



Effect of interferon alpha and cyclosporine treatment separately and in combination on Middle East Respiratory Syndrome Coronavirus (MERS-CoV) replication in a human in-vitro and ex-vivo culture model

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ABSTRACT

Middle East Respiratory Syndrome Coronavirus (MERS-CoV) has emerged as a coronavirus infection of humans in the past 5 years. Though confined to certain geographical regions of the world, infection has been associated with a case fatality rate of 35%, and this mortality may be higher in ventilated patients. As there are few readily available animal models that accurately mimic human disease, it has been a challenge to ethically determine what optimum treatment strategies can be used for this disease. We used in-vitro and human ex-vivo explant cultures to investigate the effect of two immunomodulatory agents, interferon alpha and cyclosporine, singly and in combination, on MERS-CoV replication. In both culture systems the combined treatment was more effective than either agent used alone in reducing MERS-CoV replication. PCR SuperArray analysis showed that the reduction of virus replication was associated with a greater induction of interferon stimulated genes. As these therapeutic agents are already licensed for clinical use, it may be relevant to investigate their use for therapy of human MERS-CoV infection.

1. Introduction

The past 15 years has seen the emergence of two novel coronaviruses that have infected humans resulting in a high morbidity and mortality. Until 2003 it was thought that coronavirus infections, such as OC43 and 229E were associated with mild respiratory disease and there was little reason to develop novel therapeutic options for these coronavirus infections. The global outbreak of SARS in 2003 with a 10% fatality rate, and the recent emergence of MERS-CoV infection, as well as other recently recognized coronavirus infections such as NL63 and HKU1 have demonstrated the need for investigation into treatment options of severe coronavirus infections. MERS-CoV was first identified in June 2012 from a 60 year old patient from Middle East who developed clinical symptoms and signs similar to SARS, and who eventually died from multi organ failure (Zaki et al., 2012). A novel coronavirus was isolated, initially called HCoV-EMC but renamed as Middle East Respiratory Syndrome coronavirus (MERS-CoV) (de Groot et al., 2013). Since 2012 the virus has continued to cause severe zoonotic human disease in the Middle East, sometimes associated with outbreaks of

human-to-human transmission within health care facilities (Arabi et al., 2017; Chan et al., 2014; Perlman and McCray, 2013). In May 2015 a large outbreak occurred in Korea (Korean Society of Infectious, Korean Society for Healthcare-associated Infection and Prevention, 2015), highlighting the threat to global public health security.

Previously we showed that MERS-CoV replicate in human upper and lower respiratory tract where it infected non-ciliated bronchial epithelial cells, bronchiolar epithelial cells, type I and type II alveolar pneumocytes and endothelial cells using *ex vivo* explants culture (Chan et al., 2013). Furthermore, we showed that in contrast to SARS-CoV infection, MERS-CoV infection elicited a lower pro-inflammatory cytokine response including the type I and III interferons (Chan et al., 2013). This evasion of innate immune induction and reduced interferon (IFN) response suggested that exogenous IFN may be a possible treatment options for human MERS-CoV infection. A previous study showed that pegylated IFN exhibited a more potent antiviral effect to MERS-CoV than SARS-CoV in cell culture and macaque model (de Wilde et al., 2013; Falzarano et al., 2013) and it was proposed that this was due to the lack of a MERS-CoV homolog of SARS-CoV ORF6 protein that blocks

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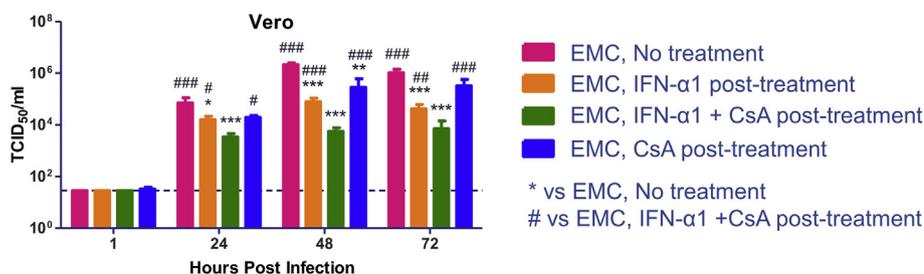


Fig. 1. Evaluation of the changes in MERS-CoV replication after the addition of IFN- α 1, CsA and a combination of the two agents in Vero cells. 9 μ M CsA and/or 2.4×10^4 U/ml of IFN- α 1 were used. The data shown are the mean \pm standard error of the mean in three representative experiments, which are analyzed by two-way ANOVA followed by Bonferroni's *post hoc* test (*/# $P < 0.05$, **/# $P < 0.01$, ***/### $P < 0.001$).

