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Targeting iRhom2/ADAM17 attenuates COVID-19-induced cytokine release from cultured lung epithelial cells

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ABSTRACT

The COVID-19 pandemic, caused by SARS-CoV-2, continues to pose a significant global health challenge, with acute respiratory distress syndrome (ARDS) being a major cause of mortality. Excessive cytokine release (cytokine storm) has been causally related to COVID-19-associated ARDS. While TNF-α inhibitors have shown potential in reducing inflammation, their broad effects on TNF-α signaling, including both pro- and anti-inflammatory pathways, present significant challenges and side effects in clinical use. Therefore, more precise therapeutic targets are urgently needed. ADAM17 is a key enzyme driving cytokine release, but its broad presence complicates direct inhibition. Targeting iRhom2, a regulator specific to immune cells that controls ADAM17's activity, offers a more focused and effective approach to reducing cytokine release. In this study, we hypothesized that targeted inhibition of ADAM-17/iRhom2 attenuates COVID-19-induced cytokine release in cultured lung epithelial cells. Human primary bronchial/tracheal epithelial cells challenged with COVID-19 pseudo-viral particles resulted in elevated cytokine release, which was attenuated following siRNA-mediated silencing of ADAM17 and iRhom2. Targeting ADAM-17/iRhom2 pathway may thus represent a strategy to overcome the COVID-19-associated ARDS.

1. Introduction

The COVID-19 pandemic, caused by the severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2), has posed a significant threat to global health [OR] COVID-19 infection affects the respiratory tract, leading to acute respiratory distress syndrome (ARDS), recognized as the leading cause of mortality in infected subjects [1,2]. ARDS is characterized by elevated levels of proinflammatory cytokines, including tumor necrosis factor-α (TNF-α), interferon-gamma (IFN-γ), granulocyte colony-stimulating factor (G-CSF), and interferons, often called a 'cytokine storm' [3–5]. Among these mediators, TNF-α has been postulated to play a pivotal role in COVID-19-associated ARDS [6,7]. In addition to precipitating a proinflammatory environment, TNF- α can activate various cell types, stimulating the release of other pro-inflammatory cytokines and augmenting the inflammatory milieu [8].

TNF-α is cleaved from the cell membrane by the metalloprotease ADAM-17 (a disintegrin and metalloprotease domain) [9–11]. In addition to TNF-α, ADAM-17 is also implicated in the cleavage of angiotensin-converting enzyme 2, interleukin receptor 6 (IL-6R), IL-8, and monocyte chemoattractant protein-1, which also contributes to COVID-19 infection [12]. Although ADAM17 inhibitors have been studied in models of lung injury [13], including COVID-19 infection-induced lung injury [12], a limitation of such an approach is its non-selectivity, owing to the ubiquitous expression of ADAM17 [14].

Consequently, there has been significant interest in regulating the activity of ADAM17, a metalloprotease that cleaves several key proteins such as TNF-α [9–11], angiotensin-converting enzyme 2, interleukin receptor 6 (IL-6R), IL-8, and monocyte chemoattractant protein-1, all of which contribute to COVID-19 infection [12]. However, targeting ADAM17 has been challenging due to its ubiquitous expression and the severe adverse effects of inhibiting other metalloproteinases sharing a similar catalytic domain [25,26]. Although ADAM17 inhibitors have been studied in models of lung injury [13], including COVID-19 infection-induced lung injury [12], their non-selectivity remains a limitation [14].

The maturation and biological activity of ADAM-17 is regulated by the rhomboid protease iRhom [15–17]. iRhoms are bound to ADAM-17

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and function as the regulatory subunit of the ADAM-17 complex [18]. An advantage of targeting iRhom is that it would specifically result in the downregulation of ADAM-17 without affecting other metalloproteases [14,17]. iRhom1 and iRhom2 have similar functions, suggesting a potential redundancy. However, while iRhom1 is expressed in all tissues, iRhom2 is specifically expressed in immune cells, which makes it an attractive target for regulating ADAM17-induced TNF-α release in immune cells [14,18,19]. Furthermore, upregulation of iRhom2 has been implicated in acute lung inflammation caused by ischemia-reperfusion injury, although its involvement in cytokine storm associated with COVID-19 infection remains unknown [20]. In this way, by focusing on the iRhom2/ADAM17 pathway, we aim to provide a more precise therapeutic approach that could mitigate the cytokine storm in COVID-19-associated ARDS without the drawbacks of current therapies. In this current study, we demonstrate that iRhom2 is necessary for COVID-19 viral particle-induced cytokine release in cultured human lung epithelial cells.

