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Delayed induction of proinflammatory cytokines and suppression of innate antiviral response by the novel Middle East Respiratory Syndrome Coronavirus: implications on pathogenesis and treatment

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ABSTRACT

The high mortality associated with the novel Middle East Respiratory Syndrome Coronavirus (MERS-CoV) has raised questions on the possible role of cytokine storm in its pathogenesis. Although recent studies showed that MERS-CoV infection is associated with attenuated interferon response, no induction of inflammatory cytokines was demonstrated during early phase of infection. To study both early and late cytokine responses associated with MERS-CoV infection, we measured the mRNA levels of eight cytokine genes (TNF-α, IL-1β, IL-6, IL-8, IFN-β, MCP-1, TGF-β and IP-10) in cell lysates of polarized airway epithelial, Calu-3, cells infected with MERS-CoV or SARS-CoV up to 30 h post infection. Among the eight cytokine genes, IL-1β, IL-6 and IL-8 induced by MERS-CoV were markedly higher than those induced by SARS-CoV at 30 h, while TNF-α, IFN-β and IP-10 induced by SARS-CoV were markedly higher than those induced by MERS-CoV at 24 and 30 h in infected Calu-3 cells. The activation of IL-8 and attenuated IFN-β response by MERS-CoV were also confirmed by protein measurements in the culture supernatant when compared to SARS-CoV and Sendai virus. To further confirm the attenuated antiviral response, cytokine response was compared to HCoV-229E in embryonal lung fibroblast, HFL, cells, which also revealed higher IFN-β and IP-10 levels induced by HCoV-229E than MERS-CoV at 24 and 30 h. While our data supported recent findings that MERS-CoV elicits attenuated innate immunity, this represents the first report to demonstrate delayed proinflammatory cytokine induction by MERS-CoV. Our results provide insights into the pathogenesis and treatment of MERS-CoV infections.

INTRODUCTION

Coronaviruses (CoVs) are known to infect humans as well as a wide variety of animals, causing respiratory, enteric, hepatic and neurological diseases of varying severity. They are now classified into four genera, *Alphacoronavirus*, *Betacoronavirus*, *Gammacoronavirus* and *Deltacoronavirus* (de Groot *et al.*, 2011; Woo *et al.*, 2009, 2012a). The high frequency of recombination and rate of mutation are believed to be responsible for the ability of CoVs to adapt to new hosts and ecological niches (Lai & Cavanagh, 1997; Herrewegh *et al.*, 1998; Hon *et al.*, 2008; Lau *et al.*, 2011; Woo *et al.*, 2006; Zeng *et al.*, 2008). This is best exemplified by the severe acute respiratory syndrome (SARS) epidemic in 2003 which has affected >8000 and killed nearly 800 people worldwide. The etiological agent, SARS-CoV, was subsequently shown to have originated from animals, with horseshoe bats as the natural reservoir and palm civet as the intermediate host (Guan *et al.*, 2003; Lau *et al.*, 2005, 2010; Li *et al.*, 2005; Ksiazek *et al.*, 2003). Except SARS-CoV, the known human CoVs usually cause self-limiting upper respiratory tract infections although pneumonia may occur in immunocompromised patients and the elderly (Woo *et al.*, 2005; Lau *et al.*, 2006).

In 2012, cases of acute severe respiratory illness were reported in Saudi Arabia, which were subsequently found to be caused by the novel Middle East Respiratory Syndrome Coronavirus (MERS-CoV), previously known as human betaCoV 2c EMC/2012 (Bermingham *et al.*, 2012; Zaki *et al.*, 2012; de Groot *et al.*, 2013). As of September 2013, 114 laboratory confirmed cases of MERS-CoV infection have been reported with 54 deaths, giving a case fatality rate of 47% (World Health Organization, 2013). So far, most cases of MERS-CoV infection presented with severe acute respiratory illness and many were associated with renal failure (World Health Organization, 2013). In addition to its disease severity, the ability to cause human-to-human

transmission has aroused the public to another possible SARS-like epidemic. While the source remains obscure, MERS-CoV is closely related to *Tylonycteris* bat CoV HKU4 (Ty-BatCoV HKU4) and *Pipistrellus* bat CoV HKU5 (Pi-BatCoV HKU5) previously discovered in bats in Hong Kong, China (Lau *et al.*, in press; Woo *et al.*, 2007, 2012b; van Boheemen *et al.*, 2012). Moreover, potential viruses with partial gene sequences closely related to MERS-CoV have also been detected in bats from Ghana and Europe (Annan *et al.*, 2013). MERS-CoV is able to infect various mammalian cell lines including primate, porcine, bat and rabbit cells (Müller *et al.*, 2012; Chan JFW *et al.*, 2013; Jiang *et al.*, 2012), which may be explained by its use of the evolutionarily conserved dipeptidyl peptidase 4 (DPP4 or CD26) as its functional receptor (Raj *et al.*, 2013). This suggested that MERS-CoV had emerged from animals and may possess broad species and tissue tropism. Nevertheless, the pathogenesis of MERS-CoV infection is still largely unknown.

