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Inhibition of SARS–CoV-2 by type I and type III interferons

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The recently emerged severe acute respiratory syndrome coronavirus-2 (SARS–CoV-2) is the causative agent of the devastating COVID-19 lung disease pandemic. Here, we tested the inhibitory activities of the antiviral interferons of type I (IFN-α) and type III (IFN-λ) against SARS–CoV-2 and compared them with those against SARS–CoV-1, which emerged in 2003. Using two mammalian epithelial cell lines (human Calu-3 and simian Vero E6), we found that both IFNs dose-dependently inhibit SARS–CoV-2. In contrast, SARS–CoV-1 was restricted only by IFN-α in these cell lines. SARS–CoV-2 generally exhibited a broader IFN sensitivity than SARS–CoV-1. Moreover, ruxolitinib, an inhibitor of IFN-triggered Janus kinase/signal transducer and activator of transcription signaling, boosted SARS–CoV-2 replication in the IFN-competent Calu-3 cells. We conclude that SARS–CoV-2 is sensitive to exogenously added IFNs. This finding suggests that type I and especially the less adverse effect–prone type III IFN are good candidates for the management of COVID-19.

The massive pandemic caused by coronavirus SARS–CoV-2 (1, 2) is calling for rapid evaluation of potential therapeutics through repurposing of drugs already in clinical use. Interferons of type I (IFN-α/β) and type III (IFN-λ) constitute an important branch of the mammalian innate immune response. These cytokines are produced by virus-infected cells and are able to establish an antiviral state in target cells by triggering the so-called JAK/STAT signaling pathway (3–5). Both type I and type III IFNs are clinically used or being tested, respectively, against a range of ailments that include viral diseases (6, 7). Previously, we and others have demonstrated the potential of IFNs to inhibit the two related, previously emerged pathogenic coronaviruses SARS–CoV-1 and MERS-CoV (8–15). Here, we investigated the potential of type I and type III IFNs against the newly emerged SARS–CoV-2.

Results

Type I IFN

We tested the effect of type I IFN against SARS–CoV-2 compared with the SARS–CoV-1 from 2003. Two different cell lines were employed, namely the human bronchial epithelial Calu-3 and the primate kidney epithelial Vero E6. The cells were first treated for 16 h with 100, 500, or 1000 units/ml of recombinant human IFN-α(B/D) and then infected with the viruses at a multiplicity of infection (MOI) of 0.01 plaque forming units (PFU)/cell to obtain multistep growth. Virus titers in supernatants were determined 24 h later, when titers are reaching a plateau (see below). The data of three biological replicates are shown in Fig. 1. Because several titers were below the detection limit of our plaque assay, a rank correlation test (Spearman's exact rank correlation test) was used for statistical dose-response correlation analysis. For SARS–CoV-2 (*dark gray bars*), statistically significant negative correlation coefficients (CC) were obtained for both cell lines, indicating that viral replication is increasingly inhibited by IFN-α. For SARS–CoV-1 (*light gray bars*), titers were also affected. However, at least in Vero E6 cells, the reduction of SARS–CoV-1 appears to be weaker than the reduction of SARS–CoV-2 (Fig. 1*B*). Observations were similar when the input MOI was reduced to 0.001 (Fig. S1), except that titers of SARS–CoV-1 in Calu-3 cells were already very low in the absence of any IFN-α, resulting in a nonsignificant effect of additional IFN. These data may suggest that the potency of IFN to reduce viral titers may be stronger and more consistent against SARS–CoV-2 than against SARS–CoV-1.

To further investigate the potential differences between the viruses, we repeated the experiment three times more with the intermediate dose of 100 units/ml and analyzed the data statistically after pooling them with the previous three replicates. Two-way ANOVA was used to simultaneously evaluate the influence of both IFN-α and virus species on virus reduction. This analysis (Fig. 2, *A* and *B*) showed again that (i) both viruses are reduced by IFN (comparison of 0 *versus* 100 units/ml IFN-α, p(IFN)) and (ii) there are differences between the SARS–CoV species (comparison of the virus experiments, p(virus)). Moreover, the "interaction" *p* value showed that, at least in Vero cells, the degree of IFN sensitivity depends on the virus species, again indicating that SARS–CoV-2 is more IFN-sensitive than SARS–CoV-1.

