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Preliminary results and a theoretical perspective of co-treatment using a miR-93-5p mimic and aged garlic extract to inhibit the expression of the pro-inflammatory interleukin-8 gene

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Abstract. The coronavirus disease-19 (COVID-19) pandemic has been a very significant health issue in the period between 2020 and 2023, forcing research to characterize severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) sequences and to develop novel therapeutic approaches. Interleukin-6 (IL-6) and IL-8 are considered significant therapeutic targets for COVID-19 and emerging evidence has suggested that microRNAs (miRNAs/miRs) serve a key role in regulating these genes. MiRNAs are short, 19-25 nucleotides in length, non-coding RNAs that regulate gene expression at the post-transcriptional level through the sequence-selective recognition of the 3'-untranslated region (3'-UTR) of the regulated mRNAs, eventually repressing translation, commonly, via mRNA degradation. For example, among several miRNAs involved in the regulation of the COVID-19 'cytokine storm', miR-93-5p can inhibit IL-8 gene expression by directly targeting the 3'-UTR of IL-8 mRNA. In addition, miR-93-5p can regulate Toll-like receptor-4 (TLR4) and interleukin-1 receptor-associated kinase 4 (IRAK4) expression, thus affecting the nuclear factor-κB (NF-κB) pathway and the expression of NF-κB-regulated genes, such as IL-6, IL-1β and other hyper-expressed genes during the COVID-19 'cytokine storm'. In the present study, the results provided preliminary evidence suggesting that the miR-93-5p-based miRNA therapeutics could be combined with the anti-inflammatory aged garlic extract (AGE) to more effectively inhibit IL-8 gene expression. The human bronchial epithelial IB3-1 cell line was employed as experimental model system. IB3-1 cells were stimulated with the BNT162b2 COVID-19 vaccine and transfected with pre-miR-93-5p in the absence or in the presence of AGE, to verify the inhibitory effects on the BNT162b2-induced expression of the IL-8 gene. The accumulation of IL-8 mRNA was assessed by RT-qPCR; the release of IL-8 protein was determined by Bio-Plex assay. In addition, the possible applications of TLR4/NF-κB inhibitory agents (such as miR-93-5p and AGE) for treating human pathologies at a hyperinflammatory state, such as COVID-19, cystic fibrosis and other respiratory diseases, were summarized.

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Abbreviations: AGE, aged garlic extract; COVID-19, Coronavirus disease 2019; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; IL, interleukin; NF-κB, nuclear factor-κB

Key words: SARS-CoV-2, garlic extracts, NF-κB, pro-inflammatory genes, COVID-19, nutraceuticals

Introduction

The coronavirus disease-19 (COVID-19) pandemic has been considered as very important health issue between 2020 and 2023. Therefore, several studies have focused on characterizing the sequences of Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) infection and developing novel therapeutic options (1-3). Despite the development, validation, approval and worldwide use of anti-SARS-CoV-2 vaccines (4,5), specific anti-viral agents targeting the life cycle of SARS-CoV-2 and improving COVID-19-related clinical manifestations are still needed (6-9). In this respect, the majority of patients with COVID-19 show moderate symptoms; however, a consistent number of patients develop severe COVID-19, which is generally characterized by a hyperinflammatory state (10-13). Additionally, inflammation is considered as a significant feature of 'long COVID-19' (14) and post-acute sequelae of SARS-CoV-2 infection (15).

It has been clearly shown that following lung infection, SARS-CoV-2 promotes immune responses, which are characterized by the high production of inflammatory cytokines. This event, also known as 'cytokine storm' (16,17), can occur due to the activation of several transcription factors, such as nuclear factor kappa-B (NF-κB) and signal transducer and activator of transcription 3 (STAT-3), which in turn regulate the expression of numerous inflammation-related genes, including vascular endothelial growth factor (VEGF), monocyte chemoattractant protein 1 (MCP-1), interleukin-8 (IL-8) and IL-6 (18-21).

Among the proteins which have been proposed to play a key role in the 'cytokine storm' phenomenon in patients with COVID-19, IL-1α, IL-1β, IL-2, IL-6, IL-7, IL-8, IL-9, IL-10, basic fibroblast growth factor, granulocyte-macrophage colony-stimulating factor (GM-CSF), G-CSF, platelet-derived growth factor, VEGF, interferon gamma, interferon-inducible protein 10, MCP-1, macrophage inflammatory protein 1-alpha (MIP-1-α), MIP-1-β and tumor necrosis factors (TNFs) are the most studied ones (22).

Examples of clinical trials on IL-6- and IL-8-targeting drugs for the treatment of patients with COVID-19 are summarized in Table I. These trials supported the concept that this approach could be considered for the development of possible therapeutic strategies against COVID-19. The above speculation has been also sustained by several published reports, such as those by Comarmond *et al* (23), Ghosn *et al* (24), Pomponio *et al* (25) and Castelnovo *et al* (26). Therefore, Comarmond *et al* (23) reported that patients suffering from inflammatory rheumatic and musculoskeletal diseases and treated with anti-IL6 therapy based on tocilizumab or sarilumab, displayed a less severe form of COVID-19. Although the association between COVID-19 outcomes and the duration of pre-existing anti-IL6 therapy was not analyzed in depth, the results of the study suggested that the anti-IL6 therapy was associated with less frequent hospitalization and need for respiratory support (23). Additionally, the pilot study by Pomponio *et al* (25) indicated that the efficacy of tocilizumab, an anti-IL6 therapy, could improve the respiratory function of patients. However, this result warrants further investigation in randomized trials. Castelnovo *et al* (26) collected data from patients with COVID-19 treated with the anti-IL6 drugs, tocilizumab and sarilumab. The analysis revealed that the anti-IL6 drugs could be effective in treating the severe forms of COVID-19. Therefore, it was hypothesized that these drugs could reduce inflammation and the risk of multi-organ failure-related mortality (26). Furthermore, a meta-analysis by Tharmarajah *et al* (27) analyzed the outcomes of IL-6 inhibition in the treatment of COVID-19. In general, the aforementioned studies on anti-IL6 therapies suggested that despite anti-IL6 monotherapy could display several benefits, this approach could be of great interest if combined with other anti-inflammatory drugs. Previous large clinical trials demonstrated that when tocilizumab was used in combination with dexamethasone to treat severe COVID-19-associated Acute Respiratory Distress Syndrome, provided significant clinical improvement. In this respect, Segú-Vergés *et al* (28) employed artificial intelligence approaches to evaluate the potential of tocilizumab in combination with corticosteroid therapy in treating COVID-19. This *in silico* study suggested that the administration of tocilizumab and dexamethasone could induce a synergistic effect, thus strongly reducing inflammation and associated pathological processes, eventually supporting the beneficial effects of the combined therapy in patients with COVID-19 in critical clinical conditions.