The relatively low virulence of human CoVs has been partly attributed to their limited cell line tropism (Yeager *et al.*, 1992; Arbour *et al.*, 1999a, 1999b; Herzog *et al.*, 2008; Dijkman *et al.*, 2013). For example, HCoV-229E was able to infect only hepatocytes (Huh-7), primary embryonal lung fibroblasts, primary human airway epithelium, neural cells, monocytes, macrophages and dendritic cells after adaptation but not human pneumocyte cell lines (Yeager *et al.*, 1992; Arbour *et al.*, 1999a; Tang *et al.*, 2005; Funk *et al.*, 2012; Dijkman *et al.*, 2013). In contrast, SARS-CoV can be readily isolated from clinical specimens in embryonal monkey kidney cell lines such as FRhK-4 or Vero E6 cells and subcultured in human lung stem cells and primary airway epithelia, hepatocytes, and intestinal cells, though it replicated poorly in human lung adenocarcinoma (A549) cells or embryonal lung fibroblasts (HFL) (Kaye *et al.*, 2006; Jia *et al.*, 2005; Sims *et al.*, 2005). MERS-CoV is unique by its even broader tissue tropism. The virus

was able to infect different human cells, including respiratory (polarized airway epithelium, Calu-3; HFL; A549), kidney (HEK), intestinal (Caco2), liver (Huh7) cells and histiocytes (His-1), with cytopathic effect (CPE) as early as day 1 upon passage in the intestinal and liver cells, and day 3 in the lower respiratory tract cell lines with frequent syncytial formation and CPE occurring even faster than that induced by SARS-CoV, which may partially explain its unusually high mortality (Müller *et al.,* 2012; Chan JFW *et al.,* 2013; Zielecki *et al.,* 2013). It could also replicate productively in human bronchial and lung *ex vivo* organ cultures, while SARS-CoV only replicated productively in lung tissue (Chan RW *et al.,* 2013).

In addition to cellular tropism, the disease severity of SARS is also related to the ability of the virus to suppress antiviral innate immune responses coupled with marked proinflammatory cytokine production (Cheung *et al.,* 2005; Spiegel *et al.,* 2005). We hypothesized that immune evasion and cytokine dysregulation may also play a role in the pathogenicity of MERS-CoV infection. To test the hypothesis, we attempted to study the cytokine expression profiles of cells infected with MERS-CoV compared to SARS-CoV. However, monocytes/macrophages only supported abortive infection by SARS-CoV and MERS-CoV (Chan JFW *et al.,* 2013), making these cells unsuitable for pathogenicity studies. On the other hand, the respiratory epithelium is the primary barrier and site of infection for respiratory viruses, and is therefore a good model to study their replication and pathogenesis. Moreover, lower airway cell lines, especially Calu-3 cells which resemble the normal human pneumocytes with tight junctions, have been shown to produce the highest viral loads with abundant N protein expression after MERS-CoV infection (Chan JFW *et al.,* 2013; Zielecki *et al.,* 2013), although these cell lines may not entirely reflect the “natural” human airway epithelium. Since Calu-3 cells are also susceptible to SARS-CoV, we compared the mRNA expression levels of eight cytokine genes in Calu-3 cells infected by

MERS-CoV and SARS-CoV at 4, 12, 24 and 30 h post infection to study both early and late cytokine response. We also measured the cytokine protein levels of the six cytokine genes which showed increase in mRNA levels in Calu-3 cells. Since results from MERS-CoV-infected Calu-3 cells suggested a delayed proinflammatory but attenuated IFN-β antiviral response, we attempted to validate the innate antiviral response of MERS-CoV as compared to that of HCoV-229E as positive control. However, as HCoV-229E did not replicate efficiently in Calu-3 cells (data not shown) or other human pneumocyte cell lines, we used HFL cells to study the cytokine profiles induced by MERS-CoV compared to HCoV-229E. The results were discussed in light of the possible pathogenesis of and treatment strategies for MERS-CoV infections.

RESULTS

Viral titers and susceptibility of cells

The titers of viruses in MERS-CoV and SARS-CoV-infected Calu-3 cells, and MERS-CoV- and HCoV-229E-infected HFL cells, at 4, 12, 24 and 30 h post infection, were shown in Fig. 1. The replication rates of the tested viruses were similar in both cell lines as shown by the rising trends in the viral loads. Cytopathic effects were visible in Calu-3 cells infected with both MERS-CoV and SARS-CoV at 24h, although more rapid and prominent cell death was observed in cells infected with MERS-CoV. Cytopathic effects were also seen in HFL cells infected with MERS-CoV at 30 h, but not HCoV-229E-infected cells, despite the lower viral titers in HFL cells infected with MERS-CoV.

Cytokine mRNA levels in human airway epithelial cells infected with MERS-CoV and SARS-CoV

To study the cytokine response associated with MERS-CoV infection as compared to SARS-CoV infection, the mRNA expression levels of eight cytokine genes were measured in Calu-3 cells infected by MERS-CoV and SARS-CoV at 4, 12, 24 and 30 h post infection. Among the eight cytokines assayed, the levels of six (IL-1β, IL-6, IL-8, TNF-α, IFN-β, and IP-10) showed marked increase in Calu-3 cells infected with MERS-CoV and/or SARS-CoV compared to uninfected cells. Among these six cytokines, the proinflammatory cytokines, IL-1β, IL-6 and IL-8, induced by MERS-CoV were markedly higher than those induced by SARS-CoV at 30 h. On the other hand, TNF-α, IFN-β and IP-10, which are important for innate antiviral immune response, induced by SARS-CoV were markedly higher than those induced by MERS-CoV at 24 and 30 h (Fig. 2). The other two cytokines, MCP-1 (a chemokine) and TGF-β (an anti-inflammatory cytokine), did not show obvious increase upon infection with MERS-CoV or

SARS-CoV.

