SARS-CoV-2 triggers an MDA-5-dependent interferon response which is unable to control replication in lung epithelial cells

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Abstract

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is the etiologic agent of coronavirus disease 19 (COVID-19), which ranges from mild respiratory symptoms to acute respiratory distress syndrome, and death in the most severe cases. Immune dysregulation with altered innate cytokine responses is thought to contribute to disease severity. Here, we characterized in depth host cell responses against SARS-CoV-2 in
primary human airway epithelia (HAE) and immortalized cell lines. Our results demonstrate that primary HAE and model cells elicit a robust induction of type I and III interferons (IFNs). Importantly, we show for the first time that melanoma differentiation associated gene (MDA)-5 is the main sensor of SARS-CoV-2 in lung cells. IFN exposure strongly inhibited viral replication and de novo production of infectious virions. However, despite high levels of IFNs produced in response to SARS-CoV-2 infection, the IFN response was unable to control viral replication in lung cells, contrary to what was previously reported in intestinal epithelial cells. Altogether, these results highlight the complex and ambiguous interplay between viral replication and the timing of IFN responses.

**Importance**

Mammalian cells express sensors able to detect specific features of pathogens and induce the interferon response, which is one of the first line of defenses against viruses and help controlling viral replication. The mechanisms and impact of SARS-CoV-2 sensing in lung epithelial cells remained to be deciphered. In this study, we report that despite a high production of type I and III interferons specifically induced by MDA-5-mediated sensing of SARS-CoV-2, primary and immortalized lung epithelial cells are unable to control viral replication. However, exogenous interferons potently inhibited replication, if provided early upon viral exposure. A better understanding of the ambiguous interplay between the interferon response and SARS-CoV-2 replication is essential to guide future therapeutical interventions.

**Introduction**

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is responsible for the current coronavirus disease 2019 (COVID-19) pandemic. This virus emerged in China in
2019 (1, 2) and has, since then, dramatically spread across the world. SARS-CoV-2 has caused more than 1,658,000 deaths worldwide, an undeniable pressure on health systems, general lockdowns in many countries and a global economic crisis. SARS-CoV-2 is related to the highly pathogenic SARS-CoV-1, which caused an outbreak in 2002-2003 in Asia (3–5) and Middle East Respiratory Syndrome coronavirus (MERS-CoV), discovered in 2012 (6). COVID-19 has many of the hallmarks of SARS-CoV disease including fever, breathing difficulty, acute respiratory distress syndrome (ARDS) and death in the most severe cases (7). Four other coronaviruses infect humans and cause common colds every winter: the human coronaviruses (HCoV)-229E, -OC43, -NL63, and -HKU1. SARS-CoV-2 is an enveloped virus with a positive-stranded RNA genome belonging to the genus *betacoronavirus*. SARS-CoV-2 mainly replicates in the respiratory tract, but can also replicate in the gastrointestinal tract (8). Similarly to SARS-CoV-1 and HCoV-NL63, SARS-CoV-2 entry into target cells is mediated by Angiotensin converting enzyme 2 (ACE2) (9–12). Cellular transmembrane protease serine 2 (TMPRSS2) is employed for Spike (S) protein priming at the plasma membrane (11, 13). Cathepsins are also involved in S cleavage and fusion peptide exposure, upon entry via an endocytic route (14–16).

Viral infections are detected through recognition of pathogen-associated molecular patterns (PAMPs) by pattern recognition receptors (PRRs). PRRs include membrane-associated Toll-like receptors (TLR) and cytosolic Retinoic acid-inducible gene I (RIG-I)-like receptors (i.e. RIG-I and melanoma differentiation associated gene (MDA)-5). The murine coronavirus mouse hepatitis virus (MHV) is sensed both by RIG-I and MDA-5 in epithelial cells, with a critical role of the latter in vivo (17–20). However, the role of known PRRs in SARS-CoV-2 sensing in epithelial cells has yet to be elucidated.

PRR activation leads to the production of type I and III interferons (IFNs), pro-inflammatory cytokines and chemokines, which act in a paracrine and autocrine manner. IFNs constitute one of the first lines of defence against viral infections, through the regulation of hundreds
of interferon-stimulated genes (ISGs), which induce an antiviral state in infected and neighbouring cells (21). Type I and III IFN treatments efficiently inhibit the replication of SARS-CoVs and MERS-CoV in vitro and in vivo (22–34). However, SARS-CoV-2 has been shown to dampen type I and III IFN induction and subsequent expression of ISGs (28, 31, 32, 37). In vivo, the recruitment of proinflammatory cells was dependent on type I IFN signalling upon SARS-CoV-2 infection in mice, but not viral clearance (38). Robust replication of SARS-CoV-1 in mice was associated with a delayed type I IFN signalling, which was linked to inflammatory responses and lung immunopathology (39). Type I IFN administration at early stages upon infection protected mice from the disease, but impairment of type I IFN signalling at later stages had the same effect, suggesting that IFNs might be more deleterious than beneficial in mice infected with SARS-CoVs. In contrast, in COVID-19 patients, a highly impaired type I IFN response seemed to characterize the most severe cases, paralleled with an exacerbated production of pro-inflammatory cytokines (40, 41). Moreover, neutralizing auto-antibodies against type I IFNs were found in at least 10% of life-threatening COVID-19 patients, as opposed to 0.33% in healthy individuals (42). Furthermore, 3.5% of the critically ill COVID-19 patients carried inborn errors in genes involved in the type I IFN pathway such as TLR3, IRF7 or IFNAR1 (43). Another recent study also linked genetic variants with severe illness, in particular in TYK2 and IFNAR1 (44). Taken together, this highlights a critical role of IFNs in COVID-19 disease severity and the importance of better understanding the interplay between SARS-CoV-2 and the IFN system.