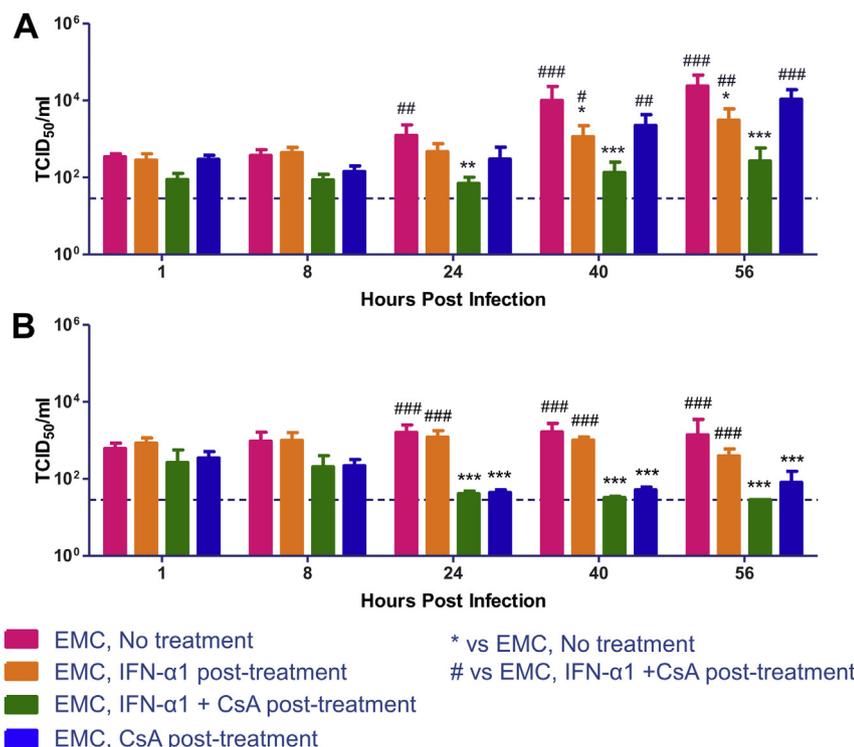


Fig. 2. Evaluation of the changes in MERS-CoV replication after the addition of IFN- α 1, CsA and a combination of the two agents in (A) human bronchus and (B) human lung explant cultures. 9 μ M CsA and/or 2.4×10^4 U/ml of IFN- α 1 were used. The data shown are the mean \pm standard error of the mean in at least three independent experiments, which are analyzed by two-way ANOVA followed by Bonferroni's *post hoc* test (*/# $P < 0.05$, **/# $P < 0.01$, ***/### $P < 0.001$).

the IFN induced translocation of STAT1 - a factor essential for signaling via the IFN receptor that leads to the induction of IFN associated antiviral genes. In the macaque model, IFN was used together with ribavirin and this led to improved clinical symptoms following MERS-CoV infection, with microarray analysis showing lower expression of inflammatory genes (Falzarano et al., 2013). Nevertheless, a number of recent clinical studies reported that the IFN and ribavirin combination did not improve long-term survival, and was not beneficial to patients who received the treatment late after infection (Al-Tawfiq et al., 2014; Omrani et al., 2014). This indicates that there is a need to consider other therapeutic combination for treating MERS-CoV infection.

Cyclosporine, such as cyclosporin A (CsA) and its derivatives has been shown to inhibit MERS-CoV replication *in vitro* (de Wilde et al., 2013). It has been demonstrated that CsA could restore type I IFN expression upon hepatitis C or rotavirus virus infection (Liu et al., 2011; Shen et al., 2013). Combined use of CsA and type I IFN was shown to inhibit hepatitis C virus replication and trigger greater virological response than IFN treatment alone (Henry et al., 2006; Inoue et al., 2003). As CsA has known immune suppressive function, non-immunosuppressive cyclophilin inhibitors have been tried in combination with ribavirin for MERS-CoV infection. Though these have an *in vitro* effect on MERS-CoV and SARS-CoV, this did not translate into a benefit in a mouse model (de Wilde et al., 2017). Here, we report the individual and combined effects of CsA and IFN- α 1 on inhibiting MERS-CoV replication in an *in vitro* and human lung and bronchus *ex vivo* explant culture model. We found the combined use of CsA and IFN- α 1 had

inhibitory effects on MERS-CoV infection and replication, as well as on the induction of interferon stimulated genes (ISG), which sheds light on the potential use of this combination in curing MERS-CoV infection.

2. Material and methods

Ex vivo explants culture of human respiratory tract was obtained from patients undergoing surgery at Queen Mary Hospital, according to previously established criteria (Hui et al., 2017). We selected areas of morphologically normal lung, and histology was performed on a control sample. The samples were subjected to virus culture and bacterial culture. If there was intrinsic disease or infection in the resected lung specimens, they were not used for research. The project was approved by the local institutional review board (UW 14-549).

