Differential interferon-α subtype induced immune signatures are associated with suppression of SARS-CoV-2 infection

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Type I interferons (IFN-I) exert pleiotropic biological effects during viral infections, balancing virus control versus immune-mediated pathologies, and have been successfully employed for the treatment of viral diseases. Humans express 12 IFN-alpha (α) subtypes, which activate downstream signaling cascades and result in distinct patterns of immune responses and differential antiviral responses. Inborn errors in IFN-I immunity and the presence of anti-IFN autoantibodies account for very severe courses of COVID-19; therefore, early administration of IFN-I may be protective against life-threatening disease. Here we comprehensively analyzed the antiviral activity of all IFNsα subtypes against severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) to identify the underlying immune signatures and explore their therapeutic potential. Proplyaxis of primary human airway epithelial cells (hAEC) with different IFNsα subtypes during SARS-CoV-2 infection uncovered distinct functional classes with high, intermediate, and low antiviral IFNs. In particular, IFNsα showed superior antiviral activity against SARS-CoV-2 infection in vitro and in SARS-CoV-2-infected mice in vivo. Dose dependency studies further displayed additive effects upon coadministration with the broad antiviral drug remdesivir in cell culture. Transcriptomic analysis of IFN-treated hAEC revealed different transcriptional signatures, uncovering distinct, intersecting, and prototypical genes of individual IFNsα subtypes. Global proteomic analyses systematically assessed the abundance of specific antiviral key effector molecules which are involved in IFN-I signaling pathways, negative regulation of viral processes, and immune effector processes for the potent antiviral IFNsα. Taken together, our data provide a systemic, multimodular definition of antiviral host responses mediated by defined IFN-I. This knowledge will support the development of novel therapeutic approaches against SARS-CoV-2.

Significance

Type I interferons (IFN-I) exhibit various biological effects during viral infections, and they have been successfully used for clinical treatment of viral diseases. Humans express 12 IFNsα subtypes, which strongly differ in their antiviral responses against different viruses. Here we analyzed the antiviral activity of all human IFNsα subtypes against severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) to identify the underlying immune signatures and explore their therapeutic potential. Our data provide a systemic pattern of antiviral host effector responses mediated by high antiviral IFN-I, which could help to identify key cellular effectors targeted in novel therapeutic approaches against SARS-CoV-2 infection.

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Without the capacity to produce or recognize interferons (IFN), mammalian hosts rapidly succumb in the case of viral infections. Accordingly, humans with loss-of-function mutations in the IFN signaling pathway even fail to control attenuated viruses. Therefore, IFNs are indispensable mediators of the first immediate intrinsic cellular defenses against invading pathogens, such as viruses. So far, three different types of IFNs, types I (IFN-I), IFN-II, and IFN-III, have been identified and classified based on their genetic, structural, and functional characteristics as well as receptor usages (1–3). IFN-I is among the first line of antiviral defense due to the ubiquitous expression of the surface receptor IFNAR consisting of two different functional classes with high, intermediate, and low antiviral IFNs. In particular, IFNsα showed superior antiviral activity against SARS-CoV-2 infection in vitro and in SARS-CoV-2-infected mice in vivo. Dose dependency studies further displayed additive effects upon coadministration with the broad antiviral drug remdesivir in cell culture. Transcriptomic analysis of IFN-treated hAEC revealed different transcriptional signatures, uncovering distinct, intersecting, and prototypical genes of individual IFNsα subtypes. Global proteomic analyses systematically assessed the abundance of specific antiviral key effector molecules which are involved in IFN-I signaling pathways, negative regulation of viral processes, and immune effector processes for the potent antiviral IFNsα. Taken together, our data provide a systemic, multimodular definition of antiviral host responses mediated by defined IFN-I. This knowledge will support the development of novel therapeutic approaches against SARS-CoV-2.

SARS-CoV-2 | type I IFNs | immunotherapy | antiviral
subunits, IFNAR1 and IFNAR2. In humans, the IFN-I family comprises IFNβ, IFNε, IFNκ, IFNω, and 12 IFNα subtypes. The latter code for the distinct human IFNα proteins: IFNα1, IFNα2, IFNα4, IFNα5, IFNα6, IFNα7, IFNα8, IFNα10, IFNα14, IFNα16, IFNα17, and IFNα21, encoded by 14 nonallelic genes, including some pseudogenes and two genes that encode identical proteins (IFNα13 and IFNα13*). The overall identity of the IFNα proteins ranges from 75 to 99% amino acid sequence identity (1, 4). Despite their binding to the same cellular receptor, their antiviral and antiproliferative potencies differ considerably (5–10). As a general event in terms of signal transduction, IFNα subtypes engage the IFNAR1/IFNAR2 receptor and initiate a signal transduction cascade resulting in the phosphorylation of receptor-associated Janus tyrosine kinases culminating in downstream signaling events including the activation of IFN-stimulated gene (ISG) factor 3 (ISGF3) consisting of phosphorylated STAT1 and STAT2 and the IFN regulatory factor 9. ISGF3 binding to the IFN-stimulated response elements (ISRE), in promotor regions of various genes, initiates the transcriptional activation of a large number of ISGs, which elicit direct antiviral, antiproliferative, and immunoregulatory properties (11). Why different IFNα proteins exhibit distinct effector functions is largely elusive (5, 6, 12). Different receptor affinities and/or interaction interfaces within the IFNAR have been discussed which may account for the observed variability in the biological activity (13, 14). Furthermore, the dose, the cell type, the timing, and the present cytokine milieu might further affect the IFN effector response (15). In the absence of specific antiviral drugs, treatment of patients with IFN-I is often considered as a first therapeutic response, given its successful therapeutic potential of IFNα subtypes, which closely resemble the in vivo physiology of the respiratory system, and differentiate into various cell types, resulting in cilialized surface barriers, IFNα/β receptor (IFNAR) clusters of high (IFNα8, IFNα10, IFNα16, IFNα17, IFNα21), moderate (IFNα7, IFNα9, and IFNα14), low (IFNα5, IFNα6, IFNα11, IFNα12, and IFNα13) expression concentrations (19 U/mL to 20,000 U/mL) antiviral activities against SARS-CoV-2 replication was assessed by quantification of viral titers (Tissue Culture Infection Dose 50 [TCID50] per milliliter) and viral antigens applying a previously described in-cell enzyme-linked immunosorbent assay (icELISA) (33) (Table 1 and SI Appendix, Fig. S1 A–D). Corroborating previous results, a striking clustering of the antiviral subtypes according to their antiviral potency was observed, which allowed their separation into classes of low (IC50 > 5,000 U/mL), intermediate (IC50 = 2,000 U/mL to 5,000 U/mL) and high (IC50 < 2,000 U/mL) antiviral activities against SARS-CoV-2 (Fig. 1 C–F and SI Appendix, Fig. S1 B–D and Table 1). Since VeroE6 cells are derived from African green monkey, expressing the nonhuman primate instead of human IFN receptor, and also lack the capacity to produce IFN-I in a natural feed-forward loop (34), we further analyzed genuine target cells of SARS-CoV-2. We utilized well-differentiated primary human airway epithelial cells (hAEC), which closely resemble the in vivo physiology of the respiratory system, and differentiate into various cell types, resulting in ciliary movement and production of mucus (35, 36). After IFN pretreatment and subsequent infection with SARS-CoV-2, apical washes were monitored concerning viral replication kinetics at 33 °C (37). Cells were lysed at 72 h postinfection (p.i.), and viral progeny (Fig. 1 G and H) as well as viral M and N gene expression (SI Appendix, Fig. S1 E–G) were determined. Again, a distinct antiviral pattern became evident (Fig. 1G) defining IFN clusters of high (IFNα5, IFNα4, IFNα14, and IFNα3), moderate (IFNα17, IFNα2, IFNα7, and IFNα21) and low antiviral activities (IFNα10, IFNα16, IFNα6, and IFNα1) (Fig. 1 H and SI Appendix, Fig. S1 G and J). Prototypical ISG expression patterns, as analyzed by qRT-PCR, revealed subtype-specific gene expression signatures (SI Appendix, Fig. S2B). In order to address whether the observed antiviral activities were SARS-CoV-2 specific, we additionally tested IAV (IAV/PR8) in hAECs. Interestingly, pretreatment of hAECs with the IFN subtypes revealed differences compared to SARS-CoV-2. In general, antiviral responses could be clustered into strong
antiviral activities for IFNα2, IFNα4, IFNα5, IFNα8, IFNα14, and IFNλ3 (Fig. 1I) and weak antiviral activities for IFNα1, IFNα6, IFNα7, IFNα10, IFNα16, IFNα17, and IFNα21 (Fig. 1J). Among the strong antiviral responses, we observed additional transient differences at 48 h p.i., with IFNα2, IFNα4, IFNα5, and IFNα14 being slightly superior to IFNα8 and IFNλ3 (Fig. 1J). These results clearly demonstrate that different IFNα subtypes mediate distinct biological and temporal activities.