Here, we aimed to characterize host cell responses to SARS-CoV-2 infection and the role of IFNs in the control of viral replication, using primary human, air-liquid airway epithelia (HAE) cultures and immortalized model cell lines. First, we showed that primary HAE cells, which supported high levels of SARS-CoV-2 replication, were capable of sensing this virus, with a potent induction of type I and III IFN genes, and production of these cytokines...
in their basal media. However, this response arrived late upon viral exposure. The naturally permissive Calu-3 lung cell line recapitulated IFN induction upon SARS-CoV-2 exposure, which validated the use of this model cell line to study viral replication and induction of innate immunity. ACE2-transduced, lung A549 and intestinal Caco-2 cell lines were also able to detect SARS-CoV-2 virus, albeit with different efficiencies. Interestingly, all these cell types could inhibit SARS-CoV-2 replication to different levels upon type I IFN pre-exposure. Using Calu-3 cells, we further showed that MDA-5 was the main innate immune sensor of SARS-CoV-2 in these epithelial cells. Finally, we demonstrated that type I and III IFN production elicited by SARS-CoV-2 infection was unable to inhibit replication, supporting the idea that the timing of IFN exposure is key to control replication.

**Results**

SARS-CoV-2 replicates efficiently in primary HAE cells while triggering type I and type III IFN responses

In order to analyse host cell responses to SARS-CoV-2 infection in physiological targets, primary HAE cells were incubated or not with SARS-CoV-2 on the apical side. Viral replication was analysed at the indicated time points, by monitoring the copy number of RNA polymerase RNA dependent (RdRp) RNAs in cells by RT-qPCR (Figures 1A and 1G). As shown previously (45), these primary cells were highly permissive to SARS-CoV-2 replication. Immunofluorescence analyses confirmed detection of double-stranded RNA in SARS-CoV-2 infected HAE cells but not in non-infected cells (Figure 1B). Cytokine production was measured in the basal media at 72 h post-infection using the Human Anti-Virus Response Panel LEGENDplex™ (Figures 1C and 1D). A strong IFN response was observed, with a substantial production of type I (IFN-β) and type III (IFN-λ1, and 2/3)
An important induction of CXCL10 (IP-10) production was also observed, along with a more modest induction of IL-6, IL-8 and TNF-α pro-inflammatory cytokines. Next, a RT² profiler analysis using the Antiviral Response panel was performed at 72 h post infection (Figures 1E and 1F). As expected, IFNB and other genes belonging to the IFN system (e.g. STAT1, TICAM1, TLR3, TLR7, TLR8, DDX58, IRF7, as well as antiviral effector ISGs, such as OAS2 and MX1) were confirmed to be upregulated. A potent induction of the inflammasome genes NLRP3 and MEFV (encoding Pyrin/TRIM20) was interestingly also observed, as well as CXCL10, CXCL11, CCL3 and CCL5 chemokine induction. Of note, HAE cells from nasal, tracheal and bronchial origins behaved globally similarly with respect to their responses to infection (Figures 1D and 1F). Finally, a RT-qPCR experiment confirmed an important induction of IFNB1, IFNL1 and L2 and parallel induction of prototype ISGs at 48 h and 72 h post-infection, but this was not observed at 24 h (Figure 1G).

Calu-3 cells recapitulate the global responses to SARS-CoV-2 observed in HAE cells

Next, a number of immortalized, epithelial human cell lines were tested for their ability to support viral replication, in comparison to simian Vero E6 cells (Figure 2). Calu-3 (lung adenocarcinoma), Caco-2 (colorectal adenocarcinoma) (both known to express ACE2 and TMPRSS2 and be permissive to SARS-CoV-2 (46)) were used in parallel to A549 (epithelial, lung carcinoma) genetically modified to express ACE2, together with TMPRSS2 or not. Caco-2 cells were also modified to express higher levels of ACE2, or ACE2 and TMPRSS2 in combination. The cells were infected with SARS-CoV-2 and lysed 48 h later to measure viral replication using RdRp RT-qPCR. Calu-3, Caco-2-ACE2 and A549-ACE2 supported SARS-CoV-2 replication to a very similar extent (Figure 2) and were therefore selected for further studies.
Responses to infection were then evaluated in Calu-3 cells. The cells were infected at the indicated MOIs and replication efficiency was assessed by RdRp RT-qPCR (Figure 3A). Whereas replication efficiency increased concomitantly with the viral input at 24 h, a plateau was reached at 48 h. Type I and III IFN production in supernatants from infected Calu-3 cells was assessed using HEK-Blue™ IFN-α/β and IFN-λ reporter cell lines, respectively (Figures 3B and 3C). This showed an important induction of both types of IFNs by SARS-CoV-2 infection, reminiscent of what was observed with primary HAE cells (Figures 3B-C and 1C). Cytokine production in Calu-3 supernatants was next measured using the Human Anti-Virus Response Panel LEGENDplex™ (Figure 3D). A very similar response than that in HAE cells was observed, with a high induction of IFN-β, IFN-λ1 and IFN-λ2/3, CXCL10 and a slight induction of IL-6 and TNF-α (Figures 3D and 1C). RT² profiler and RT-qPCR analyses confirmed a globally similar response of Calu-3 cells to SARS-CoV-2 infection as compared to HAE cells (Figures 3E-F, compared to Figures 1E-G), with a high induction of IFNB1, IFNL2 and a moderate induction of ISGs. High and moderate induction levels of IFNB1, IFNL2, and prototype ISGs ISG15 and MX1 were observed upon SARS-CoV-2 replication in A549-ACE2 and Caco2-ACE2, respectively, despite high levels of replication in both cases (Figures 4A and 4B).

Having established that SARS-CoV-2 infected cells produced high amounts of IFN, we sought to identify the PRR(s) responsible for sensing the virus. Typically, coronaviruses are sensed via RIG-I and/or MDA-5 PRRs (17, 20), which then signal through mitochondrial antiviral-signalling protein (MAVS). We therefore used CRISPR/Cas9 to generate RIG-I, MDA-5 or MAVS knockout (KO) cell populations in parallel to control (CTRL, i.e. expressing non-targeting sgRNAs) KO populations. Immunoblot analyses showed a very good KO efficiency in the different populations (Figure 5A). The KO cells were then challenged with SARS-CoV-2 and their ability to produce type I and III IFNs in
their supernatants upon infection was evaluated using HEK-Blue™ IFN-α/β and IFN-λ reporter cells, respectively (Figures 5B and 5C). We observed that MDA-5 and MAVS depletion, but not RIG-I depletion, drastically impacted the amounts of type I and type III IFNs produced, demonstrating that in Calu-3 lung epithelial cells, SARS-CoV-2 sensing mainly occurred through MDA-5.

