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Seasonal coronavirus infections trigger NLRP3 inflammasome activation in macrophages but is therapeutically targetable

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ABSTRACT

Seasonal coronaviruses widely circulate in the global population, and severe complications can occur in specific vulnerable populations. Little is known on their pathogenic mechanisms and no approved treatment is available. Here, we present anecdotal evidence that the level of IL-1β, a hallmark of inflammasome activation, appears elevated in a subset of seasonal coronavirus infected patients. We found that cultured human macrophages support the full life cycle of three cultivatable seasonal coronaviruses. Their infections effectively activate NLRP3 inflammasome activation through TLR4 ligation and NF-κB activation. This activation can be attenuated by specific pharmacological inhibitors and clinically used medications including dexamethasone and flufenamic acid. Interestingly, combination of antiviral and anti-inflammatory drugs simultaneously inhibit seasonal coronavirus-triggered inflammatory response and viral replication. Collectively, these findings show that the TLR4/NF-κB/NLRP3 axis drives seasonal coronavirus triggered-inflammatory response, which in turn represents a viable therapeutic target.

1. Introduction

Human coronaviruses (HCoVs) are enveloped, positive-sense RNA viruses that circulate widely among the global population. Their infections cause respiratory complications, ranging from mild cold to severe pneumonia and even death. To date, seven HCoVs have been identified, consisting of severe acute respiratory coronavirus (SARS-CoV), Middle East respiratory syndrome coronavirus (MERS-CoV), severe acute respiratory coronavirus 2 (SARS-CoV-2), and four endemic seasonal coronaviruses (NL63, 229E, OC43 and HKU1) (Ma et al., 2020). It is inevitable that SARS-CoV-2 is becoming the fifth endemic HCoV that will persistently circulate in human population (Katzourakis, 2022). Given the continuous evolvement of SARS-CoV-2, in particular emergence of the less pathogenic Omicron variant, it is intriguing to speculate whether these new variants would eventually resemble seasonal coronavirus to some extent.

Although infections of seasonal coronaviruses unusually cause mild to moderate symptoms in healthy individuals, severe illnesses and even mortality have been reported in vulnerable patients, such as in young children, the elderly and immunocompromised individuals (Li et al., 2021; Pinana et al., 2021). Although little is known regarding the pathogenic mechanisms of seasonal coronavirus infection, in analogy to COVID-19, we postulate that immune and inflammatory responses are crucial for determining the outcome of seasonal coronavirus infected patients.

Monocytes and macrophages are recognized as key players in hyperinflammation associated with severe COVID-19 and are the nexus of rapid activation of the inflammasome pathway in these patients (Merad and Martin, 2020). There are four major types of inflammasomes, *in casu* NLRP1, NLRP3, AIM2 and NLRC4. The NLRP3 inflammasome is most relevant in recognizing RNA virus infection (Choudhury et al., 2021). It comprises the sensor molecule NLRP3 (NLR family PYRIN domain containing-3), the adaptor protein ASC (Apoptosis-associated speck-like protein containing a caspase recruitment domain) and effector protein (Caspase-1). Two steps are required for NLRP3 activation. The priming signal upregulates the expression of inflammasome components including NLRP3 and pro-IL-1β through the recognition of pathogen-associated molecular patterns (PAMPs) or damage-associated molecular patterns (DAMPs) via activation of nuclear factor-kappa B (NF-κB). The second signal triggers the assembly of

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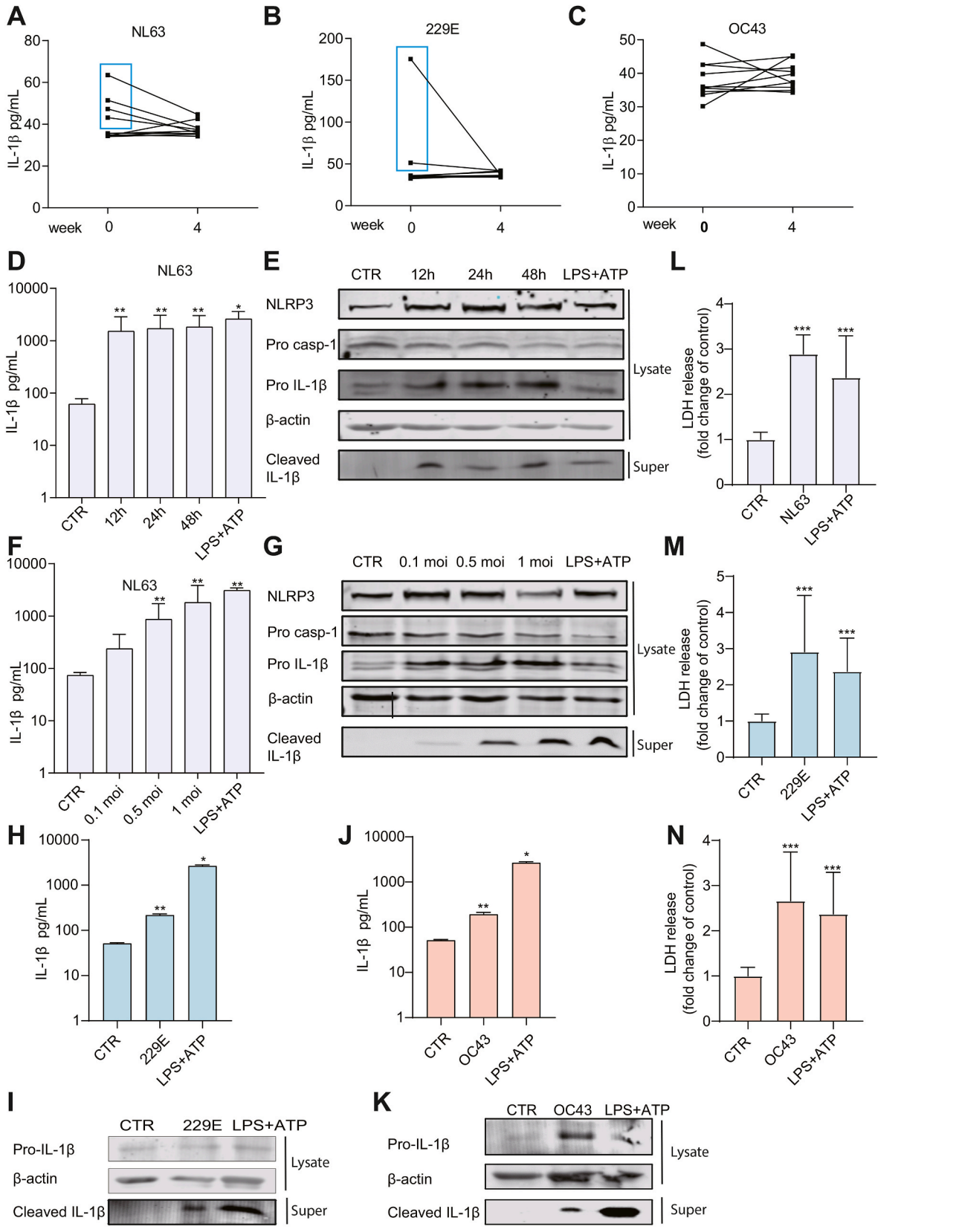

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Fig. 1. Seasonal coronavirus infection (NL63, 229E or OC43) in human macrophages triggers inflammatory response and pyroptosis. (A, B and C) IL-1β levels in the serum of seasonal coronavirus-infected patients. Samples were collected at the time of diagnosis (week 0) and post-recovery (week 4) for each individual (n = 10 for each group). THP-1 macrophages were inoculated with NL63 for 12, 24 or 48 h or treated with LPS (400 ng/mL) for 6 h followed by ATP (5 mM) for 40 min, and IL-1β protein (D) levels were quantified by ELISA (n = 4, LPS + ATP; n = 5, other groups). (E) Mature IL-1β in supernatant, and pro-IL-1β, pro-Casp-1 and NLRP3 in lysates were determined by western blot. THP-1 macrophages were inoculated with 0.1, 0.5 or 1 moi NL63 for 48 h or treated with LPS (400 ng/mL) for 6 h followed by ATP (5 mM) for 40 min, and IL-1β protein (F) levels were quantified by ELISA (n = 5, LPS + ATP; n = 7, other groups). (G) Mature IL-1β in supernatant, and pro-IL-1β, pro-Casp-1 and NLRP3 in lysates were determined by western blot. THP-1 macrophages were inoculated with 229E or OC43 for 48 h or treated with LPS (400 ng/mL) for 6 h followed by ATP (5 mM) for 40 min. IL-1β protein (H and J) levels were quantified by ELISA (n = 4, LPS + ATP; n = 6, other groups). (I and K) Mature IL-1β in supernatant, and pro-IL-1β in lysates were determined by western blot. (L, M and N) The lactate dehydrogenase (LDH) activity released from the THP-1 macrophages was measured at 48 h after NL63, 229E or OC43 infection (n = 6, LPS + ATP; n = 10, other groups). Data were normalized to the control (CTR, set as 1). Data are presented as means ± SD. *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001; NS, not significant (Mann-Whitney test). Super: Supernatant.

the NLRP3 inflammasome complex, leading to caspase-1 auto-activation, cleavage of pro-IL-1β, and release of the mature IL-1β (Cleaved IL-1β), the hallmark of inflammasome activation (Swanson et al., 2019).

Given the poor understanding of pathogenic mechanism and lacking of therapeutic option for seasonal coronavirus infection, this study aims to investigate whether these viruses can trigger inflammatory response in macrophages, the underlying mechanism-of-action, and the feasibilities of therapeutic targeting. We found that seasonal coronaviruses effectively activated NLRP3 inflammasome in human macrophages, which can in turn be pharmacologically targeted by specific inhibitors or clinically used (anti-inflammatory) medications. Combination of antiviral drug with anti-inflammatory agent simultaneously inhibited viral replication and inflammatory response.