**IFN Subtype–Specific Gene Expression Signatures.** Since we observed clear differences in the biological activities of different IFNα subtypes against SARS-CoV-2, we next aimed to identify their underlying immune signatures and mechanisms. To this end, primary hAECs were pretreated with the respective IFNs, and, 16 h poststimulation, cellular RNA was sequenced on an Illumina NovaSeq 6000, and differentially expressed genes (DEGs) were sent to Ingenuity Pathway Analysis (IPA; Qiagen) for biological analysis. In order to investigate cellular responses following viral infection, we included SARS-CoV-2–infected hAECs (18 h p.i.) in our analysis. Global transcriptomic analysis revealed unique DEGs, both up- and down-regulated upon IFN treatment (38, 39) for each IFN (SI Appendix, Fig. S3A) compared to mock-treated cells. Similar to the observed antiviral effects, a general clustering was apparent which showed similar expression patterns for low to intermediate antiviral subtypes (IFNα1, IFNα6, IFNα7, IFNα10, and IFNα21) and intermediate to high antiviral subtypes (IFNα2, IFNα17, IFNα14, IFNα4, IFNα5, and IFNλ3). Interestingly, we observed differential interferon-α subtype induced immune signatures are associated with suppression of SARS-CoV-2 infection.
a clear difference in the numbers of significantly up- and down-regulated genes after treatment with IFNα subtypes compared to mock-treated cells, which positively correlated with antiviral activity (SI Appendix, Fig. S3B). Gene ontology (GO) pathway analysis revealed higher expression of genes mostly involved in antiviral immune response among the medium and high antiviral subtypes, as well as pathways which can be associated with protein localization, translation, oxidative phosphorylation, RNA metabolism, endoplasmic reticulum (ER) stress, signaling pathways, and lymphocyte activation (Fig. 2A). Strikingly, different IFNα subtypes displayed unique GO patterns, with IFNα17, in contrast to other subtypes, regulating genes involved in translation, whereas the treatment with IFNα5 resulted in the strongest regulation of genes associated with signaling pathways and lymphocyte activation among all IFNs (Fig. 2B). We next focused on genes associated with antiviral responses (Fig. 2B). A separation based on antiviral activity could be discerned with weak antiviral IFNα subtypes (IFNα1, IFNα6, IFNα16, and IFNα10) exhibiting comparatively lower expression values of specific ISGs, whereas medium to strong antiviral IFNα subtypes induced higher expression (Fig. 2B). We observed two clusters that differed between low and intermediate to high IFNα subtypes, with ISG15, IFI27, MX1, and others showing generally lower expression values in the low antiviral IFN subtypes. Even more pronounced were expression changes of IFIT2, IFIT1, MX2, and others which resulted in a down-regulation for the low and an up-regulation for the intermediate to high antiviral IFN subtypes. As we aimed at identifying immune signatures that correlate with the antiviral activity against SARS-CoV-2 infection, we next evaluated DEGs with respect to distinct, intersecting, and common genes among and between subtypes (SI Appendix, Fig. S4A). We identified several DEGs for each subtype, with IFNα5 expressing the most unique genes (1,018 DEGs), followed by IFNα3 (670 DEGs) (Fig. 2C and SI Appendix, Fig. S4B). A comparison between high, medium, and low antiviral subtypes revealed that 19 genes were commonly differentially expressed among all subtypes, including MX1 and OAS2 (Fig. 2D). The most striking differences could be observed for MX1 and OAS2, whose expression levels clearly separated high, intermediate, and low antiviral IFN subtypes (Fig. 2D). Interestingly, a comparison between high antiviral IFNs (IFNα4, IFNα5, IFNα14, and IFNα3) and all other IFNα subtypes identified 42 distinct DEGs that were exclusively up- or down-regulated in the high antiviral group, including RNaseL and genes associated with regulation of transcription, signal transduction, and metabolic processes, as well as long noncoding (lnc) RNAs (Fig. 2E). In conclusion, we could clearly demonstrate IFN subtype–specific immune signatures that could contribute to the observed differences in antiviral activity.