2.1. Ex vivo organ culture and infection

Fresh biopsies of human bronchi and lung were sampled from human lungs that were removed at surgery as part of clinical care, but surplus for routine diagnostic requirements. *Ex vivo* infections of human bronchus and lungs was performed as previously published (Chan et al., 2013, 2014). In brief, the bronchial mucosae were placed on a surgical sponge with their apical epithelial surface facing upwards while the lung parenchymal tissues were placed into 24 well-plates directly with 1 ml of culture medium at 37 °C. Human betacoronavirus of lineage C virus (HCoV-EMC) was provided by R. Fouchier, Erasmus MC,

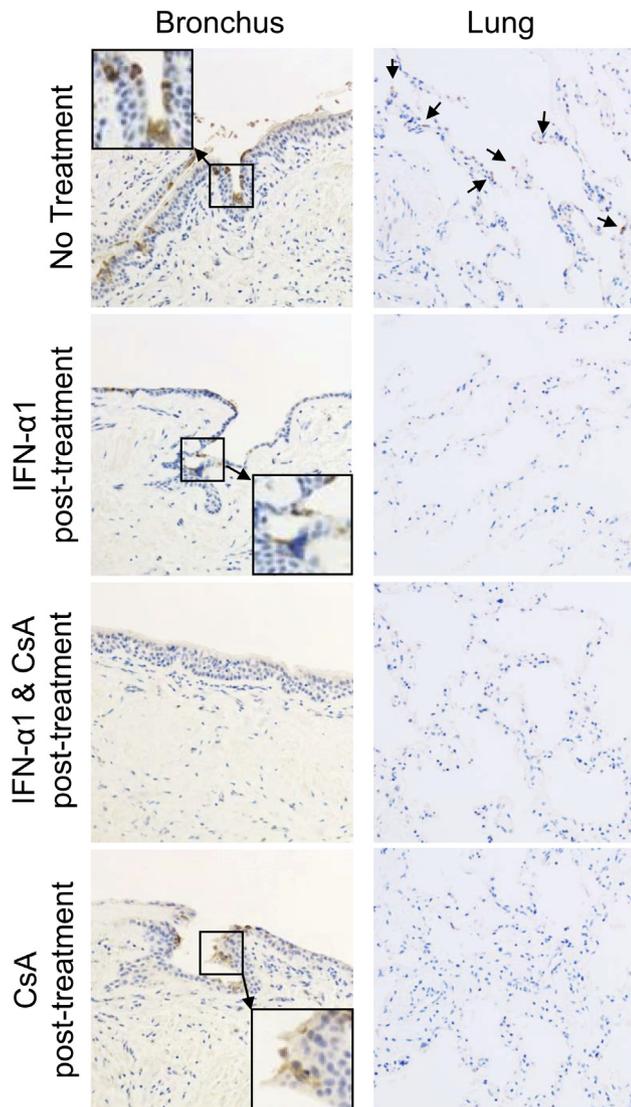


Fig. 3. IFN- α 1 and/or CsA inhibited MERS-CoV infection in bronchus & lung explant culture. 9 μ M CsA and/or 2.4×10^4 U/ml of IFN- α 1 were used. Sections of infected human bronchus and lung were stained with a polyclonal antibody against the MERS-CoV nucleocapsid protein. Positive cells were identified with a reddish brown colour. Magnification: x200. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Rotterdam, the Netherlands, and used as the prototype MERS-CoV for examining the efficacy of different treatments. Fresh bronchial and lung tissues were infected with HCoV-EMC with a viral titer of 10^6 TCID₅₀/ml for 1 h at 37 °C and then washed with 5 ml of PBS at room temperature for three times to remove unbound virus. Fresh culture medium with a regimen of 9 μ M cyclosporine A (Novartis Pharmaceuticals Corporation) and/or 2.4×10^4 U/ml of IFN alfacon-1 (Kadmon pharmaceuticals) was then added to the cultures, and the treatments were replenished in a 16-h interval. Supernatants from the infected cultures were collected at 1, 8, 24, 40 56 hpi and titrated for infectious virus using the TCID₅₀ assay for HCoV-EMC. Increasing virus titers over time provided evidence of productive virus replication. Tissues were collected at 24, and 56 hpi for RNA extraction and fixation in 10% formalin for immunohistochemical staining of MERS-CoV nucleocapsid protein (NP) using an antibody provided by Dr. R. Baric, and cleaved caspase 3 (Cell Signaling Technology). Vero cells were incubated with alpha-interferon and/or cyclosporine, for determination of optimal inhibition before application to *ex vivo* samples (Supplementary Fig. 1).

