

Online Supplement

Methods

Cell lines, primary alveolar epithelial cells and stimulation studies

Calu-3 cells used for infection and stimulation experiments and VeroE6 cells used for virus titration were cultivated using DMEM/F12 with 10% FCS or DMEM with 10% FCS. AEC and Calu-3 cells were treated with the following reagents: 10 μ M Cyclosporin A (CsA, Sigma Aldrich), 10 μ M Alisporivir (ALV, Debiopharm), 20 μ M calcineurin inhibitory peptide (CIP, Tocris), 10 μ M SB203580 (Tocris), 10 μ M SP600125 (Sigma Aldrich), 50 μ M NFAT-inhibitor (Cayman Chemical Company), DMSO solvent control or H₂O only (for CIP, exclusively).

Neutralization studies

MERS-CoV-infected Calu-3 cells were stimulated with CsA and treated with neutralizing antibodies against IFN λ 1 (100 % cross-reactivity with IFN λ 3) and IFN λ 2 (R&D, 4 or 5 μ g/ml, respectively). Neutralizing antibodies against IFN β were used as a control (R&D, 4 μ g/ml). At 24 h pi, cell culture supernatants were harvested for TCID₅₀ titration.

siRNA transfection

Silencer® Select siRNA against IRF1 (ID s7503, Invitrogen) and Silencer® Select Negative Control #1 siRNA (Invitrogen) were used for knock down studies. Calu-3 cells were transfected using Oligofectamine Transfection Reagent (Invitrogen) according to the manufacturer's protocol. 4 h post transfection (pt), cells were stimulated with CsA

and IFN-release and IFN-mRNA induction were analyzed 15 h post stimulation. For infection studies, IRF1-knockdown cells were infected 15 h pt with MERS-CoV, stimulated with CsA one hour after viral adsorption and analyzed for viral release and replication 24 h pi.

RNA extraction, cDNA synthesis and quantitative Real-Time-PCR

Total RNA was isolated using RNeasy-Minikit (Qiagen) followed by cDNA synthesis with random hexamer primers and RevertAid H Minus First Strand cDNA Synthesis Kit (ThermoFisher). Quantitative Real-Time-PCR (qPCR) using 50 ng cDNA in a total volume of 25 μ l with 2 \times QuantiFast SYBR green PCR Master Mix (Applied Biosystems) and 100 nM of forward and reverse primers (table 1) was performed (StepOne Real-Time PCR System (Applied Biosystems). β -Actin served as housekeeping gene for normalization. For determination of viral replication in cell cultures E-gene mRNA per actin ($2^{-\Delta Ct}$) was calculated. Changes in *IFN*, *IRF* and *ISG* RNA levels were determined in fold change over mock ($2^{-\Delta\Delta Ct}$) [1].

Quantification of viral RNA in lung homogenate was performed as described by Malczyk et al. 2015 [2]. Lung samples were taken from the center of the left lung lobe and were homogenized in 1 ml DMEM with ceramic beads (Lysing Matrix H 500, 2ml tube, MP Biomedicals) in a mixer mill (Retsch Schwingmühle MM 400) for 10 min at 30 Hz. To remove tissue debris, homogenates were centrifuged for 10 min at 2,400 rpm. Live virus particles in supernatant (in TCID₅₀ per milliliter) were determined on VeroE6 cells. 100 μ l of the supernatants were used for RNA isolation with the RNeasy minikit (Qiagen) according to the manufacturer's instructions. The amount of RNA was measured with a NanoDrop ND-100 spectrophotometer. Total RNA was reverse transcribed and quantified by real-time PCR using the SuperScript III OneStep RT-PCR System (Invitrogen Life Technologies) as described previously [2, 3].

Primers used in this study (all 5' to 3')

MERSupE for: GCAACGCGCGATTCAGTT, rev: GCCTCTACACGGGACCCATA

Mouse beta-Actin for: TAGCACCATGAAGATCAAGAT, rev: CCGATCCACACAGAGTACTT

Mouse IFN- λ 2/3 for: AGCTGCAGGTCCAAGAGCG, rev: GGTGGTCAGGGCTGAGTCATT

Mouse SCNN1B for: TGGTACTGCAATAACACCAACAC, rev:

AGCAGCGTAAGCAGGAACC

hActin for: CTGGGAGTGGGTGGAGGC, rev: TCAACTGGTCTCAAGTCAGTG

hIRF1 for: AGGAGCCAGATCCCAAGACGTG, rev: AGCATCCGGTACACTCGCACAG

hIFIT2 for: CTGCAACCATGAGTGAGAACAA, rev: CCTCCATCAAGTTCCAGGTGAA

hIFN λ 1 for: CGCCTTGGAAGAGTCACTCA; rev: GAAGCCTCAGGTCCCAATTC

hIFN λ 2/3 for: GCCAAAGATGCCTTAGAAGAG, rev: CAGAACCTTCAGCGTCAGG

hIFN- β for: TGCCTCAAGGACAGGATGAAC; rev: GGAAGTCTGCAGCTGCTTA

hIFIT1 for: CAGCAACCATGAGTACAAAT, rev: AAGTGACATCTCAATTGCTC

hOAS for : GCCCTGGGTCAGTTGACTGG, rev: TGAAGCAGGTGGAGAACTCGC

hMXA for: CGGTCCTCAGCCTGGTAG, rev: TGGGGTCCCGAGATATT

hViperin for: TGCCACAATGTGGGTGCTTACAC, rev: CTCAAGGGGCAGCACAAAGGAT

hIFIT3 for: GAACATGCTGACCAAGCAGA, rev: CAGTTGTGTCCACCCTTCCT

hXAF1 for: CTTACTGCCTGCGGTTCTG, rev: CGTACACCCAACCTGCTGGT

hIRF3 for: ACC AGC CGT GGA CCA AGAG, rev: TAC CAA GGC CCT GAG GCA C

hTetherin for: CCG TCCTGCTCGGCTTT, rev: CCGCTCAGAACTGATGAGATCA

hIRF7 for: GAGACTGGCTATTGGGGGAG, rev: GACCGAAATGCTTCCAGGG

hIRF 9 for: TTCTGTCCCTGGTGTAGAGCCT, rev: TTTCAGGACACGATTATCACGG

IFNλ 1/3 ELISA

Quantification of released human IFNλ1/3 in the supernatant of CsA-stimulated cells or murine IFNλ 2/3 in the bronchoalveolar lavage of CsA-fed mice was performed with DuoSet® Ancillary Reagent Kit 2 and human IFN-lambda 1/3 or mouse IFN-lambda 2/3 DuoSet ELISA (all R&D) according to the manufacturer's protocol.

IRF1 indirect immunofluorescence analysis

Intracellular localization of endogenous IRF1 protein was analyzed 3 and 4 hour post CsA stimulation. DMSO-treated cells were used as negative control. Stimulated cells were fixed with 4% PFA and permeabilized with methanol/acetone for 10 min. Cells were incubated with a rabbit monoclonal-anti-IRF1 (1:100; Cell Signaling) and an AlexaFluor 594-conjugated secondary antibody (1:400; Dianova). Cell nuclei were counterstained with DAPI. The samples were mounted in Fluoprep (Biomérieux) and images were recorded with a confocal laser scanning microscope (Leica SP5).

Epithelial integrity measurements

Calu3 cells were seeded in 0.4µm pore size transwell cell culture dishes (Corning) and cultured until achieving electrochemical resistances (TER) of $\geq 800\Omega /\text{cm}^2$ as measured by Millicell-ERS2 device. Cells were infected apically with MERS-CoV at MOI 0.1 and treated with CsA, ALV and JNK for 24 h at 37°C and then supplied with 3 mg/ml 70kDa FITC-dextran (Sigma Aldrich) labeled cell culture media including selected inhibitors. After 24 h of incubation at 37°C, apical and basal media were analyzed for FITC-dextran concentration (multi-mode-reader Synergy LX, Bio-Tek Instruments). Vectorial water transport was calculated by changes in FITC-dextran concentration between apical (C_a) and basal (C_b) media in comparison to starting conditions (C_0): $[1 - (C_0/C_a)] - [1 - (C_0/C_b)]$; as reported previously [4].

Apoptosis quantification

For quantification of MERS-CoV induced apoptosis a Caspase 3/7 Gloassay® (Promega) was performed according to the manufacturer's protocol. Confluent Calu-3 cells were infected and treated as describes above. 24 h pi Caspase 3/7 Globuffer was added to the same amount of cell supernatant (in total 300 µl) and incubated for 30 min. 100 µl of supernatant was pipetted into a whitewall 96 well plate and luminescence was analyzed using Centro LB 960 (Berthold Technologies). Duplicate determination was performed and Globuffer alone and supernatant without Globuffer were analyzed for subtraction of background. Shown are relative units to uninfected and DMSO-treated control-cells (set as 1).