Type III IFN

The primary tropism of coronaviruses typically involves epithelia of the respiratory and gastrointestinal tracts (16). On such mucosal barriers, type III IFNs rather than type I IFNs are

This article contains supporting information.

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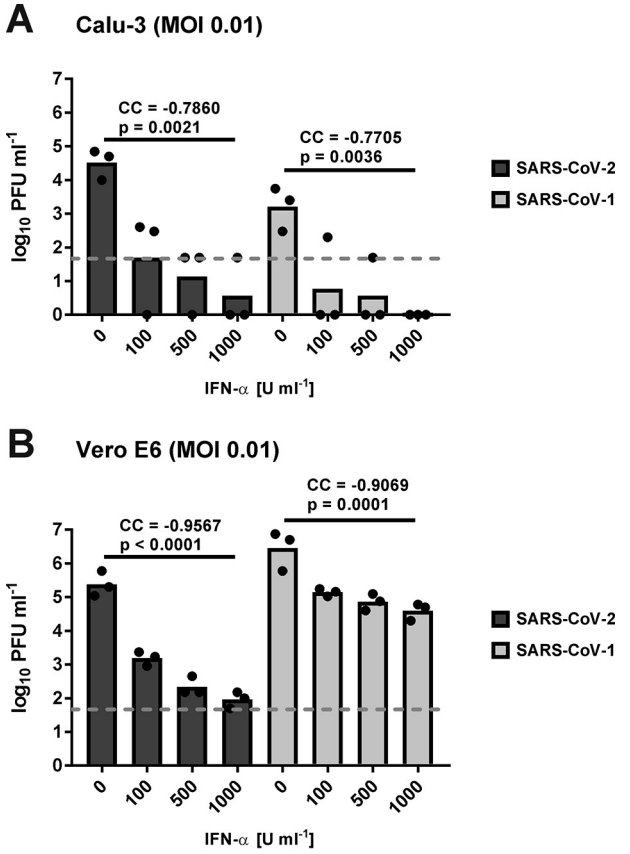

Figure 1. Sensitivity of SARS–CoV-2 and SARS–CoV-1 to type I IFN dose escalation. Calu-3 (*A*) and Vero E6 cells (*B*) were pretreated with recombinant human IFN-α and infected at an MOI of 0.01. Titers were measured at 24 h postinfection by plaque assay. Individual titers (*dots*) and geometric mean values (*bars*) from three biological replicates are shown. Log-transformed titers of each virus dose-response experiment were analyzed by Spearman's exact rank correlation test. CC and exact one-sided *p* values are provided. Note that titer values that were below the plaque assay detection level (50 PFU/ml; indicated by the *dashed line*) were set to 1 PFU/ml.