Currently, less studies have been published regarding the effect of anti-IL-8 therapies against COVID-19. Two IL-8 inhibitors are being currently evaluated for the treatment of COVID-19. HuMax-IL-8 (BMS986253), a human monoclonal antibody targeting IL-8, was studied for the first time on several types of cancer (29). Reparixin, an allosteric inhibitor of IL-8 activity, has been investigated for its safety and efficacy in patients with severe COVID-19 pneumonia (30). Promising results were obtained in a phase II trial (REPAVID-19). Reparixin was also found to effectively improve biochemical and biophysical parameters in a murine model of liposaccharide (LPS)-induced acute lung injury (31). A murine model of COVID-19 was also employed and the results demonstrated a clear clinical improvement following IL-8-like signaling inhibition with reparixin (32). Taken together, these studies supported that IL-8 could be considered as a promising therapeutic target in the treatment of severe COVID-19 and therefore more anti-IL-8 therapies should be developed in basic and applied research.

MicroRNAs (miRNAs/miRs) are short, non-coding RNAs, 19-25 nucleotides in length, which are involved in regulating gene expression at the post-transcriptional level. Commonly, miRNAs act via specifically binding to the 3'-untraslated region (3'-UTR) of their target mRNAs, thus promoting mRNA degradation (33-37). Importantly, a single miRNA can target several mRNAs, while the 3'-UTR of a single mRNA can encompass several sequences recognized by different miRNAs. It has been reported that >60% of mammalian mRNAs can be targeted by miRNAs (37). A previous study supported that the release of key proteins of the COVID-19-related 'cytokine storm' could be strongly inhibited via mimicking the biological activity of miRNAs (38). In the above study, it was hypothesized that among possible anti-inflammatory agents, 'miRNA targeting' should be carefully considered as a significant strategy (38). Therefore, the general working hypothesis was that targeting the miRNA network could serve a significant role for the development of therapeutic approaches against the SARS-CoV-2-induced pro-inflammatory responses. This hypothesis was based on several publications, showing that miRNA overexpression could inhibit the expression of 'cytokine storm'-related proteins in COVID-19 (38). The above study demonstrated that a molecule mimicking miR-93-5p could modulate the expression of IL-8 gene via targeting its RNA transcript. The first evidence of the effect of miR-93-5p on regulating IL-8 gene expression was reported by Fabbri *et al* (39). This study demonstrated that miR-93-5p could bind to the IL-8 3'-UTR. In addition, it was found that the binding sites of miR-93-5p on IL-8 3'-UTR were conserved within different species, thus supporting its molecular evolution (39). Accordingly, Gasparello *et al* (40) showed that the treatment of bronchial epithelial IB3-1 cells with the SARS-CoV-2 spike protein enhanced the secretion of IL-8. In addition, IL-8 synthesis and extracellular release could be strongly reduced by a molecule mimicking miR-93-5p. The effect of miR-93-5p on directly affecting IL-8 expression is presented in Fig. 1. This figure illustrates the possible interaction between miR-93-5p and the 3'-UTR of IL-8 RNA, and the expected (experimentally validated) effects of miR-93-5p on IB3-1 cells (39,40). This study also revealed that other genes coding for proteins involved in the COVID-19 'cytokine storm' are regulated by miR-93-5p, including IL-6, G-CSF, GM-CSF and IL-1β (40). More particularly, the results of the above study demonstrated that the expression of these genes was upregulated and downregulated following cell transfection with anti-miR-93-5p and pre-miR-93-5p, respectively, thus

Table I. Examples of clinical trials for COVID-19 based on IL-6 and IL-8 targeting.

Clinical trial number	Title	Bioactive molecule
NCT04381052	Study for the Use of the IL-6 Inhibitor Clazakizumab in Patients With Life-threatening COVID-19 Infection	Clazakizumab
NCT04343989	A Randomized Placebo-controlled Safety and Dose-finding Study for the Use of the IL-6 Inhibitor Clazakizumab in Patients With Life-threatening COVID-19 Infection	Clazakizumab
NCT04322773	Anti-IL6 Treatment of Serious COVID-19 Disease with Threatening Respiratory Failure	Two human monoclonal antibodies against IL-6 receptor, tocilizumab and sarilumab
NCT04486521	Clinical Outcome of Anti-IL6 vs. Anti-IL6 Corticosteroid Combination in Patients With SARS-CoV-2 Cytokine Release Syndrome	Cortocosteroid combinations
NCT04347226	Anti-Interleukin-8 for patients with COVID-19	BMS-986253
NCT04878055	Study on Efficacy and Safety of Reparixin in the Treatment of Hospitalized Patients With Severe COVID-19 Pneumonia	Reparixin

COVID-19, Coronavirus disease 2019; IL, interleukin; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2.

