Human Cell Tropism and Innate Immune System Interactions of Human Respiratory Coronavirus EMC Compared to Those of Severe Acute Respiratory Syndrome Coronavirus

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Infections with human coronavirus EMC (HCoV-EMC) are associated with severe pneumonia. We demonstrate that HCoV-EMC resembles severe acute respiratory syndrome coronavirus (SARS-CoV) in productively infecting primary and continuous cells of the human airways and in preventing the induction of interferon regulatory factor 3 (IRF-3)-mediated antiviral alpha/beta interferon (IFN-α/β) responses. However, HCoV-EMC was markedly more sensitive to the antiviral state established by ectopic IFN. Thus, HCoV-EMC can utilize a broad range of human cell substrates and suppress IFN induction, but it does not reach the IFN resistance of SARS-CoV.

In September 2012, a novel human coronavirus (HCoV) was isolated in association with two cases of an acute, rapidly deteriorating respiratory illness that is often connected with kidney failure (1–3). As of February 2013, 12 infections with a fatality rate of approximately 40% were reported (4, 5). The coronavirus, which was termed HCoV-EMC (EMC for Erasmus Medical Center), is phylogenetically related to the causative agent of severe acute respiratory syndrome (SARS), SARS-CoV (3, 6). The alarming parallels both in terms of taxonomy and of pathogenesis sparked the fear that HCoV-EMC could cause an epidemic similar to SARS-CoV, which in 2003 had infected more than 8,000 people, killed 800, and caused worldwide economic damages in the range of 100 billion U.S. dollars (7).

SARS-CoV is capable of propagating in primary cells and continuous cell lines of the human airway epithelium (8, 9). Moreover, SARS-CoV efficiently suppresses antiviral innate immune responses, allowing it to spread rapidly in the host (10, 11). Here, we compared these phenotypic features of HCoV-EMC and SARS-CoV in order to obtain a first assessment of the pathogenic potential of the novel human coronavirus.

First, we tested the ability of HCoV-EMC to replicate in differentiated cultures of human tracheobronchial epithelial cells (HTBE), an established primary cell model of the human airway epithelium consisting of polarized and pseudostratified ciliated, secretory, and basal cells (12). These cells were grown on 12-mm Transwell permeable membrane supports (Costar) and were fed from the basolateral side with serum-free medium containing hormones and growth factors, whereas the apical side remained exposed to air (air-liquid interface conditions). The mucins that accumulated over time on the apical side of the cultures were removed by washing the cultures 10 times with phosphate-buffered saline (PBS). Then, the cultures were inoculated with either SARS-CoV or HCoV-EMC at a multiplicity of infection (MOI) of 0.1 to enable multistep growth. Infection was conducted by incubating the apical sides of the cultures with 200 µl of viral dilutions in DMEM, and the inoculum was removed 1 h later (13, 14). The cultures were then maintained at 37°C under air-liquid interface conditions. To study the viral growth kinetics, progeny viruses were collected at 24, 48, and 72 h postinfection (p.i.) from both the apical and basolateral sides of the Transwell supports. Material from the apical side was harvested after incubating the cells with 300 µl of DMEM for 30 min. From the basolateral side, 100 µl of the maintenance medium was collected. Virus titers were determined by a 50% tissue culture infective dose ($TCID_{50}$) assay in Vero cells. Both coronaviruses were able to propagate in differentiated HTBE cultures and were released exclusively from the apical side (Fig. 1). While SARS-CoV replicated slightly faster in the beginning, HCoV-EMC reached a titer similar to the SARS-CoV titer at 72 h p.i. Thus, HCoV-EMC closely resembles SARS-CoV in the ability to replicate in differentiated primary cells of the human airway epithelium.