Cytokine expression levels in human airway epithelial cells infected with MERS-CoV and SARS-CoV. Since the mRNA levels of six (IL-1β, IL-6, IL-8, TNF-α, IFN-β and IP-10) cytokine genes were increased in Calu-3 cells infected with MERS-CoV and/or SARS-CoV, we attempted to measure their protein expression levels using Sendai virus as positive control in the culture supernatants of infected Calu-3 cells. Cytopathic effects were also visible in Calu-3 cells infected with Sendai virus though less severe than that of MERS-CoV. Among the six cytokines assayed using enzyme-lined immunosorbent assay (ELISA), only two, IL-8 (a proinflammatory cytokine) and IFN-β (an antiviral cytokine), showed detectable levels at 24 and/or 48 h in Calu-3 cells (Fig. 3). Compared to Sendai virus and SARS-CoV, MERS-CoV elicited higher level of IL-8 at 24 h. However, at 48 h, SARS-CoV showed higher level of IL-8 level than MERS-CoV, which may due to the more prominent cell death in MERS-CoV-infected cells. As for IFN-β, the levels induced by Sendai virus and SARS-CoV were markedly higher at 48 h than that induced by MERS-CoV which did not showed obvious increase at 24 or 48 h.

Cytokine mRNA levels in human lung fibroblasts infected with MERS-CoV and HCoV-229E

Since results from infected Calu-3 cells suggested that MERS-CoV elicits an attenuated innate antiviral immunity and HCoV-229E is a CoV known to be associated with strong IFN-β response (Cheung *et al.*, 2005), we attempted to validate the attenuated antiviral response of MERS-CoV using HCoV-229E as a positive control. We therefore measured the mRNA expression of the eight cytokine genes in HFL cells infected by MERS-CoV and SARS-CoV at 4, 12, 24 and 30 h post infection. Similar to that observed in Calu-3 cells infected with MERS-CoV and SARS-CoV, six of the eight cytokines assayed (IL-1β, IL-6, IL-8, TNF-α, IFN-β and

IP-10) showed marked increase in mRNA expression in HFL cells infected with MERS-CoV and/or HCoV-229E compared to uninfected cells. However, among these six cytokines, all except TNF-α showed higher induction by HCoV-229E than MERS-CoV at 24 and/or 30 h (Fig. 4). In particular, IL-6, IL-8, IFN-β and IP-10 showed markedly higher levels in HCoV-229E-infected cells than MERS-CoV-infected cells at 24 and 30 h. On the other hand, TNF-α showed higher induction by MERS-CoV than HCoV-229E at 24 and 30 h. MCP-1 and TGF-β did not show obvious increase upon infection with MERS-CoV or HCoV-229E.

DISCUSSION

The present study represents the first to demonstrate delayed proinflammatory cytokine induction by MERS-CoV. In this study, analysis of cytokine mRNA expression in Calu-3 cells infected by MERS-CoV showed marked induction of the proinflammatory cytokines, IL-1β, IL-6 and IL-8, at 30 h but lack of production of innate antiviral cytokines, TNF-α, IFN-β and IP-10, compared to SARS-CoV. This suggested that MERS-CoV elicits attenuated innate immunity while inducing a delayed proinflammatory response in human lung epithelial cells. Up to the time of writing, MERS-CoV infections were associated with a high mortality rate of approximately 50%, which is even higher than that of SARS (~10%). A macaque model for MERS-CoV infection has recently been reported, which showed that the virus caused localized-to-widespread pneumonia in all infected animals, though without systemic infection (Munster *et al.,* 2013). We therefore used human lung epithelial cells to examine the cytokine response induced by MERS-CoV compared to that of SARS-CoV, which may help understand its pathogenicity in the lungs. Calu-3 cells were used instead of primary human airway epithelium because the former continuous cell line allowed robust viral replication for both MERS-CoV and SARS-CoV with reproducible results. Analysis of mRNA expression of eight cytokines showed that MERS-CoV infection was associated with significant but delayed induction of IL-1β, IL-6 and IL-8, which were more markedly induced by MERS-CoV than SARS-CoV at 30 h post infection. The activation of IL-8 at 24 h and attenuated IFN-β response by MERS-CoV were also confirmed when the cytokine protein levels were measured in the culture supernatants of infected Calu-3 cells. The failure to detect the other cytokines in the culture supernatants is likely due to the much lower sensitivity of protein detection as compared to mRNA measurements. Proinflammatory cytokines such as IL-1β are key mediators in the innate immune response,

while IL-6 possesses both proinflammatory and anti-inflammatory functions. The induction of IL-8 may imply strong neutrophil recruitment in MERS-CoV infection as seen in reported cases (Zaki *et al.,* 2012; Albarrak *et al.,* 2012). Higher levels of IL-8 have also been shown to correlate with increased disease severity in respiratory syncytial virus infections (Mella *et al.,* 2013). However, a recent study using primary human airway epithelium showed no proinflammatory cytokine induction following MERS-CoV, SARS-CoV or HCoV-229E infections at 3, 6 and 12 h, except marginal induction of TNF-α in HCoV-229E-infected cells at 6 h (Kindler *et al.,* 2013). This may reflect the limited early transcriptional response to CoV infections in human lung epithelium, whereas delayed proinflammatory response was best observed at 30 h at the mRNA level in the present study. Yet, the use of Calu-3 cells in this study may be associated with limitations, since it cannot entirely reflect the "natural" human airway epithelium in response to MERS-CoV infection. Further studies on cytokine response in MERS patients or experimentally animals by MERS-CoV are required to more accurately delineate the proinflammatory response in the airway epithelium.