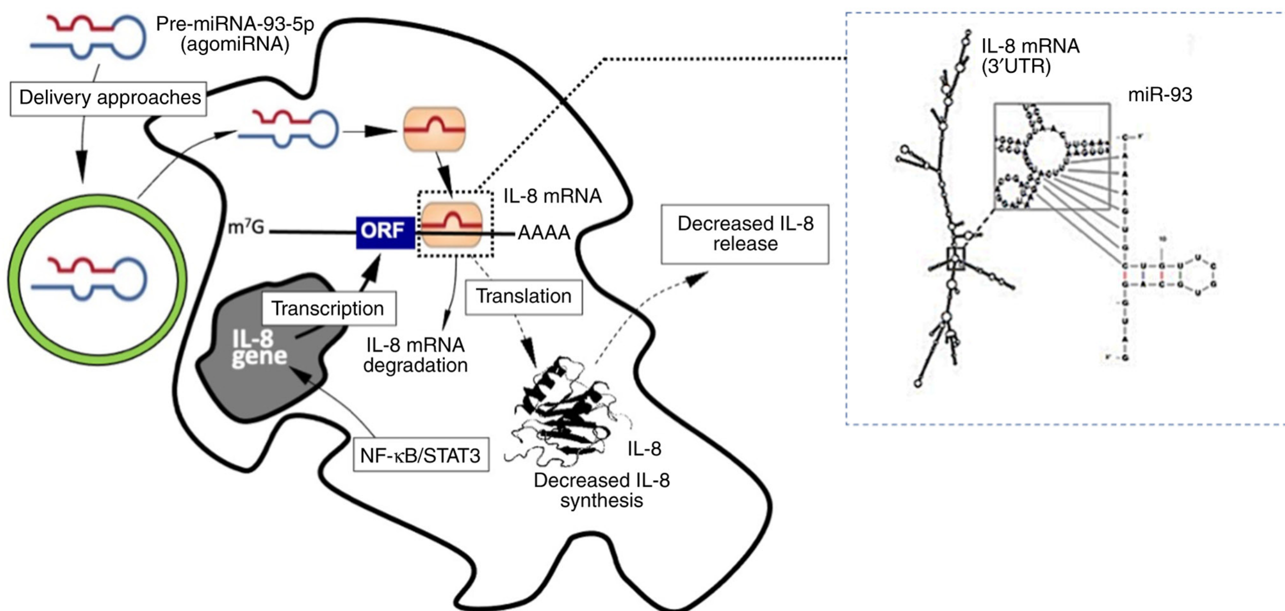

Figure 1. Pictorial representation of miRNA-therapeutics based on the use of pre-miR-93-5p molecules to inhibit the expression of IL-8. In the insert, the interaction between miR-93-5p and the 3'UTR of human IL-8 mRNA is shown [modified from Fabbri *et al*, 2014 (39)]. miR/miRNA, microRNA; IL, interleukin; UTR, untranslated region; ORF, open reading frame; NF-κB, nuclear factor-κB.

highlighting the key role of miR-93-5p in regulating the expression of these genes (40).

Fabbri *et al* (39) showed that the inhibitory effect of ago-miRNAs on the expression of pro-inflammatory genes was mediated by the interaction of these ago-miRNAs with the 3'-UTR of the target pro-inflammatory mRNAs. This finding was also verified in the miR-93-5p/IL-8 axis. In addition, the ago-miRNA molecule could interact and down-regulate the expression of other target pro-inflammatory genes. Furthermore, all IL-1α, IL-6 and IL-8 genes are upregulated by NF-κB. Eventually, all ago-miRNAs mimicking the biological activity of miRNAs on downregulating NF-κB, such as that of miR-130a, miR-146a/b, miR-218 and miR-451, could also inhibit the expression levels of NF-κB and other NF-κB-regulated genes (41). The focus of applied research on miRNA therapeutics has been discussed in several review articles, describing the results of both pre-clinical and clinical studies (42-45). However, a general and clear consensus on the potential biomedical applications of this approach is still missing (44,45). In addition, clinical studies using both