2. Materials and methods

2.1. Patient materials

Serum samples of NL63, 229E or OC43-infected patients (adults) at active infection (time of diagnosis) and recovered phase (4 weeks post-infection) were retrieved at Amsterdam UMC, University of Amsterdam, the Netherlands. The concentrations of IL-1β were measured by enzyme-linked immunosorbent assay (ELISA). This study was approved by the medical ethical committee of the Amsterdam UMC, University of Amsterdam, the Netherlands (Edridge et al., 2020).

2.2. Cell culture

The human monocytic cell lines THP-1, HL60 were cultured in RPMI 1640 Medium (Thermo Fisher) complemented with 10% (v/v) inactivated Fetal Bovine Serum with 100 IU/ml penicillin and 100 mg/ml streptomycin. For macrophage differentiation, THP-1 and HL60 cells were treated with 20 ng/ml and 40 ng/ml phorbol 12-myristate 13-acetate (PMA) at 37 °C for 48 h, respectively. Then cells were cultured for another 6 h without PMA.

2.3. Coronavirus inoculation

Seasonal coronavirus OC43 and 229E were bought from ATCC (USA) and propagated in Huh7 cells. Seasonal coronavirus NL63 was obtained from Amsterdam UMC location AMC, University of Amsterdam, The Netherlands and propagated in LLCMK2 cells. The SARS-CoV-2 Omicron variant (BA.1) was isolated from infected patients at Department of Viroscience, Erasmus MC, The Netherlands.THP-1 or HL60 monocytes and macrophages were inoculated with seasonal coronavirus particles at 33 °C. Supernatant and cell lysates were collected for ELISA, Western blot or qRT-PCR assays at indicated time points.

2.4. Reagents

Human IL-1 beta/IL-1F2 Quantikine ELISA Kit (DLB50, Bio-Techne and E4818-100, Biovision). Rabbit IL-1 beta/IL-1F2 Quantikine ELISA Kit (ml027836, MLBIO), BioLux® Gaussia Luciferase Assay Kit from Bioké. Lipopolysaccharide (LPS; L6529, Sigma) and adenosine 5-triphosphate disodium salt hydrate (ATP; A3377, Sigma) were dissolved in PBS. 12-O-Tetradecanoylphorbol 13-acetate (PMA, P1585, Sigma), BAY11 7085 (sc-202490, Santa Cruz Biotech, CA), MCC950 (inh-mcc, Invivogen) and Belnacasan (VX-765) (S2228, Bio-Connect BV) were dissolved in DMSO. Molnupiravir (MedChem Express, USA), abacavir (MedChem Express, USA), flufenamic acid (MedChem Express, USA), stavudineand (Selleckchem, USA), tiaprofenic acid (Selleckchem, USA), fenamic acid (Selleckchem, USA), pentoxifyllinewere (Selleckchem, USA), resveratrol (Selleckchem, USA), tranilast (Selleckchem, USA) were dissolved in dimethyl sulfoxide (DMSO, Sigma, Zwijndrecht, The Netherlands). VECTASHIELD® Antifade Mounting Medium with DAPI (13285184) obtained from Fisher Scientific. Antibodies including IL-1β (D3U3E) (Rabbit mAb,12703), Cleaved IL-1beta (Asp116) (D3A3Z) (Rabbit mAb, 83186s), Cleaved Caspase-1 (Asp297) (D57A2) (Rabbit mAb, 4199s), NF-κB p65 (C22B4) (Rabbit mAb, 4764), anti-rabbit IgG(H + L),F(ab') 2 Fragment (4412s, Alexa Fluor 488 conjugate) were obtained from Cell Signaling. Caspase-1 Antibody (14F468) (Mouse mAb, sc-56036), β-actin antibody (mouse mAb, sc-47778) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). NLRP3 antibody (PA5-20838) (Rabbit, pAb) was obtained from Fisher Scientific. Anti-double-stranded-RNA (dsRNA) antibody (SCIONS J2 monoclonal antibody) was purchased from English&Scientific Consulting Kft. Anti-rabbit and anti-mouse IRDye-conjugated secondary antibodies (Li-Cor Biosciences, Lincoln, NE, USA) were also used.

2.5. qRT-PCR

Total RNA was isolated with a Macherey NucleoSpin RNA II Kit (Bioke, Leiden, The Netherlands) and quantified with a Nanodrop ND1000 (Wilmington, DE, USA). During RNA isolation, DNase was added to remove genomic DNA according to the manufacturer's instructions. cDNA was synthesized from 500 ng of RNA using a cDNA synthesis kit (TaKaRa Bio, Inc., Shiga, Japan). The cDNA of all targeted genes transcript were quantified by SYBR-Green-based (Applied Biosystems) real-time PCR on the StepOnePlus™ System (Thermo Fisher Scientific Life Sciences) according to the manufacturer's instructions. Human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as reference gene to normalize gene expression. The relative expression of targeted gene was calculated as $2-\Delta\Delta CT$, where $\Delta\Delta CT = \Delta CT$ sample - ΔCT control (ΔCT = CT[targeted gene] - CT[GAPDH]).

2.6. $TCID_{50}$ assay

Cell culture supernatants from virus infected THP-1 macrophages were collected at indicated time points. Virus titer was quantified by using a 50% tissue culture infectious dose ($TCID_{50}$) assay. Briefly, ten-fold dilutions of viruses were inoculated onto LLCMK2, Huh7 or Vero-E6 cells, grown in a 96-well tissue culture plate at 2000 cells/well. The plate was incubated at 37 °C for 5–7 days, and each well was examined under a light microscope for cytopathic effect (CPE). The

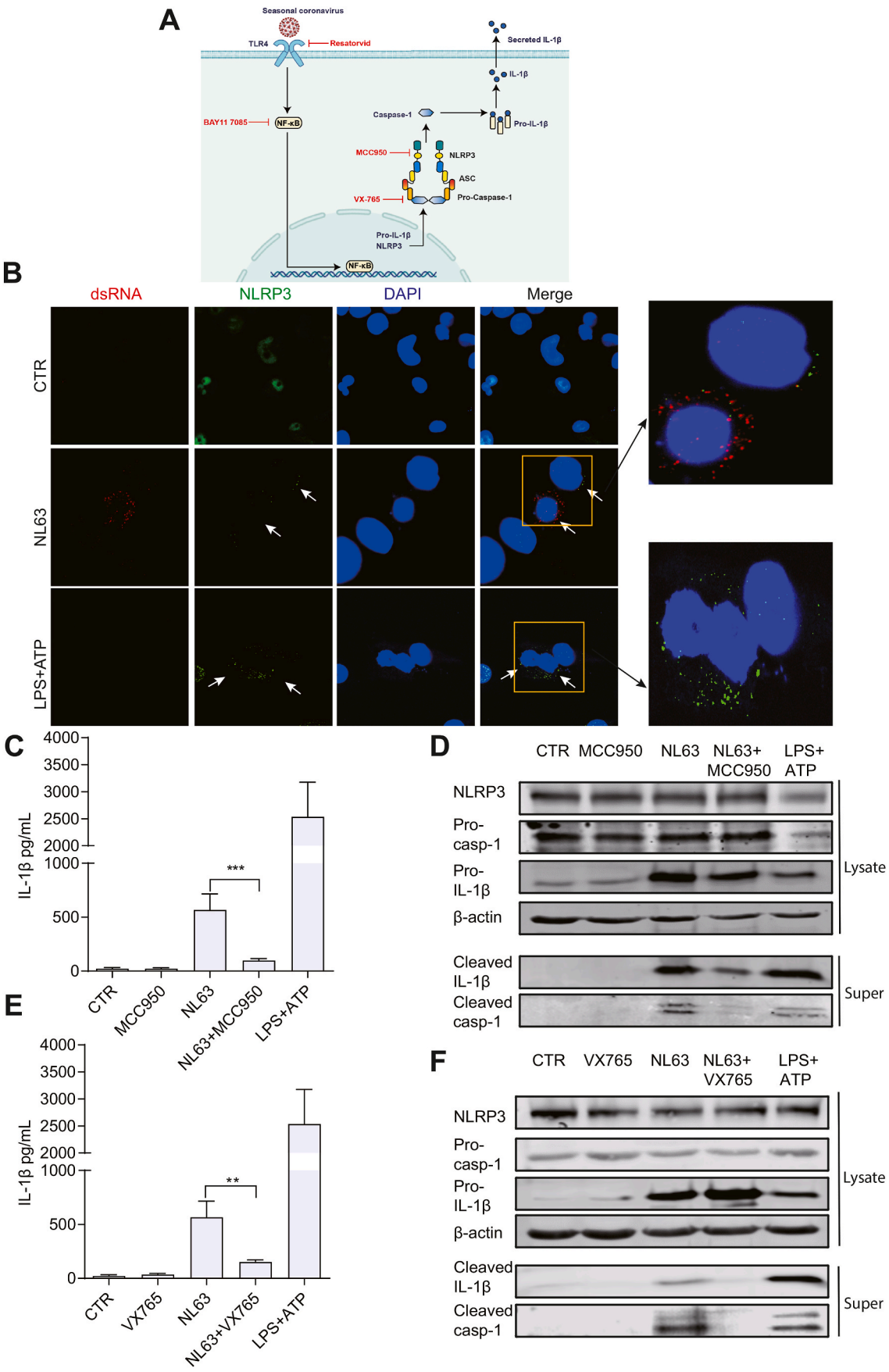

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Fig. 2. NL63 induced NLRP3 inflammasome and caspase-1 activation (A) Schematic overview of the NLRP3 inflammasome cascade and the respective targets by tested inhibitors. This illustration was prepared by using the web-based tool BioRender. (B) THP-1 macrophages were inoculated with NL63 for 48 h. NL63 double-stranded (ds) RNA (red), NLRP3 inflammasome (green) and the nucleus marker DAPI (blue) were examined under confocal microscopy. (C) THP-1 macrophages were infected by NL63 with or without treatment of 10 μM NLRP3 inhibitor (MCC950) for 48 h, or treated with LPS (400 ng/mL) for 6 h followed by ATP (5 mM) for 40 min. IL-1β level was quantified by ELISA (n = 4, LPS + ATP; n = 7, other groups). (D) Mature IL-1β and cleaved Casp-1 in supernatant, and pro-IL-1β, pro-Casp-1 and NLRP3 in lysates were determined by western blot. (E) THP-1 macrophages were infected by NL63 with or without treatment of 50 μM Casp-1 inhibitor (VX-765) for 48 h, or treated with LPS (400 ng/mL) for 6 h followed by ATP (5 mM) for 40 min. IL-1β level was quantified by ELISA (n = 4, LPS + ATP; n = 7, other groups). (F) Mature IL-1β and cleaved Casp-1 in supernatant, and pro-IL-1β, pro-Casp-1 and NLRP3 in lysates were determined by western blot. Data were normalized to the control (CTR, set as 1). Data are presented as means ± SD. *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001; NS, not significant (Mann-Whitney test). Super: Supernatant.