2. Material and methods

2.1. Cell culture and treatment

Normal human primary bronchial epithelial cells (NHBE, ATCC®, PS-300-010) were cultured to sub-confluence in a complete medium consisting of Airway Epithelial Cell Basal Media (ATCC®, PS-300-030) supplemented with Bronchial/Tracheal Epithelial Cell Growth Kit (ATCC®, PS-300-040) and 1 % penicillin-streptomycin (100 U/ml) and maintained in a 5 % CO_2 incubator at 37 °C. All reported assays were performed at P7. To evaluate the effect of SARS-CoV-2 on inflammatory responses, bronchial epithelial cells were stimulated with SARS-CoV-2 pseudo-viral particles (MyBioSource, MBS434275) with a multiplicity of infection, MOI = 10. Following 24 h, the efficacy of infection of the SARS-CoV-2 pseudo-viral particle was determined using a luciferase substrate (MyBioSource, MBS434279) that measures firefly luciferase activity using a microplate reader (TECAN, Germany). Also, the TNF-α production was accessed via ELISA. Phorbol-12-myristate-13-acetate (PMA, Sigma, P8139) at a concentration of 0.1 μM was used as a positive control to induce cytokine release.

2.2. Transfection and ADAM17/iRhom2 inhibition

Cells were seeded and cultured at 37 °C under 5 % CO_2 until they achieved 70–80 % confluence. The cell culture medium was then replaced with a serum-free medium containing the complex with 50 nM of siRNA, Lipofectamine™ RNAiMAX Reagent (ThermoFisher, 13778-150), and Opti-MEM (ThermoFisher, 31985-062) were used in accordance with the manufacturer's protocols. After 24 h, the transfection efficiency was measured using quantitative real-time PCR for the targeted mRNAs. The list of the siRNA used in this study is given in Supplementary Table 1. In addition to silencing using siRNA, pharmacological inhibition of ADAM-17 was achieved using TNF-α Protease Inhibitor-0 (TAPI-0, Sigma, SML1292).

2.3. Cytotoxicity

Different siRNA concentrations were first tested for their potential cytotoxicity. Following treatment of the cells with siRNA for 24 h, the cell culture medium was replaced with a fresh medium, followed by treatment with MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) reagent (ThermoFisher, M6494) (0.5 mg/ml) and incubated for 2 h. After incubation, the medium was removed and replaced with the same volume of DMSO and incubated for 10 min to dissolve the insoluble formazan. Absorbances were measured using a *UV/Vis* spectrophotometer at 570 nm; results are expressed as relative cell viability.

2.4. ELISA/Multiplex assay

The levels of human TNF-α (MyBioSource, MBS2502004), IL-1α, IL-1β, IL-4, IL-6, IL-8, IL-10, IL-13, MCP-1, IFN- γ, TNFα (Abcam, ab197449) were evaluated from the collected medium of different treatment groups. All the cytokines were quantified using the manufacturer's protocols.

2.5. Immunofluorescence

For immunocytochemistry, the cells were cultured at Lab-Tek II CC^2 chamber slide systems (ThermoFisher, 154941), and after 12h of different treatments, the chamber slides were fixed with pre-chilled 10 % formalin for 20 min. Cells were permeabilized and blocked with 2 % BSA for an hour at room temperature. Cells were then incubated with Anti-ADAM17 antibody (Abcam, ab57484) in 2 % BSA overnight at 4 °C. Antibody binding was visualized using Alexa Fluor-conjugated anti-goat antibody (Abcam, ab150115). Nuclei were counterstained with DAPI (Calbiochem, 268298). To visualize stained cells, a fluorescent microscope EVOS FLoid Imaging System (ThermoFisher, 4471136) was used.

2.6. Activity assay

Cells were collected, and the protein extraction was made using TRIzol reagent (ThermoFisher, 15596026) per the manufacturer's protocols. The protein concentration was determined using a BCA assay (ThermoFisher, 23227). Then, ADAM17 Fluorogenic Assay Kit (BPS Bioscience, 78000) was used to measure ADAM-17 activity in 5 μg of each protein sample. Recombinant ADAM-17 was used as a positive control.