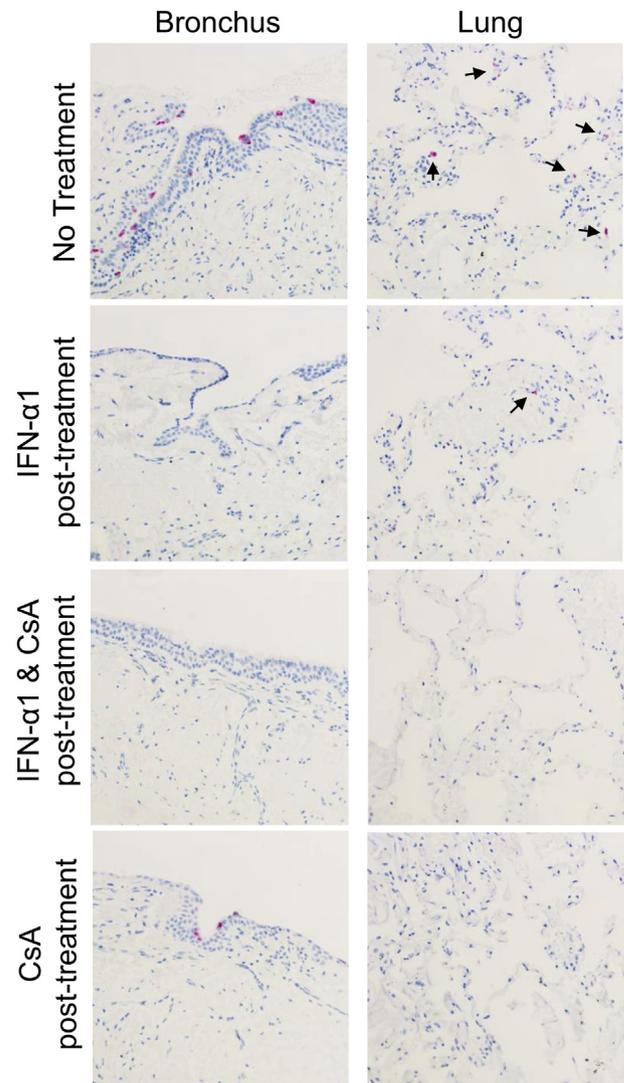


Fig. 4. IFN- α 1 and/or CsA reduced apoptosis caused by MERS-CoV infection in bronchus & lung explant culture. 9 μ M CsA and/or 2.4×10^4 U/ml of IFN- α 1 were used. Sections of infected human bronchus and lung were stained with a monoclonal anti-cleaved caspase 3 antibody. Positive cells were identified with a Vector Red staining (pink colour). Magnification: x200. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

2.2. Cell cultures

Vero (ATCC CCL-81) cell line was cultured in Minimum Essential medium (MEM, Gibco) supplemented with 10% fetal bovine serum (FBS, Gibco), 100 units/mL of penicillin and 100 μ g/mL of streptomycin. Human lung microvascular endothelial cells were prepared from fresh human lung by selection of CD31⁺ cells using anti-human CD31 antibody (Abcam) and Dynabeads cell separation system (ThermoFisher). The isolated cells were maintained in EGM-2 medium supplemented with 5% FBS (Lonza). All cells used in this study were maintained at 37 °C with 5% CO₂ humidified atmosphere.

2.3. Viral replication kinetics by 50% tissue culture infectious dose (TCID₅₀/ml) titration

Confluent 96-well tissue culture plates of Vero E6 cells were prepared 1 days before the virus titration assay. Cells were washed once with PBS and replenished with DMEM with 2% FBS, 100 units/mL of penicillin and 100 μ g/mL of streptomycin. Serial half-log₁₀ dilutions

