SARS-CoV2-mediated suppression of NRF2-signaling reveals potent antiviral and anti-inflammatory activity of 4-octyl-itaconate and dimethyl fumarate

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Antiviral strategies to inhibit Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV2) and the pathogenic consequences of COVID-19 are urgently required. Here, we demonstrate that the NRF2 antioxidant gene expression pathway is suppressed in biopsies obtained from COVID-19 patients. Further, we uncover that NRF2 agonists 4-octyl-itaconate (4-OI) and the clinically approved dimethyl fumarate (DMF) induce a cellular antiviral program that potently inhibits replication of SARS-CoV2 across cell lines. The inhibitory effect of 4-OI and DMF extends to the replication of several other pathogenic viruses including Herpes Simplex Virus-1 and-2, Vaccinia virus, and Zika virus through a type I interferon (IFN)-independent mechanism. In addition, 4-OI and DMF limit host inflammatory responses to SARS-CoV2 infection associated with airway COVID-19 pathology. In conclusion, NRF2 agonists 4-OI and DMF induce a distinct IFN-independent antiviral program that is broadly effective in limiting virus replication and in suppressing the pro-inflammatory responses of human pathogenic viruses, including SARS-CoV2.
The 2020 SARS-CoV-2 pandemic emphasizes the urgent need to identify cellular factors and pathways that can be targeted by new broad-spectrum antiviral therapies. Viral infections usually cause disease in humans through both direct cytopathic effects and excessive inflammatory responses of the infected host. This also seems to be the case with SARS-CoV2, as COVID-19 patients develop cytokine storms that are very likely to contribute to, if not drive, immunopathology, and disease severity. For these reasons, antiviral therapies must aim to not only inhibit viral replication but also to limit inflammatory responses of the host.

Nuclear factor (erythroid-derived 2)-like 2 (NRF2) belongs to the cap’n’collar basic leucine zipper family of transcription factors characterized structurally by the presence of NRF2-ECH homology domains. At homeostasis, NRF2 is maintained in an inactive state in the cytosol by association with its inhibitor protein KEAP1 (Kelch-like ECH-associated protein 1), which targets NRF2 for proteasomal degradation. In response to oxidative stress, KEAP1 is inactivated and NRF2 is released to induce NRF2-responsive genes. In general, the genes under the control of NRF2 protect against stress-induced cell death and NRF2 has thus been suggested as the master regulator of tissue damage during infection. Importantly, NRF2 is now demonstrated as an important regulator of the inflammatory response and functions as a transcriptional repressor of inflammatory genes in murine macrophages, most notably interleukin (IL-)1β.

Recent reports demonstrated that NRF2 was induced by several cell derived metabolites including itaconate and fumarate, to limit inflammatory responses following lipopolysaccharide stimulation. A chemically synthesized, cell-permeable derivative of itaconate, 4-oyctyl-itaconate (4-OI) was then shown to be a potent NRF2 inducer. Of special interest is the derivative of fumarate, dimethyl fumarate (DMF), a US Food and Drug Administration approved drug that is used as an anti-inflammatory therapeutic in multiple sclerosis (MS), with the capacity to suppress pathogenic inflammation through a Nrf2-dependent mechanism.

In addition to limiting the inflammatory response to LPS, 4-OI also inhibited the Stimulator of Interferon Genes (STING) antiviral pathway and interferon (IFN) stimulated gene expression via NRF2 induction. A recent single-cell RNA-sequencing analysis demonstrated that the NRF2 antioxidant gene signature correlated with resistance to HSV1 infection. However, whether NRF2 agonists can be used to inhibit SARS-CoV2 replication or other pathogenic viruses remains unknown.

Here we demonstrate that expression of NRF2-dependent genes is suppressed in biopsies from COVID-19 patients and that treatment of cells with NRF2 agonists 4-OI and DMF induced a strong antiviral program that limits SARS-CoV2 replication. This antiviral program extends to other pathogenic viruses including Herpes Simplex Virus-1 and-2 (HSV-1 and HSV-2), Vaccinia Virus (VACV), and Zika Virus (ZIKV). Furthermore, 4-OI and DMF limits the release of pro-inflammatory cytokines in response to SARS-CoV2 infection and to virus-derived ligands through a mechanism that involves inhibition of IRF3 dimerization. In summary, NRF2 agonists are plausible broad-spectrum antiviral and anti-inflammatory agents. Our results suggest that repurposing the clinically approved compound DMF may represent a rapidly applicable strategy for the treatment of COVID-19-associated disease.

Results
The NRF2-response is suppressed in COVID-19 patient biopsies. To identify host factors or pathways important in the control of SARS-CoV2 infection, publicly available transcriptome data sets including transcriptome analysis of lung biopsies from COVID-19 patients were analyzed using differential expression analysis. Here, genes linked with inflammatory and antiviral pathways, including RIG-I receptor and Toll-like receptor signaling, were enriched in COVID-19 patient samples, whereas genes associated with the NRF2 dependent antioxidant response were suppressed in the same patients (Fig. 1a–c). That NRF2-induced genes are repressed during SARS-CoV2 infections was supported by reanalysis of another data-set building on transcriptome analysis of lung autopsies obtained from five individual COVID-19 patients (Desai et al.15) (Fig. 1d). Furthermore, that the NRF2-pathway is repressed during infection with SARS-CoV2 was supported by in vitro experiments where the expression of NRF2-inducible proteins Heme Oxygenase 1 (HO-1) and NAD(P)H quinone oxidoreductase 1 (NqO1) was repressed in SARS-CoV2 infected Vero hTMPRSS2 cells while the expression of canonical antiviral transcription factors such as STAT1 and IRF3 were unaffected (Supplementary Fig. 1). These data indicate that SARS-CoV2 targets the NRF2 antioxidant pathway and thus suggests that the NRF2 pathway restricts SARS-CoV2 replication.