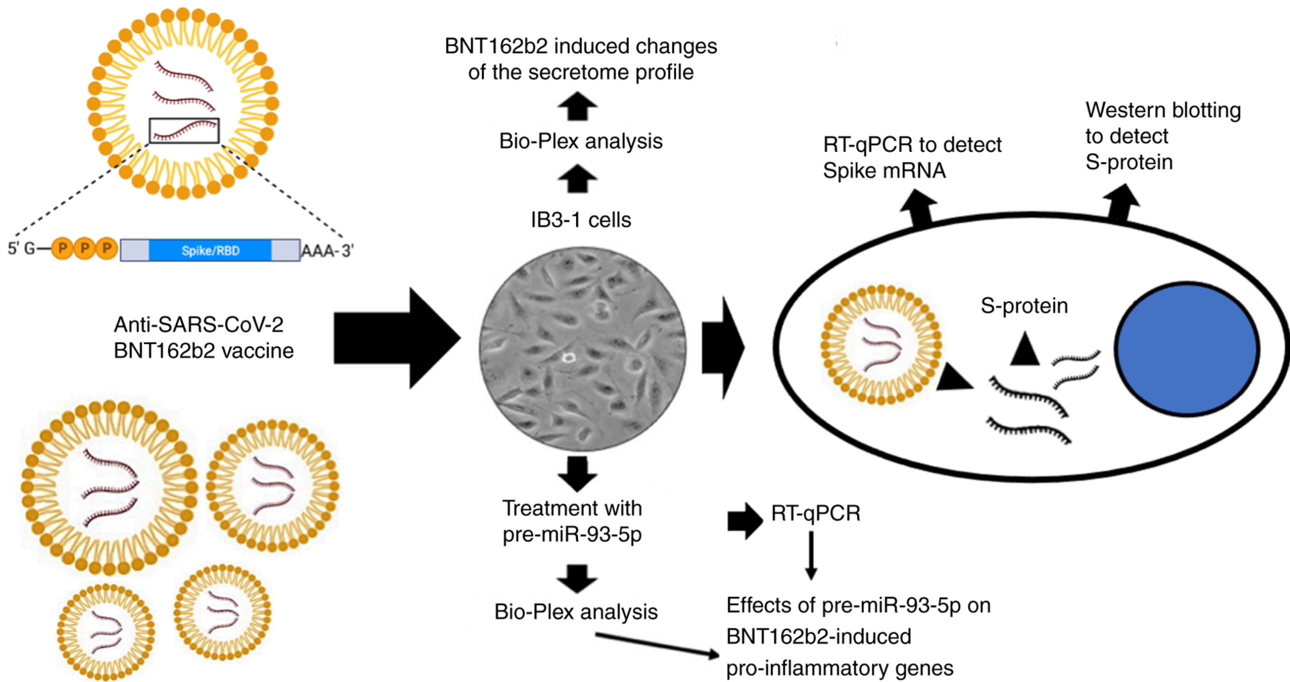

Figure 2. Pictorial representation of the experimental approach for characterizing the activity of inhibitors of pro-inflammatory genes using cystic fibrosis bronchial IB3-1 epithelial cells (39) treated with the BNT162b2 vaccine. SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; RT-qPCR, reverse transcription-quantitative PCR; miR/miRNA, microRNA; S-protein, spike protein.

PROOFS-FIG2 anti-miRNA (46) and miRNA mimicking (47) molecules are ongoing.

The experimental system, which could be employed to study the effects of potential inhibitors on the expression of pro-inflammatory genes is described in Fig. 2. According to the above experimental system, the upregulation of pro-inflammatory genes could be achieved following treatment of target cell lines, and particularly bronchial epithelial IB3-1 cells, with the spike RNA-based BNT162b2 vaccine. The approach was the same with that described in other experimental model systems, which aimed to uncover the effects of the BNT162b2 vaccine on the gene expression and functions of eukaryotic cells (48,49). The most significant characteristics of this approach are the following: i) The cellular uptake of spike mRNA could upregulate and promote the release of S-protein; and ii) the resulted NF-κB upregulation, could be also associated with the increased expression of other NF-κB-regulated genes, including those coding proteins associated with 'cytokine storm' and those involved in the hyperinflammation state of other human diseases, such as cystic fibrosis. Regarding the roles of the BNT162b2 vaccine in the expression of NF-κB and NF-κB-dependent genes, it should be noted that according to the study by Gasparello *et al* (50) on the effects of purified SARS-CoV-2 S-protein on IB3-1 cells, these should be expected. In this respect, BNT162b2 vaccine is composed of a liposomal vaccine vector which is used to carry the spike mRNA. Since lipid-based nanoparticles are known to stimulate inflammatory responses through endosomal Toll-like receptor (TLR)-7 and TLR-8 pathways (51-53), it was suggested that both lipid formulation and spike mRNA are involved in the activity of BNT162b2 on regulating the expression of pro-inflammatory genes. In any case, in the experiments based on the use of the BNT162b2 vaccine on IB3-1 cells, the effects of the anti-inflammatory compounds could be studied. Interestingly, in the majority of cases, the inducing effects of BNT162b2 on IB3-1 cells were more potent compared with those of other control stimuli, such as SARS-CoV-2 spike (40,50), *Pseudomonas aeruginosa* infection (39), TNF-a (54,55) and LPS (56). Although the experimental condition may vary in these studies, the data available strongly suggest that the BNT162b2 treatment of IB3-1 cells is an efficient strategy to induce the expression of pro-inflammatory genes.

A previous study by Gao *et al* (57) indicated that miR-93-5p could suppress inflammation in an LPS-induced acute lung injury mouse model via targeting the TLR4/myeloid differentiation factor 88 (MyD88)/NF-κB signaling pathway. In the above study, a luciferase construct, composed of a partial sequence of the TLR4 3'-UTR, encompassing the binding sites of miR-93, and sub-cloned into a dual-luciferase reporter vector (pmirGLO), was used to verify that TLR4 was a direct target of miR-93-5p (57). The results demonstrated that RAW264.7 cell transfection with miR-93 mimics inhibited the luciferase activity directed by the TLR4-3'UTR reporter plasmid. By contrast, cell co-transfection with a miR-93 inhibitor enhanced luciferase activity. These findings were consistent with those reported by Fabbri *et al* (39). This study investigated the effects of miR-93-5p on luciferase activity using a vector carrying the 3'-UTR of human IL-8 mRNA. In both studies, the control experiments demonstrated that there were no changes in luciferase activity in cells transfected with mutant TLR4 (57) and IL-8 (39) 3'-UTRs, lacking the miR-93 binding sites, thus indicating that there was a direct interaction between miR-93 and the 3'-UTR of IL-8 and TLR4 (39,57).

The possible effects of miR-93-5p on TLR4 are of great significance in applied research, regarding its possible role as inhibitor of SARS-CoV-2 infection and SARS-CoV-2-associated pro-inflammatory gene expression. In fact, several previous studies showed that the SARS-CoV-2 spike protein could activate TLR4, which in turn promoted the synthesis of proinflammatory cytokines (58-60). In this context, the molecular effect of TLR4 in COVID-19 should be considered as a significant regulatory factor of SARS-CoV-2 infection (61). The interaction between SARS-CoV-2 spike protein and TLR4, and its activation, was also reported by Zhao *et al* (62) and Patra *et al* (63). The above studies suggested that the binding of SARS-CoV-2 spike protein to TLR4, resulting in its activation, upregulates angiotensin-converting enzyme 2, thus promoting virus entry and hyperinflammation. These studies supported that targeting human TLRs could be a strategy to combat COVID-19. Accordingly, Das *et al* (64) performed *in silico* experiments to identify novel anti-SARS-CoV-2 agents from bioactive phytocompounds targeting the viral spike glycoprotein and human TLR4. The above study analyzed a group of 30 phytocompounds, previously demonstrated to retain antiviral activity. These compounds were theoretically screened, using molecular docking, MD simulations and ADME analysis, for their binding efficacy to SARS-CoV-2 spike protein and TLR4. The *in silico* results showed that cajaninstilbene acid and papaverine could block human TLR4, thus suggesting that these phytocompounds could mitigate SARS-CoV-2-induced proinflammatory responses (64). The significance of this approach was also verified by Sahanic *et al* (65), reporting that SARS-CoV-2 could activate the TLR4/MyD88 pathway in human macrophages, thus indicating that this effect could be associated with strong pro-inflammatory responses in severe COVID-19. Accordingly, Nakazawa *et al* (66) found that SARS-CoV-2 could directly impair renal cells via promoting pro-inflammatory responses, while inhibition of TLR4 and IL-1R could prevent SARS-CoV-2 mediated kidney injury. The interplay between TLR4 and SARS-CoV-2, which is involved in the complex mechanisms of inflammation and severity in COVID-19 infections, has been recently reviewed by Asaba *et al* (67). This study provided novel insights into the unique role of TLR4 in SARS-CoV-2 infection, thus highlighting its potential in the development of novel treatment strategies for COVID-19.