2.7. Quantitative real-time PCR

Total RNA was extracted from cells using RNeasy Mini Kit (Qiagen, 74104). RNA was reverse transcribed into cDNA using the e RT^2 First Strand Kit (Qiagen, 330401). Quantitative real-time PCR was performed with PowerUp™ SYBR™ Green Master Mix (ThermoFisher, A25742) following the manufacturer's instructions. Briefly, 50 ng cDNA was used as a template for each PCR amplification. All PCR reactions were conducted at an annealing temperature of 60 °C and 40 cycles. The ADAM17 primers forward and reverse were 5′- GAAGTGGGAGGCGATTA -3′ and 5' – CGGGCACTCACTGCTATTACC -3′, respectively. The iRhom2 primers were 5′- CGATTGACCTGATCCACC -3 and 5′-CAAAGTCTCCGAGCAGTCC -3′. And the GAPDH primers were 5′-CCAGCCCCAGCGTCAAAGGT -3′ and 5′ -CGGGGCTCTCCAGAACATCATCC -3′. The results were obtained as threshold cycle values (Ct), and the expression levels were calculated using the 2-ΔCt method (21).

2.8. Interactome construction

The STRING (Search Tool for the Retrieval of Interacting Genes/Proteins) platform was employed to construct a functional protein association network [21]. The STRING database integrates both experimental and predicted protein-protein interactions. The network was created using the cytokines assessed in the current study. A list of interacting proteins with associated confidence scores was identified using the default STRING settings. A confidence score threshold of 0.7 was used to filter the network, focusing solely on high-confidence interactions.

2.9. Statistical analysis

Statistical significance was evaluated using the Prism® 9.5 software (GraphPad Software Inc., San Diego, CA). Data are represented as mean ± SEM, and statistical significance was determined by applying One-way ANOVA with Tukey's multiple comparisons tests based on triplicate

samples unless otherwise stated.

3. Results

3.1. Silencing of ADAM17 and iRhom2 in cultured NHBE cells

Cells exhibited over 80 % viability after transfection with 50 nM ADAM17 siRNA (Fig. 1A) and increased viability to over 100 % with 50 nM iRhom2 siRNA (Fig. 1B). The viability increase with iRhom2 inhibition did not reach significance, which is attributable to the compensatory mechanisms that upregulate other proliferative pathways to maintain epithelial homeostasis when EGFR ligand shedding is decreased [22]. The efficacy of transfection was accessed by RT-qPCR and both ADAM17 and iRhom2 siRNA treatment resulted in the attenuation of ADAM17 and iRhom2 mRNA expression in bronchial epithelial cells (Fig. 1C and D, respectively).

3.2. ADAM17 mediates COVID-19 pseudo-virus-induced cytokine release in cultured bronchial epithelial cells

Treatment of cultured bronchial epithelial cells for 8 h with COVID-19 pseudo-viral particles (10 MOI) resulted in a robust release of TNF-α release into the cell media culture compared to vehicle treatment (Fig. 1E). Phorbol mesityl acetate (0.1 μM) used as a positive control in these experiments caused a modest increase in TNF-α release (1423.8 ± 2.2 pg/ml), although the increase was seen within the first hour of incubation.

We next examined the effects of pharmacological inhibition and silencing of ADAM-17 by using TAPI-0 and ADAM-17 siRNA on COVID-19-pseudo-viral-particle-induced TNF-α release by cultured bronchial epithelial cells. As illustrated in Fig. 1F, treatment with TAPI-0 and ADAM17 siRNA efficiently attenuated TNF-α release by the bronchial epithelial cells in the culture supernatant. In addition to TNF-α, silencing of ADAM17 resulted in the downregulation of the release of the other cytokines, including IL-1 α, IL-1β, IL-6, IL-8, MCP-1, IFN-γ, and TNF-α (Supplementary Fig. 1).