NRF2 agonists 4-OI and DMF inhibit SARS-CoV2 replication. Considering that NRF2 suppresses antiviral IFN-responses, it was surprising to discover that treatment of Vero cells with 4-OI generated before infection with SARS-CoV2 (strain #29313 FR-4286 isolated from a patient in Germany) resulted in a 10^6–10^4 reduction in SARS-CoV2 RNA levels in a dose dependent manner (Fig. 2a, b) while not affecting cell viability, as determined by lactate dehydrogenase (LDH) release assay (Supplementary Fig. 2). Furthermore, subsequent release of progeny SARS-CoV2 virus particles to the cell supernatant was equally decreased by 4-OI treatment. This was measured by TCID50 assay to quantify virus by dilution of virus-induced cytopathogenic effects and by plaque assay (Fig. 2c–f). The reduced viral replication led to reduced virus-induced cytotoxicity of the infected Vero cells as determined by LDH release assay and immunoblotting for cleaved Caspase 3 and Poly(ADP-Ribose) Polymerase 1 (PARP-1), which are hallmark indicators of apoptosis (Fig. 2g, h). Interestingly, the observation that the NRF2 pathway is inhibited in response to SARS-CoV-2 infection was recapitulated in SARS-CoV-2 infected Vero cells, as demonstrated by both the decrease in the basal level expression of NRF2-driven proteins HO-1 and NqO1 and their inability to be induced by the NRF2 agonist 4-OI (Fig. 2h). The antiviral effect of 4-OI was also demonstrated in the lung cancer cell line Calu-3, where SARS-CoV2 RNA levels were reduced by >2-logs (Fig. 2i), while release of progeny virus was reduced by >6-logs based on TCID50 analysis of cell supernatants (Fig. 2j, k). In the immortalized human epithelial cell line NuLi, total infection levels were relatively low compared to what was observed in Calu3 and Vero cells, but 4-OI treatment still reduced SARS-CoV2 RNA levels and release of progeny virus (Fig. 2l, m). We further tested the antiviral effect towards SARS-CoV2 in primary human airway epithelial (HAE) cultures (Fig. 2n). Here, 4-OI treatment also significantly reduced viral RNA levels (Fig. 2o). Interestingly, DMF treatment of Calu3 cells likewise inhibited SARS-CoV-2 replication by a similar magnitude as observed with 4-OI (Fig. 2p-q); in Vero cells, a reduced but significant inhibition of SARS-CoV-2 multiplication was also detected (Fig. 2r). To further evaluate the role of the NRF2/KEAP1 axis in SARS-CoV-2 replication, siRNA silencing of KEAP1 was used to activate NRF2. Silencing of KEAP1 decreased SARS-CoV-2 RNA levels, reduced viral protein expression, and virus titers (Fig. 2s–u). Thus, both treatment with NRF2 agonists and genetic activation of NRF2 led to restriction of SARS-CoV2 replication. Finally, the antiviral effect of 4-OI was reproduced using a different SARS-CoV-2 isolate from Japan (Fig. 2v, w).
Fig. 1 Expression of NRF2-driven genes is suppressed in COVID-19 patient biopsies. a, b, c Reanalysis of data published by Blanco-Melo et al.14 (a) Bar-chart of the number of transcripts showing significant differential expression (adjusted p value < 0.05 and log2(FC) > 1.0). Data from COVID19 lung biopsies was normalized against healthy lung biopsies, and in cell lines Calu3, NHBE, and A549 infected with either SARS-CoV2, Influenza A virus (IAV), Respiratory Syncytial virus (RSV), or human parainfluenza virus type 3 (HPIV3) against mock treated cells. Expression and p values were calculated with DESeq2 using Wald test statistic and Benjamini-Hochberg correction for multiple testing. b Heat map of the subset of genes significantly differentially expressed in COVID19 biopsies and simultaneously differentially expressed in at least 3 of the other conditions tested. The genes in each cluster were used for pathway enrichment analysis. Genes in cluster 1 are dominantly down-regulated in COVID19 biopsies while a sub-cluster of genes in cluster 1 are up-regulated in the cell lines. Conversely, genes in cluster 2 are predominantly up-regulated in biopsies and in most other test-samples. A sub-cluster of the genes in cluster 2 are down regulated in the cell lines. For each cluster, the significantly enriched pathways are listed (EnrichR). c Cloud analysis of NRF2-driven differentially expressed genes. Subsets annotated as inflammation/NFkB signaling and Type I IFN signaling exhibit different expression patterns. The experiment is a reanalysis of data from Blanco-Melo et al. [https://doi.org/10.1101/2020.03.24.004655]. d Reanalysis of the data from Desai et al.15 GEO accession code GSE150316. Heat map of NRF2 target genes of the lung autopsies from the five COVID19 patients. Healthy lung samples were used as negative control.
These data demonstrate that NRF2 inducers 4-OI and DMF inhibit SARS-CoV-2 replication by a magnitude of several logs.