$TCID_{50}$ value was calculated by using the Reed-Muench method.

2.7. MTT assay

Cells were seeded in 96-well plates, and 10 mM 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma-Aldrich) was added. The plate was incubated at 37 °C with 5% CO2 for 3 h, then the medium was removed, and 100 μL of DMSO was added to each well. The plate was incubated at 37 °C for 1 h. The absorbance was read on the microplate absorbance reader (Bio-Rad, Hercules, CA, USA) at a wavelength of 490 nm.

2.8. Confocal microscopy

PMA-differentiated THP-1 cells was infected with NL63, 229E or OC43 for 48 h. Cells were fixed in 4% paraformaldehyde at room temperature for 15 min. After washing three times with PBS, the cells were permeabilized with PBS containing 0.1% Triton X-100 for 10 min, washed three times with PBS, and finally blocked with blocking buffer (Li-Cor Biosciences) for 1 h. The cells were then incubated with the anti-NF-κB p65 (C22B4) (Rabbit mAb, 4764) antibody (1:100), anti-dsRNA (10010200) antibody (1:200) or NLRP3 antibody (PA5-20838) (1:100) overnight at 4 °C, followed by incubation with anti-rabbit IgG(H + L),F(ab') 2 Fragment (4412s, Alexa Fluor 488 conjugate) (1:1000) or anti-mouse IgG secondary antibodies (H&l Alexa Fluor®594, Abcam) (1:1000) for 1 h. Nuclei were stained with VECTASHIELD® Antifade Mounting Medium with DAPI (13285184). Images were detected using Leica SP5 cell imaging system.

2.9. Immunoblot analysis

Concentrated supernatant or lysates were heated at 95 °C for 5 min. Proteins were subjected to a 15% or 10% sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE), separated at 100 V for 100 min, and electrophoretically transferred onto a PVDF membrane (pore size: 0.45 μm; Thermo Fisher Scientific Life Sciences) for 100 min with an electric current of 230 mA. Subsequently, the membrane was blocked with blocking buffer (Li-Cor Biosciences) in PBS containing 0.05% Tween-20. Membranes were incubated with primary antibodies overnight at 4 °C. The membrane was washed 3 times, followed by incubation for 1 h with anti-rabbit or anti-mouse IRDye-conjugated secondary antibodies (1:5000; Li-Cor Biosciences) at room temperature. β-actin served as the loading standard. The membrane was scanned by Odyssey Infrared Imaging System (Li-Cor Biosciences), which has two infrared fluorescent detection channels enabling simultaneous two-color target analysis. Results were visualized with Odyssey 3.0 software. Band intensity data of each immunoblot was also quantified by Odyssey Software.

2.10. Enzyme-linked immunosorbent assay (ELISA)

The concentrations of IL-1β in cell culture supernatant were measured by the ELISA Kit (BD Biosciences, San Jose, CA, USA). The concentrations of IL-1β in the serum of patients and rabbits were measured by Human IL-1 beta/IL-1F2 Quantikine ELISA Kit (E4818-100, Biovision) and Rabbit IL-1 beta/IL-1F2 Quantikine ELISA Kit (ml027836, MLBIO), respectively.

2.11. LDH release assay

Lactate dehydrogenase (LDH) released into cell culture supernatants after different treatments was measured using the CytoTox 96® Non-Radioactive Cytotoxicity Assay Kit (Promega) according to the manufacturer's suggested protocols. Briefly, THP-1 macrophages were inoculated with NL63, 229E or OC43 for 48 h or treatment with 500 ng/mL LPS for 6 h. Then, supernatant were harvested. The LDH activity in the culture supernatant was measured with 490 nm wavelength.

2.12. Co-culture of macrophages with Huh7 or A549 cells harboring seasonal coronaviruses

THP-1 cells were treated with 20 ng/mL of PMA at 37 °C for 48 h. Then, cells were cultured for another 6 h without PMA. Coculture of THP-1 macrophages with Huh7 or A549 cells was established at a ratio of 1:1, mimicking the human tissue environment.

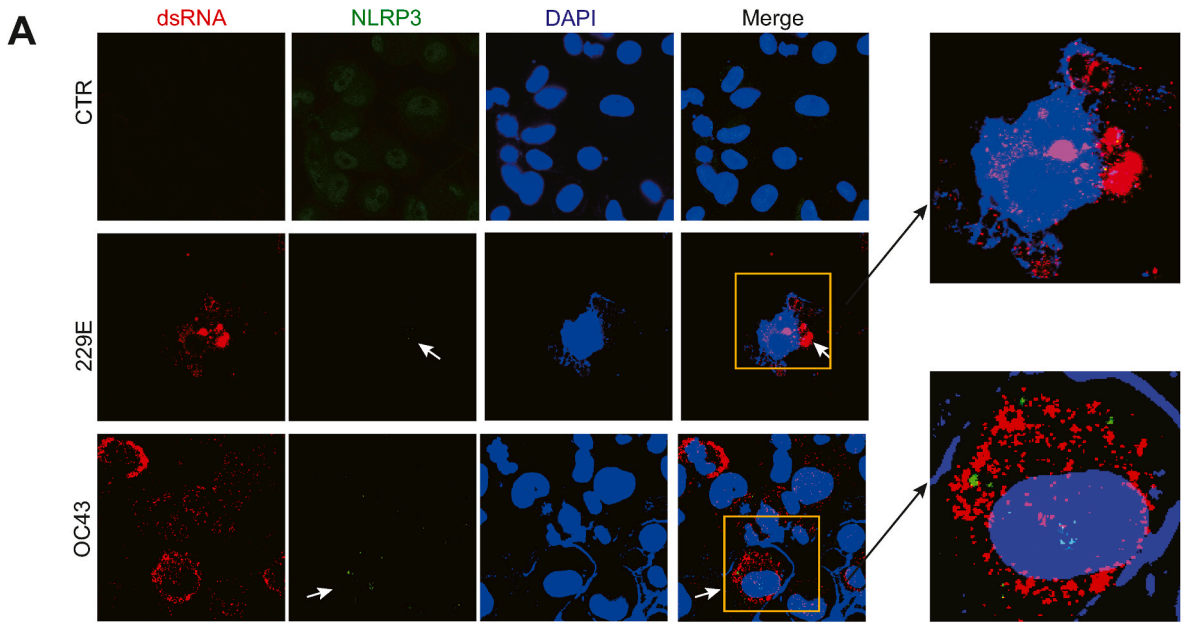
2.13. Statistical analysis

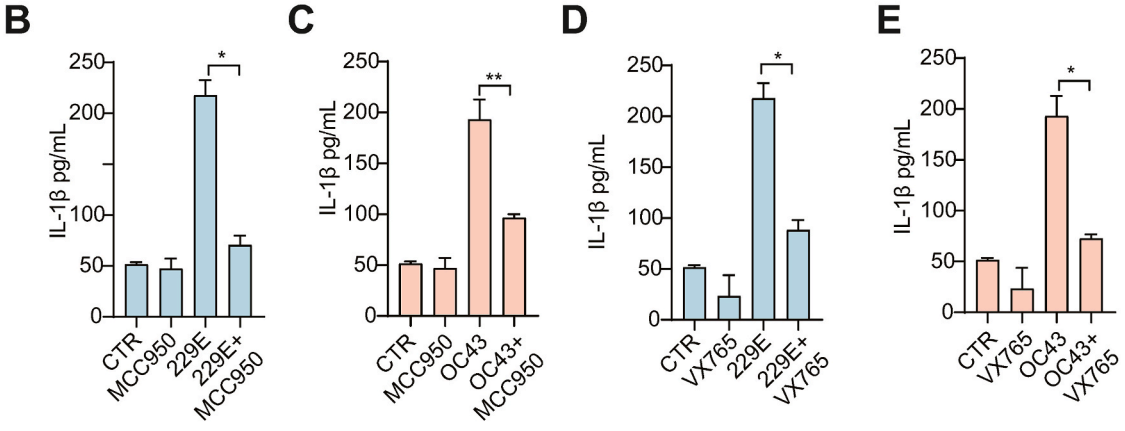
GraphPad Prism 7 software was used for data analysis using a Mann-Whitney test. All results were presented as mean ± standard deviation (SD). P values of less than 0.05 (single asterisks in figures) were considered statistically significant; whereas P values less than 0.01 (double asterisks), 0.001 (triple asterisks) and 0.0001 (four asterisks) were considered highly significant. Correlation was evaluated using Pearson correlation coefficient.