Western blot

SDS-Page and western blot was analyzed as described previously [5]. Calu-3 cells were infected with MERS-CoV using a MOI of 0.1 and stimulated with CsA 1 hour after virus adsorption. 24 h pi cells were scratched off with 500 µl PBS supplemented with protease-inhibitor mix (Calbiochem) and centrifuged for 5 min at 5,000 rpm. Cell pellets were resuspended in sample buffer [6] containing 4% SDS and boiled at 100°C for 10 min. After discharge of the probes out of the BSL4 laboratory another 10 min boiling step was performed before the samples were separated using an 7,5 % SDS-Gel. After blotting on a nitrocellulose membrane and blocking using PBSdef with 5% milk powder first antibodies (Anti-CFTR Antibody, clone MM13-4 and mouse monoclonal Anti-Vinculin antibody both Sigma-Aldrich; ENaCβ antibody (E-10), sc-48428; Santa Cruz Biotechnology) diluted in PBSdef with 1% milk powder were incubated overnight followed by secondary antibody-incubation for 1 h (Goat Anti-Mouse/HRP and Swine Anti-Rabbit/HRP; both Dako). For visualization of the signals Image Lab software was used.

Mouse in vivo experiments - sample preparation

All animal experiments were performed in accordance with the regulations of German animal protection laws and as authorized by the regional authorities (Regierungspräsidium Giessen, G73/2017). Six- to 12-week-old C57Bl/6J mice were inoculated intratracheally (i.t.) with 50 µl of an adenovirus vector encoding human DPP4 and mCherry with a final titer of 2.5×10^8 PFU per inoculum (AdV-hDPP4; ViraQuest Inc.). Starting at day 3 post transduction, mice were fed daily with CsA (50mg/kg/day) or with DMSO as control mixed with nut-chocolate creme. CsA or DMSO fed mice were challenged intranasally (i.n.) with 30 µl of MERS-CoV at a dose of 1.5×10^5 TCID₅₀ as described before [2, 3]. The mice were euthanized 4 or 7 days after MERS-CoV infection, and representative left lobe lung samples were prepared for RNA isolation.

Non-infected control mice were fed with CsA or DMSO for 6 days and bronchoalveolar lavage was performed to determine the IFN λ levels in the lung by ELISA according to manufacturer's instructions (Mouse IL-28A/B (IFN-lambda 2/3) ELISA with R&D Systems DuoSet Development Kit).

Histology

For histopathological analyses of formalin-fixed, paraffin-embedded murine lung tissues, sections of 2 µm thickness were cut from four to six evenly distributed planes throughout the entire lungs and mounted on adhesive glass slides. The slides were stained with hematoxylin and eosin and coverslipped. Histopathological evaluation was performed using an established four grade scoring scheme [7] including the following parameters: affected area, severity and distribution of interstitial inflammation, infiltration of macrophages, lymphocytes and granulocytes, necrosis, alveolar

hemorrhage and edema as well as formation of Bronchus-associated lymphoid tissue (BALT) and perivascular, lymphocytic cuffing.

Supplemental Figures

Figure E1: ENaC β expression in Calu-3 after MERS-CoV infection and CsA treatment. Calu-3 were infected with MERS-CoV using an MOI of 0.1, stimulated with DMSO or 10 μ M Cyclosporin A (CsA), and analyzed at 24h pi. Cell lysates were analyzed by western Blot for expression of ENaC β (100 kDa) and vinculin (120 kDa). Left panel shows representative western blots of n = 3 experiments. Right panel shows relative quantitation with mock samples set to 100%.