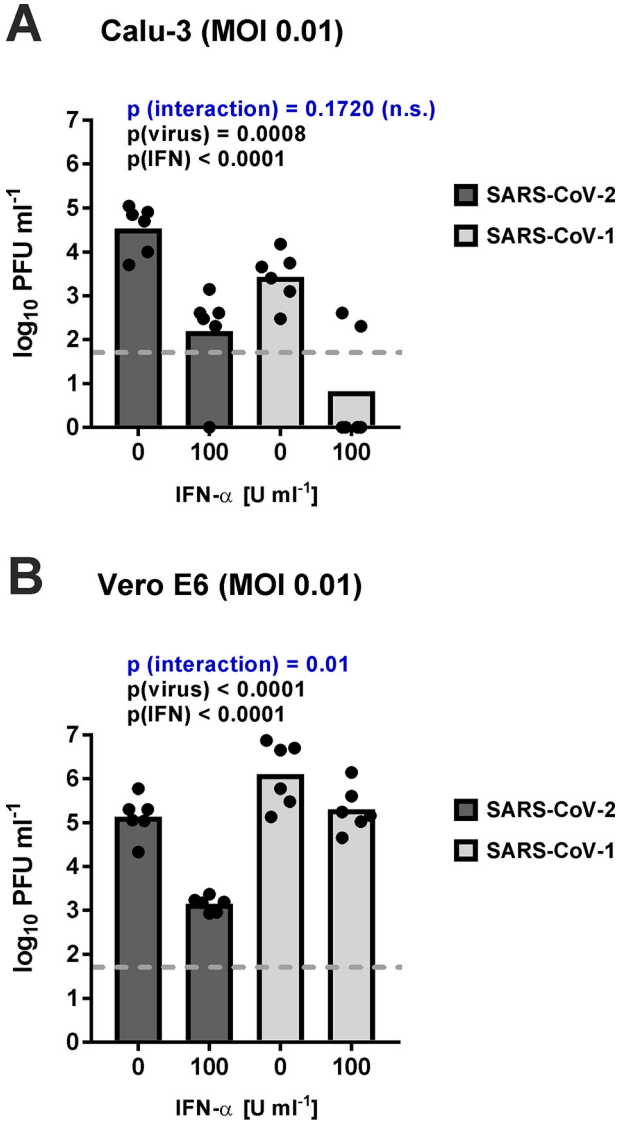

Figure 2. Sensitivity of SARS–CoV-2 and SARS–CoV-1 to intermediate-dose type I IFN. Calu-3 (*A*) and Vero E6 cells (*B*) were pretreated with 100 units/ml IFN-α, infected at an MOI of 0.01, and titrated 24 h later. Log-transformed data were analyzed by two-way ANOVA with factors "IFN" and "virus," for each of which the specific *p* values are indicated. *p (interaction)* designates the probability that IFN sensitivity depends on the virus species. Data points and geometric mean values from six independent experiments are shown. Note that three of the six biological repeats are repeats from Fig. 1.

the predominant antiviral cytokine (4, 5). Although the IFN induction as well as signaling and up-regulation of IFN-stimulated genes (ISGs) are very similar, type III IFNs engage a different receptor that is restricted to epithelial cells, and generate a weaker but longer-lasting antiviral response (5, 17). IFN-λ was previously shown to have activity against coronaviruses (11, 18, 19) and proposed as potential COVID-19 treatment (20). Hence, we compared the sensitivity of the two SARS–coronaviruses also to recombinant human IFN-λ. As shown in Fig. 3*A*, pretreatment with 10 or 100 ng/ml IFN-λ exhibited only in Vero E6 cells a dose-dependent inhibitory effect on SARS–CoV-2. For SARS–CoV-1, by contrast, no significant inhibition was noted in any of the cell lines. To further investigate the difference between the viruses, we repeated the IFN-λ experiment three times more with the intermediate dose of 10 ng/ml and analyzed the data after pooling with the previous 10 ng/ml IFN-λ experiment (Fig. 3*B*). Conventional statistical analysis (one-tailed Student's *t* test, because none of the values was below the detection limit) again revealed a significant impact of IFN-λ on SARS–CoV-2 and the lack of an effect for SARS–CoV-1. Our data thus show that IFN-λ can inhibit SARS–CoV-2 but not SARS–CoV-1.

Blocking JAK/STAT signaling by ruxolitinib

A recent study on the host cell interactome of SARS–CoV-2 identified a number of human proteins for which Food and Drug Administration–approved drugs are available (21). Ruxolitinib, a compound known to target the type I and type III IFN-triggered JAK/STAT signaling pathway (22), was among the proposed inhibitors of virus–host cell interactions (21). Because virus inhibition by an IFN inhibitor seems counterintuitive, we aimed to clarify the influence of this compound on SARS–CoV-2 replication. Cells were pretreated with 1 μM ruxolitinib for 16 h and infected at the two different MOIs, and titers were measured 24 or 48 h later. As shown in Fig. 4, with this setting titers in nontreated controls are already reaching a plateau at the 24-h time point. In Calu-3 cells, ruxolitinib had a