4-OI broadly inhibits viral replication through an IFN-independent pathway. The antiviral effect of 4-OI was not restricted to SARS-CoV-2 but also extended to other human pathogenic viruses. Using the human keratinocyte cell line HaCat, 4-OI also inhibited HSV1 and HSV2 multiplication, as measured by virus titers, intracellular viral RNA content and accumulation of viral proteins (Fig. 3a–e and Supplementary Fig. 3). In contrast, the NRF2-inducible genes HO-1, NqO1, and Sequestosome 1 (SQSTM1) were highly increased in response to 4-OI treatment (Fig. 3c and Supplementary Fig. 3); the antiviral effect of 4-OI was at least partly dependent on NRF2, since siRNA silencing of NRF2 reduced the capacity of 4-OI to inhibit HSV1 infection and viral protein expression (Fig. 3f–g).
the range of pathogenic viruses inhibited by 4-OI, HaCaT cells and bone marrow derived dendritic cells (BMDCs) were pre-treated with 4-OI and then infected with vaccinia virus (VACV) or electromalia virus (ECTV); infection and replication of both poxviruses were strongly inhibited by 4-OI (Fig. 3h-n). Further, the inhibitory effect of 4-OI extended to zika virus (ZIKV), an important human pathogenic virus causing a severe disease when transmitted in utero, where 4-OI pre-treatment reduced zika virus replication in both A549 and Huh-7 cells (Fig. 3o-p).

Interestingly, the antiviral effect of 4-OI did not extend to infection with vesicular stomatitis virus (VSV) (Supplementary Fig. 4); this result further provided indirect evidence that the antiviral program induced by 4-OI was distinct from the classical IFN-dependent antiviral response, since VSV, and especially VSVΔG1M used in this study, is highly sensitive to the effects of IFNs. The antiviral effect of 4-OI relied on intracellular restriction of replication, since HSV entry was not inhibited by 4-OI treatment across cell lines (Supplementary Fig. 5).

To address the possibility that the inhibitory effect of 4-OI was independent of type I IFN signaling, as suggested by the results in Vero cells that are deficient in type I IFN19, HaCaT cells deficient in IFN alpha receptor 2 (IFNAR2), Signal Transducer and Activator of Transcription 1 (STAT1) or STING were treated with 4-OI, followed by infection with HSV1 and VACV.

Replication of both viruses was inhibited by 4-OI in IFNAR2 and STAT1 KO cells, for HSV1 replication was also decreased in STING KO cells, as measured by plaque assay and expression of viral proteins (Supplementary Fig. 6). In conclusion, 4-OI induced an antiviral program that functioned independently of IFN signaling (Supplementary Fig. 6).

To examine what general pathways were affected by 4-OI treatment that could predict the underlying mechanism of its antiviral effect, RNA sequencing analysis was performed with HaCaT cells treated with 4-OI and then infected with HSV1. By comparing untreated to 4-OI treated cells, either with or without infection with HSV1, several pathways were identified that were induced or repressed by 4-OI, including repression of the IFN-signaling pathway by 4-OI (Supplementary Fig. 7). Amongst the top up-regulated genes induced by 4-OI was the heme oxygensase-1 (HO-1, HMOX-1), an enzyme canonically involved in stress detoxification, also reported to have antiviral activity against amongst other viruses Zika and Dengue viruses20–22. To assess whether, HO-1 had any antiviral activity in our cellular system, Vero hTMRPSS2 and Calu-3 cells were either transfected with an overexpression plasmid encoding HO-1, or genetically silenced for KEAP1 and HO-1 by siRNA, respectively before infection with SARS-CoV-2. These treatments (HO-1 overexpression or silencing) did not alter SARS-CoV-2 infection/replication, suggesting an HO-1-independent antiviral program induced by 4-OI (Supplementary Fig. 8).

In an attempt to pin-point the antiviral mode of action of 4-OI, we also used microscope-based analysis of morphology by cell-paint technology (Supplementary Fig. 9) to compare morphological changes in cells treated with 4-OI to cells treated with compounds that have known cellular targets and with cells treated with other compounds with reported antiviral activity toward SARS-CoV2 including Remdesivir and Hydroxychloroquine. In this analysis, 4-OI had a low but significant morphological activity whithout loss of cell viability. Interestingly, the activity of 4-OI did not seem to overlap with other compounds known to perturb cell morphology, indicating that the antiviral mode of action of 4-OI is distinct from known antiviral mechanisms.

4-OI and DMF suppress the inflammatory response to SARS-CoV2. In COVID-19, an uncontrolled proinflammatory cytokine storm contributes to disease pathogenesis and lung damage. For this reason, we investigated if 4-OI and DMF inhibited inflammatory cytokine gene expression induced by SARS-CoV2. In Calu-3 cells, SARS-CoV2 infection increased the expression of IFNB1, C-X-C motif chemokine 10 (CXCL10), Tumor Necrosis Factor alpha (TNFA), IL-1B, and C-C chemokine ligand 5 (CCL5). Interestingly, this induction was abolished by 4-OI pre-treatment, thus reducing the pro-inflammatory response to SARS-CoV2 (Fig. 4a-b). In contrast, expression of the Nrf2 inducible gene HMOX1 was highly increased in response to 4-OI treatment (Fig. 4c). The potential anti-inflammatory effect of 4-OI in this context was supported when using HAE cultures. Here, treatment with 4-OI also reduced the expression of IFNB1, CXCL10, TNFA, and CCL5 in the context of SARS-CoV2 infection (Fig. 4d-e), while increasing the expression of the Nrf2 inducible gene HMOX1 (Fig. 4f). A similar pattern was observed in Calu3 cells, treated with DMF before SARS-CoV2 infection; IFNB1, CXCL10, and CCL5 mRNA levels were reduced in DMF treated cells, while TNFA mRNA levels were unaffected (Fig. 4g, h).

