Since January 2020 Elsevier has created a COVID-19 resource centre with free information in English and Mandarin on the novel coronavirus COVID-19. The COVID-19 resource centre is hosted on Elsevier Connect, the company's public news and information website.

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Short communication

Exposure to particulate matter upregulates ACE2 and TMPRSS2 expression in the murine lung☆

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ABSTRACT

Coronavirus disease (COVID-19) is currently a serious global issue. Epidemiological studies have identified air pollutants, including particulate matter (PM), as a risk factor for COVID-19 infection and severity of illness, in addition to numerous factors such as pre-existing conditions, aging and smoking. However, the mechanisms by which air pollution is involved in the manifestation and/or progression of COVID-19 is still unknown. In this study, we used a mouse model exposed to crude PM, collected by the cyclone method, to evaluate the pulmonary expression of angiotensin-converting enzyme 2 (ACE2) and transmembrane protease serine type 2 (TMPRSS2), the two molecules required for the entry of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) into host cells. Multiplex immunohistochemical analysis revealed that exposure to PM increased the expression of these two molecules at the same site. Furthermore, image cytometry analysis revealed increased expression of these proteins, particularly, in the alveolar type 2 cells and macrophages, which are potential targets for SARS-CoV-2. Our findings provide an experimental evidence that exposure to PM may adversely affect the manifestation and progression of COVID-19, mediated by the impact of SARS-CoV-2 on the site of entry. The study results suggest that examining these effects might help to advance our understanding of COVID-19 and aid the development of appropriate social interventions.

1. Introduction

Coronavirus disease (COVID-19) is caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) and is currently one of the most important global health concerns. In cases of SARS-CoV-2 infection, viral entry into the host cells depends on the presence of angiotensin-converting enzyme 2 (ACE2) and transmembrane protease serine type 2 (TMPRSS2) (Hoffmann et al., 2020). It has been suggested that differences in the regulation of ACE2 and TMPRSS2 expression can affect susceptibility to COVID-19 (Saheb Sharif-Askari et al., 2020).

ACE2 and TMPRSS2 expression in the lungs can be influenced by environmental factors. Indeed, it has been suggested that tobacco smoking upregulates ACE2 and TMPRSS2 expression in the lungs (Chakladar et al., 2020). In animal models, smoke exposure has also shown to increase the expression of ACE2 in the airways (Yilin et al., 2015).

In light of this background, we focused on air pollution as one of the environmental factors modulating the expression of ACE2 and TMPRSS2. Various experimental and epidemiological studies have identified a link between air pollution and respiratory infections;

Abbreviations: COVID-19, Coronavirus disease; SARS-CoV-2, Severe acute respiratory syndrome coronavirus 2; ACE2, Angiotensin-converting enzyme 2; TMPRSS2, Transmembrane protease serine type 2; PM, Particulate matter.

☆ All experimental procedures were approved by the Ethics Committee of Animal Research at Kyoto University.

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notably, it has been found that even short-term exposure to ambient particulate matter (PM) can increase the likelihood of developing acute lower respiratory infections (Horne et al., 2018). Additionally, air pollutants, including PM, have been reported to be associated with COVID-19 morbidity and severity (Yao et al., 2020; Zhu et al., 2020). While it has been reported that PM increases the expression of ACE2 in the lungs (Lin et al., 2018), the cell types in the lungs, which are involved in the increased expression of ACE2 by the PM, and whether the PM increases the expression of not only ACE2 but also TMPRSS2, remains unknown. In this study, we assessed the expression of these proteins in the murine lung tissue exposed to PM using multiplex immunohistochemical analysis, and quantitatively using image cytometry analysis, in accordance with the previously reported methods (Tsujikawa et al., 2017).

Conventional collection methods for PM using filters require an elaborate extraction process since the PM is trapped in a filter; therefore, it is difficult to use PM in its natural form for cell- and animal-based exposure experiments (Onishi et al., 2018). On the other hand, we considered cyclonic separation in which centrifugal forces are made available for the collection of particles without the use of filters or extraction. The present study employed the cyclone technique to collect crude PM "in powder form," which permits *in vivo* studies of PM exposure that reflects realistic condition in ambient atmospheres (Okuda et al., 2018).