An important hallmark of virulence is the extent to which viruses are able to cope with the antiviral type I interferon (alpha/beta interferon [IFN-α/β]) system, a major part of the innate immune response (15). Type I IFNs are the first cytokines upregulated after infection, stimulating the expression of more than 300 antiviral and immunomodulatory genes (16). Although SARS-CoV infection is impeded to some extent by exogenously added IFN (17–19), the massively increased IFN sensitivity of an nsp3 macrodomain mutant (20) and the resistance to the IFN-stimulated antiviral kinase protein kinase R (PKR) (21) suggest the presence of active mechanisms to dampen the antiviral effect of IFN. We compared the IFN sensitivity of HCoV-EMC and SARS-CoV in a dose-response experiment. As test systems, we used two established continuous cell line models for SARS-CoV (22, 23), namely, Calu-3 (derived from human bronchial epithelium) and Vero (derived from the kidney of an African green monkey), and primary nondifferentiated HTBE cells for comparison. These cells were pretreated overnight with different amounts
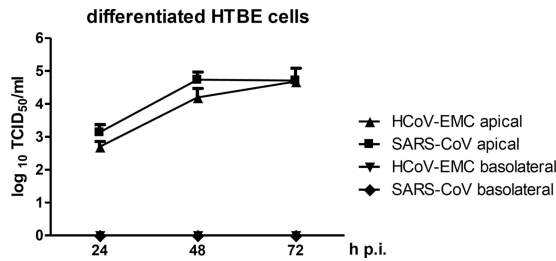