In contrast, DMF treatment increased the expression of the NRF2 inducible gene HMOX1 (Fig. 4i).
Fig. 3 4-OI broadly inhibits other pathogenic viruses including HSV, VACV, and Zika Virus. a HaCaT cells treated with 4-OI (125 μM) for 48 h and infected with HSV1-GFP (MOI 0.01). Viral titers were determined by plaque assay. Data are obtained from one experiment of at least seven independent experiments each performed in triplicates. b RNA analyzed using RNA-sequencing (n = 3) performed once. c–e HaCaT cells treated with 4-OI (125μM) and infected with HSV1-GFP (MOI 0.01). Lysates were analyzed by immunoblotting with Vinculin (VCL) as loading control and by flow cytometry (n = 7 from three independent experiments). f, g HaCaT cells were lipofected with siRNA for 72 h, subsequently challenged with 4-OI (125 μM) before HSV1-GFP infection (MOI 0.01) for 24 h. Infectivity and silencing efficiency was determined by immunoblotting (f) and flow-cytometry (g). Data obtained from one experiment representative of three independent experiments. (h–n) HaCaT cells (h–l) and BMDCs (m, n) were treated with 4-OI (125 μM) for 48 h and infected with VACV expressing either GFP or ECTV expressing mCherry for 24 h. Viral titers and infectivity were determined by plaque assay (h), flow cytometry (i–j and m–n), and visualized by confocal microscopy (k–l). Data in (i–j, m, and n) are obtained from one experiment representative of two independent experiments each performed in triplicates. o–p A549 and Huh-7 cells were pre-treated with 4-OI for 48 h (150 μM) and infected with Zika virus (ZIKV) (MOI 0.1) for 4 days. Viral genome was determined by qPCR. Data were obtained from one experiment representative of two independent experiments. For all panels, bars indicate mean ± s.e.m. Unless otherwise stated, all statistical analysis were performed using a two-tailed Mann-Whitney test to determine statistical significance where *p < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.0001. Individual p values were, a, p < 0.0001; c, p < 0.0001; d, p < 0.0002(two tailed t test, MOI 0.1) and p < 0.0001(two tailed t test, MOI 0.01); j, p < 0.0001(two tailed t test, MOI 0.1) and p < 0.0001 (two tailed t test, MOI 0.01); n, p < 0.001(two tailed t test, MOI 0.1) and p = 0.0178(two tailed t test, MOI 0.01). Source data are provided as a Source Data file.
The effect of 4-OI on peripheral blood mononuclear cells (PBMC) harvested from healthy donors was also examined; although PBMC infection by SARS-CoV2 (MOI 1) yielded a very weak induction of CXCL10 compared to sendai virus (SeV) infection (50 HAU), and no detectable induction of other cytokines, 4-OI treatment also reduced CXCL10 mRNA levels in this context (Fig. 4j). Furthermore, using PBMCs from four individual patients with severe COVID-19 and admitted to hospital Intensive Care Units, we demonstrated that in three of four patients, the expression of CXCL10 was increased compared to healthy controls; in all four patients, these levels were reduced to basal levels after treatment with 4-OI (Fig. 4k), indicating that 4-OI was able to reverse the inflammatory response in patients PBMCs ex vivo. That CXCL10 levels is a relevant readout in SARS-CoV2 was recently supported by a report from Cheemarla et al.26 ([https://doi.org/10.1101/2020.06.04.20109306])26 demonstrating that CXCL10 expression is increased in the upper airways of patients infected with SARS-CoV2.