Apart from delayed proinflmmatory response, MERS-CoV is also associated with attenuated innate antiviral response in human lung epithelial cells. TNF-α, IFN-β and IP-10, which were markedly induced by SARS-CoV at 24 and 30 h, did not show significant induction by MERS-CoV in Calu-3 cells. The lack of IFN-β response was confirmed when the protein level was measured and compared to Sendai virus which is known to be a strong IFN- β inducer and therefore used a positive control. In fact, similar trend of attenuated IFN-β response was observed at mRNA level when compared to Sendai virus (data not shown). TNF-α is an important acute phase pyrogen that can inhibit viral replication, while IFN-β is produced in

different cells after infection to induce antiviral response through upregulation of antivirally active host factors. Therefore, attenuated TNF-α and IFN-β response suggested that MERS-CoV may more readily evade the antiviral response than SARS-CoV. Since IP-10, also known as CXCL10, is an antiviral chemokine for recruiting macrophages, T lymphocytes and dentritic cells, the lack of IP10 induction after MERS-CoV infection may also help the virus to evade the innate immunity. Our results were in line with a recent study showing the failure of MERS-CoV to induce TNF-α, IFN-β and IP-10 in A549 cells, although other cytokines were not measured (Chan RW *et al.,* 2013). While contrasting results have been reported on the effects of SARS-CoV on interferon induction in various cell lines (Qian *et al.,* 2013; Okabayashi *et al.,* 2006; Yoshikawa *et al.,* 2010), a recent study comparing MERS-CoV and SARS-CoV revealed no substantial upregulation (<20-fold change) of IFN-β, ISG56 and IP-10 in Calu-3 cells infected with both viruses up to 24 h (Zielecki *et al.,* 2013), compared to approximately 40-fold change of IFN-β and IP-10 levels in SARS-CoV-infected cells at 24 h in the present study. The apparent discrepancy between different studies could be due to the different experimental conditions such as viral inocula or cell lines.

Attenuation of IFN-β and IP-10 response by MERS-CoV was also confirmed when compared to HCoV-229E in infected HFL cells. HCoV-229E is strong IFN-β inducer and therefore used as a positive control, whereas HFL cell line was used because it allowed robust viral replication of both viruses. Moreover, embryonal lung fibroblasts are connective tissue cells sharing a common mesenchymal origin with macrophages of the reticuloendothelial system, which served as a good model for studying virus-induced antiviral response. Analysis of mRNA expression of eight cytokines showed that HCoV-229E induced marked production of IL-6, IL-8, IFN-β and IP-10

compared to MERS-CoV. The marked induction of IFN-β by HCoV-229E is in line with previous observations (Cheung *et al.*, 2005), which, together with IP-10 production, may reflect the strong antiviral response against HCoV-229E elicited by reticuloendothelial cells and explain the mild respiratory illness caused by this human CoV. On the other hand, the attenuated IFN-β and IP-10 induction by MERS-CoV supported that MERS-CoV may more readily evade the innate antiviral immunity than HCoV-229E. Although MERS-CoV appeared to induce stronger TNF-α than HCoV-229E, the fold-change observed is comparable to that in Calu-3 cells, which is still far lower than that induced by SARS-CoV. The induction of the proinflammatory cytokines, IL-1β, IL-6 and IL-8, by HCoV-229E, however, is less clinically relevant, since the virus is mainly confined to the upper respiratory tract during natural infection, without infecting cells in the lower airway such as lung fibroblasts. Several non-structural proteins (NSPs) of SARS-CoV have been shown to play important roles in both subversion of host antiviral response and viral replication (Perlman & Netland, 2009). It would be interesting to elucidate the role of cytokine dysregulation during natural or *in vivo* MERS-CoV infections, and identify the viral factors that may cause cytokine activation and counteract innate immunity.

The present results provide insights on the treatment of severe MERS-CoV infections. While extensive research efforts have been made in understanding the epidemiology and pathogenesis of CoVs during the past decade, there is still no effective antiviral treatment for CoV infections. Recent studies have shown that type I and III interferons can efficiently reduce MERS-CoV replication in Vero cells, human airway epithelium and *ex vivo* respiratory cultures, providing a possible treatment option (Chan RW *et al.*, 2013; Kindler *et al.*, 2013; de Wilde *et al.*, 2013). MERS-CoV was also found to be much more sensitive than SARS-CoV to antiviral action, and

was similar to SARS-CoV in the ability to inhibit IFN regulatory factor family 3-mediated antiviral IFN response (Zielecki *et al.,* 2013). For SARS-CoV and avian influenza virus infections, immune-modulation, through reduction of cytokine storm, is considered an important element in treating severe diseases (Cheung *et al.*, 2005; Zheng *et al.*, 2008; Woo *et al.,* 2010). In the recently reported macaque model, clinical signs of disease developed in all animals within 24 hours (Munster *et al.,* 2013). As the present study showed that induction of cytokines by MERS-CoV infection only occurred at 30 h, adjunctive immuno-modulatory treatment combined with antivirals may theoretically be useful after the patient develops symptoms of infection. Our results are in line with recent studies on combination treatment with interferon-α2b and ribavirin, which was shown to suppress viral replication in vitro and reduce systemic and lung proinflammatory markers, and histopathological changes in the lungs of infected rhesus mascques (Falzarano *et al.,* 2013a; Falzarano *et al.,* 2013b). More animal and clinical studies are eagerly awaited to facilitate further pathogenesis and therapeutic studies on MERS-CoV.

METHODS

Cell lines and viruses. The polarized airway epithelial cell line, Calu-3, and embryonal lung fibroblast cell line, HFL, were used throughout this study. Calu-3 cells were incubated in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 20% fetal calf serum (FCS) and 100 IU/ml of penicillin and 100 μg/ml of streptomycin. HFL cells were incubated at 37°C in minimal essential medium (MEM) supplemented with 10% fetal calf serum (FCS) A clinical isolate of MERS-CoV was kindly provided by Fouchier and Zaki et al (Zaki *et al.*, 2012). SARS-CoV strain HKU-39849 was isolated from the lung tissue biopsy of the brother-in-law of an index patient who traveled from Guangzhou and started a superspreading event in the Hong Kong Special Administrative Region leading to the epidemic (Peiris *et al.*, 2003). The HCoV-229E strain ATCC VR-740 and Sendai virus strain ATCC VR-907 were used as positive controls. The SARS-CoV and HCoV-229E strains used in our experiments had undergone three passages in the FRhk-4 cell and MRC-5 cell lines, respectively, and were stored at −70°C. The MERS-CoV and Sendai virus strains were amplified by one additional passage in Vero cell line to make working stocks of the virus.