In agreement with previous studies (33, 47, 48), we then observed that a 16 h pre-treatment with increasing doses of type I IFN proportionally limited SARS-CoV-2 replication in Vero E6 cells, with the best dose being 1000 U/mL (Figures 6A and 6B). Interestingly, pre-exposure of HAE and Calu-3 cells with 1000 U/mL IFN potently decreased SARS-CoV-2 RNA amounts in infected cells (by 1.5-2 logs) and the production of infectious viruses (by several orders of magnitude; Figures 7A-B and 7E-F).

Immunofluorescence staining failed to detect dsRNA in SARS-CoV-2 infected cells following IFN pre-treatment in HAE cells, contrary to what was observed in non-treated cells (Figure 7C). Immunoblot analysis showed a potent inhibition of Nucleocapsid (N) and Spike expression, concomitant with ISG induction, in Calu-3 cells (Figure 7D). Intracellular Spike staining in infected Calu-3 cells confirmed a 10-fold decrease in the percentage of infected cells following IFN pre-exposure (Figure 7G). Of note, type I IFN pre-treatment had also a very strong impact on SARS-CoV-2 infection in A549-ACE2 cells but a milder effect in Caco-2-ACE2 cells (Figure 8), as seen with viral RNA quantification by RT-qPCR (Figure 8A-B), intracellular Spike staining (Figures 8C-D), and immunoblot analyses (Figures 8E-F).

A striking observation here was that the concentration of type I IFN used for pre-treatment, which was highly inhibitory in HAE and Calu-3 cells, was actually similar to what was naturally produced by these cells upon infection (Figures 1C, 3B, and 3D). However, high levels of replication were observed in these cell types in the absence of exogenous IFN.
We therefore hypothesized that the IFN produced during the course of infection did not have an impact on replication. Indeed, we observed that MDA-5 and MAVS KO in Calu-3 cells did not positively impact viral production, despite abolishing IFN production (Figures 9A and 5B-C). In order to confirm this, we used CRISPR/Cas9 to disrupt genes belonging to the common signalling pathway for type I and III IFNs, rather than the distinct type I and III IFN receptors. Hence, we generated IRF9 and control (CTRL) KO Calu-3 cell populations and observed that IRF9 KO did not substantially improved SARS-CoV-2 replication (Figure 9B), whereas the KO cells were not able anymore to induce prototype ISG expression following IFN treatment (Figure 9C). Similar data were obtained in JAK1 knockout A549-ACE2 cells and similarly unable to respond to IFN (Figures 9D-E). In line with these results, we observed that contrary to pre-exposure, exogenous IFN exposure 24 h post-infection in Calu-3 cells did not have an impact on SARS-CoV-2 replication efficiency (Figure 9F). Moreover, when added as early as 8 h post infection, IFN treatment had an impact only at the lowest MOIs used (Figure 9F). In conclusion, these data showed that the IFN naturally produced upon SARS-CoV-2 replication could only have a minimal impact, if any, on SARS-CoV-2 replication.

**Discussion**

Here, we confirmed the potent induction of innate responses following infection of primary, air-liquid HAE cultures with SARS-CoV-2 (45), with an important but somewhat late induction of type I and III IFNs. In contrast to our results, a lack of IFN response in HAE cells exposed to SARS-CoV-2 was recently reported (49). Of note, viral production in the HAE model we used was several magnitudes of order higher than what was reported in the other study (up to $10^7$ PFU/mL, Figure 7B, in comparison to $\sim2-3.10^2$ PFU/mL (49)), which could easily explain the observed difference in sensing. A lack of IFN induction was also reported upon SARS-CoV-2...
infection of normal human bronchial epithelial (NHBE) cells \( (28) \). However, in this study, only 0.1\% of total deep sequencing reads were from the virus in NHBE, which was a percentage highly similar to what was observed in wild-type A549 cells \( (28) \), known to be refractory to replication because of the lack of ACE2 expression. This strongly suggested poor replication efficiency in this particular model, again explaining the lack of sensing. In support of our data, and in addition to the aforementioned previous report in HAE cells \( (45) \), an IFN induction has also been reported in human intestinal organoids \( (34, 50) \).

Using model cell lines, we notably showed that naturally permissive, lung epithelial Calu-3 cells were a good model for innate immune responses to SARS-CoV-2 infection, with a similar pattern of innate immunity gene induction and pro-inflammatory cytokine production to what we observed in HAE cells. In addition to IFNs, SARS-CoV-2 infection induced the production of the CXCL10 chemokine and the pro-inflammatory cytokines TNF-\( \alpha \) and IL-6, in both cell types (and, in HAE only and to a lower extent, IL-8), but no production of IL-1\( \beta \), consistent with observations in COVID-19 patient samples \( (51) \). Interestingly, at the RNA level, a potent induction of inflammasome-related genes (i.e. \textit{NLRP3} and \textit{MEFV}) was observed. It will be of high interest to further explore the potential regulation of the inflammasome by SARS-CoV-2 and determine whether it is activated and, if that were the case, why there is no IL-1\( \beta \) production by the infected cells.