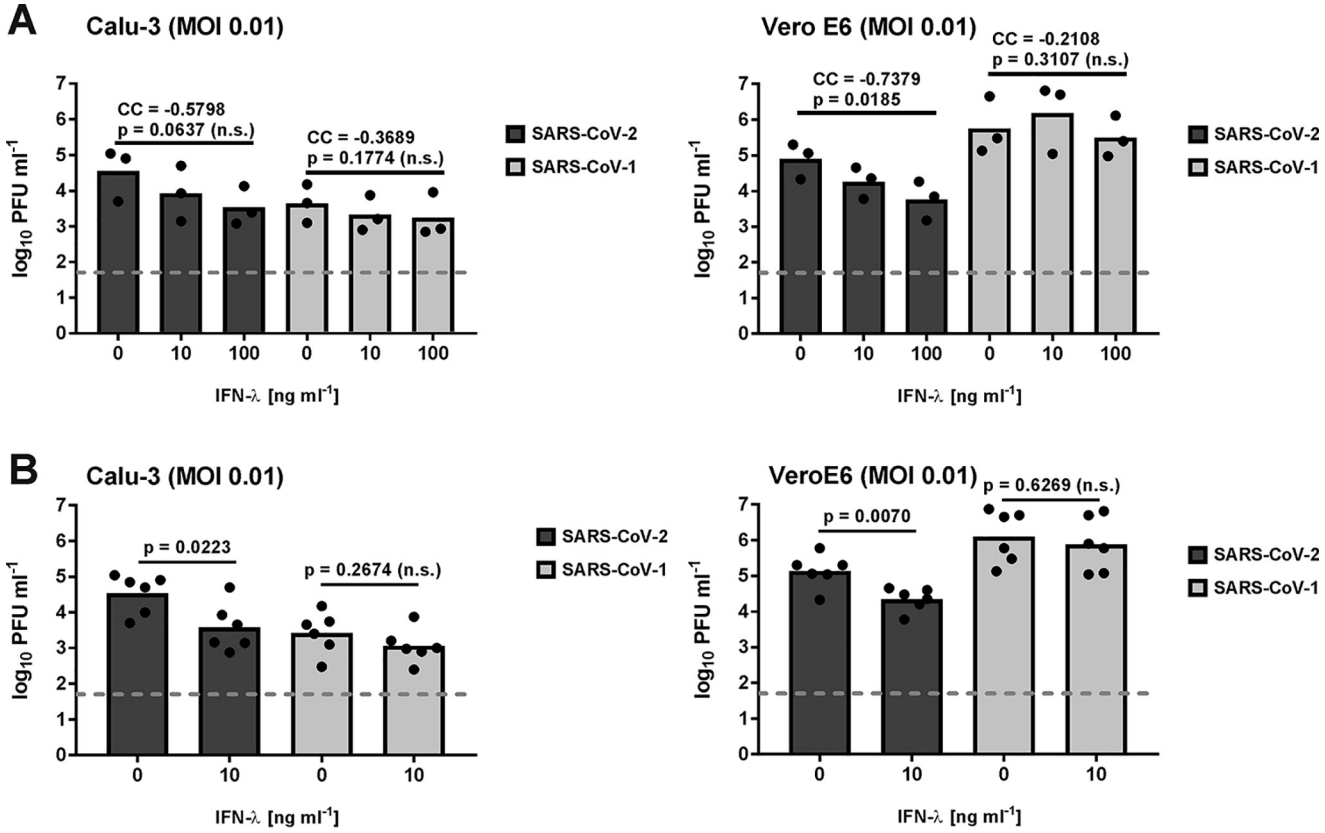

Figure 3. Sensitivity of SARS–CoV-2 and SARS–CoV-1 to type III IFN. *A*, experiments were performed as described for Fig. 1, except that recombinant human IFN-λ was used. Log-transformed titers of each virus dose-response experiment with concentrations of 10 and 100 ng/ml IFN-λ were analyzed by Spearman's exact rank correlation test. CCs and exact one-sided *p* values are provided. *B*, three additional biological replicates of the 10 ng/ml IFN-λ were performed, and the resulting titer data were pooled with the 10 ng/ml IFN-λ data from *A*. Log-transformed titers were analyzed by unpaired one-tailed Student's *t* test. *n.s.*, nonsignificant.