Proteomic Analysis Highlights Key Cellular Factors. Our transcriptional analysis revealed IFNα subtype–specific distinct, intersecting, and common expression patterns of DEGs that most likely contribute to the differential biological activity against SARS-CoV-2. To further uncover relevant cellular effector proteins for the antiviral activity against SARS-CoV-2, we additionally performed proteomic analysis on hAECs pretreated with IFNs. Since we had observed the strongest antiviral activity for IFNα5 and IFNα3, we decided to further investigate their specific proteomic profile in direct comparison with IFNα7, which exhibited a moderate antiviral effect, and IFNα16, displaying a weak effect against SARS-CoV-2 infection, in order to identify key antiviral pathways, crucial in controlling coronavirus infection. To this end, primary hAECs were pretreated with selected IFNs for 16 h. In addition to the early time point (t = 0 h), where we aim to identify key cellular factors that are expressed before viral infection, we included a late time point, 72 h post-treatment both in the presence (t = 72 h [CoV-2]) and absence of viral infection (t = 72 h [mock]), to investigate potential antiviral mechanisms and potential intervention by viral effectors (SI Appendix, Fig. S5A). Principal component analysis revealed a clustering according to donor and/or infection and time points (SI Appendix, Fig. S5 B–D). In addition to host cell proteins, various viral peptides were identified, which correlate to viral titers depending on the respective donor (SI Appendix, Table S1 and Fig. S5E). For all donors, no SARS-CoV-2 peptides could be detected following treatment with IFNα5 and IFNα3. Pretreatment of cells with IFNα subtypes resulted in up- or down-regulation of a variety of proteins compared to untreated hAECs, depending on the IFN stimulation (SI Appendix, Fig. S6 A–C). In order to perform statistical analysis, we considered proteins that were measured in a minimum of three of four donors; however, on/off analysis (defined as full absence of a protein in one group of a pairwise comparison) revealed additional proteins which might be of interest (SI Appendix, Fig. S6 D–F and Table S2). GO analysis of proteins differentially abundant between untreated and IFN-treated samples at each time point (untreated vs. IFN) identified enrichment of antiviral immune responses for all IFNs except IFNα16 (Fig. S4 and Fig. S7A). For IFNα16, only proteins associated with lymphocyte regulation were induced, which likely do not contribute to SARS-CoV-2 restriction in cell culture but may be very important in vivo. At 72 h, pathways belonging to proteolysis, metabolism, and protein localization were additionally enriched after treatment with IFNα5 and IFNα3. The most prominent up-regulated proteins, associated with IFN signaling (STAT1, MX1, ISG15, ISG20, IFI35, and others), were found to be on/off regulated and present only upon treatment with IFNα5, IFNα7, and IFNα3. Additional ISGs, including IFIT3, OAS2, and IFITM3, were on/off regulated after 72 h and SARS-CoV-2 infection, except for IFNα16 treatment (Fig. 3B and SI Appendix, Fig. S7B). Interestingly, the comparison of samples in the presence or absence of SARS-CoV-2 (mock vs. CoV-2) showed a striking trend toward down-regulation of proteins upon SARS-CoV-2 infection. Enrichment of biological processes associated with complement activation and O-glycan processing (Fig. 3C) highlighted various complement factors (e.g., CFB, C4B, and C3) as well as various mucines (e.g., MUC1 and MUC16) by SARS-CoV-2, independent of IFN treatment and resulting viral titers (Fig. 3D and SI Appendix, Fig. S7 C and E and Table S3). In contrast, the strongest biological effects on antiviral immune responses after treatment with IFNα5 and IFNα3, for example, IFN signaling as well as antigen presentation, nuclear factor κB signaling, or lymphocyte regulation, were not affected by viral infection. Interestingly, proteins belonging to other pathways, for example, antigen presentation by major histocompatibility complex (MHC) class I or proteolysis, seemed to be less abundantly represented under

Table 1. IC50 values of IFNα subtypes on VeroE6 cells obtained from endpoint dilution assay

| IFNα subtype | IC50 (U/mL) |
|--------------|-------------|
| IFNα4        | 56.91       |
| IFNα14       | 70.73       |
| IFNα5        | 79.73       |
| IFNα8        | 327.0       |
| IFNα2        | 1,026       |
| IFNα7        | 2,431       |
| IFNα21       | 4,944       |
| IFNα16       | >5,000      |
| IFNα1        | >5,000      |
| IFNα17       | >5,000      |
| IFNα6        | >5,000      |
| IFNα10       | >5,000      |
viral infection in the IFNa5-treated samples, a phenomenon which was not as prominent after treatment with IFNα3 (Fig. 3E and SI Appendix, Fig. S7D). STRING (Search Tool for the Retrieval of Interacting Genes/Proteins) analysis (Fig. 3F) highlighted the presence of antiviral key effector molecules (e.g., ISG20, ISG15, IFI44L, IFI2, IFIT3, IFI35, PML, and SP100), which are involved in IFN-I signaling pathways, negative regulation of viral processes, and immune effector processes among the most potent antiviral IFNs. In conclusion, we identified a variety of antiviral cellular effector molecules that correlate with antiviral activity and control of coronavirus infection.