In agreement with an important role of MDA-5 in host responses to MHV infection in mice \( (20) \), CRISPR/Cas9 KO approaches showed that MDA-5 was the main sensor for SARS-CoV-2 in Calu-3 lung epithelial cells, with no impact of RIG-I in this particular model. While this manuscript was being peer-reviewed, MDA-5 was also reported by Prof. Chanda’s lab to be the main sensor of SARS-CoV-2 in Calu-3 cells, confirming our data \( (52) \). Whether MDA-5 is also the main sensor in HAE cells remained to be determined, but it has so far proven difficult to genetically modify these primary cells.
As reported previously in cell lines and in models of primary bronchial epithelial, air-liquid cell cultures (28, 33, 37, 53, 54), we confirmed that type I IFN pre-treatment potently inhibited SARS-CoV-2 replication in primary HAE cells and lung cell lines. However, in the absence of IFN pre-treatment and despite an important amount of endogenous IFNs produced upon infection in HAE cells, SARS-CoV-2 replication was highly efficient in these cells. Similar data were obtained in Calu-3 cells, despite an earlier IFN response than in HAE cells. This suggested that IFNs were produced too late to efficiently prevent replication, as proposed in another study (32) and/or did not efficiently induced ISG expression. In agreement with this, we observed that knocking-out genes essential for type I and III responses (e.g. IRF9 or JAK1) had not beneficial impact on replication in model lung cell lines, contrary to what was reported in intestinal epithelial cells (34). Indeed, in the latter, a critical role for type III IFNs in replication control was observed and this dramatically highlights differences in the importance of IFN between different target cell types, which we propose are most likely due to different kinetics of IFN production. Moreover, in lung cells, adding post-infection high amounts of exogenous type I IFN had no real impact on replication (even as early as 8 h post-infection, when a high MOI was used), supporting the idea that the timing of IFN exposure is key to control replication. In line with this, SARS-CoV-2 efficiently dampens IFN responses and ISG induction through several mechanisms (19, 37, 55–57). Indeed, nsp1, nsp6, nsp13, ORF3a, M, ORF7a and ORF7b inhibit STAT1/2 phosphorylation and STAT1 nuclear translocation is inhibited by ORF6. Nonetheless, numerous clinical trials are currently evaluating the impact of IFN therapy on COVID-19 patients and should shed light on whether exogenous IFN could be useful in this context. Of note, a substantial proportion of patients with severe diseases may well be unresponsive to such treatments, due to the presence of anti-IFN autoantibodies or inborn mutations in genes belonging to the IFN pathway, such as IFNAR1 (42, 43). This highlights the importance to identify the IFN-induced antiviral effectors, which are so potently...
active against SARS-CoV-2, in order to potentially guide future, targeted therapeutic interventions.
Materials and Methods

Plasmids and constructs

The pRRL.sin.cPPT.SFFV/IRES-puro.WPRE lentiviral vector has been described (58). Human ACE2 (NM_021804) and TMPRSS2 variant 1 (herein called TMPRSS2v1; NM_001135099) were amplified using the SuperScript® III One-Step RT-PCR System with Platinum® Taq (Invitrogen) from 500 ng RNA obtained from 293T cells and Caco-2 cells, using primers 5'-AATTAATTTAGCGGCCGATGTCAGCTCTCTCGTGC-3' and 5'-AATTAATTTACTCGAGCTAAAAAGGAGGTCTGAACATCATCAGTG-3' and 5'-AATTAATTTAGCGGCCGATGCCCCCTGCCCCGCC-3' and 5'-AATTAATTTACTCGAGTTAGCCGTCTGCCCTCATTTGTC-3', respectively, and digested by Notl and Xhol. Human ACE2 was inserted into Notl-Xhol-digested pRRL.sin.cPPT.SFFV/IRES-puro.WPRE to generate pRRL.sin.cPPT.SFFV/ACE2.IRES-puro.WPRE (Addgene 145839). The IRES-puromycinR cassette was removed by Xhol-Sall digestion and either replaced by nothing, to generate pRRL.sin.cPPT.SFFV/ACE2.WPRE (Addgene 145842), or by an IRES-neomycinR cassette, to generate pRRL.sin.cPPT.SFFV/ACE2.IRES-neo.WPRE (Addgene 145840), or by an IRES-hygromycin R cassette, to generate pRRL.sin.cPPT.SFFV/ACE2.IRES-hygro.WPRE (Addgene 145841), respectively. These cassettes were obtained by overlapping PCR using primers 5'-AATTAATTCTCGAGTTAACGAATTCGCGCC-3' and 5'-GTTCATTACGTGTTGCGCCATATTATCATCGTGTATGCC-3' and 5'-GTTCATTACGTGTTGCGCCATATTATCATCGTGTATGCC-3' and 5'-ATATGGCACAACCATGATTGAACAAGATGGATTGCAGC-3' and 5'-TATATATTAGTCAGCTGAAACTCGTCAAGAAGGCGATAG-3' on the ECMV IRES sequence and the neomycin resistance gene (amplified using pRRL.sin.cPPT.SFFV/IRES-puro.WPRE and pcDNA3.1+, respectively) and using primers 5'-AATTAATTCTCGAGTTAACGAATTCGCGCC-3' and 5'-AATTAATTCTCGAGTTAACGAATTCGCGCC-3' and...
AGGCTTTTTCATGGTTGTGGCCATATTATCATCGTGTTTTTC-3’ and 5’-
ATATGGCCACAACCATGAAAAAGCCTGAACCTACACC-3’ and 5’-
TTAATTAATTGTGCACCTATTTCCGCCCTGACGAGTG-3’ on the ECMV IRES sequence and the hygromycin resistance gene (amplified using pAHM (59)), respectively.

Human TMPRSS2 was cloned into NotI-Xhol-digested pRRL.sin.cPPT.SFFV/IRES-neo.WPRE to generate pRRL.sin.cPPT.SFFV/TMPRSS2v1.IRES-neo.WPRE (Addgene 145843). Of note, a mutation (G8V) is present on the cloned CDS of TMPRSS2v1 but this does not seem to impact functionality (not shown).

The pLX_311-Cas9 and LentiGuide-Puro vectors were gifts from John Doench and Feng Zhang, respectively (60, 61) (Addgene 96924 and 52963) and we have described before the LentiGuide-Neo, LentiGuide-Neo-CTRLg1 and g2 (58) (Addgene 139449, 139450, 139451). Guide RNA coding oligonucleotides were annealed and ligated into BsmBI-digested LentiGuide-Neo vector, as described (Addgene). The gRNA coding sequences used were as follow: gRIG 5’-GGGTCTTCCGGATATAATCC, gMDA 5’-TGGTTGGACTCGGGAATTCG; gMAVS 5’-AGGTGGCCCGCAGTCGATCC; gIRF9 5’-CAGCAACTGATACACCTTGT; and gJAK1 5’-TCTCGTCATACAGGGCAAAG.