3.3. SARS-COV-2 pseudo-viral particles increase the expression of ADAM17

Next, we evaluated the ability of COVID-19 pseudo-viral particles to regulate the expression of ADAM17. As shown in Fig. 2, treatment of lung epithelial cells with COVID-19 pseudo-viral particles resulted in an increase in the mRNA levels of ADAM-17. This increase in ADAM-17 message levels was attenuated by pretreating the cells with ADAM-17 siRNA before the challenge with the viral particles (Fig. 2A and B). In addition to the mRNA levels, we also evaluated the protease activity of ADAM17, which was elevated in response to the COVID-pseudoviral challenge and rescued by pretreatment with ADAM17 siRNA (Fig. 2C).

3.4. COVID-19 pseudo-virus-induced upregulation of ADAM-17 depends on iRhom2

To evaluate the role of iRhom2 in the COVID-pseudo-virus-induced upregulation of ADAM-17, we transfected the cells with iRhom2 siRNA prior to challenge with the viral particles and assessed the levels of ADAM-17. As depicted in Fig. 2A–C, silencing of iRhom2 resulted in attenuation of both the message and activity of ADAM17 in response to the pseudo-viral particle challenge.

3.5. Silencing of iRhom2 blunts COVID-19-pseudovirus-induced cytokine release in cultured lung epithelial cells

Data presented in Fig. 3 demonstrate that silencing of iRhom2

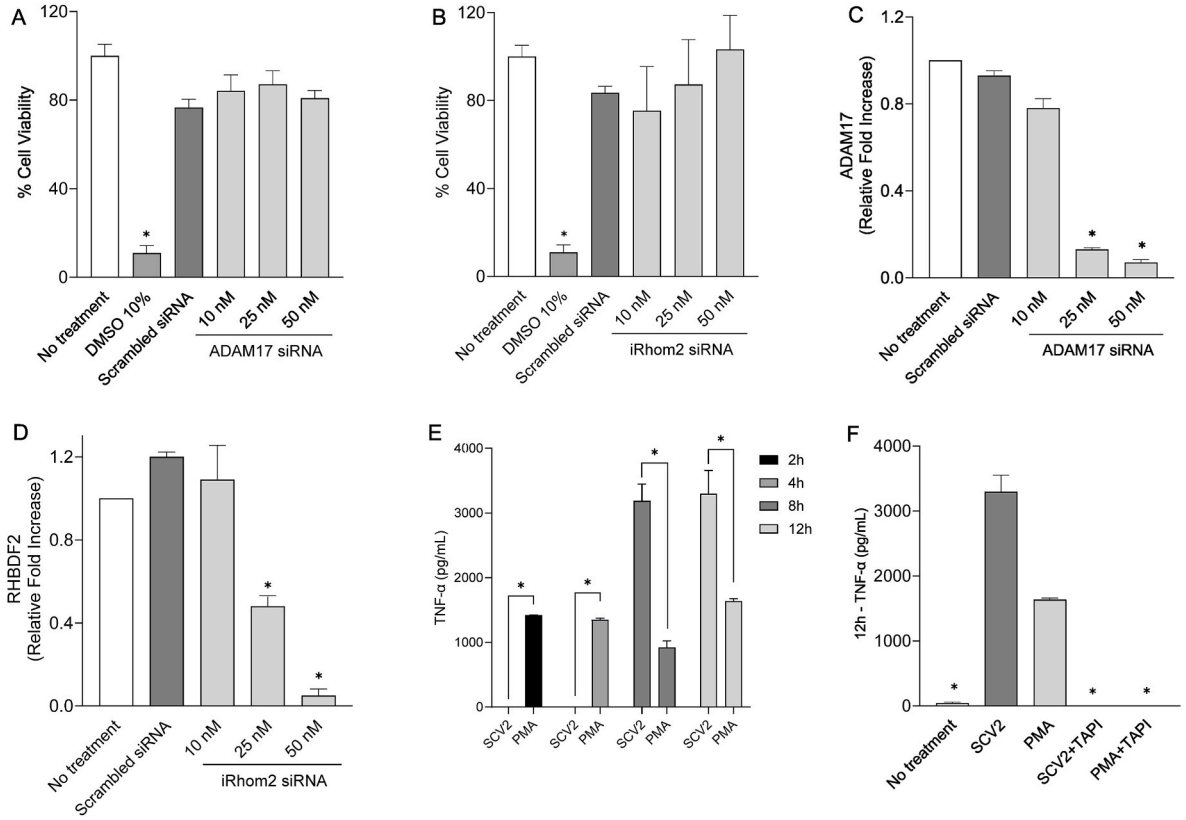

Fig. 1. (A & B) Cell viability of cultured bronchial epithelial cells in response to ADAM17 and iRhom2 siRNA treatment, respectively. (C & D) Transfection efficacy of ADAM17 siRNA and iRhom2 siRNA, respectively. (E) COVID-19 pseudo-viral particles (SCV2)-induce the release of TNF-α. (F) Pharmacological inhibition of ADAM17 with TAPI-0 inhibits the release of TNF-α. Data are represented as mean ± SEM, *p < 0.05 versus the control group (n = 4).