Materials and methods

Materials. All chemicals and reagents were of analytical grade. Aged Garlic Extract (AGE) was obtained by Wakunaga Pharmaceutical Co., Ltd. (Hiroshima, Japan). The Pre-miR™ miRNA Precursor (PM10951, hsa-miR-93-5p), and the Pre-miR™ miRNA Precursor Negative Control #1 were both purchased from Ambion-ThermoFisher (cat no. AM17100 and cat no. AM17110, respectively).

Cell culture. Human bronchial epithelial IB3-1 cells (40,50) were cultured as previously described (39). Cells were transfected with 100 nM hsa-miR-93-5p precursor and pre-miR-93 negative control (NEG93) (40) using the Lipofectamine RNAiMAX Transfection Reagent (Invitrogen; Thermo Fisher Scientific, Inc.). The AGE powder used in our experiments was prepared by lyophilization. The concentration of AGE employed in this study was 0.5 mg/ml, in agreement with the study by Gasparello *et al* (68). The AGE powder was freshly dissolved in complete cell culture medium prior to each experiment.

Treatment of IB3-1 cells with the BNT162b2 vaccine. The BNT162b2 vaccine (Comirnaty™; Lot. no. FP8191) was obtained from the Hospital Pharmacy of the University of Padova. For treatment with the BNT162b2 vaccine, IB3-1 cells were seeded at a density of 200,000 cells/ml and were then treated with 0.5 μg/ml of the vaccine for 24 h (48,49). When needed, cells were transfected with pre-miR-93-5p and/or co-treated with AGE.

mRNA quantitative analyses. For the quantification of the relative mRNA expression levels, total RNA was reverse transcribed into cDNA, as described by Gasparello *et al* (40). Quantitative (q)PCR analysis for IL-6 (assay ID, Hs.PT.58.40226675), IL-8 (assay ID, Hs.PT.58.38869678.g) and IL-1β (assay ID, Hs.PT.58.1518186) was performed using the corresponding assay kits, as previously described (40). PCR amplification was carried out as previously described using the CFX96 Touch Real-Time PCR Detection System (Bio-Rad Laboratories, Inc.) (40,69). RT-qPCR analysis for BNT162b2 Spike mRNA was performed according to the study by Fertig *et al* (70) (as reported in Data S1). Relative IL-8 gene expression was calculated using the comparative cycle threshold method ($^{\Delta\Delta Cq}$ method) and the endogenous control human β-actin was used as normalizer, described previously (39).

Statistical analysis. The differences among different groups were compared using one-way ANOVA (analyses of variance between groups, http://vassarstats.net/anova1u.html). Prism (v. 9.02) by GraphPad software was also employed (followed by Bonferroni's post-hoc tests). Differences were considered statistically significant when $P<0.05$ and highly significant when $P<0.01$ (1,4,68,70).

Results

Effect of BNT162b2 vaccine on NF-κB expression. The BNT162b2-treated IB3-1 bronchial cell model is characterized by the BNT162b2-induced expression of pro-inflammatory genes with a higher efficiency compared with SARS-CoV-2 spike protein (40). As a control, in this model spike protein is produced following cell treatment with the BNT162b2 vaccine. The intracellular content of SARS-CoV-2 spike protein should be increased based on the concentrations of BNT162b2 used to treat IB3-1 cells. Herein, a significant increase ($P<0.01$) in the content of intracellular BNT162b2 Spike mRNA was observed following cell treatment with 1 μg/ml BNT162b2 vaccine for 24 h (Fig. S1). Quantitative RT-qPCR analysis for BNT162b2 Spike mRNA was performed according to the study by Fertig *et al* (70). In this study, primers were designed to be specific to the publicly available, codon-optimised vaccine mRNA sequence (70). Fully in agreement with the RT-qPCR data shown in Supplementary Fig. S1, full-length S-protein was detected by western blot analysis (data not shown). Since previous studies demonstrated that S-protein could promote