Cell lines

Human 293T, A549, Caco-2 and Calu-3, HEK-Blue™ IFN-α/β and IFN-λ cells, simian Vero E6 cells were maintained in complete Dulbecco’s modified Eagle medium (DMEM) (Gibco) supplemented with 10% foetal bovine serum and penicillin/streptomycin. HEK-Blue™ IFN-α/β and IFN-λ cells were cultured with 100 μg/ml zeocin and 30 μg/ml Blasticidin, or 100 μg/ml zeocin, 30 μg/ml Blasticidin and 1 μg/ml puromycin, respectively. Caco-2 and Calu-3 cells were obtained from American Type Culture Collection (ATCC); HEK-Blue™ IFN-α/β and IFN-λ cells were obtained from InvivoGen; 293T, A549, Vero E6 cells were gifts from Michael Malim’s lab, Wendy Barclay’s lab, and from the CEMIPAI facility, respectively. All
339 cell lines were regularly screened for the absence of mycoplasma contamination.
A549 and Caco-2 cells stably expressing ACE2 and TMPRSS2 were generated by
transduction with either RRL.sin.cPPT.SFFV/IRES-puro.WPRE, RRL.sin.cPPT.SFFV/IRES-neo.WPRE, RRL.sin.cPPT.SFFV/IRES-hygro.WPRE or RRL.sin.cPPT.SFFV.WPRE containing-vectors (cDNA as indicated) and were maintained under 1 μg/ml puromycin, and/or 1 mg/ml G418, 50 μg/ml hygromycinB selection.

For CRISPR-Cas9-mediated gene disruption, A549-ACE2 and Calu-3 cells stably expressing Cas9 were first generated by transduction with LX_311-Cas9 followed by blastcidin selection at 10 μg/ml. Cas9 activity was checked using the XPR_047 assay (a gift from David Root, Addgene 107145) and was 79.5% and >83.4%, respectively, for Cas9-expressing A549-ACE2 and Calu-3 cells. The cells were then transduced with guide RNA expressing LentiGuide-Puro and Lentiguide-Neo vectors (as indicated) and selected with antibiotics for at least 10 days.

Air-liquide cultures of primary Human Airway Epithelial (HAE) cells of nasal, tracheal and bronchial origins from healthy donors were obtained from Epithélix (MucilAir™) and cultured with MucilAir™ media (Epithélix). The apical side of the HAE cells was washed when necessary and 1 day prior to IFN exposure, following the manufacturer’s instructions.

When indicated, universal type I IFN (PBL Interferon source) was added at the indicated concentration (e.g. 1,000 U/ml) for 16-24 hr prior to virus infection. For HAE cells, IFN was added both in the basal media and on the apical side of the cells (diluted in 20 μl of MucilAir media; 20 μl of media without IFN was added to the control cells in parallel).

**Lentiviral production and infection**

Lentiviral vector stocks were obtained by polyethylenimine (PEI; for LentiGuides) or Lipofectamine 3000 (Thermo Scientific; for ACE2 and TMPRSS2 lentiviral vectors).
mediated multiple transfection of 293T cells in 6-well plates with vectors expressing Gag-Pol, the miniviral genome, the Env glycoprotein at a ratio of 1:1:0.5. The culture medium was changed 6 h post-transfection, and vector containing supernatants harvested 36 h later, filtered and used directly or stored at -80°C.

SARS-CoV-2 production and infection

The BetaCoV/France/IDF0372/2020 isolate was supplied by Pr. Sylvie van der Werf and the National Reference Centre for Respiratory Viruses hosted by Institut Pasteur (Paris, France). The patient sample from which strain BetaCoV/France/IDF0372/2020 was isolated was provided by Dr. X. Lescure and Pr. Y. Yazdanpanah from the Bichat Hospital, Paris, France. The BetaCoV/France/IDF0372/2020 was amplified in Vero E6 cells (MOI 0.005) in serum-free media supplemented with 0.1 μg/ml L-1-p-Tosylamino-2-phenylethyl chloromethylketone (TPCK)-treated trypsin (Sigma–Aldrich). The supernatant was harvested at 72 h post infection when cytopathic effects were observed (with around 50% cell death), cell debris were removed by centrifugation, and aliquots frozen down at -80°C. Viral supernatants were titrated by plaque assays in Vero E6 cells. Typical titers were 3-5.10^6 plaque forming units (PFU)/ml.

Simian Vero E6 and human cell infections were performed at the indicated multiplicity of infection (MOI; as calculated from titers in Vero E6 cells) in serum-free DMEM and 5% serum-containing DMEM, respectively. The viral input was left for the duration of the experiment (unless specified otherwise). The viral supernatants were frozen down at -80°C prior to RNA extraction and quantification and/or titration by plaque assays.

HAE cells were incubated for 2h with SARS-CoV-2 diluted in 50 μl of PBS1X added to the apical side. The viral input was then removed and the cells washed with 100 μl PBS1X. To collect the progeny viruses at 24 h, 48 h, and 72 h post infection, 50 μl of PBS1X was added to the apical side of the cells and collected after a 20 min incubation at 37°C. The
viral supernatants were frozen down at -80°C prior to titration by plaque assays on Vero E6 cells. The cells were lysed in RLT buffer (Qiagen) followed by RNA extraction at 72 h post-infection.