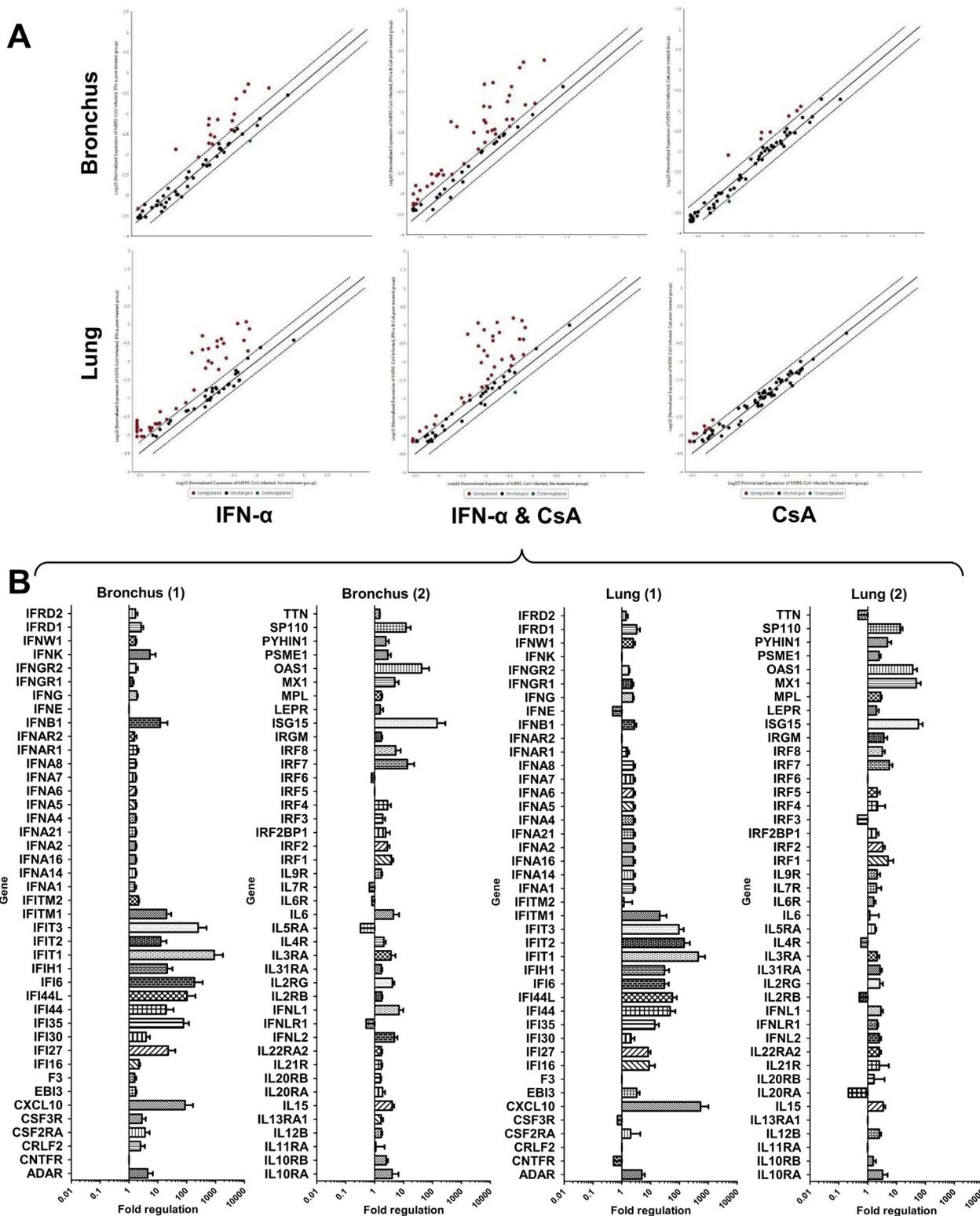


Fig. 5. Combined treatment of IFN- α 1 and CsA induced highest level of interferon stimulated genes (ISGs). 9 μ M CsA and 2.4×10^4 U/ml of IFN- α 1 were used. Distribution of gene expression was shown in the clustergram (A), the X axis indicates the normalized expression level of EMC infected lung or bronchus tissue without treatment, and the Y axis indicates the normalized expression level of EMC infected lung or bronchus tissue with different treatments; each dot on the plot represents the corresponding expression of a gene, the central line indicates no differences in gene level between two groups, while the boundary lines indicates the fold-change threshold ($> = 2$ folds). The red dots lie above the upper boundary line are the up-regulated genes in EMC infected tissues with different treatments as compared to EMC infected tissues without treatment; and the green dots in lower section of the plot are the down-regulated genes. Gene expression of the combined treatment group in human bronchus (B) and lung (C) explant culture relative to the no treatment group ($n = 3$). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