FIG 1 Virus multiplication in differentiated cultures of human tracheobronchial epithelial cells. Differentiated HTBE cultures grown on Transwell permeable membrane supports under air-liquid interface conditions were apically inoculated with SARS-CoV strain FFM-1 (34) or the HCoV-EMC isolate (3) at an MOI of 0.1. Virus yields from the apical and basolateral sides were determined at 24, 48, and 72 h p.i. by a $TCID_{50}$ assay. Mean values plus standard deviations (error bars) of 3 replicate experiments are shown.

of human IFN-β and infected with the coronaviruses at an MOI of 0.01, and virus yields were determined by a $TCID_{50}$ assay. In agreement with previous studies (8, 9), we noted that SARS-CoV was unable to grow in the nondifferentiated primary HTBE cells (Fig. 2A, top panel). Interestingly, HCoV-EMC could be propagated in these cells, albeit at a reduced rate compared to differentiated HTBE cells (Fig. 2A, bottom panel). The addition of IFN-β clearly had an antiviral effect, reducing HCoV-EMC titers from 10E3/ml to undetectable levels. On Calu-3 cells, both viruses replicated with similar efficiency (Fig. 2B). Interestingly, HCoV-EMC displayed a much more pronounced IFN sensitivity. At 24 h postinfection, 100 units of human IFN-β per ml decreased the titer of HCoV-EMC by 4 orders of magnitude, whereas the titer of
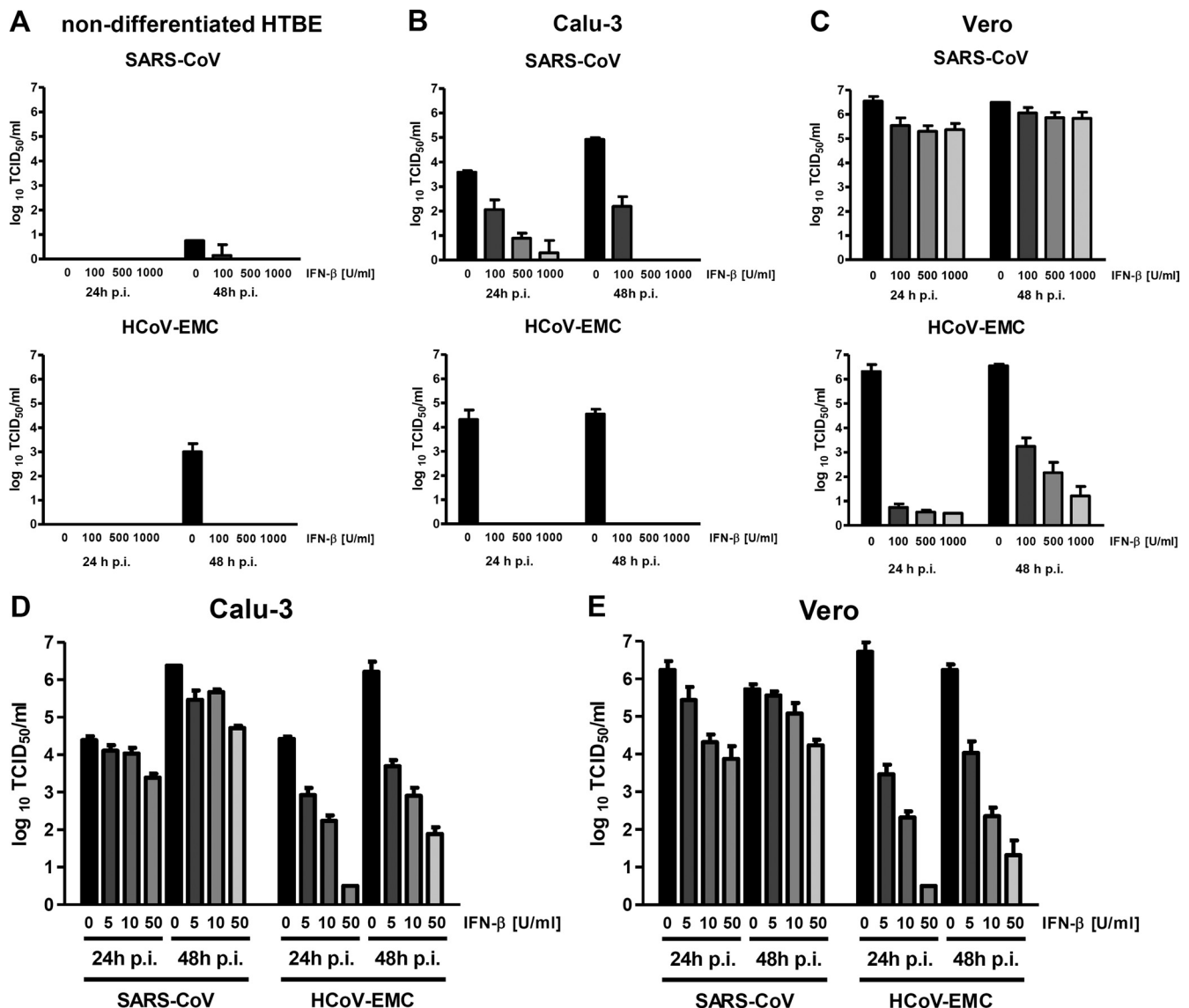