Quantification of mRNA expression

3-5 x 10^5 cells with or without treatment with IFNα and SARS-CoV-2 infection were harvested and total RNA was extracted using the RNeasy kit (Qiagen) employing on-column DNase treatment, according to the manufacturer's instructions. 140 µl of supernatants from infected cells were subjected to RNA extraction using the QIAamp Viral RNA Mini Kit (Qiagen), according to the manufacturer's instructions. 125 ng cellular RNA or 1.2 µl viral RNA were used to generate cDNAs. The cDNAs were analysed by qPCR using published RdRp primers and probe (62), as follow: RdRp_for 5’-GTGARATGGTCAATGTTGGCGG-3’, RdRp_rev 5’-CAATGTAAAAACACTATTAGCATTA-3’ RdRp_probe 5’-FAM-CAGGTGAACCTCATAGATGAC-TAMRA-3’ and/or TaqMan gene expression assays (Applied Biosystems) for ACTB (Hs99999903_m1), GAPDH (Hs99999905_m1), ISG15 (Hs01921425_s1), OAS1 (Hs00973637_m1), IFITM3 (Hs03057129_s1), MX1 (Hs00182073_m1), IFNB1 (Hs01077958_s1), IFNL1 (Hs00601677_g1), IFNL2 (Hs00820125_g1). qPCR reactions were performed in triplicate, in universal PCR master mix using 900 nM of each primer and 250 nM probe or the indicated Taqmans. After 10 min at 95°C, reactions were cycled through 15 s at 95°C followed by 1 min at 60°C for 40 repeats. Triplicate reactions were run according to the manufacturer’s instructions using a ViiA7 Real Time PCR system (ThermoFisher Scientific). For ISG expression, GAPDH and/or ACTB mRNA expression was used to normalize samples. pRdRp (which contains an RdRp fragment amplified from SARS-CoV-2 infected cell RNAs using primers RdRp_for and RdRp_rev and cloned into pPCR-Blunt II-TOPO) was diluted in 20 ng/ml
salmon sperm DNA to generate a standard curve to calculate relative cDNA copy numbers and confirm the assay linearity (detection limit: 10 molecules of RdRp per reaction).

**RT² profiler**

The RT² First Strand kit (Qiagen) was used for the synthesis of the complementary DNA strand using 400 µg of total RNA from samples extracted using the RNeasy kit (Qiagen) employing on-column DNase treatment. RT² Profiler™ PCR Array Human Antiviral Response (PAHS-122Z) was used in the present study, according to manufacturer’s instructions. The ViiA7 Real Time PCR system (ThermoFisher Scientific) was used to amplify the DNA with a thermal cycling of 95 °C for 10 min followed by 40 cycles of 15 s at 95°C and 60 s at 60°C. Five housekeeping genes (β-actin (ACTB), β-2-microglobulin (B2M), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), hypoxanthine phosphoribosyltransferase1 (HPRT1) and ribosomal protein, large, P0 (HPLP0) were used as internal controls. The average of the Ct values from these 5 controls was used to normalize gene expression. Changes in mRNA expression between the non-infected and the infected conditions were analysed using the ΔΔCt method.

**Quantification of secreted Cytokines**

The concentration of 13 secreted cytokines was measured in the supernatants and basal media of infected Calu-3 and HAE cells, respectively, at the indicated conditions, using LEGENDplex bead-based immunoassays (BioLegend, human anti-virus response panel), according to the manufacturer’s recommendations. Samples were analysed on a BD Canto II flow cytometer using the Diva software (BD Biosciences, San Jose, CA). BioLegend’s LEGENDplex Data Analysis Software was used to analyse data.

**Immunofluorescence and microscopy**
HAE cells were pre-treated or not with IFN for 20 h and infected with SARS-CoV-2, as described above, for 48 h. Cells were fixed with PBS1X containing 4% paraformaldehyde (EM Sciences) for 15 min, permeabilized with 0.5% Triton X-100 for 15 min, and blocked/quenched in buffer NGB (50 mM NH$_4$Cl, 1% goat serum, 1% bovine serum albumin) for 1 h. An overnight incubation at 4°C with mab J2 (Scicons) followed by incubation in a secondary anti-mouse antibody conjugated to Alexa Fluor 546 and in Alexa Fluor 488 Phalloidin (Thermofisher Scientific) for 2h at RT were used to visualise dsRNA and F-actin, respectively. The transwell membranes were removed from the inserts and mounted between slides and coverslips using ProLong™ Gold Antifade Mountant (Thermofisher Scientific). Images were acquired with a LSM880 confocal microscope paired with an Airyscan module (ZEISS) with a 63x lens. Post-processing of RAW Airyscan images was performed using the Zen Black software.

**HEK-Blue™ IFN-α/β and IFN-λ assays**

HEK-Blue™ IFN-α/β cells and HEK-Blue™ IFN-λ (InvivoGen) were plated at 30,000 cells per well in a 96-well plate. The following day, media from infected cells (or control cells) was added and a standard curve was generated in parallel by serial dilutions of type I or type III IFNs in complete DMEM. After 20-24 h incubation, 30 µl of HEK-Blue™ IFN-α/β supernatants was added to 120 uL of Quanti-blue™ substrate (InvivoGen) and incubated at 37°C for 15 min. Absorbance was measured at 620nm using an Envision plate reader (Perkin-Elmer). The standard curves were used to provide semi-quantitative analyses of the IFN concentrations produced by the infected cells.

**Spike intracellular staining and flow cytometry analysis**

Infected cells were harvested at the indicated time points post-infection and fixed for 30 min in PBS1X-4% PFA. Cells were washed once in PBS1X and twice in BD Perm-Wash.
buffer and permeabilized for 15 min at RT in BD Perm-Wash buffer. Cells were incubated on ice for 30-45 min in FACS buffer (PBS1X-5% FCS) containing a 1/250 dilution of Alexa 488-conjugated anti-Spike antibody (GTX632604 conjugated using the Zenon Alexa Fluor 488 Mouse IgG Labeling Kit, ThermoFisher) and washed 4 times in FACS buffer. Flow cytometry was performed using the NovoCyte™ (ACEA Biosciences Inc.).