2. Materials and methods

2.1. Sampling of PM

The fine and coarse fractions of PM used in this study were collected in Yokohama, Japan, as previously described (Onishi et al., 2018). Briefly, fine and coarse PM were separated using a virtual impactor with an aerodynamic diameter of 2.4 μm prior to entry into the cyclone device. Subsequently, the cyclone imparted a centrifugal force on the gas stream within a conical-shaped chamber, and operated by creating a vortex inside the cyclone body, enabling the collection of crude PM (US EPA, 2003). Fine particles were 0.30–2.4 μm and coarse particles were 2.4 μm or greater in size. The chemical composition of PM is as described in Supplementary Table 1.

2.2. Animal experiments

Male Institute of Cancer Research (ICR) mice (7 weeks old, n = 3 in each group) were purchased from Nihon CLEA (Tokyo, Japan). All experimental procedures were approved by the Ethics Committee of Animal Research at Kyoto University.

Particles of crude PM (either fine or coarse fractions) collected using cyclonic separation were dispersed in a sterile phosphate-buffered saline solution with 0.05% Tween 80 and ultrasonicated at a concentration of 10 mg/mL. Based on a previous report (Wang et al., 2017), a 50-μl solution of PM (either fine or coarse fractions) was administered by intratracheal instillation through a polyethylene tube under inhalational isoflurane anesthesia (i.e., 500 μg/body of PM was administered). The control mice received 50 μl of the same solution without PM. Twenty-four hours after intratracheal administration of PM, the treated mice were sacrificed, and the lungs were fixed in 10% formalin solution. The lungs were obtained and embedded in paraffin and sectioned at 4-μm thickness.

2.3. Multiplex immunohistochemistry

A multiplex immunohistochemical method was used to stain for multiple antigens in a single lung tissue section (Tsujikawa et al., 2017). After deparaffinization, the slides were stained with hematoxylin (S3301; Agilent Technologies), followed by antigen retrieval using Target Retrieval Solution (S1699; Agilent Technologies, Santa Clara, CA, USA), and thereafter, endogenous peroxidases were blocked with 0.6% H_2O_2.

Table 1
Details of multiplex immunohistochemistry.

	Round 1	Round 2	Round 3	Round 4
Primary Antibody	proSP-C	TMPRSS2	Mac-3	ACE2
Supplier	Merck	Abcam	Bio Legend	Abcam
Product#	AB3786	ab92323	108502	ab15348
Concentration	1:2000	1:1000	1:500	1:500
Reaction	RT, 60 min	4 °C, overnight	RT, 60 min	4 °C, overnight
Isotype Control	Normal rabbit IgG	Normal rabbit IgG	Normal rat IgG	Normal rabbit IgG
Supplier	Abcam	Abcam	Santa Cruz Biotechnology	Abcam
Product#	ab37415	ab37415	sc-2026	ab37415
Histofine Reaction	Anti-rabbit RT	Anti-rabbit RT	Anti-rat RT	Anti-rabbit RT
Reaction time	30 min			
Supplier	Nichirei Bioscience			
Product	Histofine Simple Stain MAX Peroxidase			
AEC[a] Reaction time	20 min			

[a] AEC = alcohol-soluble peroxidase substrate 3-amino-9-ethylcarbazole.

Subsequently, the slides were processed by iterative cycles of antibody staining and imaging. The slides were subjected to protein blocking using Blocking One Histo solution (Nacalai Tesque, Kyoto, Japan), followed by staining with primary antibodies and an horseradish peroxidase-conjugated polymer, and then, chromogenic detection by alcohol-soluble peroxidase substrate, 3-amino-9-ethylcarbazole (Vector Laboratories, Burlingame, CA, USA).

After image acquisition using a BZX-810 fluorescence microscope (Keyence, Osaka, Japan), the tissue sections were then stripped of antibodies and the chromogen, followed by subsequent rounds of antibody staining and imaging. Table 1 lists the antibodies and conditions used for antigen staining.

2.4. Image processing

In accordance with the previously reported methods (Tsujikawa et al., 2017), image preprocessing, visualization, quantitative analysis, and cytometry were performed using Cell Profiler v.2.2.0, ImageJ v.1.52, Image Scope (Leica Biosystems, Wetzlar, Germany), and FCS Express 7 Image Cytometry Version April 7, 0014 (De Novo Software, Pasadena, CA, USA), respectively. Briefly, in the image cytometry analysis, chromogenic signal intensity was quantified based on a single-cell segmentation, and positivity was determined based on the cut-off level derived from the isotype control slide.