**Therapeutic Potential of IFNα Subtypes.** Currently, there are only a few approved specific antiviral drugs (e.g., monoclonal antibodies) (40, 41) for the treatment of COVID-19, which severely limit treatment options during severe clinical courses. Remdesivir, a nucleotide-analogous RNA-dependent RNA polymerase inhibitor originally developed as antiviral against Ebola virus, received an emergency use approval against COVID-19 and has been employed in clinics. Unfortunately, due to lack of evidence for recovery of critically ill patients, it is no longer recommended by the World Health Organization as a single treatment for COVID-19 (42). Therefore, alternative therapeutic approaches such as combination therapies are urgently needed. As we have observed the strongest antiviral effect in this study for IFNa5, we explored its therapeutic potential in comparison and in combination with remdesivir. Additionally, we included IFNa2 as the clinically approved IFNα in this analysis. In regard to patients viewed as an entity, prophylactic treatment with IFNs is not a clinical option. Nevertheless, a treatment initiated following diagnosis can still “prophylactically” condition and protect cells in the body against later infection events. To monitor the kinetics of the antiviral activity of IFNα subtypes, we treated cells either before infection (“pre”) or up to 8 h p.i. (“post”) and studied the antiviral activity by determining viral titers as TCID₅₀ per milliliter and viral antigens by in-cell ELISA (iELISA) (Fig. 4A and B). As expected, the strongest reduction in viral titers was observed upon pretreatment with IFNa5 as cells become alerted toward an antiviral state, and antiviral effectors can be transcribed or even translated prior to viral infection (Fig. 4B). Intriguingly, even after
Proteomic analysis highlights key cellular mediators. Proteomic analysis of IFN-treated (1,000 U/mL or 1,000 ng/mL) and/or SARS-CoV-2-infected hAECs. (A) Biological processes induced by IFNs 16 h posttreatment (t = 0 h) or 88 h posttreatment (t = 72 h). (B) Volcano plots of IFN-treated hAECs at different time points posttreatment. Detected ISGs are colored yellow. (C) Biological processes induced by IFNs 88 h posttreatment in the presence of SARS-CoV-2 (t = 72 h): mRNA, messenger RNA; ncRNA, noncoding RNA. (D) Volcano plots of IFN-treated SARS-CoV-2-infected hAEC. Detected proteins are colored due to their biological function: red, complement activation; green, O-glycan processing. (E) Heatmaps of differentially activated biological processes by highly antiviral IFNα5 and IFNλ3 compared to untreated controls at different time points posttreatment in the presence and absence of SARS-CoV-2. (F) STRING analysis of proteins increased in IFN-treated and/or SARS-CoV-2–infected hAECs and identified abundant protein–protein interactions. Proteins are shown as circles and colors indicating biological processes. In A–F, n = 4.
viral infection was established, treatment with IFNα5 was able to significantly reduce viral titers (Fig. 4B), which was also observed with the antiviral drug remdesivir (SI Appendix, Fig. S8A). Given the clear antiviral but incomplete inhibitory effect of both treatment modalities, we next studied a potential beneficial effect of IFNα5 or IFNα2 when coadministered with remdesivir (scheme in Fig. 4A). To this end, we analyzed the antiviral effect upon pretreatment as well as posttreatment of an established infection. To quantify the interaction between the antiviral drugs, the observed combination response was compared to the expected effect, using the Loewe additivity model, with δ-scores above 10 indicating synergistic effects. Combination therapies in VeroE6 cells revealed an additive antiviral activity, with over 90% viral inhibition upon pretreatment in the highest concentrations of both combinations tested and a Loewe synergistic score of 8.504 and 4.801 for IFNα5 and IFNα2, respectively (Fig. 4 C and E), without any cytotoxicity (SI Appendix, Fig. SSB). Likewise, posttreatment in combination with remdesivir resulted in a dose-dependent, additive viral inhibition with over 70% (Fig. 4 D and F) for both IFNα subtypes. To confirm these findings, we analyzed selected combinations of IFNα5 with remdesivir postinfection in hAEC. For this, we combined low doses (0.313 μM remdesivir, 0.2444 μM IFNα5), medium doses (0.63 μM remdesivir, 15.625 μM IFNα5), and high doses (2.5 μM remdesivir, 1.953 μM IFNα5), and observed, in all combinations, an additive therapeutic effect when coadministered 8 h p.i. (Fig. 4 G–I). To further strengthen the therapeutic potential of high antiviral IFNα subtypes, we performed therapeutic treatments with IFNα2 and IFNα5 in Rag2−/−CD47−/− triple-knockout (TKO) mice, which received human fetal lung transplants (humanized lung-only mice [LoM]). Treatment for 4 d with the highly antiviral IFNα5, starting 2 h postchallenge with SARS-CoV-2, significantly reduced viral titers in human lung organoids (Fig. 4J). Taken together, we provide evidence that coadministration of direct antiviral drugs together with potent IFNα subtypes clearly impaired viral replication and might provide an alternative therapeutic approach.

Discussion
IFN-I serve as one of the first lines of defense and are induced almost immediately upon viral encounters. IFN-I foster intrinsic immunity, stimulate innate immunity, and recruit and orchestrate adaptive immunity. They can modulate the immune system in several ways, by exerting a wide range of biological activities including antiviral, antiproliferative, immunomodulatory, and regulatory activities. Importantly, impaired IFN-I activity are correlated with severe courses of COVID-19, highlighting their clinical importance (43). Accordingly, defectsiveness to IFN-I significantly contributes to disease severity, and genetic polymorphisms decreasing IFN-I production are associated with more severe cases of COVID-19 (44–46). Furthermore, pegylated IFNα2a therapy in patients with inborn errors of IFN-I immunity prevented severe COVID-19 disease (47). In addition to the impaired IFN-I response triggered by SARS-CoV-2, recent studies have demonstrated the development of autoantibodies that can neutralize IFN-I (45, 48). To evade the antiviral effects of IFN-I, viruses have evolved various strategies to suppress IFN induction. SARS-CoV-2 codes for several proteins that have been implicated in IFN-I antagonism, thereby compromising host responses and favoring viral replication (49). Thus, early administration of IFN-I might be an effective treatment option for COVID-19 patients. The IFN-I family consists of multiple IFNα subtypes, which are highly conserved, and they all signal through the same ubiquitously expressed IFNAR1/IFNAR2. Activation of various downstream signaling cascades implicates that the IFNα subtypes share some overlapping functions, but also possess unique properties. Upon pretreatment of cells with 12 distinct IFNα subtypes, we observed cluster-specific antiviral patterns which were distinct between different viruses. These differential antiviral functions cannot be explained solely by the binding affinity to both receptor subunits, as IFNα5 and IFNα6 exhibit a median affinity to IFNAR1 and IFNAR2 in the range of 0.94 μM to 3 μM and 2.1 μM to 3.8 μM, respectively (15). Furthermore, pegylated IFNα2 with the highest binding affinity to IFNAR2 (IFNα10, IFNα17, IFNα6, IFNα14, and IFNα7) did not induce significantly higher numbers of DEGs. In IFN-treated gut biopsies of chronically HIV-infected patients, the numbers of induced genes by different IFN-I (IFNα1, IFNα2, IFNα5, IFNα8, IFNα14, and IFNβ) were not associated with binding affinity or ISRE activation (12). Importantly, it has been shown that the different IFN-I induced a specific pattern of genes, which are involved in various biological processes (12). Furthermore, these distinct IFN-induced ISG expression patterns clearly differ between subtypes in different cell types as well as in response to different viruses, indicating qualitative differences in IFNα subtype targeted antiviral responses (5, 12, 50). We observed distinct antiviral patterns, that could be clearly clustered into high, intermediate, and low antiviral effects against SARS-CoV-2. Interestingly, we identified 19 genes that were common between all groups, indicative of a basal IFN response. On top of that basal response, we identified several genes that were distinct, intersecting, or commonly differentially regulated between the high and/or medium group. Our dataset enabled us to identify expression patterns that can be correlated with antiviral activity against SARS-CoV-2. Foremost, antiviral immune responses were significantly dysregulated in the moderate and high antiviral groups. Nevertheless, several biological processes, for example, such as associated with protein localization, translation, or ER stress, displayed variable induction patterns depending on the IFNα subtype. Proteomic analysis confirmed expression of IFN effector molecules in high and moderate antiviral subtypes. We mostly identified factors involved in IFN-I signaling pathways, negative regulation of viral processes, and immune effector processes. These results clearly demonstrate unique and overarching properties of different IFNα subtypes. Another group recently reported that saturated concentrations (1,000 pg/mL) of IFNα5 and IFNα2, respectively, significantly reduced viral titers in human lung organoids (Fig. 4F). Taken together, we provide evidence that coadministration of direct antiviral drugs together with potent IFNα subtypes clearly impaired viral replication and might provide an alternative therapeutic approach.
arbidol or IFNβ-1b showed faster recovery from SARS-CoV-2 infection and decreased levels of inflammatory cytokines (21, 22). Furthermore, prophylactic intranasal application of IFNα2a/IFNα2b in healthcare workers in China completely prevented new SARS-CoV-2 infections (53). A recent report from SARS-CoV-2 infection in golden hamsters demonstrated a systemic inflammation in distal organs like brain or intestine (54). The authors hypothesized that virus-derived molecular patterns and not infectious SARS-CoV-2 were disseminated to the periphery, leading to systemic inflammation and increased IFN signatures.