**Immunoblot analysis**

Cells were lysed in lysis buffer (10 mM TRIS 1M pH7.6, NaCl 150 mM, Triton X100 1%, EDTA 1 mM, deoxycholate 0.1%) supplemented with sample buffer (50 mM Tris-HCl pH 6.8, 2% SDS, 5% glycerol, 100 mM DTT, 0.02% bromphenol blue), resolved by SDS-PAGE and analysed by immunoblotting using primary antibodies against SARS-CoV Nucleocapsid (Bio-Techne NB100-56683), SARS-CoV Spike (GeneTex GTX632604), Actin (Sigma-Aldrich A1978), IFITM3 (Proteintech 11714-1-AP), MX1 (ThermoFisher Scientific PA5-22101), RIG-I (Covalab mab10110), MDA-5 (Ozyme D74E4), and MAVS (ProteinTech 14341-1-AP), followed by secondary horseradish peroxidase-conjugated anti-mouse or anti-rabbit immunoglobulin antibodies and chemiluminescence Clarity or Clarity max substrate (Bio-Rad). A Bio-Rad ChemiDoc imager was used.

**Requests for materials**

Requests for material should be addressed to Caroline Goujon or Olivier Moncorgé at the corresponding address above, or to Addgene for the plasmids with an Addgene number.

**Data availability**

The datasets generated during and/or analysed during the current study are available from the corresponding authors on reasonable request.
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The authors have no conflicts of interest to declare in relation to this manuscript.
A.R., O.M., and C.G. conceived and designed the experiments; A.R., A.L.C.V., M.T., O.M., and C.G. performed the main experiments; G.M. and S.N. performed the LegendPlex experiments and analyses; B.B. designed the molecular cloning strategies; J.M. did the immunofluorescence and microscopy analyses; R.P. and M.A. provided technical help; A.R., O.M. and C.G. wrote the manuscript with input from all authors.
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Figure legends.

Figure 1. Primary human airway epithelial host cell responses to SARS-CoV-2 infection. A. Human HAE cells (MucilAir™, Epithelix) were non-infected (N.I.) or incubated with SARS-CoV-2 on the apical side at MOI 0.01 and 0.1 for 2 h. Cells were harvested and lysed for RNA extraction and RT-qPCR analysis using RdRp primers and probe 48 h and 72 h post-infection. B. Human HAE cells were N.I. or incubated with SARS-CoV-2 on the apical side at MOI 0.1 for 2 h. 48 h later, cells were stained for Actin with Phalloidin (magenta) and an anti-double stranded RNA antibody (green). Representative images, acquired with an LSM880 Airyscan microscope, are shown; scale bar 10 μm. C. Human HAE cells were N.I. or incubated with SARS-CoV-2 (MOI 0.1), as in A. Cytokine concentrations in the basal media were measured using the Human Anti-Virus Response Panel LEGENDplex™ 72 h later (top) and the heat map (bottom) represents the fold difference in cytokine concentrations (log2 scale) in basal media from infected compared to N.I. cells. D. Data from Human Anti-Virus Response Panel LEGENDplex™ as performed in C. with supernatants from cells of nasal, tracheal and bronchial origins. E. An Antiviral Response RT² profiler PCR array analysis was performed using the RNAs from A. extracted at 72 h (N.I. and MOI 0.1). Relative expression is shown for the indicated genes. F. Data from Antiviral Response RT² profiler PCR array analysis as in D. obtained with RNAs from HAE cells of nasal, tracheal and bronchial origins. G. Human HAE cells were mock infected (N.I.) or incubated with SARS-CoV-2 on the apical side at MOI 0.01 for 2 h, harvested at the indicated time points and lysed for RNA extraction and RT-qPCR. Differential ISG expression was measured using the indicated taqmans, and data were normalized to both ActinB and GAPDH (left y-axis), and viral replication was analysed using RdRp primers and probe (right y-axis). The light blue line (sets at 1) indicates no change in cytokine production or in gene expression (D, F, G). The mean of 4 (A), 3 (C-F), or 6 (G; apart for the 24 h time point, n=3) independent experiments is shown, with error bars representing the standard deviation (s.d.).

Figure 2. Replication of SARS-CoV-2 in genetically modified human cell lines. Caco-2 and A549 cells were transduced or not with lentiviral vectors to stably overexpress ACE2, or ACE2 and TMPRSS2. The indicated (unmodified and modified) cell lines were infected with SARS-CoV-2 at MOI 0.05 and lysed 48 h later for RNA extraction and RdRp RT-qPCR analysis. A representative experiment (with technical triplicates) is shown.

Figure 3. Calu-3 model cell line responses to SARS-CoV-2 infection. A. Human Calu-3 cells were N.I. or incubated with SARS-CoV-2 at the indicated MOIs. Cells were harvested and lysed for RNA extraction and RdRp RT-qPCR analysis. B-C. Cell supernatants from (A) were harvested at the indicated time points and type I (B) and type III (C) IFN concentrations were measured using HEK-Blue™ IFN-α/β and IFN-λ reporter cells, respectively. D. Cell supernatants from (A) were harvested and cytokine concentrations were measured using the Human Anti-Virus Response Panel LEGENDplex™ at 24 h and 48 h. Concentrations are shown (top), and the fold difference in cytokine concentration in supernatants from infected compared to N.I. cells was represented as a heat map (bottom; log2 scale). E. An Antiviral Response RT² profiler PCR array analysis was performed using the RNAs from (A) extracted at 48 h (MOI 0.005). F. Relative expression levels of the indicated IFN genes and...
ISGs were analysed by RT-qPCR analysis at the indicated time points using both ActinB and GAPDH for normalization. The mean of 4 (A and F) or 3 (B-E) independent experiments is shown, with error bars representing the s.d. from the mean.

**Figure 4.** A549-ACE2 and Caco-2-ACE2 model cell line responses to SARS-CoV-2 infection. **A-B.** Human A549-ACE2 (A) and Caco-2-ACE2 (B) cells were non-infected or incubated with SARS-CoV-2 at the indicated MOIs. Cells were harvested, lysed for RNA extraction. Relative expression levels of the indicated IFN genes and ISGs were analysed by RT-qPCR analysis using both ActinB and GAPDH for normalization (left y-axis), and viral replication was analysed using RdRp primers and probe (right y-axis). The mean of 3 independent experiments is shown, with error bars representing the s.d. from the mean.