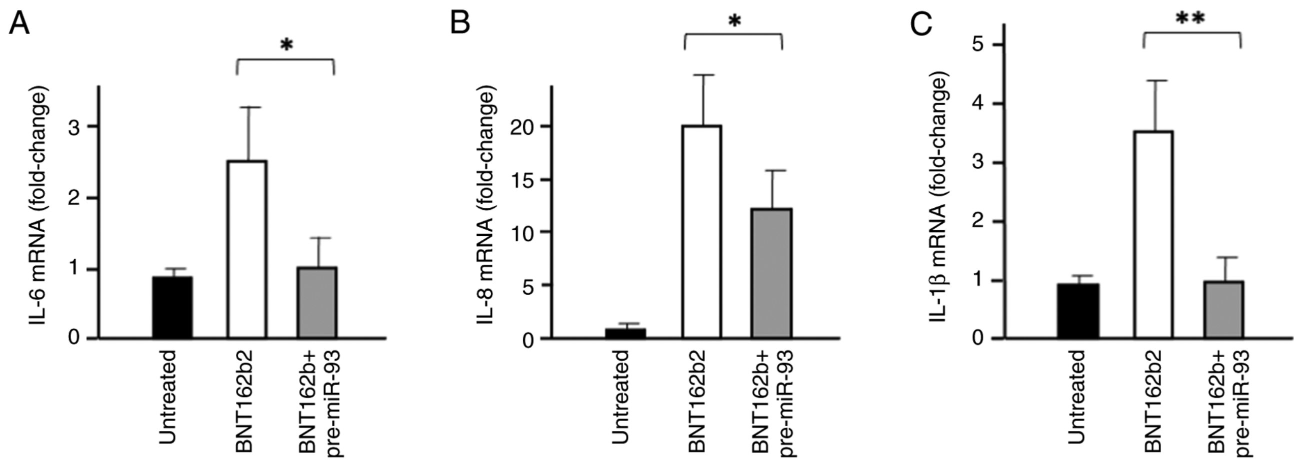

Figure 3. Representative examples showing the increase of the expression of pro-inflammatory genes upon treatment of IB3-1 cells with BNT162b2 and the effect of transfection with 100 nM pre-miR-93-5p. The expression of (A) IL-6, (B) IL-8 and (C) IL-1β. Results represent the average ± standard deviation of four independent experiments. *P<0.05 and **P<0.01. miR, microRNA; IL, interleukin.

pro-inflammatory gene expression via activating the NF-κB pathway in several cellular systems (69,71,72), in the present study NF-κB was upregulated in IB3-1 cells treated with the BNT162b2 vaccine (data not shown).

AGE as a possible anti-inflammatory agent for tackling 'cytokine storm' in COVID-19. The AGE powder used in our experiments was prepared by lyophilization. The concentration of AGE employed in this study was 0.5 mg/ml, in agreement with the study by Gasparello *et al*, who found that this concentration is optimal to obtain inhibition of pro-inflammatory genes in bronchial epithelial IB3-1 cells stimulated with either the SARS-CoV-2 S-protein or the BNT162b2 vaccine (68). The AGE powder was freshly dissolved in complete cell culture medium prior to each experiment. The analysis of the organosulfur compound content in the AGE powder was performed using a GC-MS chromatography and has been reported by Gasparello *et al* (68). The relative expression levels of the pro-inflammatory genes, IL-6, IL-8 and IL-1β, were detected after 3 days treatment by reverse transcription-qPCR. As shown in Fig. 3, the mRNA expression levels of the aforementioned cytokines were increased following IB3-1 cell treatment with 0.5 µg/ml BNT162b2 (white bars). However, cell transfection with 100 nM pre-miR-93-5p displayed the opposite effect (grey bars).

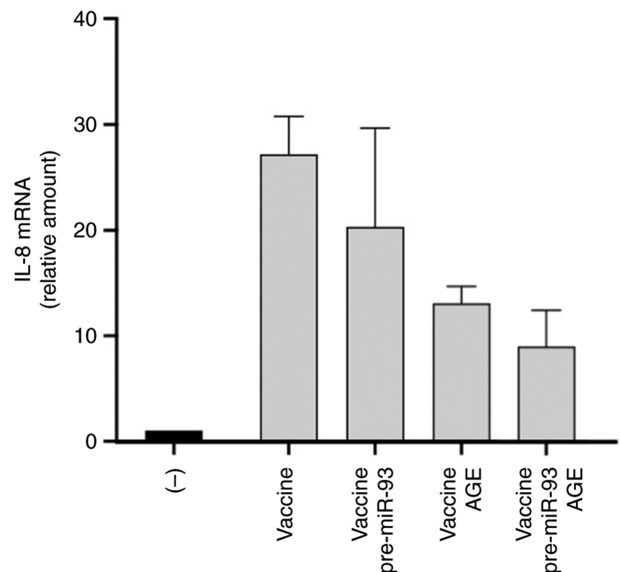

Figure 4. Increase of the expression of IL-8 gene upon 3-days treatment of IB3-1 cells with 1 µg/ml BNT162b2 vaccine and inhibition of IL-8 gene expression upon culturing BNT162b2-treated IB3-1 cells with 100 nM pre-miR-93-5p, 0.5 mg/ml AGE or 100 nM pre-miR-93-5p plus 0.5 mg/ml AGE, as indicated. Results represent the amount of RNA (average ± standard deviation; n=3). IL, interleukin; miR, microRNA; AGE, aged garlic extract.

AGE as a potential anti-inflammatory agent for tackling 'cytokine storm' in COVID-19: A pilot study exploring the effects of a combined treatment with pre-miR-93-5p. The present study aimed to explore the possible effects of the combination of pre-miR-93 and AGE in mitigating 'cytokine storm' in COVID-19, since a previous study demonstrated that the AGE constituent S-allyl-cysteine (SAC) could interact with TLR4 (71). For example, docking analysis performed by Gasparello *et al* (68), predicted that the SAC/TLR4 interaction was based on hydrogen bonds, involving His685, Tyr657, Ser659 and Arg722. This effect could affect, at least in principle, TLR4 activity, since TIR domain is essential for NF-κB activation (73-76). The results of the pilot experiments, where cells were co-treated with AGE and pre-miR-93-5p, are shown in Fig. 4. In preparing this experiment, we first verified the transfection efficiency of Lipofectamine-delivered pre-miR-93-5p, according with the procedure described by Gasparello *et al* (40); this validation was performed by RT-qPCR analysis of the miR-93-5p sequences accumulated in pre-miR-93-5p transfected IB3-1 cells. The list of the assays employed for miRNA detection is reported in Table SI. A representative experiment is shown in Supplementary Fig. S2. We always found high transfection efficiency similar to that shown in Fig. S2, with no exception. Another important control was the formal demonstration of the specificity of the effects of transfection of IB3-1 cells with the pre-miR-93-5p. This was demonstrated by comparing the effects of