The observed decrease in antiviral and proinflammatory cytokine response can be explained by the 4-OI mediated reduction in intracellular viral RNA, with subsequent reduced induction of cytokines through cellular RNA sensors such as...
Fig. 4 4-OI and DMF limit SARS-CoV2 and HSV induced inflammatory responses. a-c Calu-3 treated with 4-OI (125 µM, 48 h) before SARS-CoV2 infection (MOI 0.5, 48 h) and qPCR analysis. a, b, and c are pooled data from three independent experiments in triplicates. d-f HAE-cultures treated with 4-OI before SARS-CoV2 infection for 24 h and analysis by qPCR. Data are from two donors. g-i Calu-3 cells with DMF (48 h) before SARS-CoV2 infection. Analysis by qPCR. h, i, and l, display pooled data from three independent experiments in duplicates and triplicates. (d-f), data are representative of four independent HAE-cultures. (j) Healthy PBMCs treated with 4-OI before infection and qPCR analysis. Data are representative of two donors. (k) PBMCs from four COVID-19 patients (k) and 2 healthy controls (HC) treated with 4-OI before qPCR. (l-n) HaCaTs treated with 4-OI before stimulation with M8 for 6 h followed by qPCR. Data represent one experiment in triplicate. n HaCaT cells with siRNAs before M8 treatment for 3 h followed by immunoblotting. Data are representative of two independent experiments. o HaCaTs treated with siRNA for 72 h before 4-OI and stimulation with M8. Analysis by immunoblotting. Data representative of two independent experiments. p, q HEK293 and HaCaTs transfected with expression-plasmids before 4-OI. In (q), HaCaTs were treated with siRNAs for 72 h before plasmid transfection. Analysis by qPCR and immunoblotting. Data in (p) are from one experiment performed in triplicate. Data displayed in (q) is representative of two independent experiments. (r-t) HaCaTs treated with 4-OI before infection with HSV1 or transfection with dsDNA. Analysis by qPCR and immunoblotting in 6 h and 3 h respectively. (n-t), data are from one experiment representative of two independent experiments. Data in (r) and (s) are representative of two independent experiments in triplicates. Bars indicate mean ± s.e.m. Unless otherwise stated, all statistical analysis by two-tailed Mann-Whitney test where *p < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.0001. P values: a: p = 0.0001(IFNβ) and p = 0.0001(CXCL10), b: p < 0.0001(CCL5) and p < 0.0001(TNFα) and p < 0.0001(IL1β), c: p < 0.0001, d: p = 0.0286 (IFNβ) and p = 0.0286(CXCL10), e: p = 0.0286(CCL5), f: p = 0.0286, g: p = 0.0003(IFNβ) and p = 0.0002(CXCL10), h: p = 0.0002(CCL5) and p = 0.0003(TNFα), i: p = 0.001, j: p = 0.0005 (two-tailed t test, SARS-CoV2) and p = 0.0053 (two-tailed t test, SeV), p: p = 0.0092 (two-tailed t test). Source data are provided as a Source Data file.

Discussion

Altogether, this study demonstrates that the expression of NRF2 dependent antioxidant genes is significantly inhibited in COVID-19 patients, and that the NRF2 agonists 4-OI and DMF inhibit both SARS-CoV2 replication, as well as the expression of associated inflammatory genes. Significantly, DMF is currently used as an anti-inflammatory drug in relapsing-remitting MS and could easily be repurposed and tested in clinical trials as a small molecule inhibitor of SARS-CoV-2 replication and inflammation-induced pathology in COVID19 patients.

The ability of NRF2 inducers to also reduce potentially pathogenic IFN- and inflammatory responses while retaining their antiviral properties is unique to these compounds and highlights their potential to control virus-induced pathology. That NRF2 might be a natural regulator of IFN-responses in the airway epithelium is supported by a recent report demonstrating that NRF2 activity is high while IFN activity is low in the bronchial epithelium. Our discovery that NRF2 seems to affect stability of IRF3 dimerization adds to the notion that NRF2 is a more universal regulator of type I IFN induction, while keeping in mind that this is not the only mechanism through which this could be achieved. Other examples are the suppression of type I IFNs by NRF2 through inhibition of STING expression and the regulation of IFN-responses to VSV through NRF2-mediated autophagy. That VSV is highly sensitive to IFNs and its outgrowth promoted by autophagy could be part of the explanation as to why VSV, in contrast to other viruses tested, was insensitive to or even seemed to benefit from a chemical pre-treatment with 4-OI.

The observation that 4-OI strongly inhibited the IFN antiviral response to both cytosolic DNA and RNA but retained the capacity to block viral replication also supports the existence of an unidentified cellular program that restricts viral replication independently of IFNs. This is supported by the negative correlation between NRF2-inducible gene expression and infection with HSV1 discovered by Wyler et al., through single cell transcriptome analysis. By analogy with the induction of hundreds of IFN-stimulated effector genes, an NRF2 antiviral program may also use a variety of mechanisms to restrict viral replication by targeting distinct stages of viral replication. Our transcriptome analysis of the cells treated with 4-OI identified several cellular pathways or programs that each could contribute to the antiviral state of the 4-OI treated cells. These cellular pathways included Hypoxia-inducible-factor 1 alpha-controlled gene expression as well as basal cellular glycolipid and nitrogen metabolism. Further studies will be needed to unravel the contribution of each of these cellular pathways to antiviral immunity.

Considering the dual effect of NRF2 agonists on both viral replication and inflammatory markers, it would be interesting to investigate if patients that develop severe SARS-CoV2 pathology also have an underlying dysregulation of NRF2 or of central pathways or programs that each could contribute to the antiviral response to VSV through NRF2-mediated autophagy. That VSV is highly sensitive to IFNs and its outgrowth promoted by autophagy could be part of the explanation as to why VSV, in contrast to other viruses tested, was insensitive to or even seemed to benefit from a chemical pre-treatment with 4-OI.

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Methods

Cell lines, reagents and culture conditions. Human lung adenocarcinoma epithelial A549 cells, immortalized human HaCaT keratinocytes, Calu-3 epithelial
lung cancer cells, and human embryonic kidney HEK293T cells were kindly provided by S.R.P. (Aarhus University, Denmark) and cultured in DMEM (Lonza) supplemented with 10% heat inactivated fetal calf serum, 100 U/mL penicillin, 100 µg/mL streptomycin and 200 µg/mL glutamine (hereafter termed DMEM complete). Vero E6 cells expressing hTMRPR52 were a kind gift of Makoto Takeda (University of Tokyo, Japan) and were cultured in DMEM (Lonza) supplemented with 10% heat inactivated fetal calf serum, 200 U/mL penicillin, 100 µg/mL streptomycin, 600 µM 4-Octyl-itaconate (4-OI) and 1% blasticidin. All cell lines were regularly tested for mycoplasma contamination by sequencing from GATC Biotech (Germany). 4-Octyl-itaconate (4-OI) was chemically synthesized by Thomas B. Poulsen (Aarhus University, Denmark) and was dissolved in DMSO12.