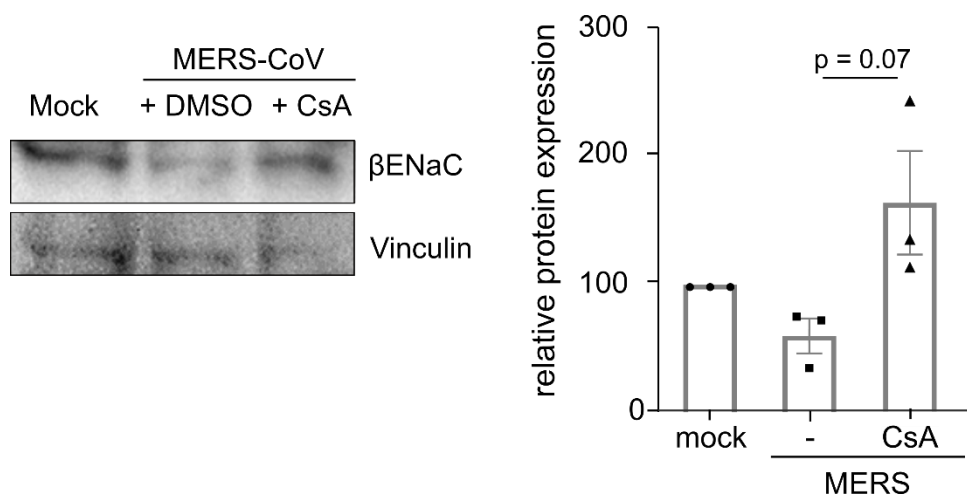


Figure E2: ALV treatment improves epithelial integrity upon MERS-CoV infection. Calu-3 were infected with MERS-CoV using an MOI of 0.1, stimulated with DMSO or 10 μ M CsA or 10 μ M ALV, and analyzed at 24h pi. **(A)** MERS-CoV-induced CPE and foci formation was documented in live cells by phase contrast microscopy at a magnification of 100x. **(B)** Epithelial integrity was quantified by transepithelial resistance measurements **(C)** and vectorial water transport evaluated by FITC-Dextran quantification 48h pi. Bar graphs in **(B, C)** represent means \pm SEM of n = 4 experiments. Statistical significance was analyzed by one-way-ANOVA and Bonferroni's multiple comparisons test, where all groups were compared to each other. *P < 0.05; **P < 0.01. Shown micrographs **(A)** are representative of n = 3 - 4 experiments.