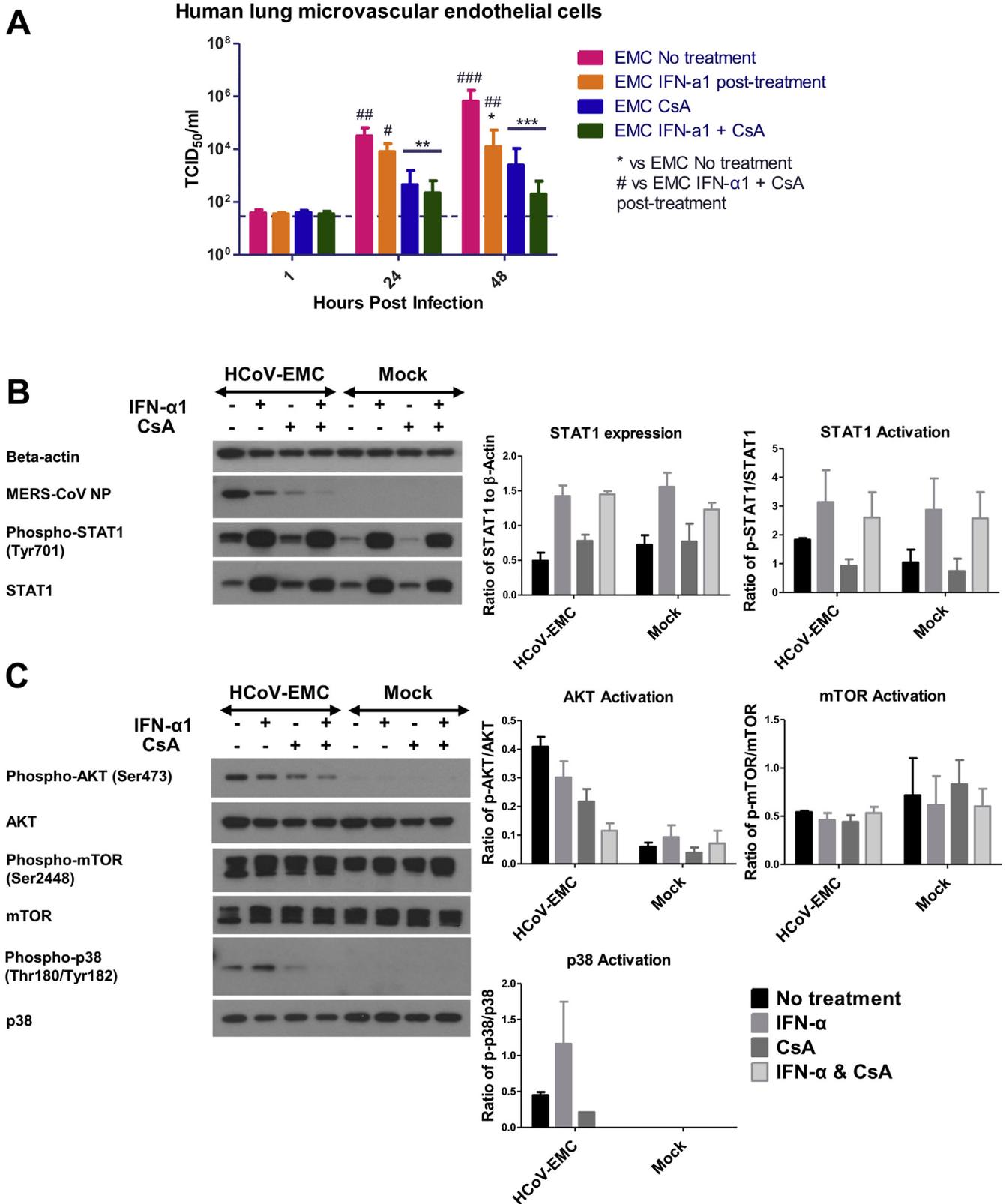


Fig. 6. Replication of MERS-CoV with different treatments in human microvascular endothelial cells (A), 9 μM CsA and/or 2.4 × 10⁴ U/ml of IFN-α1 were used. Effect of IFN-α1 and/or CsA on protein expression level of MERS-CoV nucleocapsid and phosphorylation/activation of STAT1 protein (B); phosphorylation/activation level of protein involve in PI3K/Akt/mTOR and p38 MAPK pathways (C) were shown. Bands from three independent experiments were quantified by densitometry using ImageJ software, normalized expression/activation levels were indicated on the histogram. 9 μM CsA and/or 2.4 × 10⁴ U/ml of IFN-α1 were used.

(from 0.5 log to 7 log) of virus-infected culture supernatants was added onto the wells in quadruplicate. The plates were observed for CPE daily, for seven days. The endpoint of viral dilution leading to CPE in 50% of inoculated wells was estimated by using the Karber method and designated as one TCID₅₀/ml.

2.4. Quantification of viral and host cytokine and chemokine mRNAs by quantitative RT-PCR

Bronchial and lung fragments were homogenized using a TissueRuptor (Qiagen, Hilden, Germany) in 700 µl RLT lysis buffer with beta-mercaptoethanol on ice. RNA extraction was carried out using RNeasy Mini kit (Qiagen, Hilden, Germany) following manufacturer's instruction with the addition of DNase-treatment and eluted in 50 µl RNase free water. 25 ng of total RNA was used for the first-strand cDNA synthesis with PrimeScript RT reagent Kit System (TaKaRa).

2.5. Evaluation of interferon pathway profiles by superarray

The expression of 84 key genes involved in cell signaling mediated by interferon receptors and ligands was profiled by RT-PCR-based RT² Profiler Interferon and receptors Arrays (Qiagen). RT-PCR reactions were performed in 96-well plate format using the ViiA7 Real-Time PCR System (Thermo Fisher Scientific). Fold changes of IFN gene expression in experimental samples relative to the control samples (e.g. mock-infected) were calculated using the $\Delta\Delta Ct$ method. The $\Delta\Delta Ct$ value of each sample was normalized by up to a total of 5 housekeeping genes (β -2-microglobulin [B2M], hypoxanthine phosphoribosyltransferase 1 [HPRT1], ribosomal protein L13a [RPL13A], glyceraldehyde-3-phosphate dehydrogenase [GAPDH] and β -actin [ACTB]). All data was analyzed by the RT² Profiler PCR Array Data Analysis Template v3.5 and all gene expression changes greater than 5 fold was considered significant, and significant gene changes were confirmed by individual qPCR.

2.6. Western blotting

Cell lysate were prepared using RIPA buffer, which were heated for 10 min at 95 °C. The protein were then resolved in SDS-PAGE and transferred to PVDF membrane. Mouse anti-actin (Millipore) and rabbit anti-MERS-CoV nucleocapsid protein (kindly provided by Dr. R. Baric) were used as loading and infection controls. Other proteins of interest, including phosphor-STAT1 (Tyr701), STAT1, phosphor-AKT (Ser473), AKT, phosphor-mTOR (Ser2448), mTOR, phosphor-p38 (Thr180/Tyr182), and p38, were also detected (all from Cell Signaling Technology). The membranes were incubated with the respective HRP conjugated secondary antibody, and signals of different protein of interest were detected by enhanced chemiluminescence method.