Fig. 4. Therapeutic potential of highly antiviral IFNα subtypes. (A) Schematic depiction of treatment. (B) Pretreatments and posttreatments with IFNα5 of VeroE6 cells by icELISA (gray bars) and TCID₅₀ assay (white bars). Each data point represents a biological replicate measured at an optical density at 450 nm (OD₄₅₀). (C) Inhibition of SARS-CoV-2 infection by IFNα5 and analysis of drug combination experiments using SynergyFinder web application 8 h p.i. (Pre-Treatment). (D) Inhibition of SARS-CoV-2 infection and analysis of drug combination experiments using SynergyFinder web application 16 h before infection (Pre-Treatment). (E) Inhibition of SARS-CoV-2 infection by clinically approved IFNα2 and analysis of drug combination experiments using SynergyFinder web application 16 h before infection (Pre-Treatment). (F) Inhibition of SARS-CoV-2 infection and analysis of drug combination experiments using SynergyFinder web application 8 h p.i. (Post-Treatment). (G–I) Remdesivir and IFNα5 combinational treatment 8 h p.i. of hAECs with low doses (0.313 µM remdesivir, 0.2444 U/mL IFNα5; G), medium doses (0.63 µM remdesivir, 15.625 U/mL IFNα5; H) and high doses (2.5 µM remdesivir, 1.953 U/mL IFNα5; I). (J) Therapeutic effect of IFNα5 and IFNα2 in SARS-CoV-2-infected LoM. In B–I, n = 3. In J, n = 9. *P < 0.05; **P < 0.01.
These observations might further highlight the need to apply IFN-I via intranasal route or inhalation, as the IFN response in the periphery is already highly stimulated, and systemic administration would not further increase the antiviral host immune response. We clearly demonstrated the additive benefit of combining treatment of IFN-I with a direct acting antiviral, for example, remdesivir, as well as a direct therapeutic effect of high antiviral IFNα in humanized LoM. Taken together, most of the data so far support the administration of IFN-I early during infection to curb viral infection and lessen disease severity. Next to involvement of various cellular pathways, both on the transcriptomic and the proteomic levels, we identified unique signatures in primary hAEC after infection with SARS-CoV-2. Strikingly, despite reduced viral replication in the presence of highly antiviral IFNα subtypes, infection with SARS-CoV-2 resulted in a down-regulation of O-glycan processing. Mucus plays a vital role in protecting the respiratory tract from various factors, and serves as first line of defense against invading pathogens. Goblet cells secrete soluble mucus whose major components are heavily O-glycosylated mucin glycoproteins (55). Inflammatory conditions result in an increase of soluble and transmembrane mucins, and alteration of their glycosylation to boost mucosal defense (56, 57). Therefore, it is striking that we observed a consistent down-regulation of various mucins upon SARS-CoV-2 infection. Some recent studies have highlighted the highest level of expression of ACE2 and TMPRSS2, entry factors utilized by SARS-CoV-2, in the nasal goblet and ciliated cells in healthy individuals, cells which are also associated with high MUC1 and MUC5A expression levels (58, 59). Therefore, it is likely that these cells represent the initial infection route for the virus. It is tempting to speculate that virus infection of these cells triggers mucin down-regulation in order to impede cellular defense mechanisms. Interestingly, a significant proportion of COVID-19 patients present with dry cough, indicating that down-regulation of mucins could contribute to this clinical characteristic. In contrast, a recent study has described elevated MUC1 and MUC5AC protein levels in airway mucus of critically ill COVID-19 patients (60). However, the authors speculated that elevated mucin levels could originate from detached and disrupted epithelial cells. It will be interesting to further analyze the role of mucins and their glycans during the initial infection route for the virus. It is tempting to speculate that virus infection of these cells triggers mucin down-regulation in order to impede cellular defense mechanisms. Interestingly, a significant proportion of COVID-19 patients present with dry cough, indicating that down-regulation of mucins could contribute to this clinical characteristic. In contrast, a recent study has described elevated MUC1 and MUC5AC protein levels in airway mucus of critically ill COVID-19 patients (60). However, the authors speculated that elevated mucin levels could originate from detached and disrupted epithelial cells. It will be interesting to further analyze the role of mucins and their glycans during COVID-19 pathogenesis and study the influence of viral replication on mucin expression. In conclusion, in this study, we provide a global characterization of the antiviral response of different IFNα subtypes on various levels and uncover immune signatures which are able to significantly reduce SARS-CoV-2 infection, as well as identifying unique features after virus infection of primary cell types. Our study contributes to an enhanced understanding of the molecular landscape controlling SARS-CoV-2 infection and could thereby pave the way toward novel therapeutic approaches upon identification of key cellular pathways and factors involved in SARS-CoV-2 infection.