3. Results

3.1. Human macrophages support productive infection of seasonal coronaviruses

To investigate potential involvement of monocytes and macrophages in seasonal coronavirus infection, we first tested whether these cells support the infection of NL63, 229E and OC43. Since HKU1 is still not cultivable, it is not included in this study. We differentiated the THP-1 human monocyte cell line into macrophages by PMA treatment. Upon inoculation of thus generated macrophages with NL63, 229E or OC43 virus particles, cellular viral RNA levels dramatically increased over time (Fig. S1A, S1E and S1I). Further evidence of productive infection was obtained from experiments in which viral double-stranded RNA (dsRNA), the replication intermediate in the coronavirus life cycle, was visualized (Fig. S1B, S1F and S1J). We also found that HL60 macrophages are also susceptible for NL63, 229E and OC43 infections





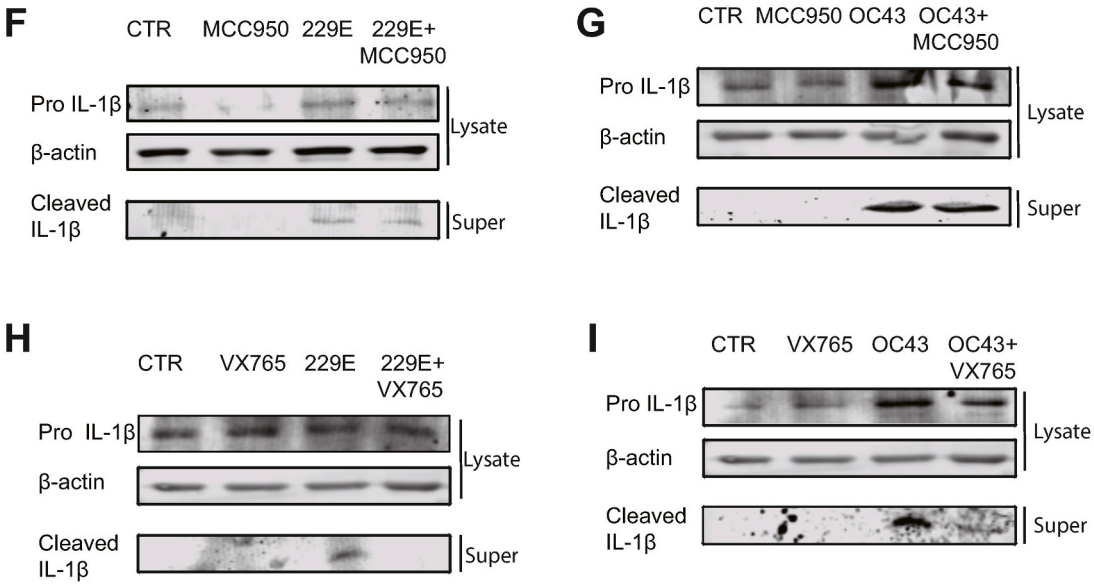


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Fig. 3. NLRP3 inflammasome assembly and caspase-1 activation are essential for seasonal coronavirus-triggered IL-1β secretion (A) THP-1 macrophages were inoculated with 229E or OC43 for 48 h 229E or OC43 double-stranded (ds) RNA (red), NLRP3 inflammasome (green) and the nucleus marker DAPI (blue) were examined under confocal microscopy. (B, C) THP-1 macrophages were infected by 229E or OC43 with or without treatment of 10 μM NLRP3 inhibitor (MCC950) for 48 h. IL-1β level was quantified by ELISA (n = 4–5) (n = 4, mcc950 group; n = 5, other groups). (F, G) Mature IL-1β in supernatant and pro-IL-1β in lysates were determined by western blot. (D, E) THP-1 macrophages were infected by 229E or OC43 with or without treatment of 50 μM Casp-1 inhibitor (VX-765) for 48 h. IL-1β level was quantified by ELISA (n = 4). (H, I) Mature IL-1β in supernatant and pro-IL-1β in lysates were determined by western blot. Data were normalized to the control (CTR, set as 1). Data are presented as means ± SD. *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001; NS, not significant (Mann-Whitney test). Super: Supernatant.

(Figs. S2A–C). Similarly, THP-1 monocytes and HL60 monocytes also supported seasonal coronavirus infection, albert to a lesser extent (Figs. S2D–I). Next, we investigated whether seasonal coronaviruses infect and produce infectious particles in human macrophages. Naïve LLCMK2 or A549 cells were inoculated with conditioned cell culture medium from NL63-, 229E- or OC43-infected THP-1 cells. Detection of dsRNA indicated active infection with NL63, 229E or OC43 (Fig. S1C, S1G and S1K) upon secondary inoculation in these cell systems. Moreover, infectious particles are increased in supernatant over time (Figs. S1D and S1H and S1L). Collectively, these results showed that human monocytes and macrophages support the full life cycle of seasonal coronaviruses, but the levels of susceptibility and viral particle production vary among the NL63, 229E and OC43 strains in these cell types.

3.2. Anecdotal evidence indicates possible activation of inflammasome in a subset of seasonal coronavirus infected patients

Inflammasome activation in macrophages by SARS-CoV-2 infection has been observed in COVID-19 patients (Rodrigues et al., 2021; Sefik et al., 2022). To gauge whether this also occurs in seasonal coronavirus infected patients, we measured IL-1β levels in serum of patients infected with NL63 (n = 10; Fig. 1A), 229E (n = 10; Fig. 1B) or OC43 (n = 10; Fig. 1C), as IL-1β release is the hallmark of inflammasome activation. We compared the level in samples collected at the time of active infection and following recovery (4 weeks post-infection). Although there is no statistical significance when considering the overall groups, elevation of IL-1β during infection appears evident in four NL63 (Fig. 1A), and two 229E (Fig. 1B) infected patients. This appears in accordance with the clinical feature that only a small subset of these patients develop severe complications (Callaway, 2021). These preliminary results encouraged us to further explore the presence of more concrete evidence of seasonal coronavirus-triggered inflammasome activation in preclinical models.

3.3. Seasonal coronaviruses trigger inflammatory response and pyroptosis in cultured human macrophages

To determine whether seasonal coronaviruses activate inflammatory response in human macrophages, we measured the expression and production of IL-1β. We found that NL63 infection robustly induced IL-1β mRNA expression (Fig. S3A) and protein expression (Fig. 1D), comparable to the positive control treated with lipopolysaccharides (LPS) together with ATP. The expression and secretion of IL-1β induced by NL63 was consistent with the time lapsed following initial infection (Fig. 1D, S3A). Western blotting confirmed an increase in pro-IL-1β and the secretion of mature IL-1β in cell lysate and supernatant respectively (Fig. 1E). In apparent agreement, we observed that NL63 triggered IL-1β mRNA expression (Fig. S3B) and secretion of the corresponding protein (Fig. 1F) in a dose-dependent manner. Production of both pro-IL-1β and cleaved IL-1β was increased with inoculation of increased titers of NL63 (Fig. 1G).

Similarly, 229E and OC43 infections also significantly induced mRNA expression (Figs. S3D and S3E) and protein secretion of IL-1β (Fig. 1H and J) in THP-1 macrophages. Robust production of pro-IL-1β and cleaved IL-1β were also induced by 229E or OC43 (Fig. 1I and K). In HL60 macrophages, NL63, 229E or OC43 infection moderately induced IL-1β secretion (Fig. S3F, S3G and S3H). Interestingly, expression of pro-IL-1β was significantly induced by 229E and OC43 but not NL63 (Figs. S3G and S3H). In addition, NL63, 229E and OC43 also induced the expression of other pro-inflammatory cytokines (Figs. S3I and S3J).

Pyroptosis is an inflammatory and lytic form of programmed cell death that is triggered through activation of the inflammasome. Thus, we measured LDH level, a hallmark of pyroptosis, in supernatant of seasonal coronavirus-infected cells. Our results showed that NL63, 229E or OC43 infection induced LDH release in human macrophages, comparable to that by LPS treatment (Fig. 1L-N). Taken together, NL63, 229E and OC43 infections induced IL-1β secretion and LDH release in human macrophages.

3.4. NLRP3 inflammasome assembly and caspase-1 activation are essential for seasonal coronavirus-triggered inflammatory response

The NLRP3 inflammasome response during RNA virus infection is mediated through caspase-1 activation (Fig. 2A) (Mangan et al., 2018). We observed that NL63 infection in human macrophages triggered NLRP3 inflammasome assembly (Fig. 2B). Consistently, IL-1β production was profoundly inhibited by treatment of an NLRP3 inhibitor (MCC950) (Fig. 2C). Western blotting results showed that cleaved IL-1β was also inhibited (Fig. 2D). Production of cleaved caspase-1 was induced by NL63 but inhibited by MCC950 treatment (Fig. 2E). As expected, IL-1β expression was effectively blocked by VX765, a caspase-1 inhibitor (Fig. 2E and F). However, neither MCC950 nor VX765 treatment impaired pro-IL-1β expression and cell viability (Fig. 2D and F and Figs. S4A and S4B). Our results demonstrate that NL63 infection triggers NLRP3 inflammasome-mediated inflammatory response via caspase-1 activation. Similar results were observed in 229E and OC43 infections (Fig. 3).

OC43 and 229E infection in human macrophages also triggered NLRP3 inflammasome assembly (Fig. 3A). Consistently, IL-1β production was profoundly inhibited by treatment of the NLRP3 inhibitor (MCC950) or caspase-1 inhibitor (VX765) (Fig. 3B, C, 3D and 3E). Western blotting results showed that cleaved IL-1β was also inhibited (Fig. 3F, G, 3H and 3I). However, neither MCC950 nor VX765 treatment impaired the expression of pro-IL-1β (Fig. 3F, G, 3H and 3I). Interestingly, the NLRP3 inhibitor and caspase-1 inhibitor exerted variable effects on the replication of different coronaviruses (Figs. S4C and S4D). Taken together, seasonal coronaviruses trigger inflammatory response via NLRP3 inflammasome and caspase-1 activation in human macrophages.