2.7. Statistical analysis

Experiments were performed independently with three different donors. Results shown in figures included the calculated mean and standard error of mean. Comparison among three or more groups was analyzed using two-way analysis of variance (ANOVA) with Bonferroni's multiple-comparisons as *post-hoc* test between groups. Statistical significance is defined when $p < 0.05$.

3. Results

3.1. Interferon- α 1 and/or cyclosporine A inhibit the replication of HCoV-EMC

Viral replication in Vero cells culture was determined with regimens of 9 µM cyclosporine A and 2.4×10^4 U/ml of IFN alfacon-1 separately or in combination. Although there was a significant reduction of viral

titres with IFN-alfacon or CsA used separately, cultures treated with both agents in combination had significantly lower viral titres at 24, 48 and 72 h when compared with cultures treated with a single drug as well as untreated cells (Fig. 1).

While HCoV-EMC replicated in untreated *ex vivo* lung and bronchial tissues, titres in the bronchus were higher than those in the lung, especially at 40 and 56 hpi. Similar to the replication in cell lines, viral replication was reduced by all treatments at 40 and 56 h post-infection (Fig. 2A and B). In *ex vivo* cultures of bronchus (Fig. 2A), combination of CsA and IFN alfacon-1 treatment had significantly lower viral titres compared to CsA or IFN treatment alone. In the *ex vivo* lung cultures, combination therapy or CsA therapy resulted in significantly lower viral titres than untreated cultures or IFN treatment alone (Fig. 2B). However, the effect of CsA alone was comparable with the combination therapy. These data suggested additive or synergistic effects of IFN and CsA in limiting HCoV-EMC replication in the bronchus.

3.2. Immunohistochemistry for viral antigen and apoptotic cell death in interferon- α 1 and/or cyclosporine A treated *ex vivo* cultures of human lung and bronchus

Lung and bronchus explants tissues were collected and fixed with 10% formalin after infection. MERS-CoV nucleocapsid protein (NP) was used as an indicator of HCoV-EMC infectivity (Fig. 3). Extensive HCoV-EMC infection was found in both bronchial ciliated and non-ciliated cells and alveolar pneumocytes without any treatment. In bronchial explants, it has been found that IFN- α 1 treatment could inhibit HCoV-EMC infection, and the level of inhibitory effect in infection was greatest in the combined treatment group; CsA alone inhibited the least HCoV-EMC infection. Furthermore, in *ex vivo* lung explants infection, HCoV-EMC infection was significantly inhibited in all treatment groups when compared to the control treatment.

Next, we determined the ability of different treatments to reduce cellular apoptosis induced by HCoV-EMC infection by staining for cleaved caspase 3 (Fig. 4). Extensive staining of cleaved caspase 3 were found in the *ex vivo* bronchial and lung explants without treatment, while the level of staining was reduced in all treatments groups in bronchus tissues. IFN- α 1 and CsA combined treatment group had the greatest impact on reducing cleaved caspase 3 staining, and the effect by CsA alone was the lowest in bronchus tissues. For lung tissues, the level of apoptosis was minimal in both IFN- α 1 and CsA combined treated and CsA treated groups.

3.3. Induction of interferon stimulated genes but not interferon receptors by the treatment of Interferon- α 1 and cyclosporine A

After showing the ability of IFN- α 1 and CsA combined treatment in inhibiting HCoV-EMC replication, we investigated the potential antiviral mechanisms underlying such inhibition using an interferon and receptor PCR array. The data showed that the combined treatment of IFN- α 1 and CsA had the most potent effect on inducing interferon-stimulated genes (ISGs) in both lung (24 hpi) and bronchial (56 hpi) tissues (Fig. 5A). The combined treatment group also induced the expression of IFN beta-1 but not IFNAR1, 2 and IFN gamma receptors in both lung and bronchus (Fig. 5B). ISGs such as ISG15, MX1, IRF7, IFI44, IFI44L, OAS1, SP110, IFIT1, IFIT2, IFIT3, and IFI27 were highly induced and these data were verified in independent qPCR using lung and bronchus tissues (Supplementary Figs. 2–3), and in Vero E6 cell line (Data not shown), the induction of ISGs were significant compared with the no treatment group.