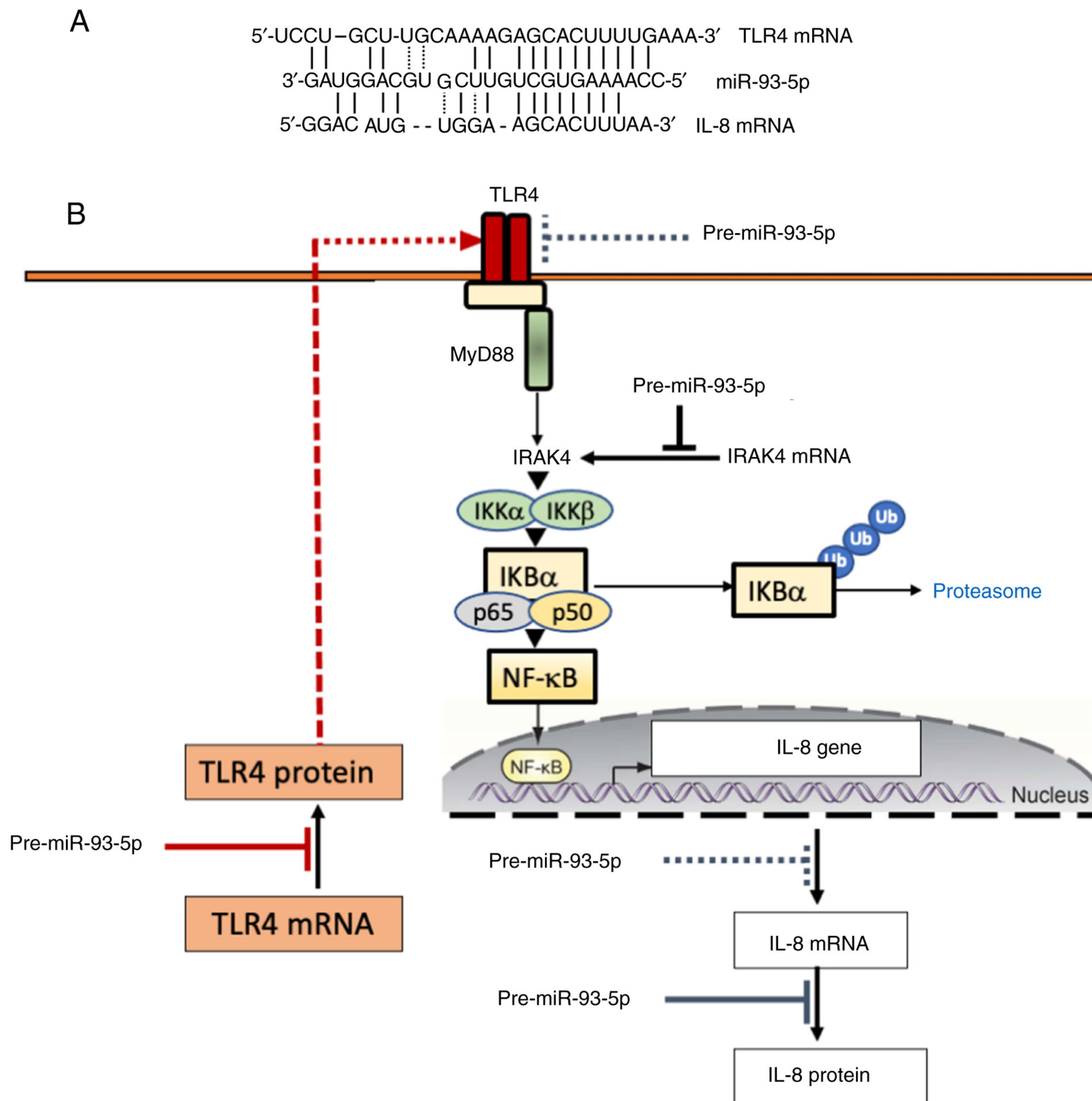

Figure 5. Binding of miR-93-5p to the 3'-UTR of TLR4 and IL-8 mRNAs. (A) Interactions of miR-93-5p sequences with TLR4 and IL-8 mRNAs (39,45). (B) Proposed mechanism of action of miR-93-5p, leading to inhibition of IL-8 gene expression. The proposed mechanism of action of miR-93-5p on TLR4 and IL-8 mRNAs is based on the studies published by Gao *et al* (57) and Fabbri *et al* (39). In both cases, a luciferase construct was used to demonstrate functional binding of miR-93-5p to the target RNA sequences; the constructs were constituted by a partial sequence of TLR4 3'-UTR or IL-8 3'UTR (both containing miR-93 binding sites) cloned into the dual-luciferase reporter vector pmirGLO (38,45). Control constructs containing mutated miR-93 binding sites were employed, to demonstrate that the effects found on luciferase activity were related to functional miR-93 binding sites. The proposed mechanism of action of miR-93-5p on IRAK4 mRNA is based on the studies published by Xu *et al* (77) and Tian *et al* (78). IL, interleukin; miR, microRNA; TLR4, Toll-like receptor 4; UTR, untranslated region; IRAK4, IL-1 receptor-associated kinase 4; NF-κB, nuclear factor-κB.

pre-miR93-5p to those of the pre-miR93 negative control, as reported in the representative experiments shown in Fig. S3 and in Table SII. The results concerning the effects of a combined treatment based on AGE and pre-miR-93-5p is shown in Fig. 4. The results obtained revealed a sharp increase in IL-8 gene expression levels following IB3-1 cell treatment with the BNT162b2 vaccine for three days. However, the BNT162b2-induced IL-8 gene expression was reduced after treatment of cells with pre-miR-93-5p, AGE or pre-miR-93-5p plus AGE. Interestingly, the inhibition of IL-8 gene expression was more potent in cells treated with the combination of pre-miR-93-5p and AGE.