clear boosting effect on SARS–CoV-2 replication, mostly at 48 h postinfection, and at both MOI 0.01 and 0.001 (Fig. 4*A* and Fig. S2*A*). By contrast, in Vero E6 there was neither a positive nor a negative effect discernible (Fig. 4*B* and Fig. S2*B*). Of note, Calu-3 cells are capable of inducing IFN in response to virus infection, whereas Vero cells are not (15). Our data thus indicate that (i) if anything, ruxolitinib is an enhancer rather than an inhibitor of SARS–CoV-2 multiplication, and (ii) the boosting effect is most likely due to inhibition of the antiviral JAK/STAT signaling pathway, because it is not present in the IFN induction–deficient Vero E6 cells.

Comparison of the cell lines

Our observations so far suggest that SARS–CoV-2 is consistently more sensitive to IFNs than SARS–CoV-1. Moreover, type I IFN seems to have a more profound effect than type III IFN. To see whether principal differences in signaling or subsequent gene expression could account for these phenomena, we tested the ability of the cell lines to respond to the IFNs. The immunoblot analysis (Fig. 5) shows that Calu-3 cells have a very similar reaction to both types of IFN concerning phosphorylation of STAT1 and STAT2 and expression of the IFN-stimulated MxA and ISG15. Vero E6 cells also responded to IFN-λ as expected (23), but the ISG response was lower than to IFN-α. Moreover, in nontreated Calu-3 cells, there was already a background ISG expression, which was not observed in Vero cells. Ruxolitinib was in principle able to influence these ISG responses, as expected, but it was more potent against IFN-λ than against IFN-α, and its effects on IFN-stimulated genes were more evident in the Vero E6 compared with the Calu-3 (Fig. 5). Thus, both cell lines are capable to respond to the different types of IFN, but IFN-λ was less potent, which is in agreement with our observations on SARS–CoV sensitivity, as well as with previous studies (5, 17).

Discussion

The recently emerged SARS–CoV-2 is responsible for major health crises all over the world. Here, we show that type I and type III IFNs are able to inhibit SARS–CoV-2 replication, with effects that in our hands were consistently more profound than against the SARS–CoV-1 from 2003. It should be noted however, that the differences between the viruses could be due to the cell types used or due to the observed differences in virus replication (which could result in higher production of IFN antagonists). Thus, the question whether SARS–CoV-2 is intrinsically more sensitive to IFNs remains to be solved.

PEGylated IFN-α was the standard of care against chronic infection with hepatitis C virus until the recent introduction of other, directly acting antiviral drugs (24). Although associated with some side effects, IFN-α is well-characterized, has been used to treat millions of patients, is considered safe, and is available