To obtain BMDCs, a cell suspension from femurs of C57BL/6 mice (Charles River) was cultured for 6-8 days at 37 °C in RPMI-1640 medium supplemented with 3% FBS and 10% of J558 cell line supernatant containing GM-CSF. Cells were seeded at a density of 10^6 cells/mL and medium was partially replaced every 2 days. B10.B cells (ECACC) were maintained in DMEM supplemented with 5% FBS, 2 mM glutamine, 200 U/mL penicillin, and 100 µU/mL streptomycin at 37 °C 5% CO2.

For generation of KO cell line clones in HaCaT cells specific guide RNA sequences targeting STING (5’-AGGACACATGTCGCTG-3’) or STAT1 (5’TATAATGTGACATGTGAGC-3’) were cloned into the plasmids pX461 (Addgene) (STING) or LentiCRISPR v2 (Addgene) (STAT1). Wildtype HaCaT cells were transfected with the plasmids using the Lipofectamine 2000 Reagent (Invitrogen, Life Technologies). 72 h post transfection, the GFP expressing cells were sorted as single cells by FACS and clones were grown to larger cultures (STING). Or 24 h post transfection, the cells were seeded in a dilution sufficient to obtain about one to two colonies per membrane. The 2 µg/ml puromycin selection was initiated 48 h post transfection and continued for 72 h (STAT1). Hereafter, single cell clones were grown to larger cultures which were validated for absence of protein by western blotting and functional analysis to confirm the biological effect of the gene deficiency.

**Viruses.** We used the SARS-CoV-2 strain #291.3 FR-4286 isolated from a patient in Germany, and kindly donated by professor Georg Kochs (Freiburg). The virus was propagated in Vero E6 cells expressing human TMPRSS2. SARS-CoV-2 genome detection was performed with Taqman based qPCR using SARS-CoV-2 specific primers and probes with the following sequences: Forward primer: AAATTTGGGGACACAGAAG, reverse primer: TGGCACCTGTGTAGGTC. AAc. Probe: FAM-ACTGACATGGTCCTGGATTGTC. The virus was propagated in Vero E6 cells expressing human TMPRSS2. Vero E6 cells expressing human TMPRSS2 were seeded in 6-well plates and at 80% confluence ALI cultures was washed for 5 min using DMEM-1% Pen/Strep supplemented with 2 packs of Airway Epithelial Cell Growth Medium Supplement (PromoCell, 4C-39160) containing either 150 µM 4-OI or DMSO. Baso-lateral medium containing 4-OI was added to the apical compartment overnight. At time of infection, apical medium was removed and 100 µl DMEM low glucose (SageVax Inc., D5921) was added to all membranes for 1 h and placed in 37 °C incubator. After 1 h apical infection medium was removed and membranes placed in 37 °C incubator for 24 h before harvest.

Upon initiation of treatment ALI cultures was washed for 5 min using DMEM (low glucose, no additives) and baso-lateral medium changed for ALI medium containing either 150 µM 4-OI or DMSO. Baso-lateral medium containing treatment was left overnight. A 100 µl of DMEM (low glucose) with 150 µM or DMSO was additionally added to the apical compartment overnight. At time of infection, apical medium was removed and 100 µl DMEM low glucose (SageVax Inc., D5921) was added to all membranes for 1 h and placed in 37 °C incubator. After 1 h apical infection medium was removed and membranes placed in 37 °C incubator for 24 h before harvest. At time of harvest, baso-lateral medium was removed and 500 µl DMEM/EDTA was added bao-laterally and 200 µl was added apically. After ~5 min cells were harvested using 5% FBS/DMEM (low glucose). Cells were lysed for RNA isolation using lysibuffer from High Pure RNA Isolation Kit (Roche Diagnostics, #11828665001). For Western blot cells were lysed in RIPA buffer containing 1/10 Protease inhibitor (Roche), 1/1000 Benzonase (Sigma, #E1014) and 24 µg of hydrocortisone (StemCell, #07925) and 0.2 mg heparin (StemCell, #07980). Membranes were located at least 21 days of differentiation verified by extensive cilia beating and mucus covering.

**Short-interfering RNA (siRNA)-mediated knock down.** For short interfering RNA experiments, HaCat cells were transfected in 6-well plates with 80 pmol of bidirectional siRNA (sc-37007) or control siRNA (sc-37006) in serum and antibiotic free DMEM and using Lipofectamine RNAi Max as per manufacturer’s instructions. HaCat cells were incubated for 72 h in the presence of the siRNA before being processed. For experiments in Calu-3 cells, the same protocol conditions were used with control si RNA (sc-37007), Keap1 siRNA (sc-43878) and HO-1 (sc-35554) that were lipofected for 48 h.