2.5. Statistical analysis

Statistical analysis using Mann-Whitney *U* test and Fisher's exact test was performed to determine the significant differences between the groups. A *P*-value < 0.05 was considered to indicate a significant difference. All statistical analyses were performed with EZR (version 1.36) (Saitama Medical Center, Jichi Medical University, Saitama, Japan), which is a graphical user interface for R (The R Foundation for Statistical Computing, Vienna, Austria).

3. Results

Based on the anatomical distribution and shape, we considered that in the control lung tissues, ACE2 and TMPRSS2 were expressed in the bronchial epithelium, alveolar type 2 (AT2) cells, and macrophages, and that ACE2, in addition to these cells, was expressed in the vascular endothelium (Fig. 1A–C). Compared with the control lung tissues, the

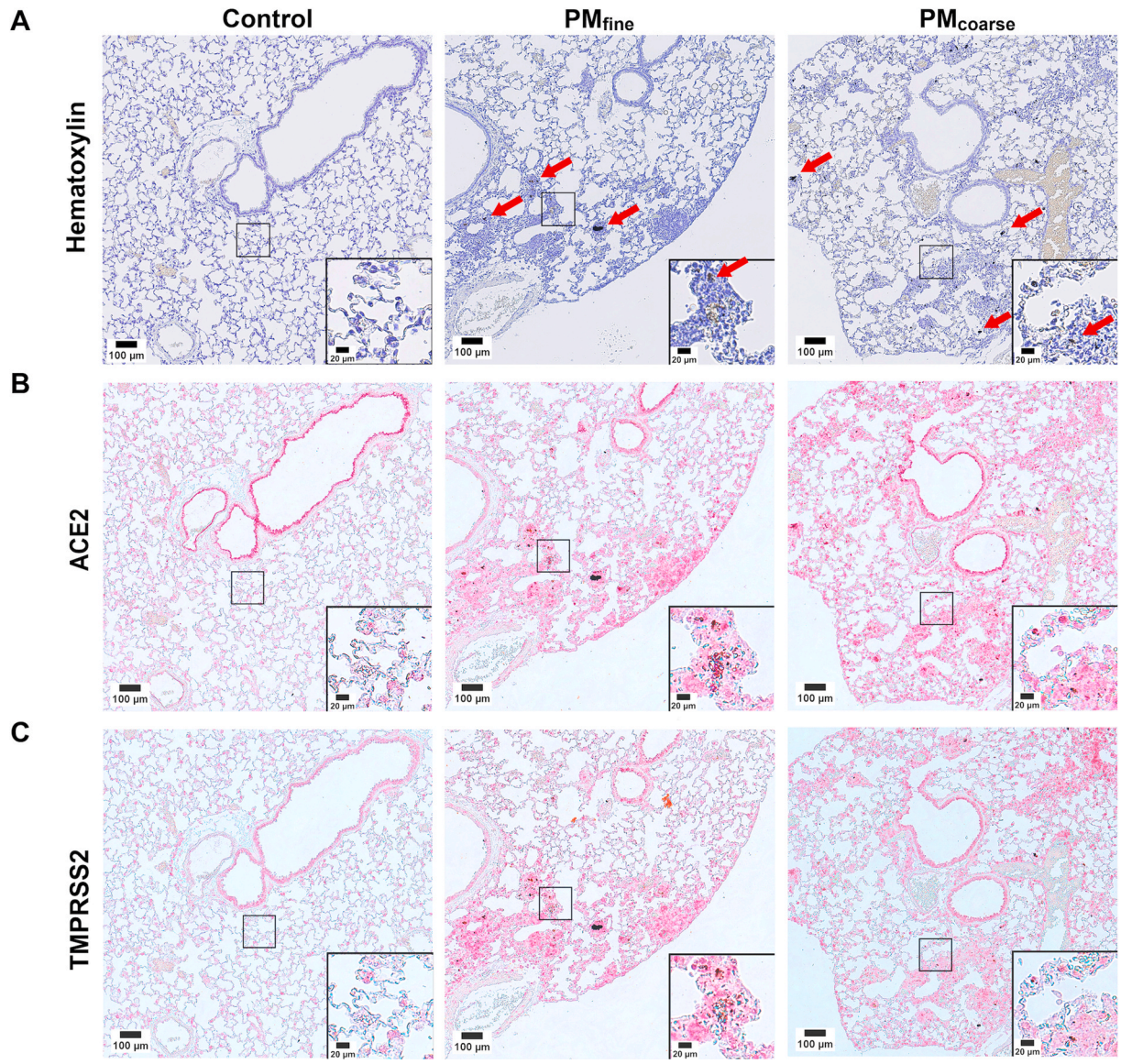

Fig. 1. Representative images of lung tissue sections obtained from experimental mice 24 h after intratracheal instillation of fine particulate matter, coarse particulate matter, or vehicle control solution. **(A)** Hematoxylin staining. The red arrows indicate particulate matter deposition in the lung. (**B–C**) Representative images of immunohistochemical staining for ACE2 (**B**) and TMPRSS2 (**C**). In each image, the box outlines the area magnified in the inset. Scales are shown. ACE2 = angiotensin-converting enzyme 2; TMPRSS2 = transmembrane protease serine type 2; PM_{fine} = Particulate matter (fine fraction); PM_{coarse} = Particulate matter (coarse fraction).

PM-exposed lung tissues showed increased ACE2 and TMPRSS2 expression in the alveolar regions, particularly in the area surrounding PM deposition (Fig. 1B and C, Fig. 2A and B).