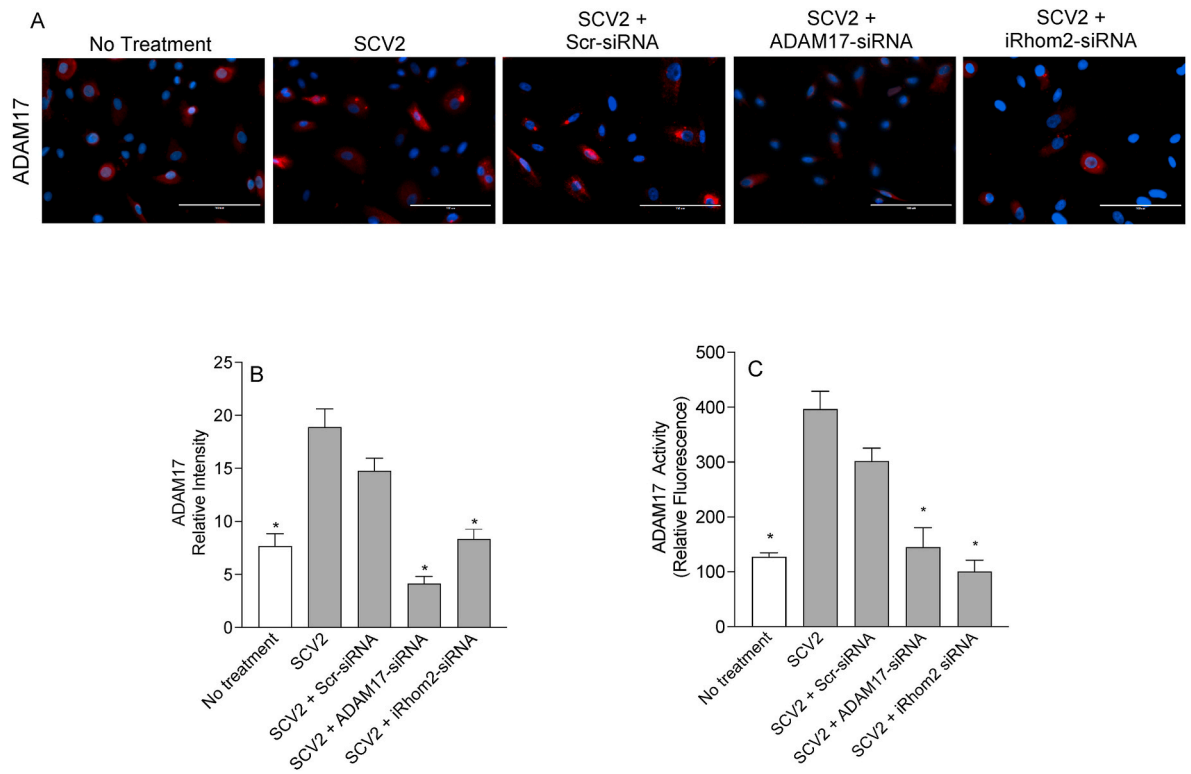

Fig. 2. (A) Silencing of ADAM17 and iRhom2 attenuates COVID-19 pseudo-virus (SCV2)-induced expression of ADAM17 in cultured bronchial epithelial cells (representative immunohistochemical images). Scale bar-100 μm (B) Quantification of the relative intensity of ADAM17 staining (n = 6), (C) Silencing of ADAM17 and iRhom2 attenuates ADAM17 activity cultured bronchial epithelial cells challenged with COVID-19 pseudo-virus mean ± SEM, *p < 0.05 versus the control group.

inhibits the release of major cytokines implicated in the 'cytokine storm' by the bronchial epithelial cells when subjected to the COVID-19 pseudo-viral particle.