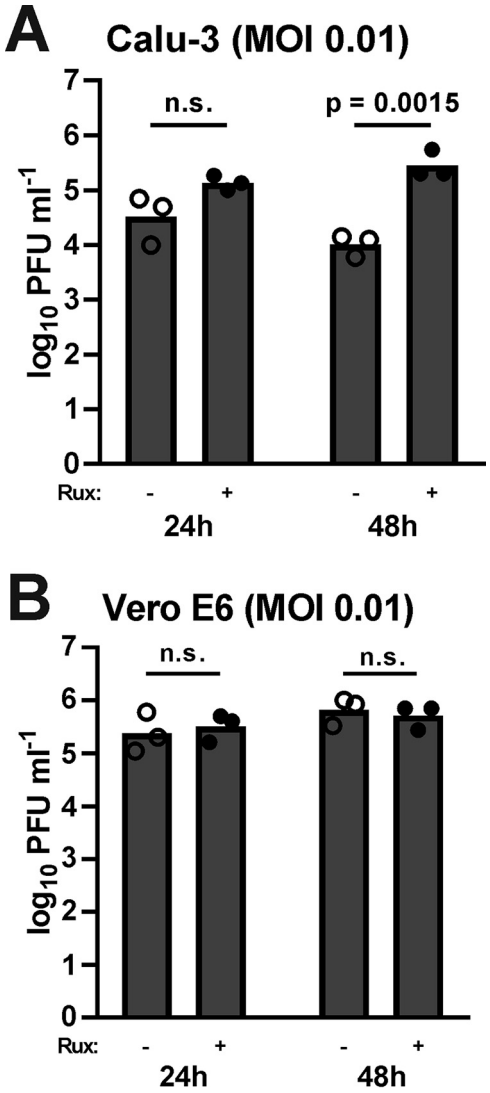

Figure 4. Effect of the JAK/STAT inhibitor ruxolitinib on SARS–CoV-2 replication. Calu-3 (*A*) and Vero E6 (*B*) cells were pretreated with 1 μM ruxolitinib (*Rux*) and infected with SARS–CoV-2 at an MOI of 0.01, and titers were determined at 24 and 48 h postinfection. Individual titers (*dots*) and geometric mean values (*bars*) from three biological replicates are shown. Log-transformed titers were analyzed by unpaired two-tailed Student's *t* test. *n.s.*, nonsignificant.

immediately. IFN-λ has undergone phase I and II clinical trials with hepatitis C virus (25). It exhibited excellent tolerance as well as efficacy, but the phase III trials were abandoned because of the availability of effective direct antivirals. IFN-λ holds promise as having fewer side effects because of its restriction to mucosal tissue and the less sudden but more prolonged antiviral response it triggers (5, 17). In line with our results, a series of preprints show that also others found type I and type III IFNs to be effective against SARS–CoV-2 replication in cell culture (26–28). Also in earlier *in vivo* studies with SARS–CoV-1, both type I and type III IFNs were shown to be important for the control of infection or the associated disease (29–33). Clinical data on the usage of type I IFN against SARS–CoV-1 or the related MERS-CoV, however, are limited, are not always conclusive, or did not show a clear benefit (34–38). Thus, type III IFN-λ rather than the side effect–prone type I IFNs (39) might be considered for clinical testing against SARS–CoV-2.

Ruxolitinib was proposed as a potential treatment against SARS–CoV-2 (21, 40), and a small clinical trial is underway (clinicaltrials.gov, NCT04334044), although case reports were discouraging (41). The replication boost obtained with ruxolitinib on the IFN-competent Calu-3 cells indicates that ruxolitinib is not at all inhibiting SARS–CoV-2 replication. Thus, drugs that interfere with viral host interactors may not necessarily be antiviral but rather boost the infection.

Experimental procedures

Cells and viruses

Calu-3 and Vero E6 cells were cultivated in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (Thermo Fisher Scientific) in a 5% CO_2 atmosphere at 37 °C. SARS–CoV-2 (strain SARS–CoV-2 /München-1.2/2020/984, p.2) (42) and SARS–CoV-1 (strain SARS-FRA1, p.2) (43) were grown on Vero E6 cells and purified via VivaSpin columns (Sartorius Stedim Biotech). The viruses were titrated on Vero E6 cells. Infection experiments were done under biosafety level 3 conditions with enhanced respiratory personal protection equipment. Of note, all cells were tested mycoplasma-negative.

Inhibitor assays

The cells were pretreated for 16 h with the indicated amounts of pan-species IFN-α(B/D) (PBL Assay Science) (44), purified recombinant IFN-λ3 (18, 45), or with 1 μM ruxolitinib (Selleckchem). Infections were performed at a MOI of 0.01 and 0.001. At the indicated times postinfection, cell supernatants were collected and titrated by plaque assay on Vero E6 cells.