**dsDNA, cGAMP and optimized RIG-1 agonist stimulation of cells.** HSV-60 naked, a viral dsDNA motif, 23’ cGAMP, a STING ligand, and M8, a sequence optimized RIG-1 agonist15 were obtained from Invivogen and John Hiscott (Pasteur Institute, Rome), respectively. Intracellular delivery of dsDNA and cGAMP was achieved using Lipofectamine 2000 (Invitrogen) diluted in serum-free medium with a ratio of Lipo.dsDNA/cGAMP of 1:1. Final concentration for both dsDNA and cGAMP was 4µg/mL. Intracellular delivery of M8 was achieved using Lipofectamine RNAiMax (Invitrogen) diluted in serum-free medium with a ratio of LipoRNA of 1:1. Final concentration of M8 was 10 ng/mL.

**VACV infection assays.** BMDCs, HaCat and HaCat Stat1 KO were incubated or not with 150 µM 4-OI for 48 h before infection with vtag2GFP using 0.1 or 0.01 pfu per cell at 37 °C for 60 min. Treated cells were infected into the nasal turbinations and twisted. To determine the proportion of vtag2GFP infected cells GFP expression was detected at 16 hpi by flow cytometry using triplets. Briefly, cells were harvested, washed with FACS buffer (PBS, 0.01% sodium azide, and 0.1% BSA) and fixed with paraformaldehyde 4% in PBS (1 h). After FACS buffer wash, 2x10^4 cells were scored and analyzed in a FACS Calibur flow cytometer (BD Sciences) per experimental condition in triplicates. To determine VACV virus...
titres, HaCaT and HaCaT STAT1 KO cells were previously stimulated for 48 h or not with 150 µM 4-OI and then infected with VACV-WR using 0.1 or 0.01 pfu per cell at 5 × 10^5. Cells were harvested in their own centrifuged at 1800 × g for 5 min, and resuspended in 0.5 mL of fresh medium. In all cases, samples were frozen, thawed three times and titrated using duplicates in BSC-1 cells. Briefly, preconfluent monolayers of BSC-1 cells were infected with tenfold serial dilutions of viral inoculums for 1 h at 37 °C. Then, inoculum was replaced with semi-solid carboxy-methyl cellulose (Sigma) media with 2% FBS and cells fixed in 10% formaldehyde at 3 d pi. Plates were stained with 0.1% (w/v) crystal-violet. Two independent experiments were performed.

Zika virus infections. A549 cells (kind gift from G. Kochs, Freiburg) and Huh-7 were cultured at 37 °C in DMEM, supplemented with 10% FCS and 2mM L-Glutamine (Sigma-Aldrich) for 48 h. Cells were infected with ZikaV (moi 0.1) for 1 h. 4-OI was freshly added when the medium was changed. Cells were lysed and total RNA was extracted at 96hpi using the QIAshredder (Qiagen) and RNeasy Mini Kit (Qiagen) according to the manufacturer’s instructions. RNA was reverse transcribed using SuperScript II Reverse Transcriptase (Invitrogen) into cDNA that was then used for qPCR with SYBR green PCR kit (Life Technologies). C_copy values were normalized to GAPDH (ΔCt). SYBR green primer probes included use GAPDH (for: CTGCGTTCGTCGTTCTTA, rev: CTCGCTTCACCCCTCTTGA) and ZIKV (for: CGAGGAAATCCAGCAGC, rev: ATTGAGGACTTGGATGTC).

SARS-CoV2 TCD50% assay. The assay was performed as follows. 2 × 10^4 Vero E6 TMPRSS2 cells were seeded in 96 well DMEM (Gibco, + 2% FCS (Sigma-aldrich) + 1% Pen/Strep (Gibco) + 0.1% nonessential amino acids) per well in 96-well plates. 24 h after, samples were titrated onto the cells by addition of 10ul of a 10-fold serial dilution. One full plate was used per sample analyzed. Each dilution was repeated 8 times. The assay was repeated in at least 2 independent experiments. Titration was performed by reading the 50% tissue culture infective dose (TCID_50) of each independent experiment.

Western blot. Cells were lysed in 100 µL of ice-cold Pierce RIPA lysis buffer (Thermo Scientific) supplemented with 10 mM NaF, 1x complete protease cocktail inhibitor (Roche) and 5 µL -1 benzanole (Sigma), respectively. Protein concentration was determined using a BCA protein assay kit (Thermo Scientific). Whole-cell lysates were denatured for 3 min at 95°C in presence of 1x XT Sample loading control. After three washes in PBST, secondary antibodies, peroxidase-conjugated F(ab)2 donkey anti-rabbit IgG (H+L) (1:10,000) (Jackson ImmunoResearch) were added to the membrane in PBST 1% milk for 1 h at room temperature. All membranes were washed three times and exposed using either the SuperSignal West Pico PLUS chemiluminescent substrate or the SuperSignal West Femto maximum sensitivity substrate (Thermo Scientific).

Cytoine qPCR analysis. Gene expression was determined by real-time quantitative PCR, using TaqMan detection systems (Applied Biosciences). RNA was extracted using the High Pure RNA Isolation Kit (Roche) and RNA quality was assessed by Nanodrop spectrometry (Thermo Fisher). RNA levels were analyzed using premade TaqMan assays and the RNA-to-Ct-1-Step kit according to the manufacturer’s recommendations (Applied Biosciences). Taqman assays for qPCR were purchased from Applied Bioscience; IFNiB1 (Hs01077958), CXCL10 (Hs00982282), IL1B (Hs01555410), HMOXI (Hs01102590), ACTB (Hs01060665), and TNFA (Hs00174128).