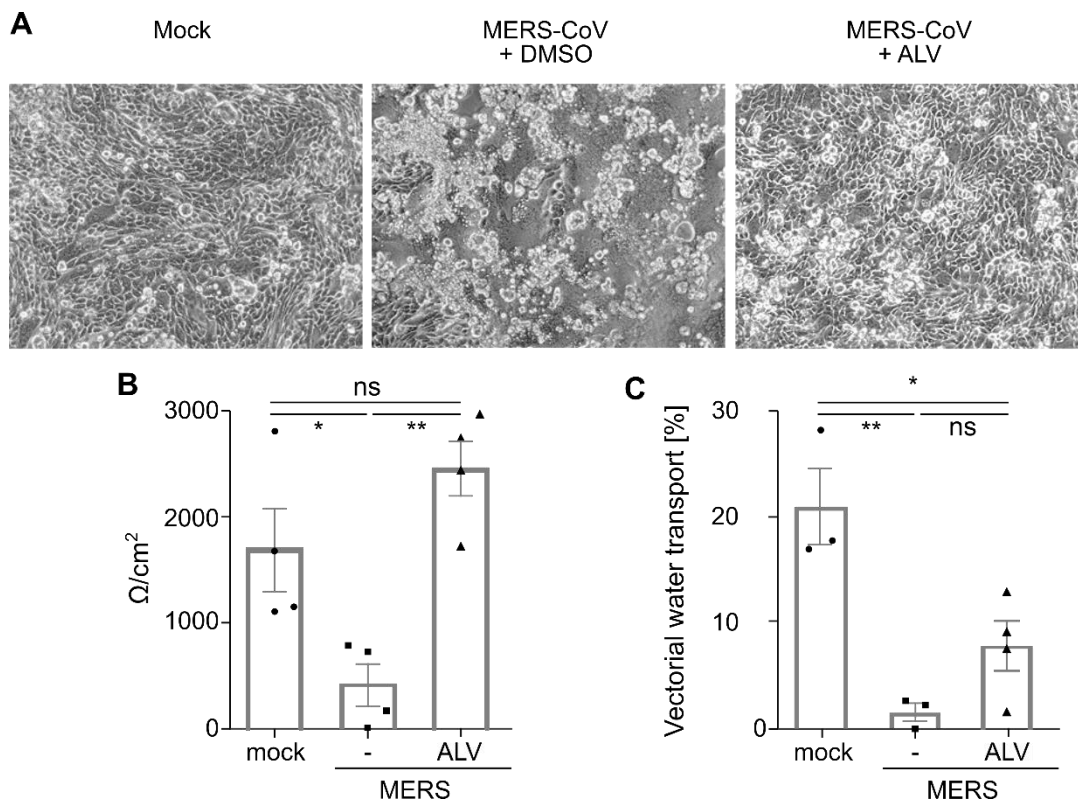


Figure E3: Inhibition of JNK does not affect cell foci formation or epithelial integrity. Calu-3 were infected with MERS-CoV using an MOI of 0.1, stimulated with DMSO or 10 μ M JNK inhibitor (SP600125), and analyzed at 24h pi. **(A)** MERS-CoV-induced CPE and foci formation was documented in live cells by phase contrast microscopy at a magnification of 100x. **(B)** Epithelial integrity was quantified by transepithelial resistance measurements **(C)** and vectorial water transport evaluated by FITC-Dextran quantification 48h pi. Bar graphs in **(B, C)** represent means \pm SEM of $n = 3 - 4$ experiments. Statistical significance was analyzed by one-way-ANOVA and Bonferroni's multiple comparisons test, where all groups were compared to each other. * $P < 0.05$. Shown micrographs **(A)** are representative of $n = 3 - 4$ experiments.