**Figure 5.** MDA-5 is the main sensor of SARS-CoV-2 in Calu-3 model cells. Calu-3-Cas9 cells were transduced with lentiviral vectors expressing CRISPR non-targeting single-guide (sg)RNAs (Ctrl #1, #2) or sgRNAs targeting RIG-I, MDA-5 or MAVS, and selected for 2 weeks. **A.** Expression levels of RIG-I, MDA-5 and MAVS were assessed in the different populations by immunoblot, Actin served as a loading control (a representative immunoblot is shown). **B-C.** Cells were challenged with SARS-CoV-2 at MOI 0,05 and their supernatants harvested at 24 h and 48 h post-infection. Concentrations of type I (B) and type III (C) IFNs produced in the supernatants were analysed using HEK-Blue™ IFN-α/β and IFN-λ reporter cells, respectively. The mean of 3 independent experiments is shown, with error bars representing the s.d. from the mean.

**Figure 6.** Inhibition of SARS-CoV-2 replication by type I IFN in Vero E6 cells. **A-B.** Vero E6 cells were pre-treated or not with increasing concentrations of type I IFN, as indicated, for 16 h prior to SARS-CoV-2 infection at MOI 0.0005. 72 h later, the cells were lysed and the supernatants collected, the RNAs were extracted and viral replication was monitored in cells (A, left panel) and viral production in the supernatants (B, left panel) by RdRp RT-qPCR. Right panels. The fold inhibition by IFN is shown (A and B, right panels). The mean of 3 independent experiments is shown, with error bars representing the s.d. from the mean.

**Figure 7.** Inhibition of SARS-CoV-2 replication by type I IFN pre-treatment in primary HAE cells and immortalized Calu-3 cells. **A.** Human HAE cells (origin as indicated) were pre-treated or not with type I IFN for 20 h, and N.I. or incubated with SARS-CoV-2 on the apical side at MOI 0,1 for 1-2 h. Cells were harvested at 72 h post-infection, lysed for RNA extraction and RdRp RT-qPCR analysis. **B.** Plaque assays were performed on washes of the apical side of the HAE cells from (A) at 24 h, 48 h and 72 h to determine the number of plaque forming units (PFU) per mL of supernatant (grey dotted line: detection threshold). **C.** Human HAE cells were pre-treated or not with IFN for 20 h, and N.I. or incubated with SARS-CoV-2 on the apical side at MOI 0,1 and 0,25, for 2 h. 48h later, cells were stained for Actin with Phalloidin (magenta) and an anti-double stranded RNA antibody (green). Representative images, acquired with an LSM880 Airyscan microscope, are shown; scale bar 10 μm. **D.** Calu-3 cells were pre-treated or not with IFN for 16-20 h, the cells were N.I. or incubated with SARS-CoV-2 at the indicated MOIs, and lysed 24 h post-infection for immunoblot analysis of SARS-CoV-2 Nucleoprotein (N) and Spike, and IFITM3, RIG-I, MX1 and Actin expression levels. A representative immunoblot is shown. **E.** Human Calu-3 cells were pre-treated or not with...
IFN, and infected as in D. Cells were harvested and lysed for RNA extraction and RdRp RT-qPCR analysis. **F.** Production of infectious viruses on supernatants from E was determined by plaque assays. **G.** Calu-3 cells were pre-treated or not with IFN and infected as in D, and cells were stained with an anti-Spike antibody. The percentage of Spike positive (+) cells was scored by flow cytometry. The mean of 3 (A-B) or 4 (E-G) independent experiments is shown, with error bars representing the s.d. from the mean.

**Figure 8.** Inhibition of SARS-CoV-2 replication by type I IFN in A549-ACE2 and Caco-2-ACE2 cells. Human A549-ACE2 (A-C-E) and Caco-2-ACE2 (B-D-F) cells, as indicated, were pre-treated or not with IFN for 16-20 h, the media was replaced and cells were mock-infected (N.I.) or incubated with SARS-CoV-2 at the indicated MOIs. **A-B.** Cells were harvested and lysed for RNA extraction and RT-qPCR analysis using RdRp primers and probe. **C-D.** Cells were fixed with PFA, permeabilized and stained with an anti-Spike antibody conjugated to an Alexa fluorochrome. The percentage of Spike + cells was scored by flow cytometry. **E-F.** Cells were lysed for Immunoblot analysis of SARS-CoV-2 Nucleoprotein (N) and Spike, IFITM3, RIG-I and MX1 ISG expression levels, Actin serving as a loading control. Representative immunoblots are shown. Of note, MX1 was not detected in Caco-2-ACE2 cell lysates. The mean of 3 independent experiments is shown (A-D), with error bars representing one standard deviation (s.d.) from the mean.

**Figure 9.** IFN production upon SARS-CoV-2 replication does not protect Calu-3 and A549-ACE2 cells against infection. **A.** CTRL, RIG-I, MDA-5 and MAVS Calu-3 knockout cells were infected with SARS-CoV-2 at MOI 0.05 (as in Figure 5B-C) and viral production was measured 48 h later by plaque assays on Vero E6 cells. **B.** CTRL and IRF9 Calu-3 KO cells were generated and selected. Cells were infected with SARS-CoV-2 at the indicated MOIs and viral replication was evaluated 48 h later by RdRp RT-qPCR. **C.** CTRL and IRF9 KO cells were pre-treated or not with IFN for 48 h, lysed and the expression levels of IFITM3, RIG-I, MX1 and Actin were analysed by immunoblot. **D.** CTRL and JAK1 A549-ACE2 knockout cells were generated and selected. Cells were infected with SARS-CoV-2 at MOI 0.0005 and viral replication was measured 48 h later using RdRp RT-qPCR. **E.** CTRL and JAK1 knockout cells were pre-treated or not with IFN for 48 h, lysed and the expression levels of IFITM3, RIG-I and MX1 were analysed by immunoblot, Actin served as a loading control. **F.** Calu-3 cells were infected with SARS-CoV-2 at the indicated MOIs after a 24h pre-treatment with IFN or not, or were subsequently treated with IFN at 4 h, 8 h or 24 h post infection. Viral replication was measured 48 h post-infection using RdRp RT-qPCR. The mean of 2 (A-D) or 3 (B-F) independent experiments is shown, with error bars representing the s.d. Representative immunoblots (C, E) are shown.