**Methods**

**Generation of Infectious SARS-CoV-2 Stocks.** The SARS-CoV-2 strain used in this study was isolated from patient material as described previously (61). For propagation, 2 × 10⁸ VeroE6 cells were seeded in a 75 flask and maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS), L-glutamine, penicillin, and streptomycin. The next day, the cells were infected with SARS-CoV-2 stock and, after 3 d of incubation, the supernatant was harvested, and cell debris was removed by centrifugation. Aliquots of the supernatant were prepared and stored at −80 °C. Viral titers were determined by performing an end-point dilution assay or a plaque assay in order to calculate the TCID₅₀ or the plaque-forming units (PFU), respectively. Virus stock was sequenced and assigned to B.1.1.10 according to the Pangolin database (62), accession number EPI_ISL_602518.

**Stimulation with Different Human IFNα Subtypes.** IFNα subtypes were produced and purified as previously described (63). Briefly, recombinant IFNs were expressed in Escherichia coli after M13 phage transduction. To harvest the proteins, the bacteria were pelleted, and the protein-containing inclusion bodies were denatured by sonication, dissolved in 6 M guanidine-hydrochloride, and refolded in arginine. The recombinant proteins were further purified by ion exchange chromatography and size exclusion chromatography; specificity and purity of the proteins were verified after each step via a sodium dodecyl sulfate (SDS) gel. By phase separation of the products with Triton X-114, the remaining endotoxin was removed from the solution. Endotoxin levels were tested using ToxinSensor (GenScript) and are below 0.25 EU/mL. The activity of each subtype was determined using the human ISRE-Luc reporter cell line, a retinal pigment epithelial cell line transfected with a plasmid containing the Firefly Luciferase gene, stably integrated under control of the ISRE. Following stimulation with IFNα, chemiluminescence can be detected and used to calculate the respective activity in units against commercially available IFNα (PBL Assay Science) (7). Protein concentrations (in milligrams per milliliter) and specific activities (in units per milliliter) are shown in Table 2.

**End-Point Dilution Assay.** VeroE6 cells were seeded at a density of 10,000 cells per well in a 96-well plate and maintained in 200 μL of DMEM supplemented with 10% FBS, L-glutamine, and penicillin and streptomycin overnight. The next day, 22 μL of virus stock or apical washes of hAEC were added to the first row of the plate (six replicates). Then, the virus was diluted 1:10 by mixing the media and pipetting 22 μL to the next row repeatedly, followed by 72 h incubation in 37 °C in a 5% CO₂ atmosphere. Thereafter, the supernatant was aspirated, and the cells were washed with 100 μL of crystal violet solution (0.1% crystal violet [Roth] in phosphate-buffered saline [PBS], 10% ethanol, 0.37% formalin) for 5 min. Subsequently, the crystal violet solution was aspirated, cells were washed with PBS, and the number of wells with intact or damaged cell layer was determined. The TCID₅₀ per milliliter was calculated by the Spearman & Karber algorithm.

**IFN Titration Assay.** VeroE6 cells were seeded at a density of 10,000 cells per well in a 96-well plate and maintained in DMEM supplemented with 10% FBS, L-glutamine, penicillin, and streptomycin overnight. Then, the medium was aspirated, and serially diluted IFNα subtypes and IFNα3 (R&D Systems) and virus with a final concentration of 350 PFU/mL were added to the cells in a total volume of 100 μL of cell culture media, followed by 72 h incubation in 37 °C in a 5% CO₂ atmosphere. Thereafter, the supernatant was aspirated, and the cells were stained with 100 μL of crystal violet solution (0.1% crystal violet in PBS, 10% ethanol, 0.37% formalin) for 5 min. Subsequently, the crystal violet solution was aspirated, cells were washed with PBS, and the number of wells with intact or damaged cell layer was determined. The IC₅₀ was calculated using GraphPad Prism 6.

**icELISA.** The icELISA was performed based on the previously published protocol (33). Briefly, VeroE6 cells were seeded at a density of 20,000 cells per well in a 96-well plate and maintained in DMEM supplemented with 10% FBS, L-glutamine, penicillin, and streptomycin overnight. At indicated time points, the medium was aspirated, and serially diluted IFNα subtypes and IFNα3 (R&D Systems) and virus with a final concentration of 350 PFU/mL were added to the cells in a total volume of 100 μL of cell culture media, followed by 24 h incubation in 37 °C in a 5% CO₂ atmosphere. Thereafter, 100 μL of 8% ROTI-Histofix (Roth) (equals 4% of total paraformaldehyde [PFA]) was added for a minimum of 2 h at room temperature to fix the cells and inactivate the virus.

**Table 2. Concentration and specific activity of IFNα preparations used in this study.**

| Human IFNα | Nanodrop 2000 Spectrophotometer, concentration (mg/mL) | RPE-ISRE-Luc reporter cell, specific activity (U/mg) |
|------------|-------------------------------------------------------|--------------------------------------------------|
| 1          | 0.21                                                  | 3.24 × 10⁵                                    |
| 2          | 1.11                                                  | 7.06 × 10⁵                                    |
| 4          | 0.21                                                  | 4.20 × 10⁵                                    |
| 5          | 1.79                                                  | 1.12 × 10⁶                                    |
| 6          | 0.17                                                  | 2.56 × 10⁵                                    |
| 7          | 0.12                                                  | 2.42 × 10⁵                                    |
| 8          | 0.62                                                  | 3.29 × 10⁵                                    |
| 10         | 0.17                                                  | 3.42 × 10⁵                                    |
| 14         | 0.35                                                  | 1.63 × 10⁵                                    |
| 16         | 0.32                                                  | 4.67 × 10⁵                                    |
| 17         | 0.21                                                  | 4.50 × 10⁵                                    |
| 21         | 0.19                                                  | 2.32 × 10⁵                                    |
Afterward, the plate was washed thrice with PBS. The PBS was aspirated, 200 μL of freshly prepared permeabilization buffer (PBS, 1% Triton X-100 [Roth]) were added to the cells, and the plate was incubated for 30 min at room temperature under constant shaking. Subsequently, the permeabilization buffer was aspirated, and 200 μL of blocking buffer (PBS, 3% FBS) were added for 1 h. Then, the blocking buffer was aspirated, and 50 μL of primary antibody solution (anti-SARS-COV-2-NP [RRID: AB_288025] 1:5,000 diluted in PBS + 1% FBS) was added to each well. The plate was incubated overnight at 4 °C. The next day, the primary antibody solution was aspirated, and the plate was washed thrice with wash buffer (PBS, 0.05% Tween 20 [Roth]). Thereafter, 50 μL of the secondary antibody solution (Peroxidase-AffiniPure Goat Anti-Mouse IgG [H+L] [RRID: AB_10015289]) was added to the wells, and the plate was incubated for 2 h at room temperature. After the incubation period, the wells were washed four times with 250 μL of wash buffer. Afterward, 100 μL of tetramethylrhodamine (TMRE) substrate solution (BioLegend) were added, and the plate was incubated for about 20 min at room temperature in the dark. The reaction was stopped by addition of 100 μL of 2 nH2SO4 (Roth). The absorbance was measured at 450 nm with a reference wavelength of 620 nm using Spark 10M multimode microplate reader (Tecan).