Subsequently, we performed multiplex immunohistochemistry using proSP-C and Mac-3, which have been reported as the protein marker for AT2 cells and lung macrophages, respectively (Hasegawa et al., 2017; Mora et al., 2006). Overlayed pseudo-colored images revealed that co-expression of ACE2 and TMPRSS2 proteins were upregulated in both proSP-C^+ AT2 cells and Mac–3^+ macrophages after exposure to fine or coarse fractions of PM (Fig. 2C).

Additionally, we performed image cytometry analysis to quantitatively assess the co-expressing ACE2 and TMPRSS2. Fig. 3A shows a representative analysis for ACE2 and TMPRSS2 co-expression in the same region as shown in Fig. 2C. Applying the same gating strategy to a wider range of all samples, we observed that these proteins were increased mainly in the proSP-C^+ AT2 cells and partly in the Mac–3^+ macrophages, when exposed to fine or coarse fractions of PM (Fig. 3B).

Combining the data of chromogen signal intensity per cell in all mice of each group, and analyzing them based on cell type, we found that ACE2 and TMPRSS2 expression significantly increased on PM exposure (Supplementary Fig. 1). Furthermore, when the data from all mice in each group obtained in Fig. 3B were combined and analyzed by cell type, the number of cells positive for both ACE2 and TMPRSS2 was found to be significantly increased by exposure to fine or coarse fractions of PM, mainly in the proSP-C^+ AT2 cells and partly in the Mac–3^+ macrophages (Fig. 3C).

4. Discussion

In this study, we examined the status of ACE2 and TMPRSS2 expression in the lung tissue of mice exposed to PM; the results of this study provide histological evidence for increased ACE2 and TMPRSS2 expression in AT2 cells and macrophages.

Given that increased gene expression of ACE2 and TMPRSS2 in lung tissues of smokers and COPD patients is thought to be associated with susceptibility to COVID-19 (Saheb Sharif-Askari et al., 2020), the results