Immunoblot analysis

The cells were treated for 24 h with the indicated amounts of IFNs or ruxolitinib (added 1 h before IFN) and lysed in T-PER protein extraction reagent (Thermo Fisher) supplemented containing 1× Protease inhibitor mixture (c0mplete, Roche), 1× phosphatase Inhibitor mixture set II (Calbiochem), and sample buffer (35.8 mM Tris-HCl, pH 6.8, 7.15% glycerol, 1.43% SDS, 1.08 mM bromphenol blue). Protein samples were run on 12% acrylamide gels and transferred to polyvinylidene fluoride membranes (Millipore) via semidry blotting. After blocking in TBS with 5% BSA (for detection of phospho-STATs, MxA, and total STAT2) or milk powder (all other detections), primary antibody staining was performed overnight at 4 °C. The membranes were washed in TBS with 0.1% Tween 20, stained with secondary antibodies for 45 min, and washed again in TBS with 0.1% Tween 20 and once in TBS. Finally, the membranes were developed with a SuperSignal West Femto kit (Pierce), and the bands were visualized using a ChemiDoc imaging system (Bio-Rad).

The primary antibodies used were phospho-STAT1, Tyr^{701} (7649S, Cell Signaling) (1:1000); phospho-STAT2, Tyr^{690} (88410S, Cell Signaling) (1:1000); STAT1 (610186, BD Biosciences) (1:1000); STAT2 (610188, BD Biosciences) (1:1000); ISG15 (sc-166755, Santa Cruz) (1:4000); MXA (MABF938, Sigma–Aldrich) (1:1000); and β-tubulin (ab6046, Abcam) (1:2000). The secondary antibodies used were peroxidase-conjugated goat anti-mouse IgG (31430; Thermo Fisher)