3.6. Effect of ADAM17/iRhom2 on TNF-α and downstream effectors of pro-inflammatory mediators

To better understand the interconnection between the iRhom2/ADAM17 pathway and cytokine release, we performed computational analysis of key cytokines and related signaling pathways involved in COVID-19 infection, such as NF-kB, JAK/STAT, and NLRP3 utilizing the STRING interaction database [21]. As shown in Fig. 4 ADAM-17/iRhom2 pathway contributes to initiating pro-inflammatory responses during infections through its direct activity on TNF-α. TNF-α has downstream effects that increase the production of various pro-inflammatory cytokines and chemokines, including IL-1α, IL-1β, IL-6, IL-8, IL-10, IL-13, and MCP-1 [23]. Additionally, TNF-α serves as the primary inducer of the NF-kB pathway, which activates cytokines and promotes the pro-inflammatory pathway. Moreover, as illustrated in Fig. 4B, TNF-α plays a pivotal role in connecting cytokine among the three main cellular signaling pathways associated with inflammation in COVID-19-induced cytokine storms, namely NF-kB, JAK/STAT, and NLRP3. It achieves this through direct interactions with NF-kB and JAK/STAT pathways, influencing the activation of NF-kB and IL-1β and subsequently affecting the regulation of the NLRP3 pathway.

4. Discussion

A growing body of evidence suggests that excessive release of cytokines is associated with the severity and mortality of COVID-19 infection. Consequently, the cytokine blockade has been evaluated as a potential option to counter the cytokine storm associated with COVID-19. In this study, we show that targeted silencing of iRhom2, responsible for the maturation and activation of protease ADAM17, attenuates cytokine release from cultured bronchial epithelial cells challenged with COVID-19-pseudo-viral particles. To our knowledge, this is the first study to demonstrate the role of iRhom2 in the upregulation of cytokine release in response to COVID-19 infection of lung epithelial cells. Furthermore, we show that the mRNA and activity levels of ADAM17, which are elevated following treatment with COVID-19 particles, are attenuated by silencing iRhom2. Our data suggest that targeting iRhom2 is a potential strategy to blunt cytokine storm associated with COVID-19 infection. These observations are in accordance with previous reports of Adrain and coworkers showing that amelioration of lung injury in iRhom2 deficient mice following intestinal ischemia-reperfusion is associated with a decrease in TNF-α expression, while IL-6 remained unaffected [17].

To understand the role of the ADAM17/iRhom2 pathway in diverse inflammation markers, we used the STRING database [21] to visualize interactions. The ADAM17/iRhom2 pathway potentially triggers pro-inflammatory responses via direct interaction with TNF-α, IL-1β, and IL-6R, which leads to the cytokine cascade and activation of the NF-kB pathway. This pathway will link the JAK/STAT and NLRP3 pathways, elucidating the storm effect of iRhom2/ADAM17 upregulation.

Despite the positive effects of controlling dysregulated inflammation in vitro, iRhom2 inhibition presents controversial results in infectious diseases. Kubo and coworkers found that Rhbdf2−/− mice were more susceptible to lung bacterial infection than wild-type mice, underscoring the importance of the iRhom2/ADAM17 pathway in innate immunity [24]. Future studies should focus on the optimal timing and concentration for iRhom2 inhibition to regulate ADAM17 response without impairing innate immunity.