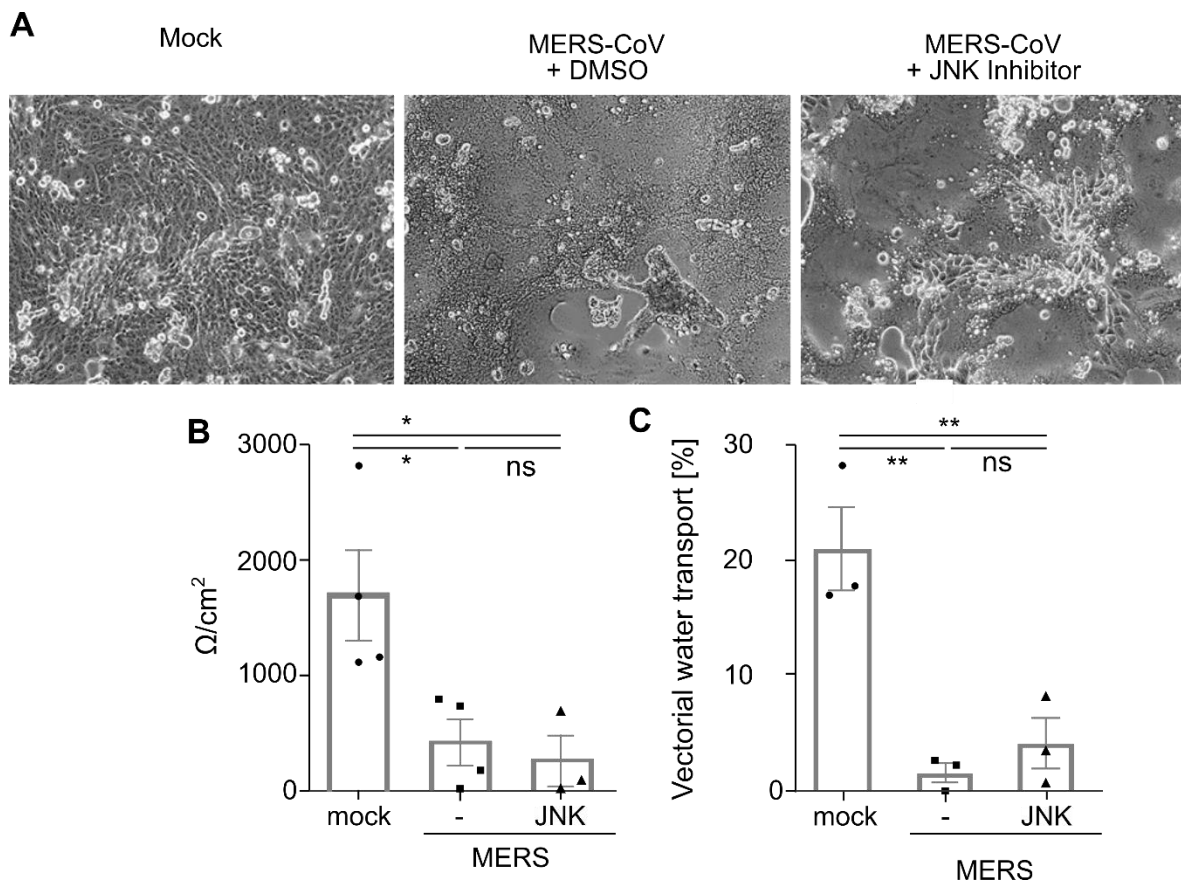


Figure E4: ALV induces interferon lambda to a similar extent as CsA. Calu-3 cells were stimulated with 10 μ M Alisporivir or 10 μ M CsA for 18, 24, 48 h, and 72 h, respectively. The amount of released IFN λ was measured by IFN λ 1/3 ELISA (R&D Systems DuoSet Development Kit). Bar graphs represent mean \pm SEM of n = 3 - 4 experiments.

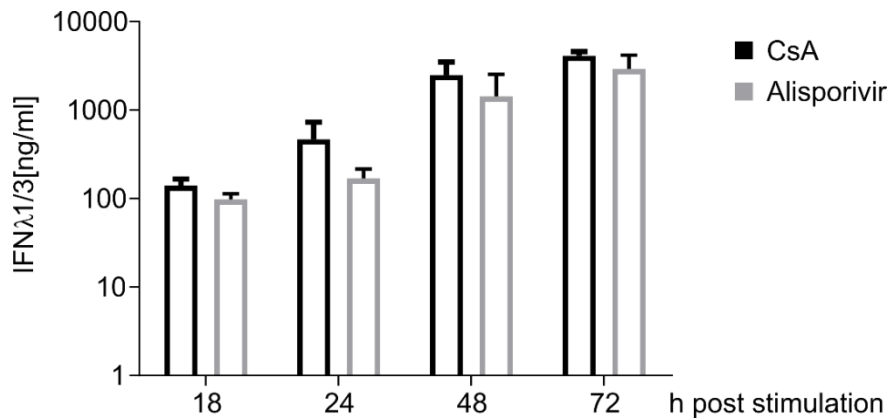


Figure E5: CsA serum levels after oral CsA application *in vivo*. Mice were fed daily with 50 mg/kg/day CsA for 6, 9 or 12 days. CsA serum levels were determined from blood sera by mouse Cyclosporin A ELISA-Kit (MyBioSource) according to the manufacturer's protocol. Single data points and means \pm SEM are given.

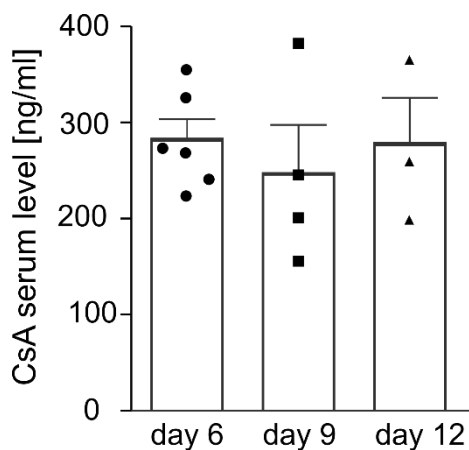


Figure E6: mCherry expression levels after oral CsA application *in vivo*. The amount of mCherry was determined as an evidence for successful and stable hDPP4 transduction. Mice were intratracheally infected with recombinant adenovirus encoding for human DPP4 and mCherry (Ad-hDPP4). Oral application of CsA (50 mg/kg/day) or DMSO as solvent control was started at day 3 post transduction, while mice were infected with 1.5×10^5 TCID₅₀/ml MERS-CoV via the intranasal route at day 5 post transduction. Mice were euthanized and lungs were isolated 4 days post MERS-CoV infection. mCherry RNA content was analyzed from lung homogenates using OneStep RT-PCR kit as described previously [2, 3]. Quantification was carried out using a standard curve based on 10-fold serial dilutions of appropriately cloned RNA ranging from 10^2 to 10^5 copies. Bar graphs in represent mean \pm SEM of n = 4 - 6 experiments.