Viral cultures. Calu-3 cells were infected with MERS-CoV and SARS-CoV, whereas HFL cells were infected with MERS-CoV and HCoV-229E as described previously with modifications (Chan JFW *et al.*, 2013). Viral titers were determined as median tissue culture infective dose ($TCID_{50}$) per ml in confluent cells in 96-well microtiter plates, which standardized the viral inoculum and measured the relative susceptibility of the respective cell line to these viruses. Calu-3 and HFL cells were seeded onto 24-well tissue culture plates, at 4×10^4 cells per well with the respective medium and incubated at 37°C and 5% CO_2 for 72 h prior to experiment. Cells were inoculated with 2 multiplicity of infection (M.O.I.) of each virus for 1 h, with DMEM

supplemented with 1% FCS for Calu-3 cells and MEM supplemented with 1% FCS for HFL cells. After 1 h of viral adsorption, the medium was removed and cells were washed twice with medium before further incubation for 4, 12, 24 and 30 h respectively. Cell lysates were collected for cytokine mRNA expression assays. All experiments were performed in triplicate. All work with infectious viruses was performed inside biosafety level-2 cabinet with standard operating procedures in approved biosafety level-3 facilities (Zheng *et al.*, 2008).

Viral replication rates. To study viral replication kinetics, progeny viruses from cell lysates collected at 4, 12, 24 and 30 h post-infection (p.i.) respectively were subject to reverse transcription-quantitative PCR (RT-qPCR) according to our previous protocol (Lau *et al.*, 2005; Tang *et al.*, 2005). Briefly, total RNA extracted from cell lysates with RNeasy Mini Spin Column (QIAgen, Hilden, Germany) was reverse transcribed and amplified with MERS-CoV primers (forward primer 5' -CAAAACCTTCCCTAAGAAGGAAAAG -3'; reverse primer 5'-GCTCCTTTGGAGGTTCAGACAT -3'), SARS-CoV primers (forward 5'-ACCAGAATGGAGGACGCAATG-3'; reverse 5'-GCTGTGAACCAAGACGCAGTATTAT-3′) and HCoV-229E primers (forward 5'-CAGTCAAATGGGCTGATGCA-3'; reverse 5'-AAAGGGCTATAAAGAGAATAAGGTATTCT-3') using real-time one-step quantitative RT-PCR assay as described previously with modifications (Chan JFW *et al.*, 2013; Poon *et al.*, 2004; van Elden *et al.*, 2004). Probes for MERS-CoV [5'-(FAM)ACAAAAGGCACCAAAAGAAGAATCAACAGACC(BHQ1)-3'], SARS-CoV [5'-(FAM)ACCCCAAGGTTTACCC(NFQ)-3'] and HCoV-229E [5'-(FAM)CCCTGACGACCACGTTGTGGTTCA(BHQ1)-3'] were used. Reactions were first incubated at 50^{o}C for 30 min, followed by 95^{o}C for 2 min, and were then thermal cycled for 50 cycles (95^{o}C for 15 s, 55^{o}C for 30 s). A series of 6 $\log_{10}$ dilutions equivalent to 1 x 10^{1} to 1 x 10^{6}

copies per reaction mixture were prepared to generate calibration curves and were run in parallel with the test samples.

RT-qPCR for cytokine gene expression analysis. To study virus-induced cytokine profiles, cell lysates of Calu-3 and HFL cells infected with MERS-CoV, SARS-CoV or HCoV-229E were collected at 4, 12, 24 and 30 h respectively. Total RNA was extracted from the cell lysates using RNeasy Mini Spin Column (QIAgen). The RNA was eluted in 50 μl of RNase-free water and was used as the template for RT-qPCR. Reverse transcription was performed using the oligo(dt) primer with the SuperScript III kit (Invitrogen). RT-qPCR assays for tumor necrosis factor (TNF)-α, interleukin (IL)-1β, IL-6, IL-8, interferon (IFN)-β, monocyte chemotactic protein (MCP)-1, transforming growth factor (TGF)-β and IFN-γ-induced protein 10 (IP-10) were performed as described previously with primers listed in Table 1, using glyceraldehyde 3-phosphate dehydrogenase (*gapdh*) for normalization (Woo *et al.,* 2010). cDNA was amplified in a LightCycler 2.0 (Roche) with 20 μl reaction mixtures containing FastStart DNA Master SYBR Green I Mix reagent kit (Roche), 2 μl cDNA, 2 or 4 mM $MgCl_2$ and 1 μM primers at 95°C for 10 min followed by 50 cycles of denaturation, annealing and extension (Table 1). Melting curve analysis was performed for each primer pair at the end of the reaction to confirm the specificity of the assay. Experiments were performed in duplicates, and the result for an individual sample was expressed as the mean expression level of a specific gene/*gapdh* relative to the reference cDNA. The relative expression between each infected sample and the uninfected control was then calculated and expressed as fold change.

Cytokine assays. To measure the protein levels of cytokine expression, Calu-3 cells were infected with MERS-CoV, SARS-CoV and Sendai virus (control) as described above. After 1 h of viral adsorption, the medium was removed and cells were washed twice with medium before

360 further incubation for 24 and 48 h respectively. Culture supernatants collected at 24 and 48 h
361 respectively were assayed for IL-1β, IL-6, IL-8, TNF-α, IFN-β, and IP-10. The concentrations of
362 the six cytokines in the culture supernatants were measured by ELISA according to
363 manufacturer's instruction (Invitrogen). The assay plate was analyzed using the ELISA plate
364 reader (PerkinElmer) at O.D. 450nm.
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LEGENDS TO FIGURES

Fig. 1. Viral loads of (A) MERS-CoV and SARS-CoV in Calu-3 cells and (B) MERS-CoV and HCoV-229E in HFL cells.