FIG 2 Cell tropism and IFN sensitivity. Multiplication and type I IFN sensitivity of HCoV-EMC in comparison to SARS-CoV were studied by applying high or low doses of IFN. (A to C) Cultures of primary nondifferentiated HTBE cells (A), the human bronchial epithelial cell line Calu-3 (B), and the primate kidney cell line Vero (C) were pretreated with 0, 100, 500, or 1,000 units per ml of recombinant human IFN-β (Betaferon; Schering). After 18 h of incubation, cells were infected with SARS-CoV (top panel) or HCoV-EMC (bottom panel) at an MOI of 0.01. Viral titers in the supernatants were determined at 24 h and 48 h p.i., using the $TCID_{50}$ assay in Vero cells. (D and E) Application of low doses of IFN. IFN sensitivity of the viruses was tested in Calu-3 (D) and Vero (E) cells, using 5, 10, and 50 units per ml of recombinant human IFN-β. Mean values plus standard deviations (error bars) of 3 replicate experiments are shown.
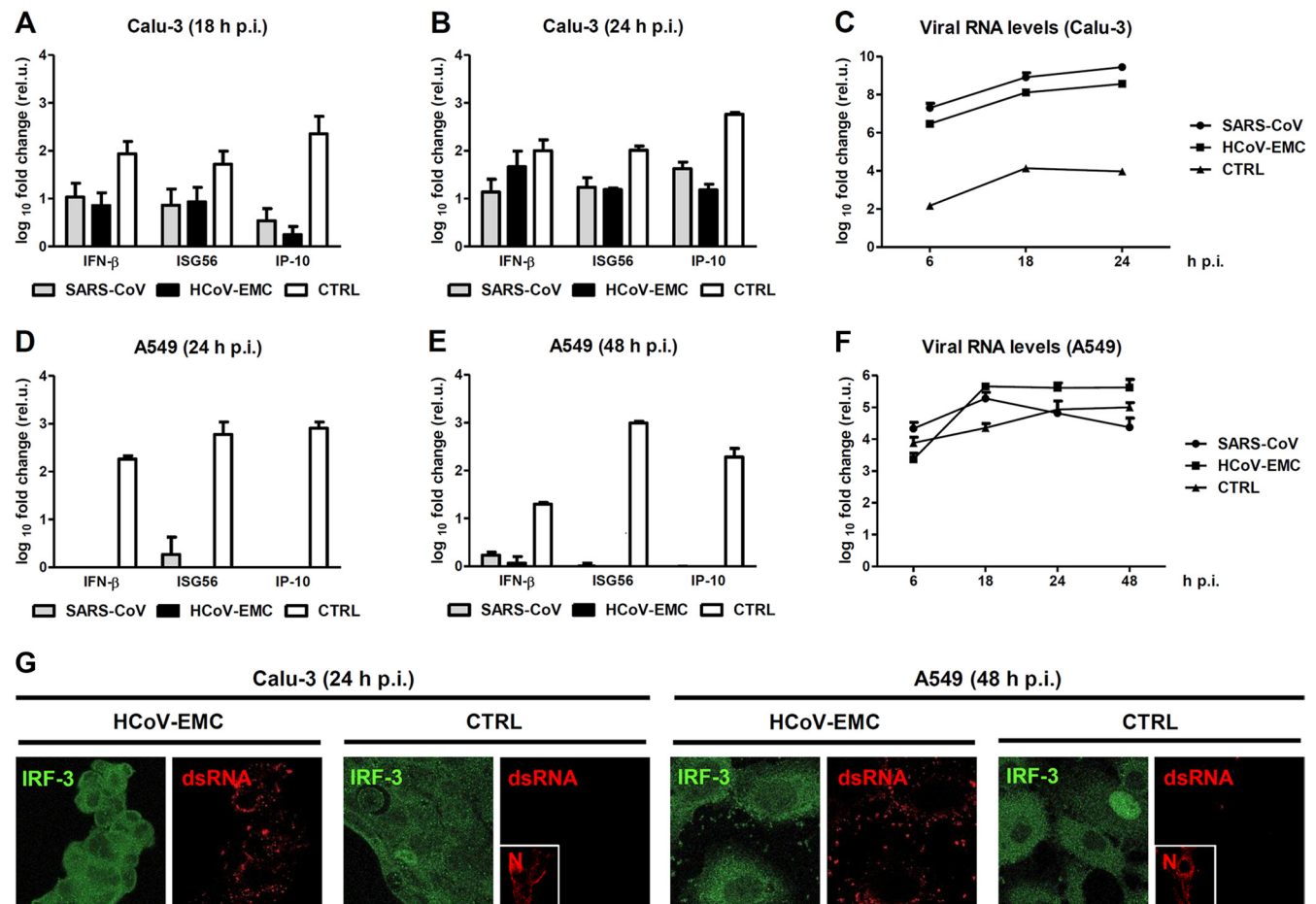
FIG 3 Cytokine responses and IRF-3 activation. (A to C) Real-time RT-PCR analyses for cytokine induction (26) and viral RNA production (35–37). The human bronchial epithelium cell line Calu-3 was infected with SARS-CoV, HCoV-EMC, or the recombinant Rift Valley fever virus (RVFV) mutant RVFVΔNSs::Ren (control [CTRL]) at an MOI of 1. Total cell RNA was assayed at the indicated time points for changes in the levels of RNAs for IFN-β, ISG56, and IP-10 (A and B) or viral RNAs (C). rel.u., relative units. (D to F) A parallel experiment measuring cytokine induction and viral RNA detection in the human lung adenocarcinoma cell line A549 at the indicated time points p.i. Mean values plus standard deviations (error bars) of 3 replicate experiments are shown. (G) Activation of IRF-3. Calu-3 cells (left panels) or A549 cells (right panels) were infected with the indicated viruses at an MOI of 1, fixed, and stained for endogenous IRF-3 (24), viral dsRNA (29), and RVFV N protein as described previously (38). Note that for reasons of antibody compatibility, the RVFV N signals shown in the small insets are from different coverslips which were infected in parallel. In all IRF-3 images, the contrast was enhanced using the autocontrast feature of Adobe Photoshop.