Effects of miRNA therapeutics combined with approaches based on the use of natural products: Proposed mechanism of action of miR-93-5p and future perspectives. The extent of complementarity between miR-93-5p and the 3'-UTRs of TLR4 and IL-8 mRNAs are shown in Fig. 5A (39,57). The number of hydrogen bonds generated between miR-93-5p and the 3'-UTRs of TLR4 and IL-8 mRNAs were very similar, thus supporting that both TLR4 and IL-8 mRNAs could be direct targets of miR-93-5p. In addition, miR-93-5p could significantly affect NF-κB signaling via directly interacting with IL-1 receptor-associated kinase 4 (IRAK4), a kinase known to activate NF-κB in the TLR signaling

pathways (77,78). Accordingly, the possible mechanism of action of miR-93-5p is depicted in Fig. 5B. In this theoretical model, the mRNA expression levels of IL-8 could be directly inhibited by miR-93-5p, as suggested by Fabbri *et al* (39). Additionally, IL-8 expression could be indirectly suppressed via the inhibition of the TLR4/NF-κB pathway. This finding could be due to the direct miR-93-5p/TLR-4-3'-UTR-mediated TLR4 downregulation (57) and the direct interaction (and consequent inhibition) with IRAK4 (77). In this case, NF-κB inhibition resulted in the inhibitory effects of miR-93-5p on NF-κB-regulated genes, such as IL-1β and IL-6. Data sustaining the direct interaction between miR-93-5p and the 3'-UTRs of IL-8, TLR4 and IRAK4 have been also reported in the studies by Fabbri *et al* (39), Gao *et al* (57) and Xu *et al* (77).

Discussion

The present study reported preliminary evidence suggesting that the miR-93-5p based 'miRNA therapeutics' combined with AGE could be considered as an efficient approach for inhibiting IL-8 expression. This could be of significant interest, since the TLR4/NF-κB-related inhibitory agents, such as miR-93-5p, could be applied in the treatment of human pathologies associated with a hyperinflammatory state, such as COVID-19 (45-49) and cystic fibrosis (62,63), which are characterized by IL-8 upregulation.

Emerging evidence has suggested that IL-8, TLR4 and IRAK4 can be directly targeted by miR-93-5p (39,57,77). Accordingly, the proposed mechanism of action of miR-93-5p (Fig. 5B), indicated that the direct targeting of the IL-8 3'-UTR by miR-93-5p could inhibit IL-8 expression. At the same time, the indirect suppression of IL-8 expression could be achieved via blocking the TLR4/NF-κB pathway, possibly through the direct interaction between miR-93-5p and IRAK4 or TLR4. Although several studies have supported the direct interaction between miR-93-5p and the 3'-UTR of IL-8, TLR4 and IRAK4 (39,57,77), future studies are still needed to verify the possible interactions of miR-93-5p with TLR4 and IRAK4 in the BNT162b2 based experimental model system, particularly in cells also treated with pre-miR-93-5p and AGE.

The most significant limitation of the current study was that the expression levels of only a few pro-inflammatory genes (IL-1β, IL-6 and IL-8) were detected in BNT162b2-treated IB3-1 cells and cells co-treated with both miR-93-5p mimics and AGE (IL-8). Therefore, the present study should be considered as an exploratory pilot study based on a proof-of-principle, suggesting that a combined treatment with pre-miR-93-5p and AGE could be most effective in inhibiting the expression of pro-inflammatory genes. Future studies, focusing on more proteins involved in the 'cytokine storm' in COVID-19 and on the hyperinflammation state of other human pathologies, such as cystic fibrosis and chronic obstructive pulmonary disease, should be performed. In addition, the current preliminary study could promote the design of other experimental studies on the effects of pre-miR-93-5p in combination with other extracts from medicinal plants, including the bioactive constituents of AGE (SAC and S1PC), on regulating IL-8 expression. Although the BNT162b2 treated cells are not a stringent model system for viral infection, the results of this study are expected to stimulate experimental efforts to determine whether the combination of pre-miR-93-5p and AGE exerts the same effects in SARS-CoV-2-infected cells (68).

The preliminary data of the present study suggested that pre-miR-93-5p could be considered as a double-acting agent, since it could directly inhibit IL-8 mRNA translation and indirectly block NF-κB signaling via inhibiting TRL4 and IRAK4. Further studies on other pro-inflammatory genes could determine whether the inhibitory effects of the pre-miR-93/AGE combined treatment on IL-8 expression could be also generalized to other genes involved in the COVID-19-related hyper-inflammatory state. This experimental plan could also verify the association between the inhibitory effects on pro-inflammatory genes and corresponding alterations on the NF-κB pathway. Another significant limitation of the current study was that only one ago-miRNA molecule, namely pre-miR-93 ago-miRNA, was employed. However, it has been reported that other miRNAs can interact with TLRs, such as miR-145-5p, and NF-κB, such as miR-130a, miR-146a/b, miR-218 and miR-451 (79-82). Studies based on molecules mimicking the function(s) of these miRNAs should be also considered to fully understand the interplay between miRNAs, NF-κB, TLR4 and 'cytokine storm'-related pro-inflammatory genes. In addition, these future studies could allow the identification of novel pre-miRNA molecules, which could be useful for verifying the hypothesis that pre-miRNA molecules in combination with natural products, such as AGE and other compounds isolated from AGE, could possibly enhance their inhibitory activity on inflammation.

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Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

AF, EA and RG designed the experimental plan. JG, CP, AF were involved in the methodology development. JG and CP performed the experiments. AF, CP, JG and RG curated the data and analysed the results. AF and RG were responsible for funding acquisition. AF and RG supervised the investigation. RG wrote the original draft. AF, RG and EA reviewed and edited the draft. All authors read and approved the final version of the manuscript. RG and AF confirm the authenticity of all the raw data.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The company Wakunaga Pharmaceutical Co., Ltd. both provided the aged garlic extract used in this study and also paid for the publication charges.

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