In summary, the current study gives credence to the view that

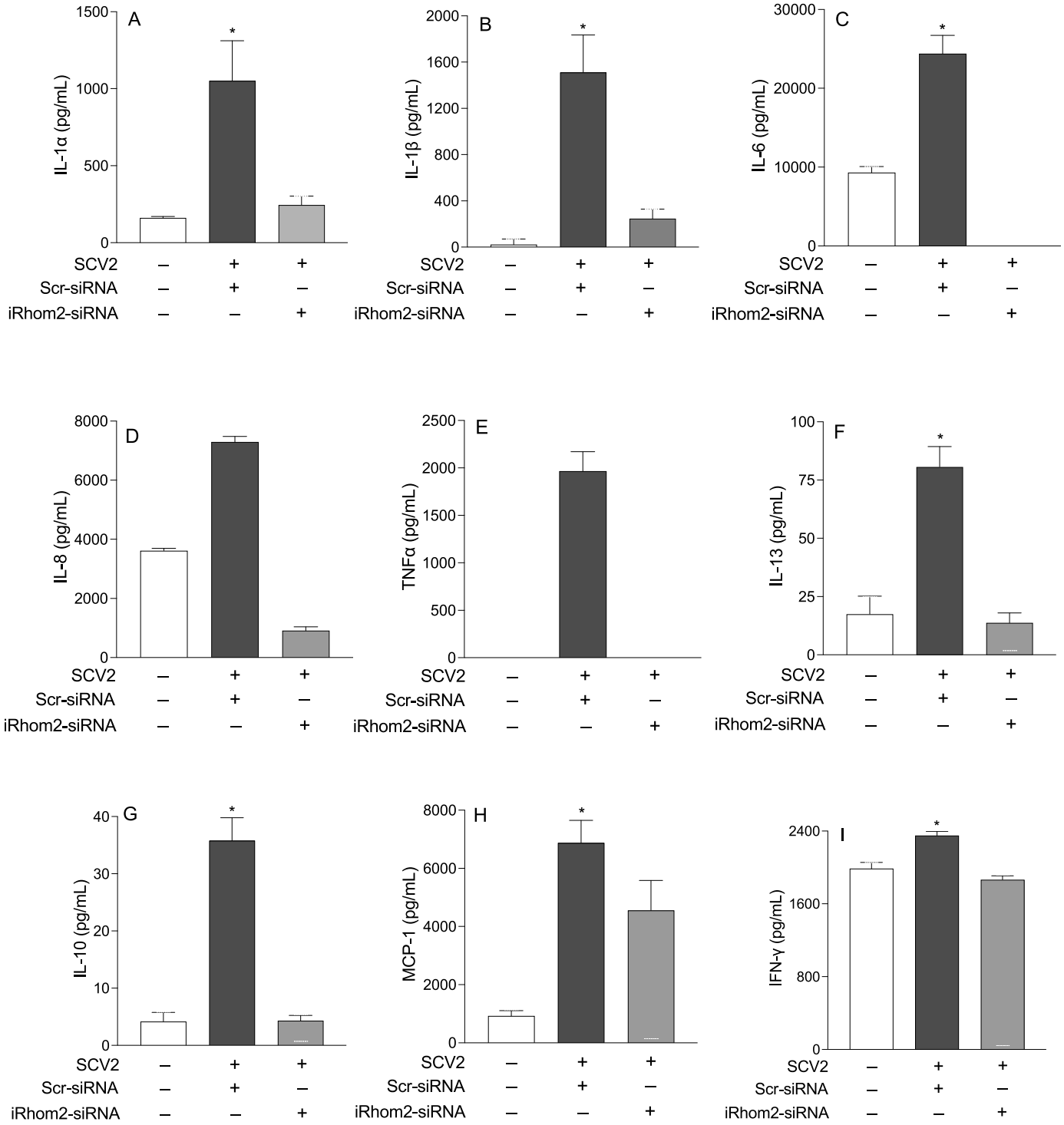

Fig. 3. Silencing of iRhom2 attenuates cytokine release in bronchial epithelial cells challenged with COVID-19 pseudo-virus (SCV2) (A) IL-1α, (B) IL-1β, (C) IL-6, (D) IL-8, (E) TNF-α, (F) IL-13, (G) IL-10, (H) MCP-1, and (I) IFN-γ. Data are presented as mean ± SEM, *$p < 0.05$ versus the control siRNA + COVID-19 group ($n = 3$).

targeting iRhom2 can effectively reduce ADAM-17 activity and consequent release of cytokines. It further suggests that inhibition of iRhom2 may represent a bonafide treatment target for COVID-19-induced ARDS.