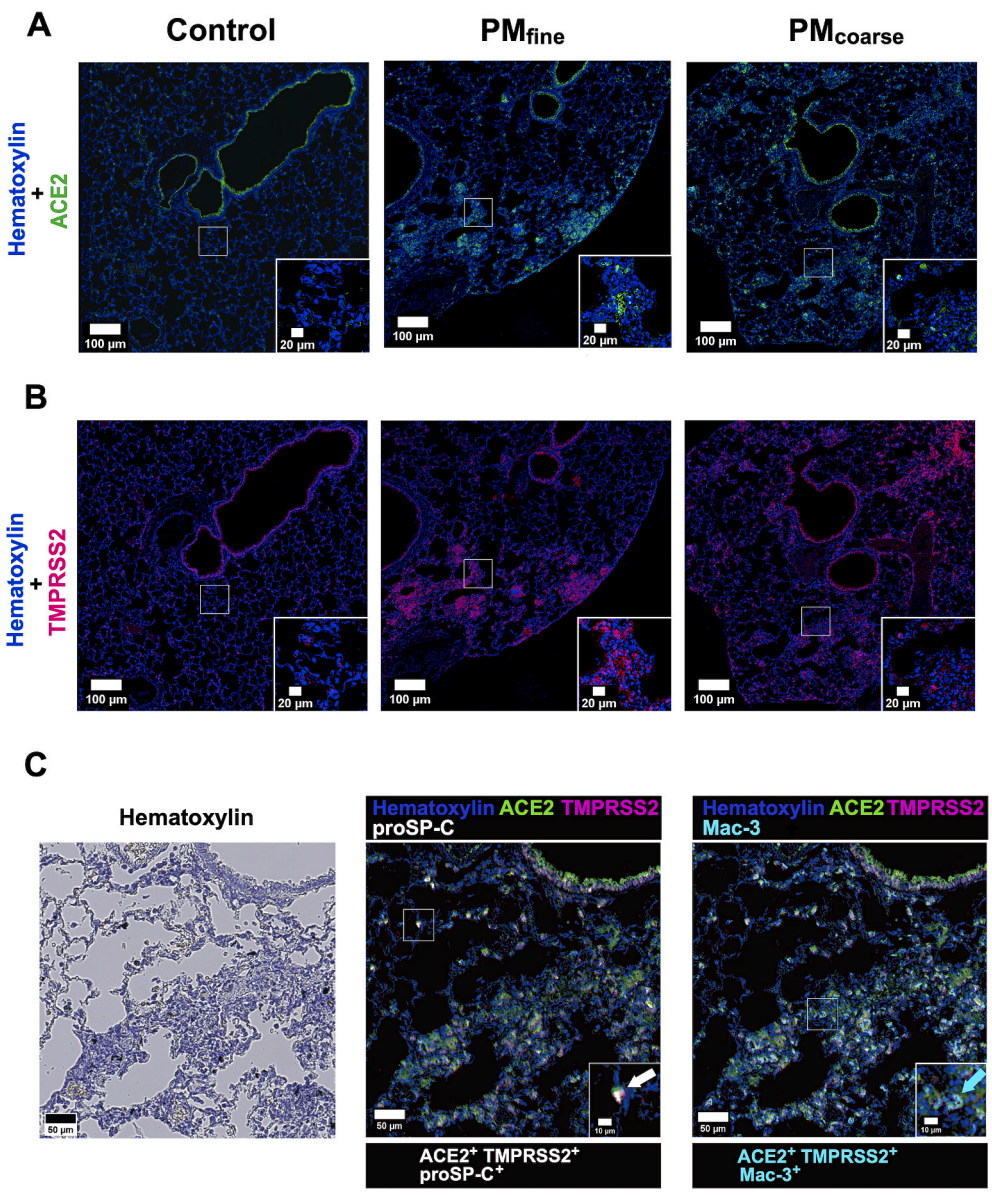

Fig. 2. (**A-B**) Representative images of lung tissue section obtained from experimental mice 24 h after intratracheal instillation of fine particulate matter, coarse particulate matter, or control solution. Pseudo-colored images of immunohistochemical staining for ACE2 (**A**) and TMPRSS2 (**B**). In each image, the box outlines the area magnified in the inset. Color annotations and scales are shown. **(C)** Representative images of lung tissue sections obtained from mice 24 h after intratracheal instillation of coarse fraction of particulate matter. Hematoxylin staining and multiplex immunohistochemical staining showing the expression of ACE2 and TMPRSS2 in the proSP-C^{+} cells and Mac-3^{+}. The boxes outline the areas magnified in the insets. ACE2 = angiotensin-converting enzyme 2; TMPRSS2 = transmembrane protease serine type 2; PM_{fine} = Particulate matter (fine fraction); PM_{coarse} = Particulate matter (coarse fraction).

of this study suggest that PM may have a similar adverse effect on COVID-19. It is crucial to discuss which components of PM are responsible for the phenomena observed in this study. In fact, PM is composed of a wide range of chemicals and biological materials such as elemental carbon; organic carbon, including polycyclic aromatic hydrocarbons (PAHs); inorganic salts; metals, and lipopolysaccharide (LPS) (Honda et al., 2017; Montgomery et al., 2020). Notably, LPS has been associated with deleterious effects on respiratory health and immune function (Onishi et al., 2018). However, a mouse study found that ACE2 expression was reduced in mice with LPS-induced acute lung injury (Ye and Liu, 2020). In the present study, it is interesting to note that PM, which contains LPS (57.2 EU/mg in PM fine and 87.9 EU/mg in PM coarse [Onishi et al., 2018]), increased ACE2 expression in AT2 cells and macrophages. Therefore, it could be inferred that other components of PM may upregulate ACE2 expression in the lung. Regarding the increase in TMPRSS2 expression in the lung, it is not clear which components of PM are responsible for this effect.