Fig. 2. Cytokine gene expression levels in Calu-3 cells infected with MERS-CoV or SARS-CoV (MOI=2). Expression of (A) IL-1β, (B) IL-6, (C) IL-8, (D) TNF-α, (E) IFN-β, (F) IP-10, (G) MCP-1, and (H) TGF-β were assayed by real-time quantitative RT-PCR. Data shown are n-fold changes of gene expression relative to mock-infected cells, after normalizing to GAPDH in each sample. Means of triplicate assays are shown.

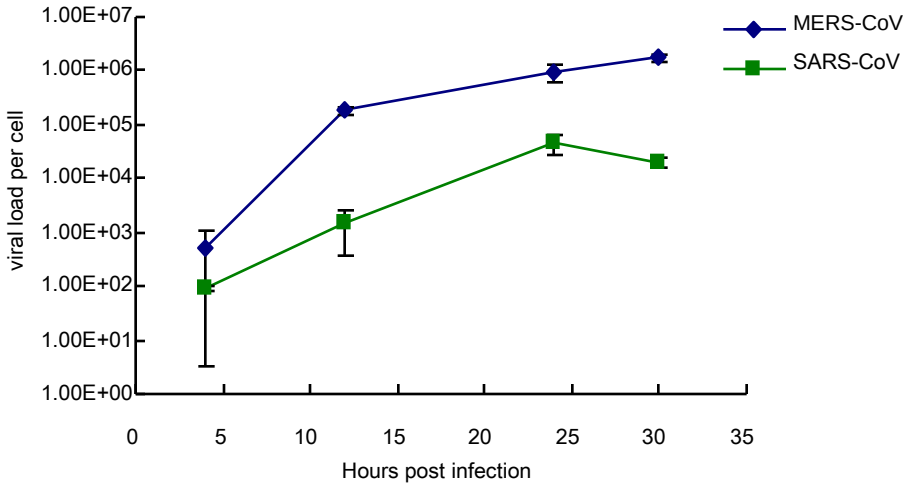
Fig. 3 Cytokine protein levels in culture supernatants of Calu-3 cells infected with MERS-CoV, SARS-CoV or Sendai virus (MOI=2). Expression of (A) IL-8 and (B) IFN-β were assayed by ELISA. Means of triplicate assays are shown.

Fig. 4. Cytokine gene expression levels in HFL cells infected with MERS-CoV or HCoV-229E (MOI=2). Expression of (A) IL-1β, (B) IL-6, (C) IL-8, (D) TNF-α, (E) IFN-β, (F) IP-10, (G) MCP-1, and (H) TGF-β were assayed by real-time quantitative RT-PCR. Data shown are n-fold changes of gene expression relative to mock-infected cells, after normalizing to GAPDH in each sample. Means of triplicate assays are shown.

Table 1. Primers and conditions for real-time RT-PCR of the eight cytokine genes

Cytokines	Primers		PCR conditions			
	Forward	Backward	Mg conc.	denaturation	annealing	extension
TNF-α	GCCAGAGGGCTGATTAGAGA	CAGCCTCTTCTCCTTCCTGAT	2mM	95˚C 10s	60˚C 5s	72˚C 5s
IL-8	AGCTGGCCGTGGCTCTCT	CTGACATCTAAGTTCTTTAGCACTCCTT	2mM	95˚C 10s	55˚C 5s	72˚C 5s
MCP-1	GCAATCAATGCCCCAGTCA	TGCTGCTGGTGATTCTTCTATAGCT	2mM	95˚C 10s	55˚C 5s	72˚C 5s
TGF-β	CCCAGCATCTGCAAAGCTC	GTCAATGTACAGCTGCCGCA	2mM	95˚C 10s	55˚C 5s	72˚C 5s
IL-6	GGTACATCCTCGACGGCATCT	GTGCCTCTTTGCTGCTTTCAC	2mM	95˚C 10s	55˚C 5s	72˚C 5s
IFN-β	GCCGCATTGACCATCT	CACAGTGACTGTACTCCT	4mM	95˚C 10s	55˚C 5s	72˚C 11s
IP-10	CTGACTCTAAGTGGCATT	TGATGGCCTTCGATTCTG	4mM	95˚C 10s	55˚C 5s	72˚C 9s
IL-1β	TCCCCAGCCCTTTTGTTGA	TTAGAACCAAATGTGGCCGTG	2mM	95˚C 10s	55˚C 5s	72˚C 5s
GAPDH	ATTCCACCCATGGCAAATTC	CGCTCCTGGAAGATGGTGAT	2mM	95˚C 10s	55˚C 5s	72˚C 5s

A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1.00E+06
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viral load per cell
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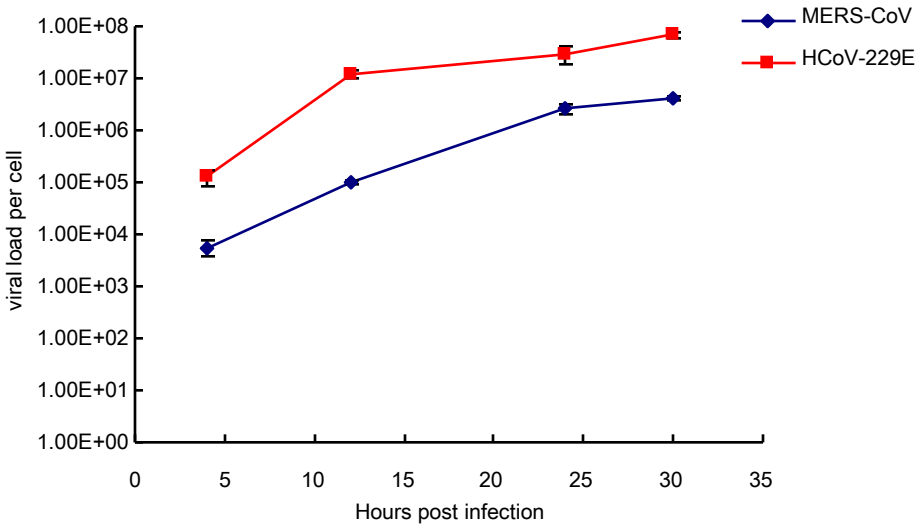
10
15
20
25
30
35
Hours post infection
MERS-CoV
SARS-CoV