3.4. Activation of pathways related to the increase in ISGs

To evaluate the possible mechanisms related to the augmented ISG levels associated with IFN and CsA, we further examined the phosphorylation level of STAT1, AKT, mTOR, and p38 MAPK, which are the

molecules linked to the transcriptional activation of interferon-sensitive response element (ISRE). We then performed these experiments in primary human cells in addition to continuous cell-lines. Therefore, we used human lung microvascular endothelial cell (HMVEC-L) which has been associated with extra-pulmonary dissemination of HCoV-EMC, as HCoV-EMC does not replicate well in primary human alveolar epithelial cells *in vitro* and we have demonstrated that HCoV-EMC could infect lung endothelial cells in our previous study (Chan et al., 2013). Our data showed that HMVEC-L was highly susceptible to HCoV-EMC infection and the IFN- α 1 and CsA combined treatment group had the greatest impact on reducing HCoV-EMC replication (Fig. 6A). The treatment dosage was not cytotoxic to the cell we used (Supplementary Fig. 1B). In line with the virus replication data, we confirmed that the IFN- α 1 and CsA treated group was more potent at reducing HCoV-EMC NP expression than either IFN or CsA alone. STAT1 expression and phosphorylation were comparably increased in the IFN treated and combine IFN- α 1 and CsA treated groups (Fig. 6B). This suggested that the effect of the combined treatment was independent of the JAK-STAT pathway. We next examined the p38 MAPK and AKT/mTOR pathways (Fig. 6C). The phosphorylation level of p38 in the combined treatment group was the lowest, which was similar to the CsA treated group. On the contrary, phosphorylation level of AKT at the s473 was the lowest in the combined treatment group, and the phosphorylation level of mTOR were comparable among all groups (Fig. 6C). This indicated that the beneficial effects of combination therapy may not be linked to the AKT/mTOR translational control of ISGs.

4. Discussion

MERS continues to cause disease in the Arabian Peninsula with high case fatality in hospitalized patients. There are still no proven specific antiviral therapies for this disease. Despite the apparent benefit of interferon therapy in rhesus macaques and marmosets, interferon therapy has not translated into clinical benefit in clinical cases of MERS (reviewed in (Al-Tawfiq and Memish, 2017; Arabi et al., 2017)). This may be in part related to the late presentation of patients compared to laboratory settings. The lack of a good experimental animal model has further hampered progress on antiviral therapies for MERS. Our *ex vivo* cultures of the human bronchus and lung provides an alternative and complementary option for investigating therapeutic agents but still have a limitation in severe CoV infection studies, because patients may present to a health care setting 5 or more days after exposure, which is currently beyond our ability to maintain the *ex vivo* tissues. In this study, we find that a combination of interferon with short term cyclosporine (Fig. 2) may be worthy of pursuit in a clinical trial setting. Previously, these drugs have been used singly but not in combination (de Wilde et al., 2013). The *ex vivo* cultures showed a significant decrease in virus replication when this combination treatment was used compared to single treatment and this was also reflected in the greater number of ISG upregulated, compared to mock or single treatments. This increase in ISG may thus dampen the potential immune suppressive effect of CsA if used as a single agent. The combined treatment of type I interferon with cyclosporine also mitigated the extent of apoptosis caused by HCoV-EMC in our study (Fig. 4). As different therapeutic modalities are considered for evaluation in clinical trials, we suggest that IFN and CsA combination therapy should be considered. A recent review has also noted the need to evaluate combination therapies (Al-Tawfiq and Memish, 2017).

CsA is a small molecule immunosuppressant while IFN- α 1 is an immuno-stimulating protein favoring cell conversion to an antiviral state. Since the mechanism underlying the increment of ISG by this combination is unclear, we tried to identify the possible mechanisms behind the higher induction levels of ISG in the combined treatment of type I IFN and CsA over the other treatment groups. In canonical type I IFN signaling, IFN- α / β activates the JAK-STAT pathway in which STAT1 is an essential member to form the ISGF3 complex, which binds

to the ISRE and controls the ISG expression (Ivashkiv and Donlin, 2014). Since the activation level of STAT1 in the combined treatment was similar or even slightly lower than the IFN- α treatment alone (Fig. 6A), we believe that the extra ISG induction could be the result of the other signaling cascades independent of the JAK-STAT signaling. Therefore, we tried to determine whether the combined treatment would have effects on the AKT/mTOR pathway, which in turn has translational controls on the ISGs (Kaur et al., 2008; Kroczyńska et al., 2009; Saleiro et al., 2015). Our results showed that mTOR activation was not affected by the combined treatment; and the decreased phosphorylation level of AKT in the combined treatment group may be related to the other signaling cascades that linked to AKT, thereby it was not likely that AKT/mTOR was involved in the enhanced ISGs levels by the double treatment. Furthermore, we also examined the p38 MAPK activation level, which also linked to the ISRE separately (Platanias, 2005; Saleiro et al., 2015). From our results, HCoV-EMC induced p38 phosphorylation (Lim et al., 2016) was reduced due to the effect from CsA, which may contribute to the inhibition of MER-CoV replication levels, while this result did not match with the elevated ISG level. Therefore, it is likely that CsA and type I IFN combination therapy uses alternative pathways to enhance antiviral effect and in enhancing ISG induction by acting directly or indirectly on the ISRE (Supplementary Fig. 4).

In conclusion, we have demonstrated that CsA and IFN- α 1 is a potent therapeutic combination for inhibiting HCoV-EMC replication *in vitro* and *ex vivo*, which can result in higher ISG expression. This combination may be worth considering in future clinical trials.

Declarations of interest

None.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.antiviral.2018.05.007>.

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