3.5. NF-κB signaling activation is required for seasonal coronavirus-induced NLRP3 inflammasome response

NF-κB, as a transcription factor, is responsible for upregulating the expression of a range of genes that mediate interferon and inflammatory responses. To determine whether the NF-κB pathway is activated, THP-1 macrophages were inoculated with NL63, 229E or OC43 virus particles respectively. All the infections induced nuclear translocation of RelA, a

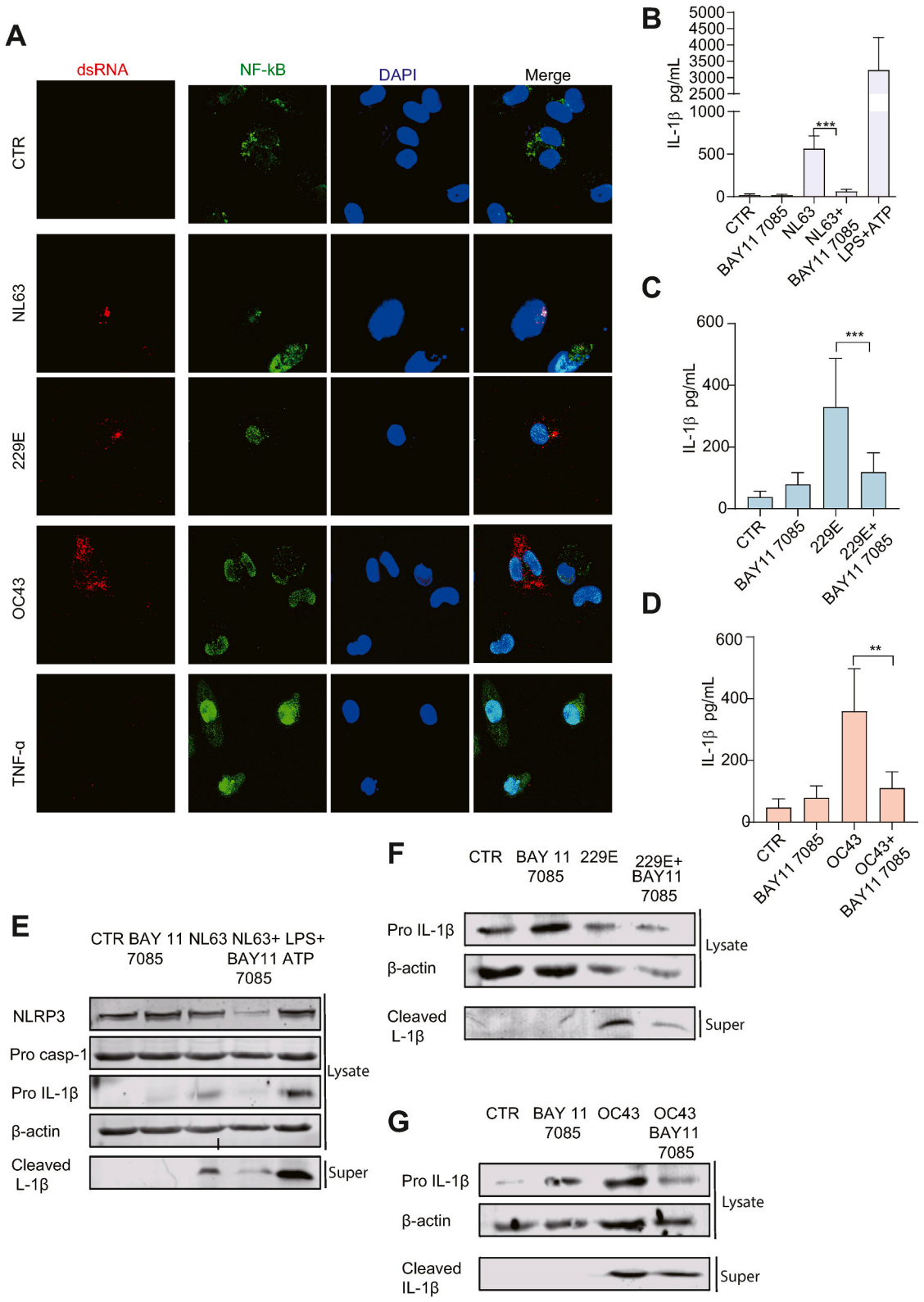

Fig. 4. NF-κB activation is essential for seasonal coronavirus-induced NLRP3 inflammasome response (A) THP-1 macrophages were inoculated with NL63, 229E or OC43 for 48 h or treated with TNF-α (100 ng/mL) for 24 h. Subcellular localization of NF-κB (green), NL63, 229E or OC43 double-stranded (ds) RNA (red) and the nucleus marker DAPI (blue) was examined under confocal microscopy. (B, C and D) THP-1 macrophages were inoculated with NL63, 229E or OC43 with or without treatment of 10 μM NF-κB inhibitor (BAY11 7085) for 48 h, or treated with LPS (400 ng/mL) for 6 h followed by ATP (5 mM) for 40 min. IL-1β level was quantified by ELISA (n = 4, BAY11 7085 group; n = 8, other groups). (E, F and G) Mature IL-1β in supernatant and pro-IL-1β, pro-Casp-1 or NLRP3 in lysates were determined by western blot. Data were normalized to the control (CTR, set as 1). Data are presented as means ± SD. *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001; NS, not significant (Mann-Whitney test). Super: Supernatant.

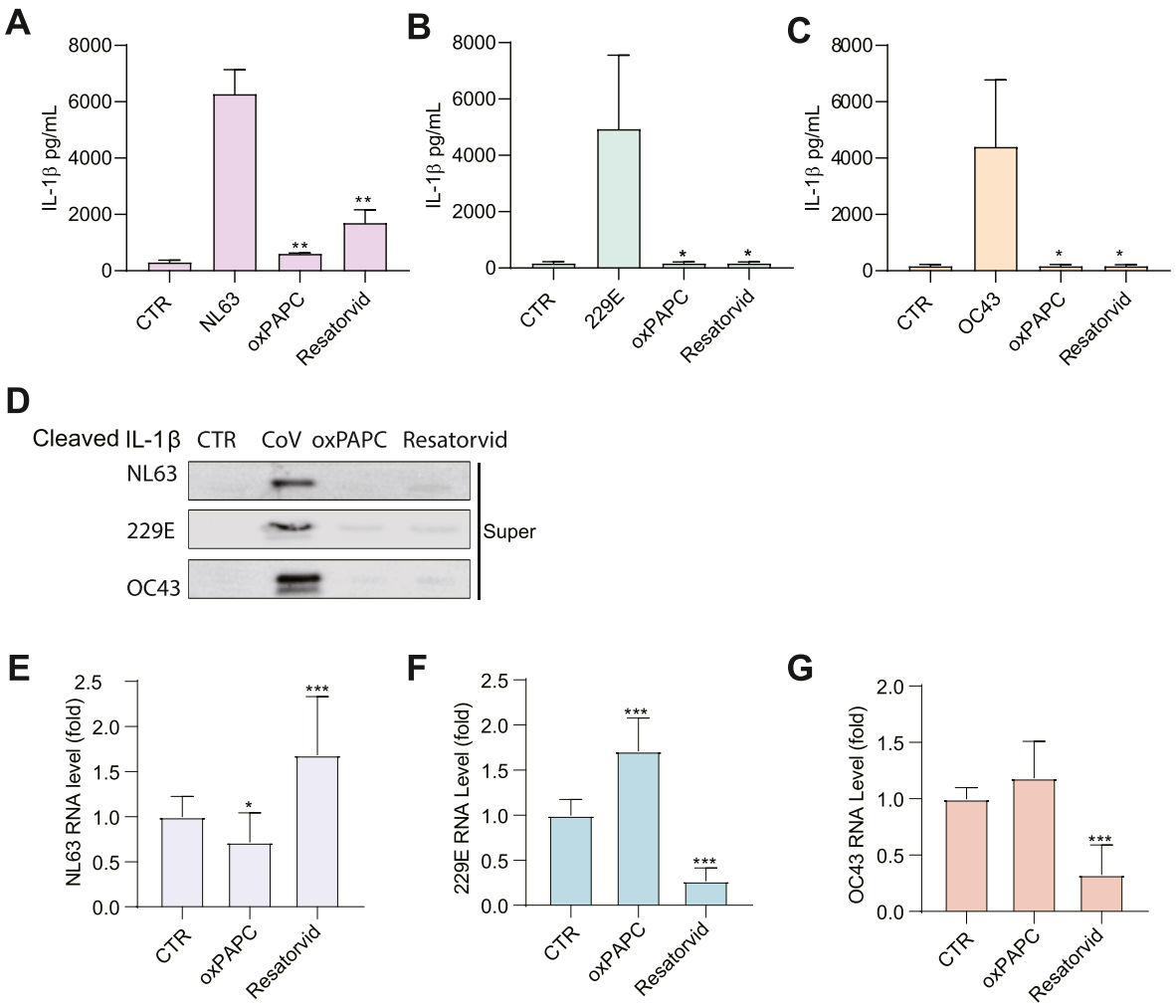

Fig. 5. TLR4 senses seasonal coronaviruses to trigger IL-1β secretion. (A, B and C) THP-1 macrophages were inoculated with 30 μg/mL oxPAPC or 10 μM resatorvid for 2 h, following NL63, 229E or OC43 infection for 48 h with or without treatment of 30 μg/mL oxPAPC or 10 μM resatorvid. IL-1β in the supernatant was quantified by ELISA (n = 5, NL63 group; n = 4, OC43 and 229E groups). (D) THP-1 macrophages were inoculated with 30 μg/mL oxPAPC or 10 μM resatorvid for 2 h, following NL63, 229E or OC43 infection for 48 h with or without treatment of 30 μg/mL oxPAPC or 10 μM resatorvid. Mature IL-1β in supernatant was determined by western blot. (E, F and G) THP-1 macrophages were incubated with oxPAPC or Resatorvid for 2 h, following NL63, 229E or OC43 infection for 48 h with or without oxPAPC or Resatorvid, and viral RNA level in the cell lysate was quantified by qPCR (n = 12, NL63 group; n = 8, OC43 and 229E groups). Data were normalized to the control (CTR, set as 1). Data are presented as means ± SD. *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001; NS, not significant (Mann-Whitney test). Super: Supernatant.

hallmark of NF-κB activation, comparable to the positive control (TNF-α treatment) (Fig. 4A). Generally, two steps are needed for IL-1β activation, the first step being the induction of pro-IL-1β and NLRP3 expression by NF-κB activation and the second step being the IL-1β cleavage by the NLRP3 inflammasome. Consistently, pharmacological inhibition of NF-κB by BAY11 7085 inhibited NL63-induced IL-1β secretion (Fig. 4B). The expression of both NLRP3 and pro IL-1β was dramatically inhibited (Fig. 4B and E). Of note, BAY11 7085 treatment has some mild effect on cell viability (Fig. S5A).