B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viral load per cell
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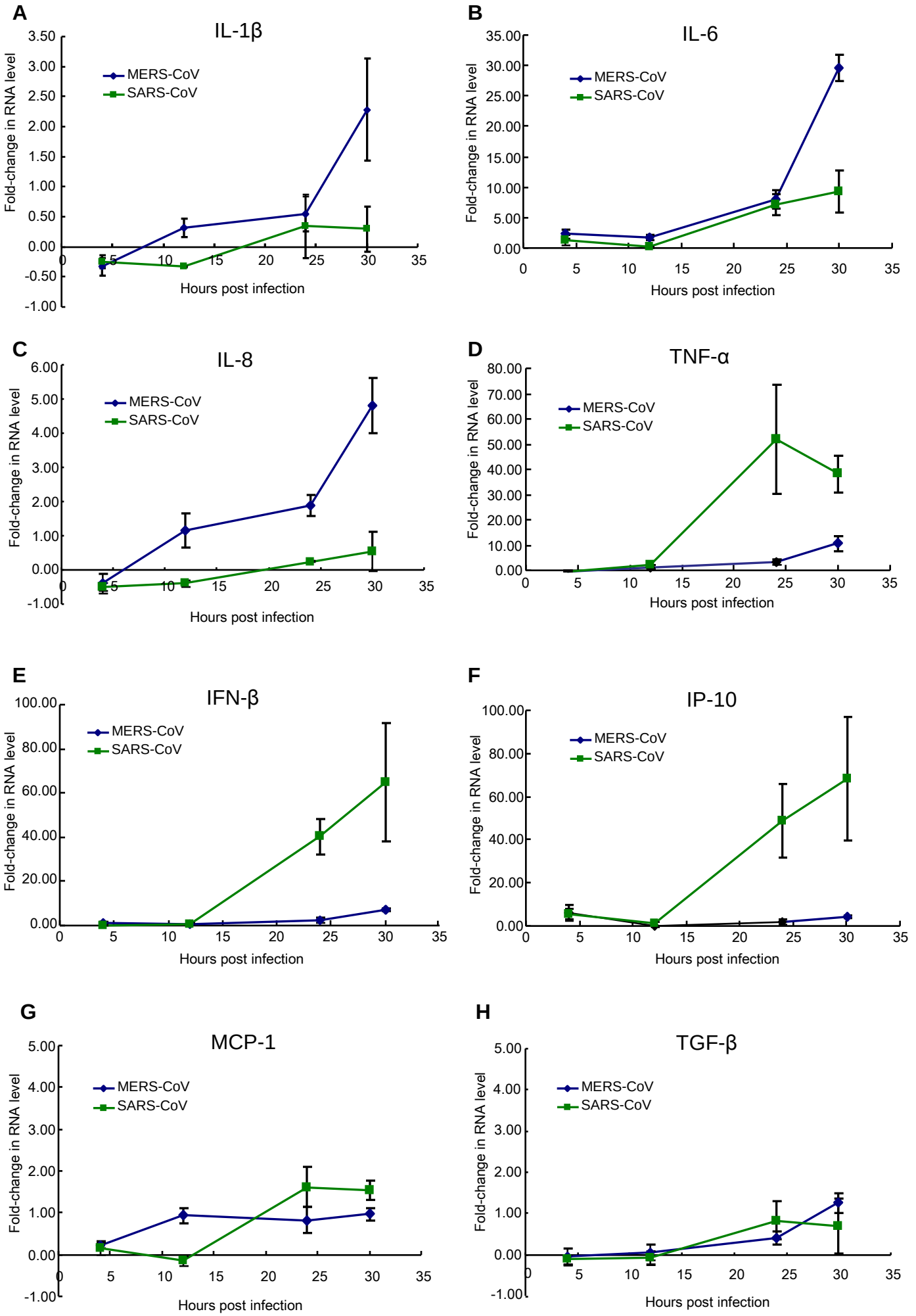
Hours post infection
MERS-CoV
HCoV-229E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IL-1β
MERS-CoV
SARS-CoV
Fold-change in RNA level
Hours post infection
B
IL-6
MERS-CoV
SARS-CoV
Fold-change in RNA level
Hours post infection
C
IL-8
MERS-CoV
SARS-CoV
Fold-change in RNA level
Hours post infection
D
TNF-α
MERS-CoV
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Hours post infection
E
IFN-β
MERS-CoV
SARS-CoV
Fold-change in RNA level
Hours post infection
F
IP-10
MERS-CoV
SARS-CoV
Fold-change in RNA level
Hours post infection
G
MCP-1
MERS-CoV
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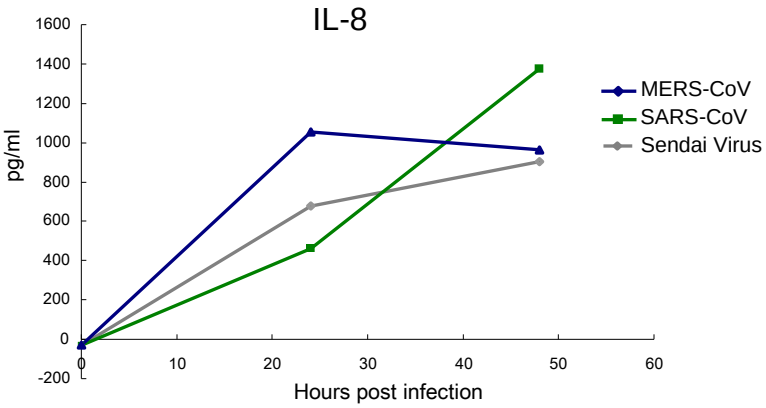
A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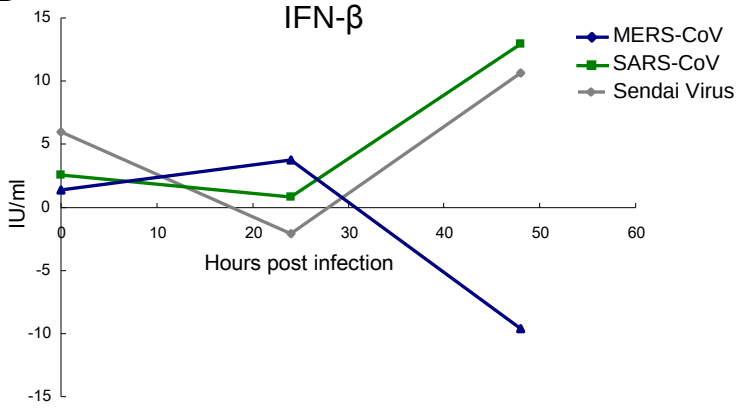
Hours post infection
MERS-CoV
SARS-CoV
Sendai Virus