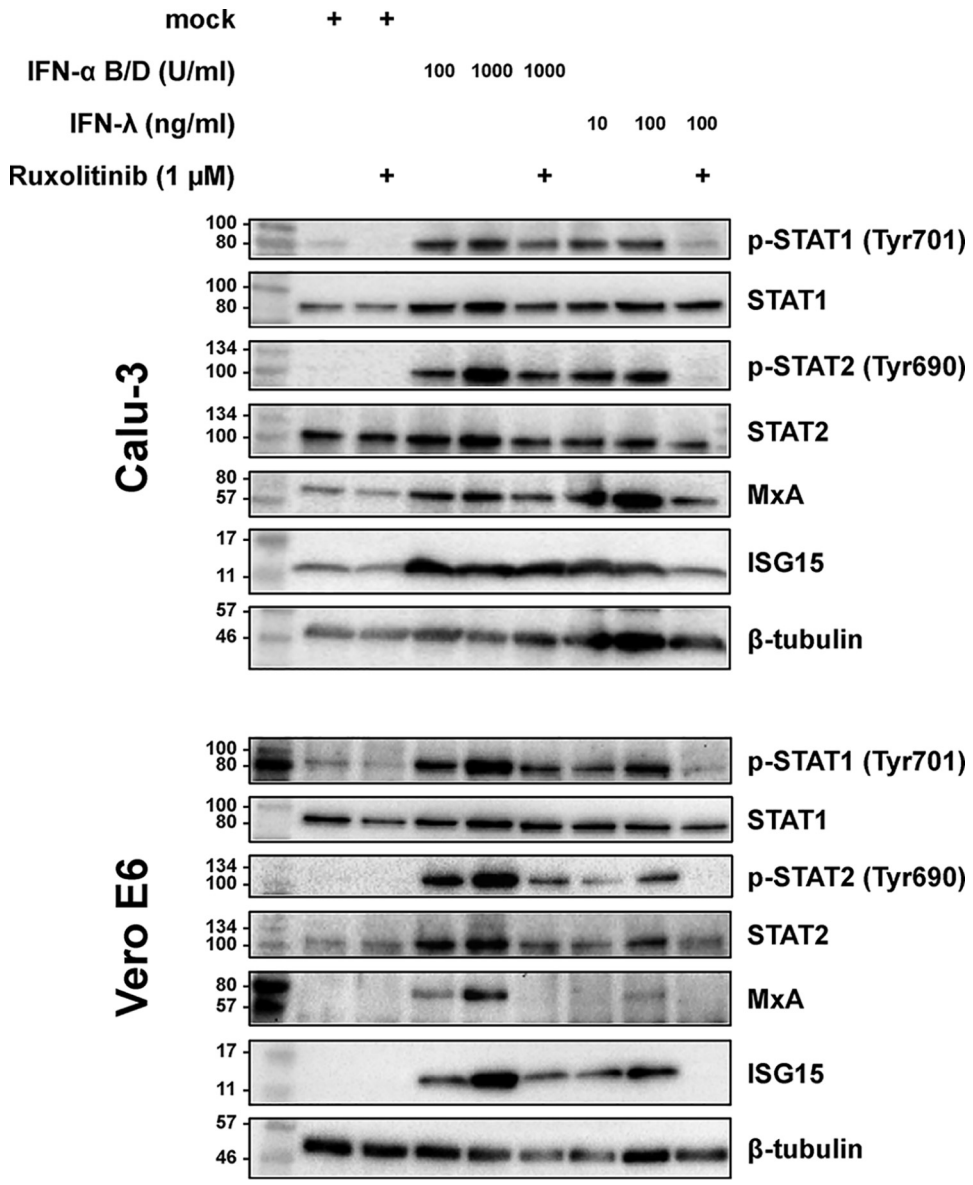

Figure 5. Effect of IFNs and ruxolitinib on Calu-3 and Vero E6 cells. Calu-3 and Vero E6 cells were incubated with the indicated amounts of IFNs and ruxolitinib (added 1 h before IFN) and 24 h later were analyzed for the indicated antigens using immunoblotting. The data are representative of three independent experiments. Molecular markers are shown on the *left sides* of the blots.

(1:10,000) and peroxidase-conjugated goat anti-rabbit IgG (31460; Thermo Fisher) (1:10,000).

Statistical analyses

The statistical analysis of the data were done by means of the statistical program packages BMDP (46) and StatXact® (version 9.0.0). For the statistical testing of the dose-response effect of IFN (type I and III) against SARS–coronaviruses, the typical regression procedures were not applicable because of several values below the detection limit and some ties in the data. Instead of this, the nonparametric Spearman rank CC was used in the exact version (software StatXact). Because the scientific question was clearly one-sided formed (only PFU reduction under application of IFN), one-sided p values were given.

If only two IFN concentrations were to compare with no data below the detection limit, then the t test for independent samples was used (program BMDP3D). For testing the effect of IFN and virus type simultaneously, the two-way ANOVA (program BMDP7D) was applied especially considering a possible interaction between the two tested factors.

In the parametric statistical analyses as well as the graphical representations, the response variable PFU was logarithmically transformed because of its right skewed statistical distribution. In all cases a statistical significance level of $\alpha = 0.05$ was applied.

Data availability

All data presented and discussed are contained within the article.

Author contributions—U. F., A. S., R. H., A. R. S., K. F., C. D., and F. W. conceptualization; U. F., C. D., and F. W. validation; U. F., A. S., A. R. S., and K. F. investigation; U. F. and F. W. visualization;

U. F., A. S., H. H. G., R. H., A. R. S., K. F., C. D., and F. W. methodology; H. H. G., R. H., and F. W. resources; R. H. and F. W. writing-review and editing; C. D. software; C. D. and F. W. supervision; C. D. and F. W. funding acquisition; F. W. formal analysis; F. W. writing-original draft; F. W. project administration.

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Conflict of interest—The authors declare that they have no conflicts of interest with the contents of this article.

Abbreviations—The abbreviations used are: ANOVA, analysis of variance; CC, correlation coefficient; CoV, coronavirus; COVID-19, coronavirus disease 2019; IFN, interferon; JAK, Janus kinase; MERS, Middle East respiratory syndrome; PFU, plaque forming units; Rux, ruxolitinib; SARS, severe acute respiratory syndrome; STAT, signal transducer and activator of transcription; MOI, multiplicity of infection; ISG, IFN-stimulated gene.

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