Transcriptional analysis COVID19. COVID19 data set analysis (Fig. 1): RNA-seq data were obtained from an already available dataset from Blanco-Melo (doi: https://doi.org/10.1101/2020.03.24.046653). From the raw read-counts differential expression values were calculated using DESeq2. Significantly differentially expressed genes (SDEGs) were selected based on the thresholds of adjusted p value < 0.05 and absolute fold change of 2 (Fig. 1a). In order to focus on commonality across the different conditions with respect to generating clinically relevant hypotheses, the 15 SDEGs obtained in the bioprospecting process were considered if these were also present in the list of SDEGs in the other conditions. All genes that occur in at least 3 or more conditions were included in the final list, resulting in 113 genes. Finally, the expression values for all genes in the final list across all conditions were assembled, clustered using Euclidean distance metric and Ward’s variance minimization cluster analysis. The heatmap was generated using Python3.7 and seaborn cluster-map tools. Then the gene-sets from each of the outlined clusters were used for pathway enrichment analysis using Enrichr. Finally, the STRING database was used to construct the cloud network starting from lists of genes manually annotated for NFR2, inflammation and IFN signaling. Edges and nodes were extracted from STRING and imported to Cytoscape version 3.7 for further visualization (Fig. 1c).

Transcriptional analysis of HSV1-infected HaCaT cells. RNA sequencing was performed in collaboration with BGI Europe Genome Center (Copenhagen, Denmark) following the standard operational procedure as described before. Briefly, the quality of total RNA was checked using the Agilent 2100 bioanalyzer. To construct the sequencing library for MGISEQ-2000, ~1 µg of polyA enriched RNA was used for library construction using the MGIEasy RNA Directional Library Prep Kit (MGI Tech). Next, paired-end sequencing with 100 cycles was performed using the MGISEQ-2000 sequencing instrument, according to the manufacturer’s instructions. We generated an average of 63 million raw reads for each sample. The clean RNA reads were first aligned to the hg9 UCSC RefSeq (human reference genome, GRCh37) using BWA-MEM. Families of proteins used to derive the virus, the unmapped reads were then aligned to the coding sequence of the human herpesvirus 1 (KOS strain). The expression of human genes and virus genes were performed by transforming mapped transcript reads to TPM using RSEM. The normalized expression were estimated and normalized by DESeq2. Differentially expressed genes were defined as genes with fold change over twofolds and adjusted p value < 0.01 using DESeq2.

Cell painting assay. The procedure from Bray et al. was followed with adaption to 96-well plates and optimized fluorophore concentrations for Vero E6 cells and the Celldiscoverer 7 imaging system: 5000 wt Vero E6 cells were seeded into the inner 60 wells of a 96-well plate with open bottom (Corning #3690) in 7 µL full growth medium. After 24 h, compounds were dosed as a 25 µL 4X final solution (dMSO = 0.5%). After 24 h, 75 µL of medium was replaced and replaced with 75 µL medium containing 500 nM Mitotracker Deep Red (final C = 325 nM) and plates were incubated (37 °C, 5% CO2, humid) in the dark for 30 min. Wells were then aspirated and 75 µL of medium were added, before adding 25 µL 16% paraformAldehyde (PFA). After fixation, cells were permeabilized by permeabilization buffer (200 mM Glycine, pH 7.5, 0.1% Triton X-100, 1 Phalloidin-AF568; 5.75 µM M SYTO 14; 5 µM Hoechst 33342 in 1% (wt/vol) bovine serum albumin (Sigma-Aldrich, #A9647)) and incubation for 30 min in the dark. Plates were washed twice with 1X HBSS (Invitrogen, #41065-065) and 75 µL 0.1% (vol/vol) Triton X-100 (BDH, #306324 N) in 1X HBSS was added and incubated for 15 min in the dark. Plates were washed twice with 1X HBSS before addition of 75 µL 2 µM MitoTracker staining solution (final concentration: 0.75 µg·mL−1 WGA-AF555; 8.75 µg·mL−1 Concanavalin-AF488; 2.5 µg·mL−1 Phalloidin-AF568; 0.75 µM SYTO 14; 5 µg·mL−1 Hoechst 33342 in 1% (wt/vol) bovine serum albumin (Sigma-Aldrich, #A9647)) and incubation for 30 min in the dark. Plates were then washed three times with 1X HBSS, with no final aspiration and imaging.
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Author contributions
D.O. and C.H. conceived the project and prepared figures. D.O., A.L.H., J.T., J.C., B.H., M.L., E.B.S., A.K., L.R., M.L., M.S., S.F., M.T., M.L., H.H., V.G., A.H., A.H., A.K., C.G., D.V.D.H., C.M., L.B., A.T., T.A., J.H., R.O., S.E.J., C.G.N., L.B., Y.L., and A.A. performed experiments, analyzed data, and prepared figures. E.F. performed bioinformatics and prepared figures. T.M. was responsible for including COVID-19 patients and achieving patient material. C.H. and D.O. drafted and finalized the manuscript. J.H. planned experiments and edited the manuscript. E.F., S.P., S.B., and M.L. performed bioinformatics and prepared figures. M.J. facilitated SARS-CoV2 laboratories.

Competing interests
The authors declare no competing interests.

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