An expression profiling meta-analysis of ACE2 and TMPRSS2 in human lung tissue shows that these genes tend to be co-regulated, and smoking and androgens as factors that enhance their expression (Gkogkou et al., 2020). Interestingly, the increased expression of ACE2 and TMPRSS2 by tobacco smoke, which is also a component of PM (Invernizzi et al., 2004), has been implicated in the androgen pathway (Chakladar et al., 2020). Furthermore, PAHs, components in both tobacco smoke and PM, are known to act as endocrine disrupting chemicals (Hayakawa et al., 2008). Therefore, it may be worth investigating the involvement of the androgen pathway in considering the mechanisms by which PM increases the expression of ACE2 and TMPRSS2.

Since the components of PM vary according to collection areas, seasons, and collection methods (Chowdhury et al., 2018), it is an extremely complicated task to elucidate the mechanisms underlying the increased expression of ACE2 and TMPRSS2 and to identify the specific components responsible. However, because of the current situation caused by COVID-19, future research will need to identify the exact mechanisms and factors mediating the effects of PM on the manifestation and/or progression of COVID-19.

In human lung tissue, AT2 cells and macrophages are known to co-express ACE2 and TMPRSS2 (Bertram et al., 2012; Z. Z. Abassi et al., 2020; Z. A. Z.A. Abassi et al., 2020), and these cells can be infected by SARS-CoV-2 (Adachi et al., 2020). Furthermore, SARS-CoV-2 has been found to infect AT2 cells and macrophages in hACE2 transgenic mice, as well as in humans (Bao et al., 2020). Although SARS-CoV-2 is not