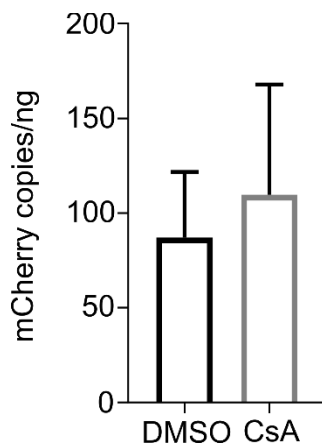


Figure E7: CsA induces interferon lambda and restricts MERS-CoV mRNA expression at day 4 pi *in vivo*. Mice were intratracheally infected with recombinant adenovirus encoding for human DPP4 and mCherry (Ad-hDPP4). Oral application of CsA (50mg/kg/day) or DMSO as solvent control was started at day 3 post transduction, while mice were infected with 1.5×10^5 TCID₅₀/ml MERS-CoV via the intranasal route at day 5 post transduction. Mice were euthanized and lungs were isolated 4 days post MERS-CoV infection. Lung homogenate was used to quantify **(A)** viral load by TCID₅₀. Bar graphs represent means \pm SEM of n = 6 - 8 experiments. Statistical significance was calculated using student's t-test. *P < 0.05. In **(B)** correlation between MERS-CoV titers and IFN λ 2/3 mRNA expression levels was calculated using Pearson correlation efficient.

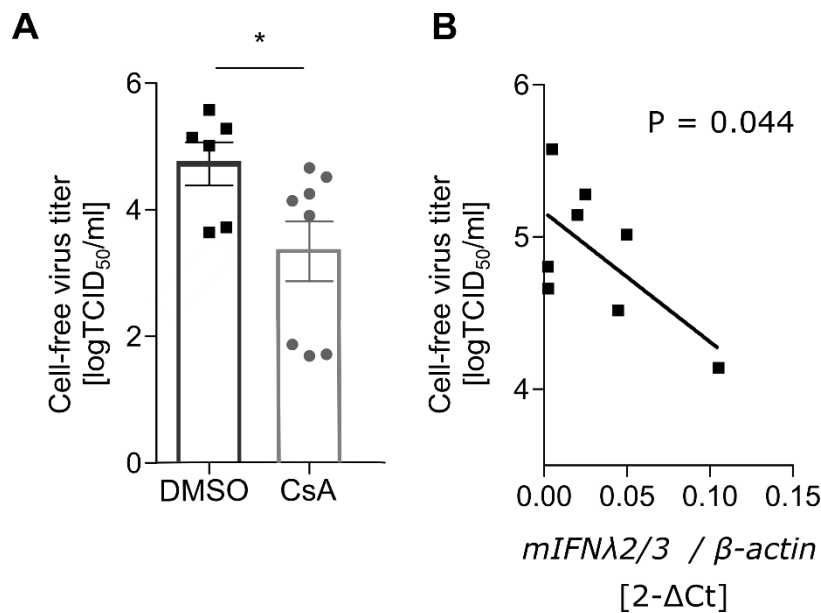
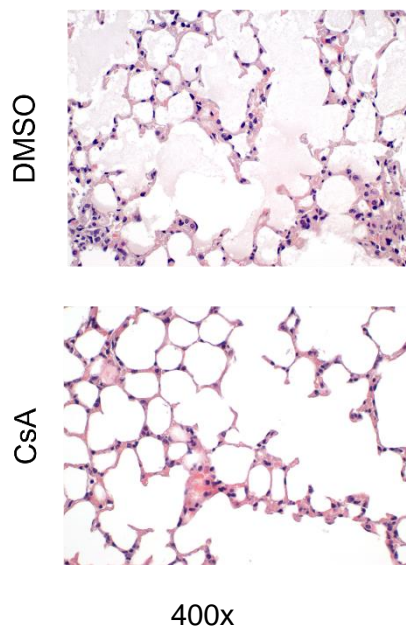


Figure E8: CsA prevents extensive edema formation in MERS-CoV-infected mice. Mice were intratracheally infected with recombinant adenovirus encoding for human DPP4 and mCherry (Ad-hDPP4). Oral application of CsA (50mg/kg/day) or DMSO as solvent control was started at day 3 post transduction, while mice were infected with 1.5×10^5 TCID₅₀/ml MERS-CoV via the intranasal route at day 5 post transduction. Mice were euthanized and lungs were isolated 7 days post MERS-CoV infection. Fixed lungs were processed for histology as described and stained with H&E. Extensive alveolar edema formation was only apparent in DMSO-treated mice (upper panel), while only mildly to moderately seen in CsA-treated mice (lower panel).



Supplemental Literature

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