SARS-CoV was reduced by only 1.5 orders of magnitude. A similar pattern was observed in Vero cells, in which IFN reduced SARS-CoV titers by a maximum of 1 order of magnitude after 24 h of infection, whereas 100 U/ml IFN-β were sufficient to suppress HCoV-EMC by 5 orders of magnitude (Fig. 2C). These surprising differences in IFN sensitivity prompted us to test smaller amounts of IFN. Indeed, even 5 U/ml IFN-β had a pronounced effect on HCoV-EMC both on Calu-3 cells and on Vero cells, whereas SARS-CoV was much less affected (Fig. 2D and E). Even if the viruses were allowed to replicate for another 24 h, low doses of IFN substantially reduced titers of HCoV-EMC, and higher doses were more effective. Collectively, these results indicate that (i) HCoV-EMC is capable of multiplying in human primary cells and continuous cell lines derived from the target organs (lung and kidney), and (ii) HCoV-EMC is much more sensitive to the antiviral action of type I IFNs than SARS-CoV is.

SARS-CoV not only counteracts the IFN-stimulated antiviral state but also downmodulates the initial production of IFN and other innate immune cytokines (24, 25). To compare the antiviral

cytokine induction by HCoV-EMC, we performed real-time reverse transcription-PCR (RT-PCR) analysis for two sensitive markers of the IFN response, IFN-β and ISG56 (interferon-stimulated gene 56) (26). In addition, IP-10 (IFN-γ-induced protein 10) (also named CXCL10 [chemokine {C-X-C motif} ligand 10]) was included as a marker of antiviral chemokines. In the first series of experiments, we infected Calu-3 cells with the two coronaviruses at an MOI of 1 (to obtain nearly simultaneous infection of all cells), or as positive control with the strong IFN inducer Rift Valley fever virus mutant RVFVΔNSs::Ren (27). Eighteen hours postinfection, total cell RNA was isolated and tested for innate immunity induction as described previously (20, 26). As expected, SARS-CoV infection did not substantially upregulate IFN-β, ISG56, or IP-10 (Fig. 3A). Interestingly, HCoV-EMC displayed a similar phenotype, as neither innate immune marker was induced above 10-fold. We made similar observations for the 24-h time point of infection (Fig. 3B). It must be mentioned, however, that at this later time point of infection, HCoV-EMC caused a cytopathic effect in Calu-3 cells (data not shown). Of note, production

of genomic RNA, the main IFN-inducing element of viruses, is much higher for SARS-CoV and HCoV-EMC than for the IFN-inducing mutant virus used as a control (Fig. 3C). We therefore extended our analyses to A549 cells (a cell line of human alveolar adenocarcinoma), an established system for sensitive measurement of IFN responses (26, 28). Also in these cells, only the positive control, but none of the coronaviruses induced a strong IFN response (Fig. 3D), even at 48 h postinfection (Fig. 3E), and levels of viral RNAs were comparable for all three viruses (Fig. 3F). The A549 system has the disadvantage that SARS-CoV and HCoV-EMC cannot produce infectious particles (data not shown). However, the production of double-stranded RNA (dsRNA), a major viral IFN elicitor (29), by HCoV-EMC (see below) implies that an active downregulation of the IFN response is taking place. Infection experiments with nondifferentiated HTBE cells and with the human embryonic kidney cell line 293 confirmed the absence of IFN induction by replicating HCoV-EMC (data not shown).

Interferon regulatory factor 3 (IRF-3) is the key transcription factor for IFN-β, ISG56, IP-10, and other antiviral genes (30). IRF-3 is normally located in the cytoplasm but transported to the nucleus upon infection. We have previously shown that SARS-CoV inhibits IRF-3 by retaining it in the cytoplasm (24). The immunofluorescence analysis shown in Fig. 3G (green channel) demonstrates that, even after a long period of infection with HCoV-EMC, IRF-3 remains located in the cytoplasm. As mentioned above, the demonstration of virally produced dsRNA in the cytoplasm again argues for the presence of an active IFN suppression strategy by HCoV-EMC (Fig. 3G, red channel). Thus, apparently, HCoV-EMC shares with SARS-CoV the ability to dampen human innate immune responses by avoiding the activation of IRF-3 and the upregulation of the IFN response.

In summary, our results demonstrate that the novel coronavirus HCoV-EMC has a human cell type range similar to or even broader than that of SARS-CoV. We found robust virus replication in differentiated and nondifferentiated primary airway epithelial cells, in the lung-derived cell line Calu3, and in the kidney cell lines Vero and 293, whereas the lung cell line A549 is abortively infected. In line with this, it was recently reported that, unlike SARS-CoV, HCoV-EMC can also infect cells of bat or pig origin (31). With respect to the innate immune suppression capacity, we found that HCoV-EMC is similar to SARS-CoV in the ability to inhibit IRF-3 and prevent an antiviral IFN response, but the novel coronavirus is much more sensitive to the antiviral action of IFN. This apparent difference from SARS-CoV raises hopes that the current isolate of HCoV-EMC will not spread at the same speed and scale as SARS-CoV did. Given that there is a range of human genetic disorders which lead to the impairment of the IFN response (32), it would be interesting to know the IFN status of the HCoV-EMC-positive individuals who were afflicted with severe respiratory symptoms (2, 4). In any case, treatment with IFN-β, which is an approved drug against a variety of viral, malignant, and autoimmune diseases (33), appears to be a promising therapeutic option against HCoV-EMC. Future investigations on the IFN-related differences between the related coronaviruses HCoV-EMC and SARS-CoV may allow shed light on the virulence determinants of emerging coronaviruses.

ACKNOWLEDGMENTS

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