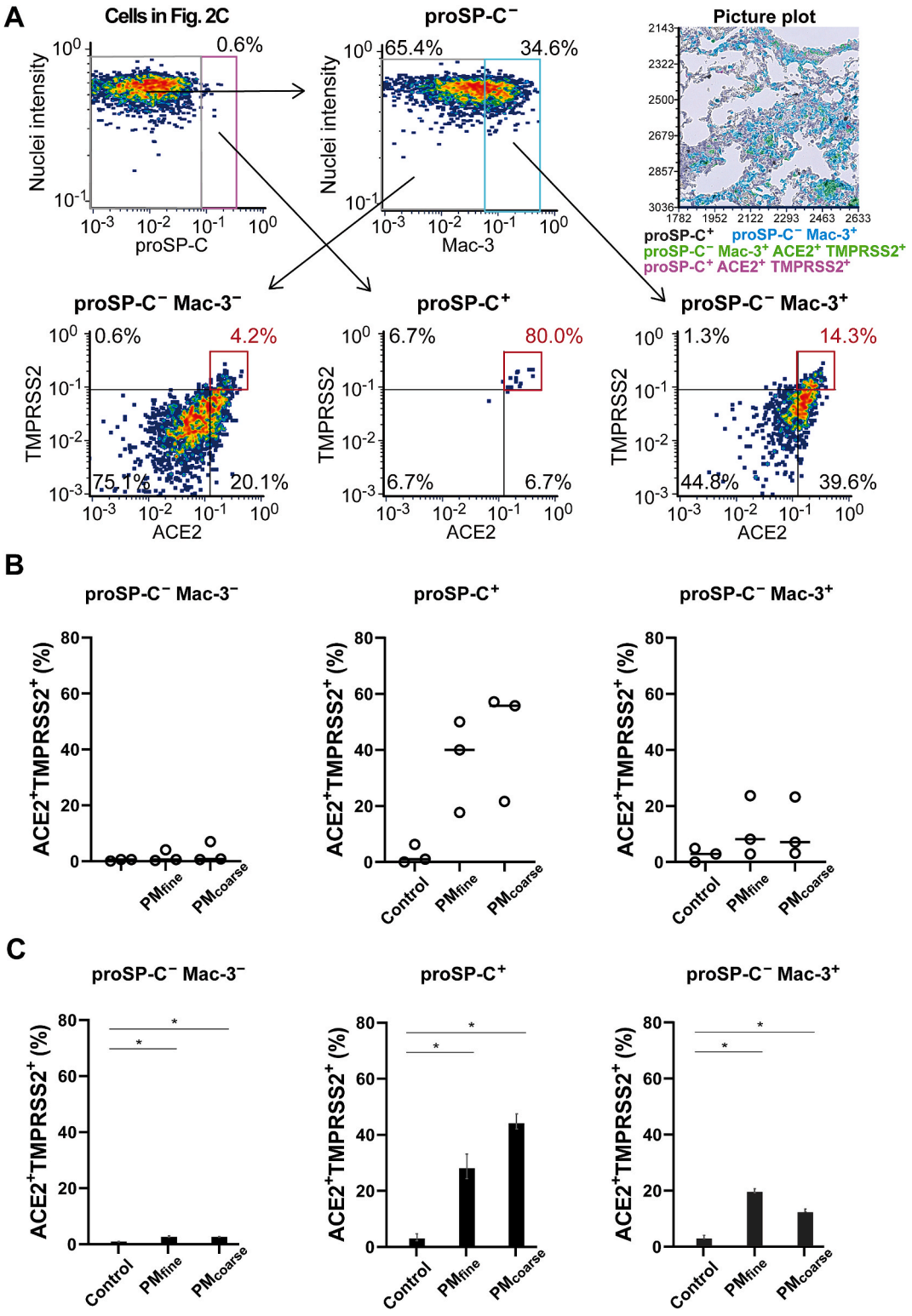

Fig. 3. (**A**) The proportion of proSP-C$^-$ Mac-3$^-$, proSP-C$^+$, and proSP-C$^-$ Mac-3$^+$ cells that are positive for both ACE2 and TMPRSS2 were quantitatively evaluated using image cytometry. The gating strategies used and a representative picture plot of the same area of lung tissue as that in Fig. 2C are shown. (**B**) The percentages of proSP-C$^-$ Mac-3$^-$, proSP-C$^+$, and proSP-C$^-$ Mac-3$^+$ cells positive for both ACE2 and TMPRSS2 are shown for each of the treatment and control groups. Each datapoint represents the mean value calculated from a wider range of one mouse, applying the gating strategy as that used in Fig. 3A. Data are shown for three mice per group. Horizontal bars indicate the median values.(C) The percentages of proSP-C$^-$ Mac-3$^-$, proSP-C$^+$, and proSP-C$^-$ Mac-3$^+$ cells positive for both ACE2 and TMPRSS2 are shown for each of the treatment and control groups. Each graph represents the results of combining the individual mouse data from Fig. 3B and analyzing them by cell type, with standard errors. *$P < 0.001$. ACE2 = angiotensin-converting enzyme 2; TMPRSS2 = transmembrane protease serine type 2; PM_{fine} = particulate matter (fine fraction); PM_{coarse} = particulate matter (coarse fraction).

considered to be pathogenic in wild-type mice, it is an important finding that the cells we identified in this study are identical to those identified in the experiments described above in human subjects and hACE2 transgenic mice.

5. Conclusion

The increased expression of both ACE2 and TMPRSS2 in AT2 cells and macrophages in the lung tissue of mice exposed to PM is considered to represent a part of the mechanisms underlying the association between PM and COVID-19. Focusing on air pollution, including PM, may help to understand the causes of the severity and susceptibility of COVID-19 and to develop countermeasures that could reduce the impact of the disease.

Credit author statement

Tomoya Sagawa: Conceptualization, Data curation, Formal analysis, Investigation, Visualization, Writing – original draft. Takahiro Tsujikawa: Data curation, Formal analysis, Investigation, Visualization, Writing – review & editing. Akiko Honda: Investigation, Supervision, Writing – review & editing. Natsuko Miyasaka: Investigation, Writing – review & editing. Michitaka Tanaka: Investigation, Writing – review & editing. Takashi Kida: Supervision, Writing – review & editing. Koichi Hasegawa: Supervision, Writing – review & editing. Tomoaki Okuda: Resources, Supervision, Writing – review & editing. Yutaka Kawahito: Supervision, Writing – review & editing. Hirohisa Takano: Project administration, Funding acquisition, Supervision, Writing – review & editing.

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.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envres.2021.110722.

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