±0.59
	100	3.53±0.41	3.27±0.04	2.77±0.54	2.67±0.53
	10	3.53±0.41	3.27±0.44	2.77±0.54	2.85±0.54

*Virus titer (log_{10} $TCID_{50}$/ml) after treatment with IFN-β (Avonex, Biogen, Cambridge, MA, USA) and IFN-λ1 (Interferon Lambda 1, human cell expressed, PBL assay science, Piscataway, USA). Data are expressed as mean value ± standard deviation. VSV titers (VC *vs* treated) were compared using One way ANOVA followed by Student's *t* test with Bonferroni correction.