Cell Viability Assay. To exclude cytotoxic effects of the compounds used in our assays, a cell viability assay was performed using the Quanti Cell Counting Solution (SeraLab) according to the manufacturer’s instructions. The cells were seeded and treated equally to the protocol that was used before without any viral infection. Afterward, 10 μL of Quanti Cell Counting Solution were added to each well, and the plate was incubated for 2 h. Then, the absorbance was measured at 450 nm with Spark 10M multimode microplate reader.

Immunofluorescence. VeroE6 cells were seeded and treated as described for the iCLLSA. After incubation with the primary antibody solution, 50 μL of secondary antibody solution (Goat IgG anti-Mouse IgG [H+L]-Alexa Fluor 488, MinX none 1:2,000 [RRID: AB_2338840], Phalloidin CF647 1:100 [Biotium] in secondary antibody solution (Goat IgG anti-Mouse IgG [H+L]) was added for 1 h. Then, the blocking buffer was aspirated, and the cells were washed thrice with 150 μL of wash buffer (PBS). Afterward, 100 μL of tetramethylrhodamine (TMRE) substrate solution (BioLegend) were added, and the plate was incubated for about 20 min at room temperature in the dark. The reaction was stopped by addition of 100 μL of 2 nH2SO4 (Roth). The absorbance was measured at 450 nm with a reference wavelength of 620 nm using Spark 10M multimode microplate reader (Tecan).

Ethics Statement. Fetal tissues for reconstitution of humanized mice were obtained through anonymous donations with informed written consent via Advanced Bioscience Resources under the University of Saskatchewan Research Ethics Board Bio ID-371. All animal studies were performed under approval of the University of Saskatchewan’s Animal Research Ethics Board protocols 20180079 and 20200016 and adhered to Canadian Council on Animal Care guidelines.

Humanized TKO-LoM Mice. TKO-LoM were generated as previously described (64, Advanced Bioscience Resources under the University of Saskatchewan Research Ethics Statement.

THUNDER Imager 3D Cell Culture.

μL of 17– to 22-wk-gestation human fetal lung tissue were implanted subcutaneously onto the backs of mice. Subcutaneous lung organoids were weighed and placed in 1 mL of DMEM supplemented with 5% FBS and 1% penicillin-streptomycin. Sample volumes of 50 μL were added to 96-well plates of 95% confluent VeroE6 cells in triplicate and incubated at 37 °C with 5% CO2 before scoring for the presence of cytopathic effects.

Infection of hAEC. The hAEC were obtained from lung transplant donors postmortem (ethics of University Duisburg-Essen 18-0024-BO and 19-8717-BO) or from explanted lungs (ethics of Hannover Medical School 3346/2016). Selection criteria for donors are listed in the Eurotransplant guidelines. The hAECs from explanted lungs were cultured and differentiated as previously described (66). The hAEC from lung transplant donors postmortem were obtained by the following protocol: During the adaptation of the donor lung, a small tracheal ring was removed and stored in PBS supplemented with antibiotics (penicillin 100 U/mL, streptomycin 100 μg/mL, 10 μg/mL ciprofloxacin [Kabi]). The hAEC were isolated from the mucosa within 24 h after transplantation by enzymatic digestion (Protease XIV [Sigma-Aldrich]) and scraping. Cells were expanded for 7 to 14 d in KSM (keratinocyte-SFM-medium [Gibco]), supplemented with human epidermal growth factor [Gibco] (2.5 nM/mL), bovine pituitary extract [Gibco] [BPE 25 μg/mL, Gibco], isoproterenol [Sigma-Aldrich] (1 μM), penicillin, streptomycin, ciprofloxacin, and amphotericin B [PanBiotech] (2.5 μg/mL) and, after trypsinization, stored in liquid nitrogen (10% dimethyl sulfoxide, 90% KSM+FBS 0.3 mg/mL). All plastic surfaces during hAEC isolation and air–liquid interface (ALI) culture were coated with human fibronectin (PromoCell) (5 μg/mL), type I bovine collagen (Advanced Bioscience Resources) eTrexo30 30 mg/mL, and bovine serum albumin (BSA) (10% v/v). For ALI cultures, cells were thawed, expanded in KSM for 5 to 7 d, and transferred to transwell inserts (PE Membrane, 12-well plates, 0.4-μm pore size, Corning). A monolayer of hAECs were grown submerged in KSM (1:1 mixture of DMEM [StemCell] and BPECMo [StemCell]), supplemented with penicillin and streptomycin, Hepes (Gibco) (12.5 μL/mL, 1×), 1× Bronchial Epithelial Cell Growth Supplement (StemCell), and EC-23 (Tocris) (5 mM) until confluent. Humanized ALI confluent hAEC monolayers were further grown in submerged culture media without air–liquid interface for 2 wk. Infection was started after cells were fully differentiated as measured by movement of cilia, secretion of mucus, and transepithelial electrical resistance (>1,000 Ω/cm²).

Fully differentiated hAECs were washed with Hanks’ balanced salt solution (HBSS) apically for 10 min before infection. For SARS experiments, the cells were infected apically with 30,000 PFU diluted in HBSS; for influenza, the cells were infected apically with 100 PFU of A/PR/8/34 (H1N1). To determine SARS-CoV-2 and influenza, respectively, human bronchial epithelial cell cultures were further grown in submerged culture media under air–liquid interface for 2 wk. Infection was started after cells were fully differentiated as measured by movement of cilia, secretion of mucus, and transepithelial electrical resistance (>1,000 Ω/cm²).

To determine relative SARS-CoV-2 M- or N-gene expression, 500 ng of total RNA were reverse transcribed using the PrimeScript RT Master Mix (Takara). Promega’s GoTaq Probe qPCR Master Mix was used according to the manufacturer’s instructions, with gene-specific primers and probes (S4 Appendix, Table S4). RT-qPCR was performed on a LightCycler 480 II (Roche) instrument, with the following conditions: Initial denaturation was 2 min at 95 °C and a ramp rate of 4.4 °C/s, followed by 40 cycles of denaturation for 15 s at 95 °C and a ramp rate of 4.4 °C/s and amplification for 60 s at 60 °C and a ramp rate of 2.2 °C/s. To assess M- and N-gene copy numbers, the M- and N-genes were partially cloned into pCR2.1 (ThermoFisher Scientific) or pMiniT 2.0 (NEB), respectively, and a 2× plasmid dilution series was used as a reference.