Similar results were observed in 229E- or OC43-infected macrophages (Fig. 4C, D, 4F and 4G), but the effect was less profound in OC43 infection (Fig. 4D and G). Unexpectedly, NF-κB inhibition increased NL63 replication but decreased 229E and OC43 replication in macrophages (Fig. S5B). Taken together, NF-κB activation is likely a key upstream event priming seasonal coronavirus-triggered NLRP3 inflammasome response.

3.6. *TLR4 senses seasonal coronaviruses to produce IL-1β*

Toll-like receptors (TLRs) are pattern recognition receptors (PRRs) for the defense against invading pathogens. TLR2 and TLR4 located at cell surface and have been reported to recognize SARS-CoV-2 infection and mediate inflammatory response. Moreover, it has been reported that TLR4 is elevated in patients with severe and critical COVID-19, and associated with disease progression (Khanmohammadi and Rezaei, 2021; Mukherjee, 2022). To investigate whether TLR2 or TLR4 are involved in inflammatory response during seasonal coronavirus infection, we employed pharmacological inhibitors to block TLR2 or TLR4. We found that oxPAPC treatment, an inhibitor of TLR2 and TLR4, effectively blocked NL63-induced IL-1β protein secretion into supernatant and IL-1β gene expression in macrophages (Fig. 5A and D and S6A). To identify which TLR is involved in NL63-induced inflammatory response, we used resatorvid, a specific inhibitor of TLR4 to block its

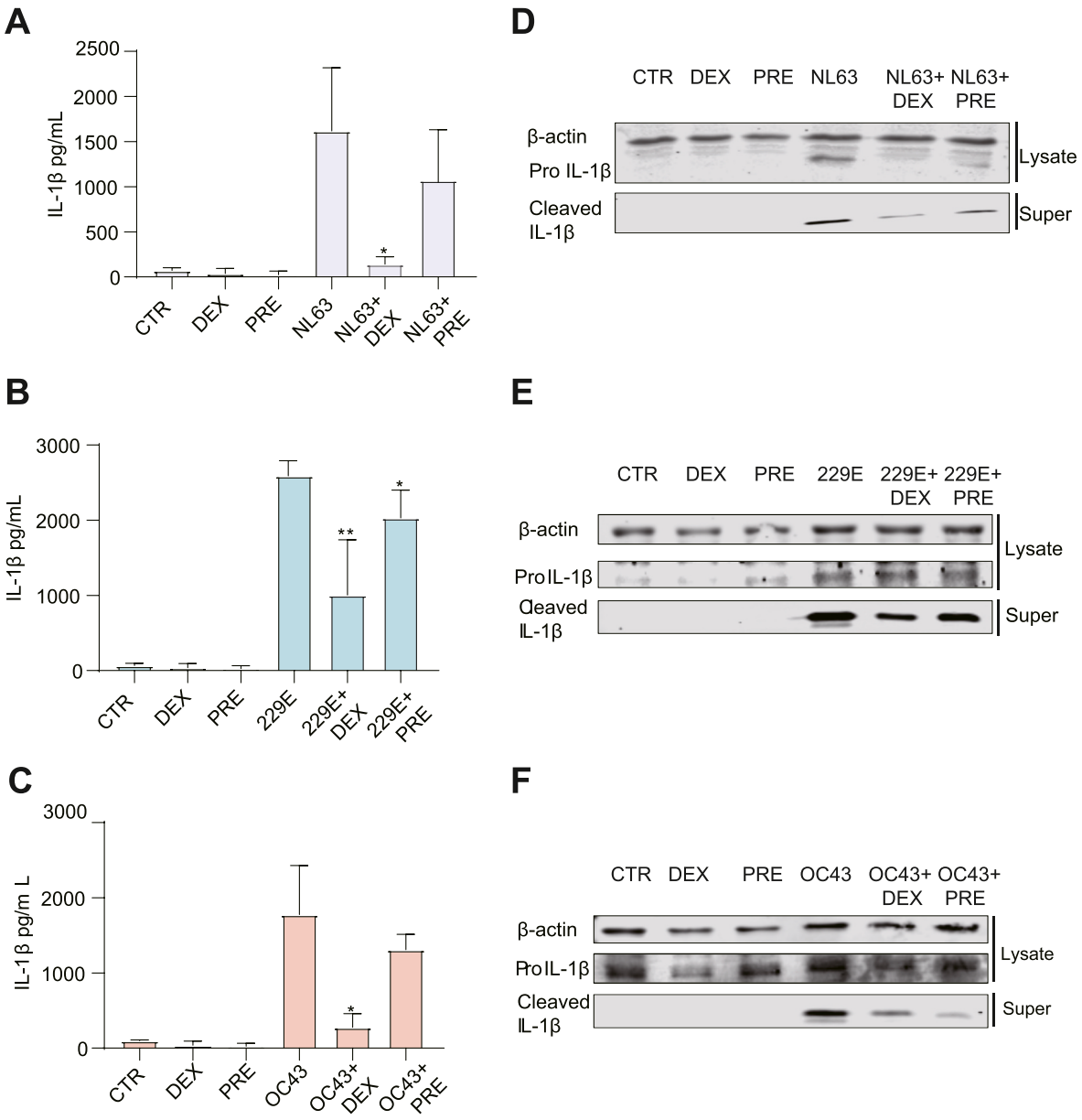

Fig. 6. Steroids inhibited seasonal coronavirus-triggered inflammatory response. (A , B and C) THP-1 macrophages were inoculated with NL63, 229E or OC43 for 48 h with or without treatment of 10 μM DEX or 10 μM PRE, and IL-1β in the supernatant was quantified by ELISA (n = 4, DEX or PRE group; n = 6, other groups). (D, E and F) THP-1 macrophages were inoculated with NL63, 229E or OC43 for 48 h with or without treatment of DEX or PRE, and IL-1β in the supernatant and pro IL-1β in cell lysate was quantified by Western blot. Data are presented as means ± SD. *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001; NS, not significant (Mann-Whitney test). Super: Supernatant.

function. We found that TLR4 inhibition markedly inhibited IL-1β production comparable to that of oxPAPC treatment (Fig. 5A, S6A). We also demonstrated that TLR4 essentially mediates 229E- or OC43-induced IL-1β production and secretion in human macrophages (Fig. 5B, C, 5D and S6A). Interestingly, oxPAPC and resatorvid appear to have differential effects on the replication of these three viruses (Fig. 5E, F and 5G). Taken together, TLR4 senses seasonal coronaviruses to induce IL-1β production in human macrophages.

3.7. *Drug profiling identifies dexamethasone and flufenamic acid as inhibitors of seasonal coronavirus-triggered inflammasome response*

Steroids, a classical anti-inflammatory drug, have shown to reduce mortality in hospitalized COVID-19 patients (Group et al., 2021). We found that both dexamethasone and prednisone treatment attenuated seasonal coronavirus-induced IL-1β secretion in human macrophages (Fig. 6A–F), although the effect of prednisone is moderate. Both steroids also attenuated virus-induced pro-IL-1β expression in cell lysate (Fig. 6D–F).

Next, we profiled different types of potential anti-inflammatory drugs including nucleoside reverse transcriptase inhibitors and non-steroidal anti-inflammatory drugs. We found that flufenamic acid inhibited NL63-, 229E- or OC43-induced IL-1β production in human macrophages (Fig. 7A–C and S7A) without affecting cell viability (Fig. S7C). We further demonstrated that flufenamic acid inhibited IL-1β production in a dose-dependent manner (Fig. 7D–G). Flufenamic acid also inhibit 229E replication but not NL63 or OC43 (Fig. S7B).