CRediT authorship contribution statement

Vitoria Mattos Pereira: Writing – original draft, Validation, Methodology, Investigation, Formal analysis, Data curation. **Amit Thakar:** Writing – review & editing, Conceptualization. **Sreejayan Nair:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

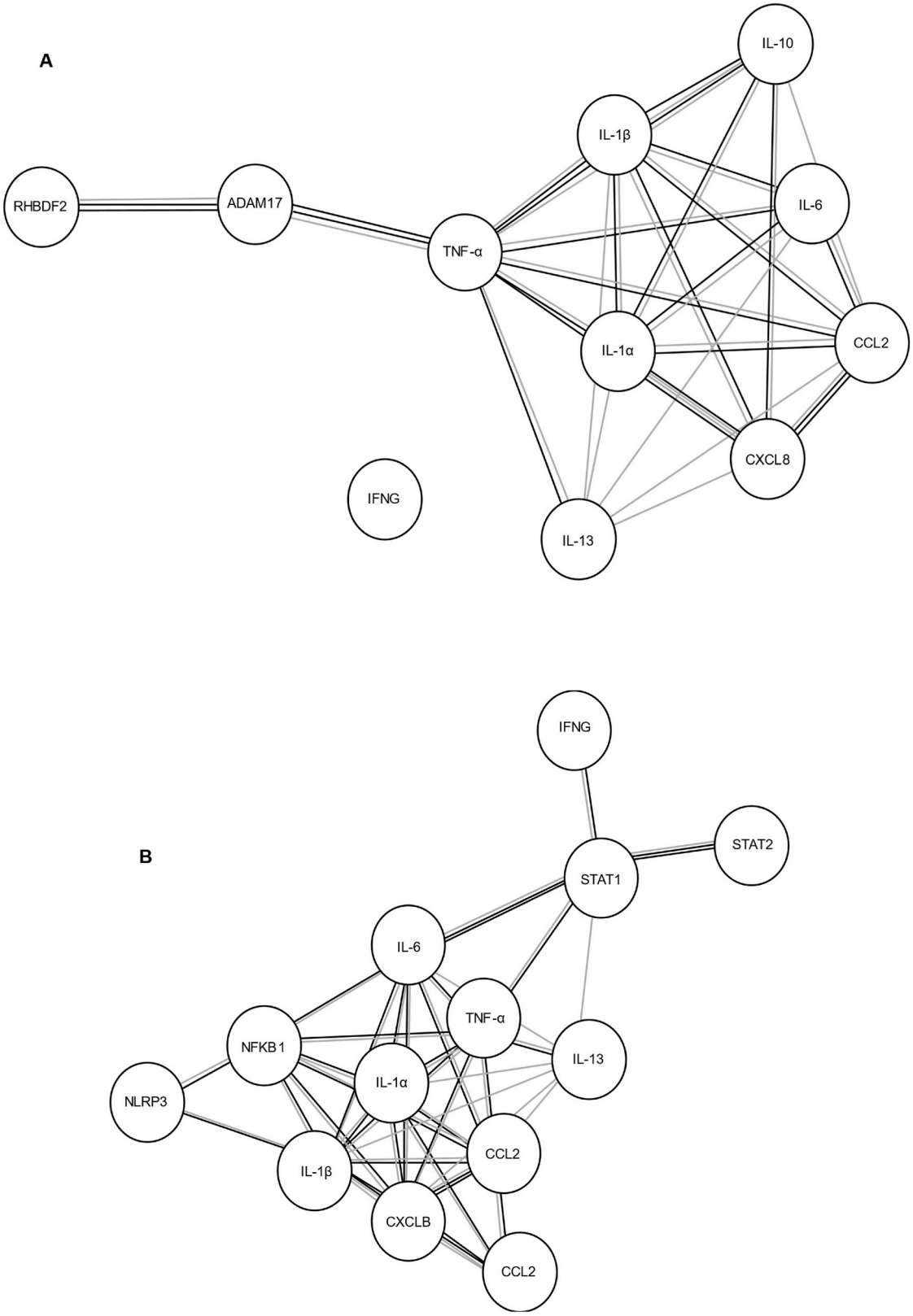

Fig. 4. (A) Network construction of ADAM17 and iRhom2 and nine key pro- and anti-inflammatory molecules, using the default STRING parameters. (B) Network construction by including nuclear factor Kappa B (NF-kB), Jannus kinase/Signal Transducer and Activator of Transcription (JAK/STAT), nucleotide-binding domain, leucine-rich–containing family, pyrin domain–containing-3 inflammasome (NLRP3) signaling pathways to the cytokines of interest. The lines indicate interactions between the proteins of interest. The black line represents experimentally determined interactions, a grey line represents interactions from curated databases, and a dotted line indicates co-expression interactions. The presence of two lines connecting the same proteins typically indicates multiple types of evidence supporting the interaction.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrep.2024.101811.

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