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InV Plaque Assay. MDCk-II cells were seeded in six-well plates, and cultured in DMEM supplemented with 5% FBS and 1% penicillin-streptomycin until 100% confluent. On the day of infection, 10-fold dilutions of apical washes were prepared in infection PBS (PBS supplemented with 1% penicillin-streptomycin, 0.01% CaCl2, 0.01% MgCl2, and 0.2% BSA). Cells were washed once with infection PBS, infected with 500 μL of diluted samples (virus inoculum), and incubated at 37 °C, 5% CO2 for 30 min. The inoculum was removed, and the infected monolayer was overlaid with plaque medium (prepared immediately before use by mixing 14.2% 10x MEM [Gibco], 0.3% NaHCO3, 0.014% DEAE-Dextran...
[Sigma-Aldrich], 1.4% penicillin–streptomycin, 0.2% BSA, 0.09% agar, 0.01% MgCl₂, 0.01% CaCl₂, and 0.15 mg of TPCK–Trypsin [Sigma]). Plates were kept at room temperature until the agar solidified, and were incubated upside down at 37 °C, 5% CO₂ for 72 h. Plaques were quantified in terms of infectious IAV particles, and were represented as plaque-forming units per milliliter.

**ISG Expression.** The 500,000 VeroE6 cells were seeded and stimulated with 1× 10⁶ U/mL of IFNα, IFNβ, and IFNγ for 16 h. Afterward, the cells were lysed using DNA/RNA Shield for RNA isolation.

RNA was isolated from cell lysates with Quick-RNA Miniprep Kit (Zymo Research) according to the manufacturer’s instruction. Complementary DNA was synthesized from isolated RNA using HiScript II RT SuperMix for qPCR (Vazyme) according to the manufacturer’s instructions. ISG expression levels were quantified by qPCR with Luna Universal qPCR Master Mix and the respective primer pairs (SI Appendix, Table S5). Expression levels were normalized by 2⁻⁰⁰⁰⁰⁰⁰⁰⁰⁰⁰⁰ method (67) using GAPDH as reference gene.

**Proteomics Sample Preparation.** Cells were washed with ice-cold PBS and harvested in urea buffer (30 mM Tris HCl, 7 M Urea, 2 M Thiourea, 0.1% NaN₃, pH 8.5). Cells were centrifuged for 15 min at 16,100 × g at 4 °C, and the supernatant was further processed. Tryptic digestion was performed on 20 μL of cell lysate. Disulfide bonds were reduced by adding a final 5 mM dithiothreitol for 15 min at 50 °C before thiol was alkylated by a final 15 mM iodoacetamide for 15 min in the dark. Hydrophilic and hydrophobic Cytiva Sera-Mag Carboxyl-Magnet-Beads (GE Healthcare) were mixed 1:1, and 2 μL of beads (25 μg/μL) were added per sample. The samples were filled up to 70% ACN (acetonitrile) and incubated for 15 min to ensure protein binding to the beads. Subsequently, beads were washed two times with 70% ETOH followed by washing with 100% ACN. Beads were resuspended in 100 mM ammonium bicarbonate containing 0.2 μg of trypsin (SERVA) per sample and incubated overnight at 37 °C. The peptides were transferred into a new reaction tube, vacuum dried, and dissolved in 0.1% trifluoroacetic acid.

**Liquid Chromatography–Tandem Mass Spectrometry (LC-MS/MS Analysis).** Then 400 ng of tryptic peptides per sample were analyzed using an Ultimate 3000 RSLCnano HPLC (Dionex) coupled to a Q Exactive HF Orbitrap (Thermo Fisher Scientific). Samples were preconcentrated on a C18 trap column (Acclaim PepMap 100, 100 μm × 2 cm, 5 μm, 100 Å; Thermo Fisher Scientific) within 7 min at a flow rate of 30 μL/min with 0.1% trifluoroacetic acid and subsequently transferred to a Nano Viper C18 analytical column (Acclaim PepMap RSLC, 75 μm × 50 cm, 2 μm, 100 Å; Thermo Fisher Scientific). Peptide separation was performed by a gradient from 5 to 30% solvent B over 120 min at 400 nL/min (solvent A: 0.1% formic acid; solvent B: 0.1% formic acid, 84% acetonitrile). Full-scan mass spectra were acquired in profile mode at a resolution of 70,000 at 400 m/z and a mass range of 350 m/z to 1,400 m/z. The 10 highest abundant peptide ions were fragmented by higher-energy collisional dissociation and normalization energy = 27), and MS/MS spectra were acquired at a resolution of 35,000.

**Proteomics Data Analysis.** Peptide identification and quantification were performed using MaxQuant (v.1.6.17) searching UniprotKB/SwissProt (2020.05, 563,552 entries) restricted to either Homo sapiens or Homo sapiens and SARS-CoV-2. Search parameters were default, label-free quantification was used for peak quantification, and normalization was enabled. Peptides were considered for quantification irrespective of modifications. Match between runs was enabled when the analysis was performed considering human proteins only. Statistical data analysis was conducted using R (v.3.6.2). Differences between the experimental groups were assessed using t tests (paired, two-sided), and proteins quantified in a minimum of three out of four donors per group with a minimum of two unique peptides, a P value ≤ 0.05, and a ratio of mean abundances ≥ 1.5 or ≤ 0.67 were considered statistically significant. Proteins that were quantified in one experimental group but not detected at all in an opposed group were defined as On-Offs between these groups. GO annotation and enrichment analyses were performed using STRING (v.11). Data visualization was done using R and Cytoscape (v.3.2.2).

**Transcriptomics.** Quality and integrity of total RNA was controlled on 5200 Fragment Analyzer System (Agilent Technologies). The RNA sequencing libraries were generated from totRNA using NEBNext Single Cell Low Input RNA Library to manufacturer’s protocols. The libraries were treated with Illumina Free Adapter Blocking and were sequenced on Illumina NovaSeq 6000 using NovaSeq 6000 S1 Reagent Kit (100 cycles, paired end run 2 × 50 bp) with an average of 3 × 10⁶ reads per RNA sample.

**Data Availability.** The authors declare that the data supporting the findings of this study are available within the article and SI Appendix. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD026079. The RNA sequencing data discussed in this publication have been deposited in National Center for Biotechnology Information’s Gene Expression Omnibus (GEO) (71) and are accessible through GEO Series accession number GSE199613.

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