B


IFN-β
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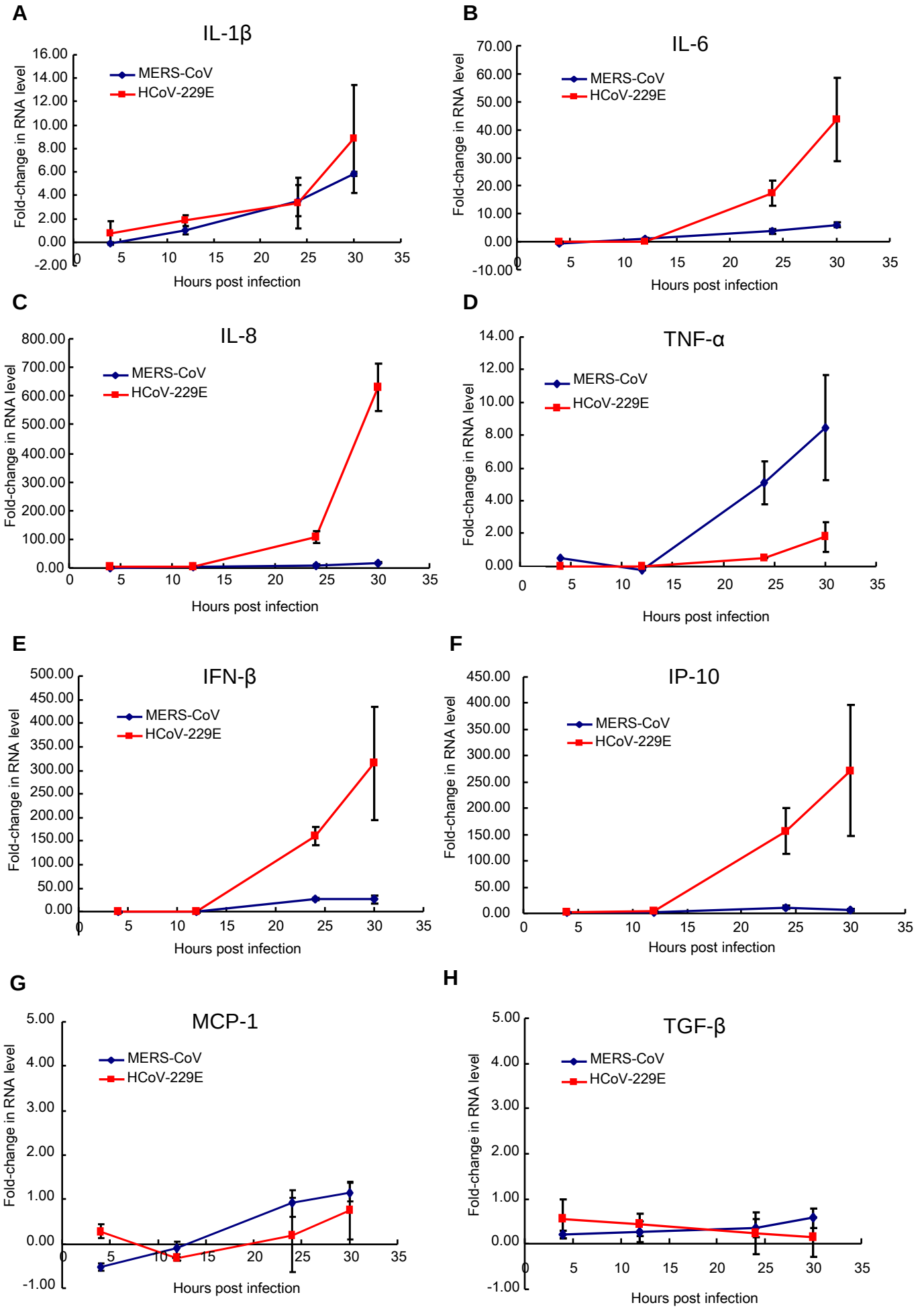
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Fold-change in RNA level
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