3.8. *Combining antiviral and anti-inflammatory treatment: a proof-of-concept*

Because of pathological hyperinflammation in severe coronavirus

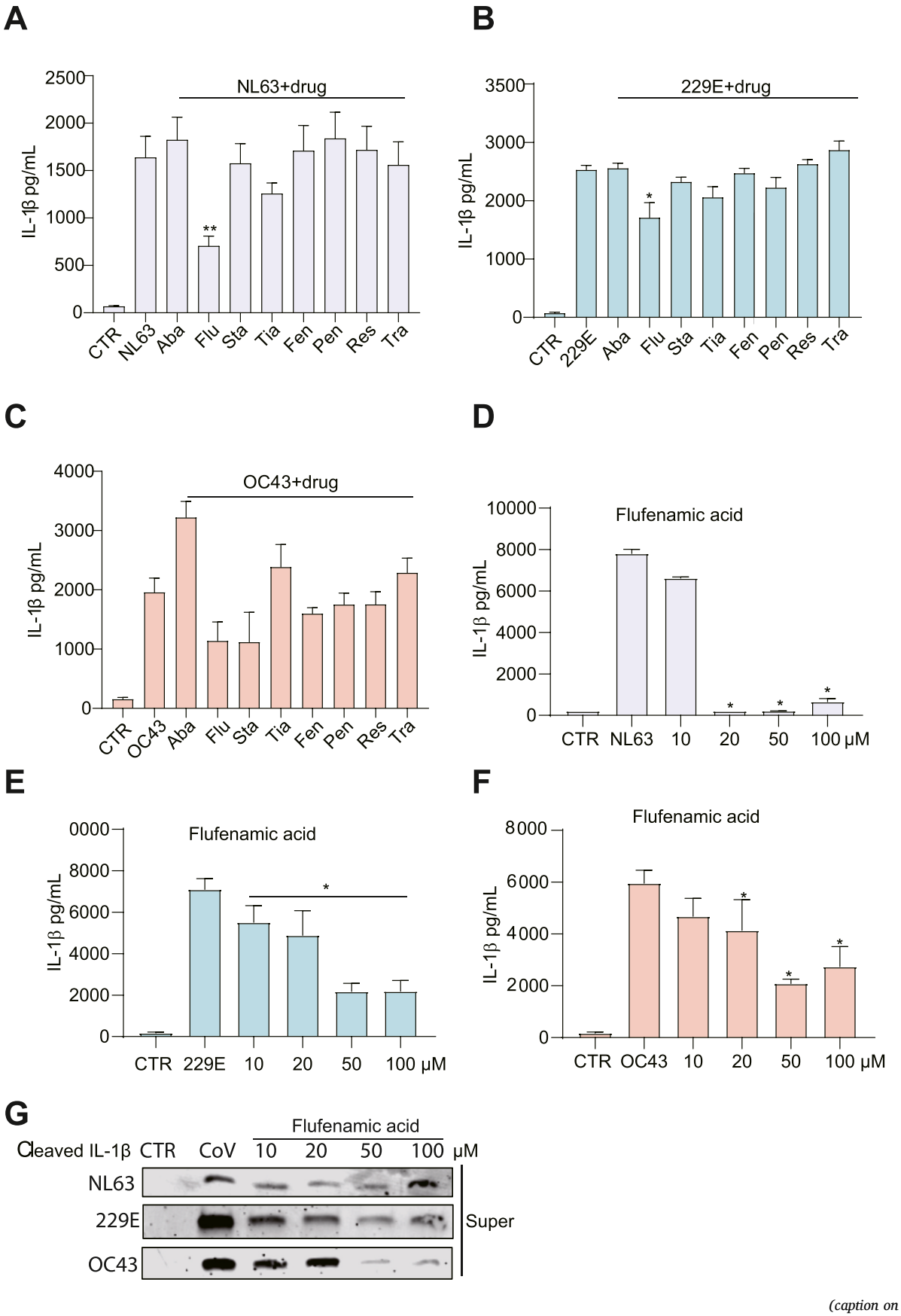

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Fig. 7. Drug profiling identifies inhibitors of seasonal coronavirus-triggered inflammatory response. (A , B and C) THP-1 macrophages were inoculated with NL63, 229E or OC43 for 48 h with or without treatment of indicated agents (20 μM), and IL-1β in the supernatant was quantified by ELISA (n = 6, NL63 group; n = 4, OC43 and 229E groups). (D, E and F) THP-1 macrophages were inoculated with NL63, 229E or OC43 for 48 h with treatment of 10 μM, 20 μM, 50 μM or 100 μM flufenamic acid, and IL-1β in the supernatant was quantified by ELISA (n = 4). (G) THP-1 macrophages were inoculated with NL63, 229E or OC43 for 48 h with treatment of 10 μM, 20 μM, 50 μM or 100 μM flufenamic acid, and mature IL-1β in supernatant was determined by western blot.Aba, abacavir; Flu, flufenamic acid; Sta, stavudine; Tia, tiaprofenic acid: Fen, fenamic acid; Pen, pentoxifylline; Res, resveratrol; Tra, tranilast. DEX, dexamethasone; PRE, prednisone. Data were normalized to the control (CTR, set as 1). Data are presented as means ± SD. *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001; NS, not significant (Mann-Whitney test). Super: Supernatant.

infection, we hypothesize that antiviral therapy alone is insufficient, whereas further combining inflammasome-targeted anti-inflammatory treatment may achieve better outcome. We first confirmed that molnupiravir, a clinically used antiviral drug for treating COVID-19, effectively inhibited 229E and OC43 infection in A549 cell lines (Fig. S8A) without affecting cell viability (Fig. S8B). Given that macrophages are not the primary target of seasonal coronavirus infection, we first established a co-culture system of huh7 or A549 epithelial cells with THP-1 macrophages to mimic the tissue environment for assessing combination therapy (Fig. 8A). In this system, molnupiravir treatment alone significantly inhibited NL63 replication, but not IL-1β production (Fig. 8B–D). Anti-inflammatory drugs (dexamethasone or flufenamic acid) treatment alone significantly inhibited NL63-induced IL-1β production but not NL63 replication (Fig. 8B–D). The combination of molnupiravir with dexamethasone or flufenamic acid simultaneously inhibited NL63-induced IL-1β production and NL63 replication (Fig. 8B–D), without evidence of cross-interference. Similar results were observed in models of 229E or OC43 infection (Fig. 8E–J).

3.9. The relevance of NLRP3 inflammasome response in SARS-CoV-2 Omicron infection, a "prototype" of seasonal coronavirus

Considering the evolvement of SARS-CoV-2 in particular the emergence of Omicron variants that may resemble seasonal coronavirus to some extent, we finally tested the impact of the Omicron variant on inflammasome response. We found that inoculation cultured human macrophages with the BA.1 Omicron sub-variant effectively activated IL-1β expression at both protein and RNA levels (Fig. S9A, S9B and S9C). This activation was attenuated by treatment with NF-κB or NLRP3 inhibitors (Figs. S9D and S9E). Combination of molnupiravir with dexamethasone or flufenamic acid simultaneously inhibited Omicron-induced inflammatory response and viral replication (Fig. S9F, S9G and S9H). Thus, these results are largely in lines with the observations of seasonal coronavirus infections in human macrophages.

4. Discussion

It has been well-described that massive inflammation accompanied with a cytokine storm is a prominent feature of severe COVID-19 (Fajgenbaum and June 2020). This pathology is closely related to intense, rapid activation of inflammasome pathway and release of its products including the pro-inflammatory cytokine IL-1β (Sefik et al., 2022). In general, the symptoms of seasonal coronavirus infection are mild, but severe complications including fatality have been reported for specific populations, in particular young children and immunocompromised patients (Li et al., 2021; Pinana et al., 2021). However, the pathogenic mechanism has been hardly studied. In this study, we first probed the clinical evidence of possible inflammatory response in seasonal coronavirus infected patients from a previously established cohort (Edridge et al., 2020; Loens et al., 2012). By measuring the serum levels of IL-1β, a hallmark of inflammasome activation, we observed that a subset of patients appears to have elevated levels at the time of infection when compared with 4-week post-infection (recovered). This is in line with the general clinical feature that only a small proportion of seasonal coronavirus infected patients develop severe complications (Li et al., 2021). However, we do not have the clinical data of these patients, and also samples from pre-infection were not available which would be the better control. Although future clinical studies are required to better investigate host response to seasonal coronavirus infection, this anecdotal evidence suggesting possible activation of inflammasome in some infected patients encouraged our further investigation in experimental models.

Macrophages are key, although not the only, players in hyper-inflammation of severe COVID-19, resulting in tissue damage of multiple organs besides the lung. SARS-CoV-2 can infect and activate NLRP3 inflammasome in macrophages (Sefik et al., 2022). In this study, we found that human macrophages support productive infection of three cultivable seasonal coronaviruses (NL63, OC43 and 229E) in cell culture. However, the level of infection in macrophages is likely not comparable to that in the primary targeting cell type such as lung epithelial cells. Nevertheless, this is sufficient in triggering robust activation of NLRP3 inflammasome, evidenced by a variety of assays including NLRP3 complex assembly, caspase-1 auto-activation, pro-IL-1β cleavage, mature IL-1β production, LDH release and inflammatory gene expression. This study only focused on NLRP3 inflammasome, since both previous studies (Sefik et al., 2022) and our findings point to the predominant role of NLRP3 inflammasome in sensing coronaviruses in macrophages. However, we do not exclude the possible involvement of other inflammasome receptors in particular when considering the response in other cell types, which is interesting to be further studied.

Different pattern recognition receptors (PRRs), including TLRs, nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs), and retinoic-acid-inducible gene I (RIG-I)-like receptors (RLRs), located on the cell surface or in the cytoplasm are responsible for recognizing viral elements and subsequent triggering inflammatory response or/and interferon response. Several PRRs have been reported to be involved in sensing coronaviruses, including TLR2, TLR4 and TLR7 (Channappanavar et al., 2019; Khan et al., 2021; Zhao et al., 2021; Zheng et al., 2021). TLR7 is essential for MERS-CoV induced interferon production in plasmacytoid dendritic cells (Channappanavar et al., 2019). TLR2 and TLR4 have been shown to sense SARS-CoV-2 envelope or spike protein to trigger the production of inflammatory cytokines (Khan et al., 2021; Zhao et al., 2021; Zheng et al., 2021). To figure out whether TLR2 and/or TLR4 are involved in seasonal coronavirus triggered inflammasome response, we first employed oxPAPC, a pharmacological inhibitor blocking both TLR2 and TLR4. We found that oxPAPC treatment profoundly attenuated the induction of IL-1β. Next, we used resatorvid, an specific inhibitor of TLR4, and comparable level of attenuation was observed. These results suggest that seasonal coronavirus-induced inflammasome activation is primarily through TLR4 ligation. Nevertheless, this study did not investigate which element of seasonal coronaviruses serves as the ligand, and this question should be studied in the future.

NLRP3 activation requires two cascades: the priming signal via NF-κB activation and the second signal of assembling the NLRP3 inflammasome complex (Swanson et al., 2019). Indeed, treatment with pharmacological inhibitors of NF-κB or NLRP3 dramatically blocked seasonal coronavirus triggered inflammatory response in macrophages. This provides the rationale of therapeutic intervention to treat infection caused pathological inflammation. Here, we observed an intriguing finding that blocking TLR4 or NF-κB activation differentially regulate the infection of NL63, OC43 and 229E. Treatment with resatorvid (targeting TLR4) or BAY11 7085 (NF-κB inhibitor) promoted NL63 but inhibited OC43 and 229E replication. It has been reported that

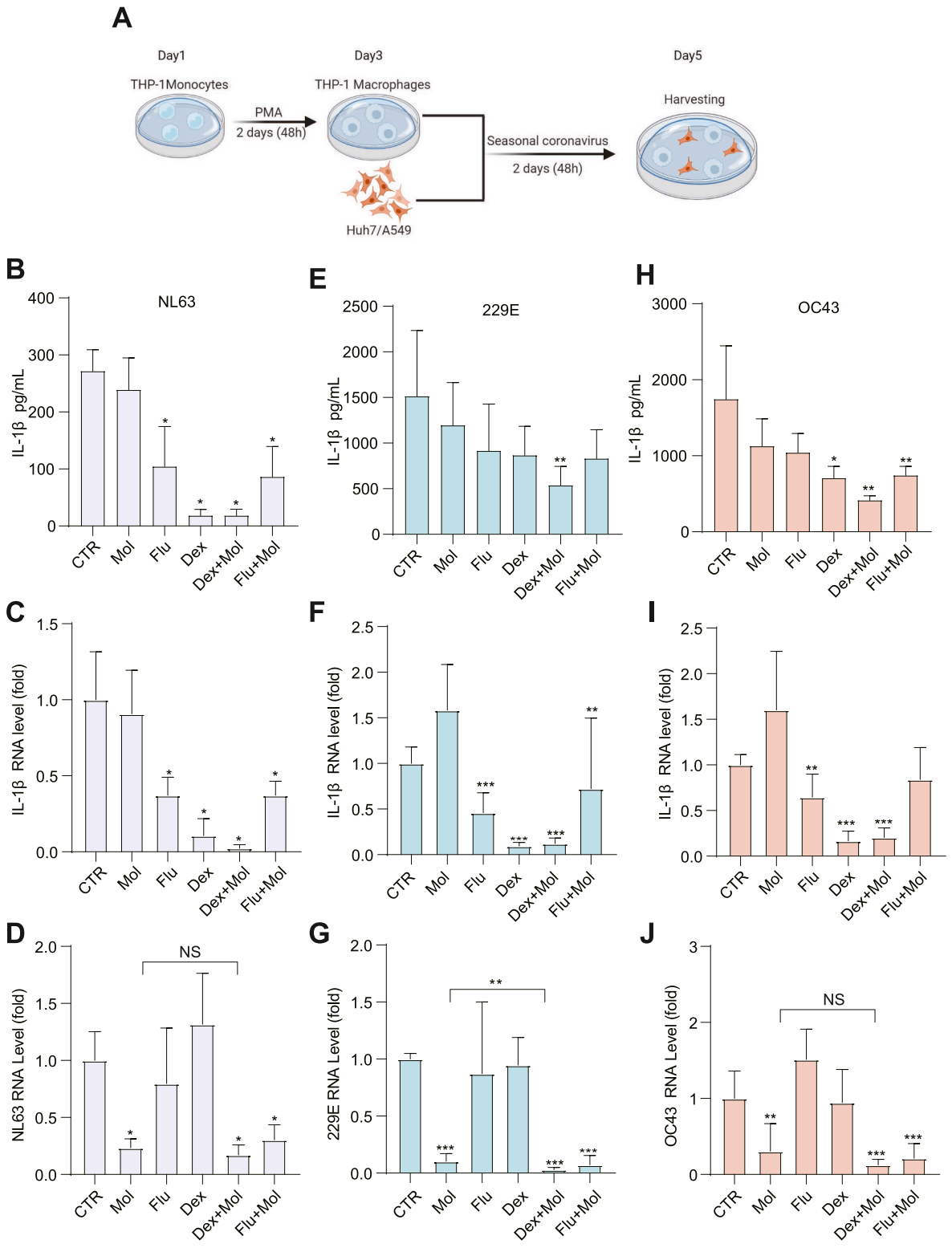

Fig. 8. Combining anti-inflammatory and antiviral treatment against seasonal coronavirus infection in a co-culture system. (A) Schematic illustration of the co-culture system of epithelial cells with THP-1 macrophages infected with seasonal coronaviruses. This illustration was prepared by using the web-based tool BioRender. The co-culture system was inoculated with NL63, 229E or OC43 particles, and traeted with10 μM molnupiravir, 10 μM Flu, 10 μM Dex, 10 μM Dex plus 10 μM molnupiravir or 10 μM Flu plus 10 μM molnupiravir for 48 h (B, E and H) IL-1β levels in supernatant were quantified by ELISA (n = 6, NL63 group; n = 4, OC43 and 229E groups). (C, F and I) IL-1β mRNA levels were quantified by qPCR (n = 8, NL63 group; n = 4, OC43 and 229E groups). (D, G and J) Viral RNA levels were quantified by qPCR (n = 10, NL63 group; n = 4, OC43 and 229E groups). Data were normalized to the control (CTR, set as 1). Mol, Molnupiravir; Flu, Fflufenamic acid; Dex, Dexamethasone. Data are presented as means ± SD. *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001; NS, not significant (Mann-Whitney test).

NF-kB-mediated inflammatory response can antagonize interferon response during viral/bacterial infections (Burke et al., 2020; Li et al., 2022). However, whether this can partially explain our observations remain questionable, and further research is required to understand the underlying mechanisms.

Currently, there is no clinically approved specific inhibitors of NLRP3 inflammasome. Encouragingly, repurposing the classical anti-inflammatory steroid drug, dexamethasone, has been indicated to inhibit SARS-CoV-2 triggered inflammasome activation (Hooftman and O'Neill, 2021), and reduce mortality of hospitalized COVID-19 patients (Group et al., 2021). Our results showed that both dexamethasone and prednisolone can inhibit seasonal coronavirus-triggered inflammasome activation, although the potency of prednisolone is very moderate. However, steroids are far from satisfactory for treating viral diseases. For example, they can directly promote the replication of SARS-CoV-2 and many other viruses (Yuan et al., 2022). Therefore, we attempted testing a panel of other potential anti-inflammatory drugs including nucleoside reverse transcriptase inhibitors and non-steroidal anti-inflammatory drugs. Several nucleoside reverse-transcriptase inhibitors, which are drugs approved to treat human immunodeficiency virus and hepatitis B virus infections, have been shown to block NLRP3 inflammasome activation (Ambati et al., 2020). Our profiling identified that flufenamic acid, a member of the nonsteroidal anti-inflammatory drugs, dose-dependently inhibited seasonal coronavirus-induced inflammasome activation in macrophages. Flufenamic acid is a cyclooxygenase inhibitor, inhibiting the formation of prostaglandins. It is available as a generic medicine in some Asian and European countries. Although the inhibition remains partial, our results demonstrated the feasibility of identifying and repurposing existing medications for treating virus-triggered inflammasome activation. There are several specific NLRP3 inhibitors being developed, with a few entering early clinical trials (Coll et al., 2022). Although the results from most of these clinical studies are not yet available (Coll et al., 2022), this new development does offer a future perspective of treating virus-caused hyper-inflammation with potent and specific NLRP3 inhibitors.

For patients with severe infection of SARS-CoV-2 or seasonal coronavirus, we postulate that antiviral or anti-inflammatory treatment alone would not be sufficient. Ideally, a single drug would potently inhibit both the virus and pathological inflammation. Through drug screening, a recent study identified niclosamide inhibiting both inflammasome activation and SARS-CoV-2 replication (de Almeida et al., 2022). Similar observations have been reported for MEK inhibitors (targeting the MAPK pathway) in experimental models of SARS-CoV-2 infection (Schreiber et al., 2022). These studies have demonstrated proof-of-concept, but their effectiveness remains to be further improved, for example through combination treatment. In this study, we extensively evaluated the combination of anti-inflammatory drugs with the clinically used oral antiviral drug for COVID-19, molnupiravir (Jayk Bernal et al., 2022). We recently demonstrated that molnupiravir has pan-coronavirus antiviral activity, including against the three tested seasonal coronaviruses (Wang et al., 2021). Here, in the co-culture of human epithelial cells with macrophages, we found that these combinations resulted in simultaneous inhibition of viral replication and inflammatory response. We believe this approach bears great potential of improving the current treatment for severe coronavirus infection, although further validation in animal models is required before clinical testing.

It is inevitable that SARS-CoV-2 is joining with the four seasonal coronaviruses to become the fifth endemic human coronavirus (Katzourakis, 2022). The rapid evolvement of new variants has offered clues to how SARS-CoV-2 is adapting and how the pandemic may eventually play out over the next years (Callaway, 2021). The currently circulating Omicron (sub-)variants harbor a large number of mutations and most of them are located around the receptor binding domain of the spike protein (Armando et al., 2022; Solanki et al., 2022). Overall, both clinical and experimental results indicated that Omicron primarily targets the upper respiratory track with attenuated pathogenesis compared to the previous variants, although remains highly transmissible (Shuai et al., 2022; Suzuki et al., 2022). However, infection with the Omicron variant particularly in immunocompromised patients can still cause severe complications including fatality (de Prost et al., 2022). In this study, we found that inoculation of human macrophages with the Omicron BA.1 (sub-)variant is capable of activating inflammasome response. We speculate that activation of NLRP3 inflammasome may be a pan-coronavirus mechanism of causing pathological inflammation, whereas the levels of activation are likely distinct among different patient populations and/or having the infection of different coronaviruses.

In summary, this study demonstrated that seasonal coronaviruses are capable of activating NLRP3 inflammasome in human macrophages, which may represent a key mechanism of causing hyperinflammation in infected patients. Nevertheless, coronavirus-triggered inflammasome activation can be pharmacologically targeted by specific inhibitors or clinically used (anti-inflammatory) medications. Importantly, combination of antiviral drug with anti-inflammatory agent simultaneously inhibited viral replication and inflammatory response. These findings shall help to develop improved strategies for managing patients severely infected with seasonal coronaviruses. Furthermore, it also bears implications for the COVID-19 pandemic, as the new variants of SARS-CoV-2 may evolve towards seasonal-like coronavirus.

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Author contributions

Y.L. and Q.P. conceived and designed the research. Y.L., Y.W. Y (unlong) L. and P.L. performed experiments and analyzed data. M.P.P. and A.C.V. discussed the project design and data interpretation. Y.L., Y. W. and Q.P. interpreted the data and drafted the manuscript. All authors critically revised the manuscript.

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.

Data availability

No data was used for the research described in the article.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.antiviral.2023.105674.

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