"Impact of inhaled swine CAFO dust on COVID-19 pathogenesis," #PR-005296

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Industry Summary:

Agricultural workers are exposed to many occupational environments that can produce inhalable dusts. Long term inhalation of both organic and inorganic dusts in the workplace without the proper use of respirators can result in inflammatory lung disease. One of the most common results of a lifetime of dust inhalation is chronic obstructive pulmonary disease (COPD). To date, individuals working around livestock dusts demonstrate the greatest incidence of occupationally-induced COPD. At the beginning of the COVID-19 pandemic, meatpacking workers were significantly impacted with transmission of SARS-CoV-2, the virus causing COVID-19. We hypothesized that other workers exposed to livestock dusts may also be at higher risk for infection due to inhalation of organic dust.

Settled dust from area swine confinement barns was collected and used to expose lung epithelial cells, the cells that line our lungs and are exposed to the outside air. Next, the cells were infected with SARS-CoV-2 or a purified surface protein from this virus. We then evaluated aspects of lung epithelial cell function including infectivity, inflammation, and injury, as well as some of the known intracellular enzymes that regulate these processes to determine whether any dust-mediated injury enhancement occurs in the presence of the virus.

In mice, dust exposure increased lung shedding of the receptor that binds the virus (ACE2). After repeated daily dust exposure for 3 weeks, this increased receptor correlated to higher amounts of virus in the lungs. Dust increased the activation of an important enzyme (Protein Kinase C) in cells. Correspondingly, this dust-induced enzyme activity increased ACE2 expression in cells. Increasing cell ACE2 levels by treating with enzyme inhibitors and a low dose of dust enhanced virus entry into the cells. Following viral entry, an important inflammation response cytokine was reduced, suggesting a lowered innate defense.

The benefit of this research to the Industry is to determine if worker COVID-19 infection and severity of recovery is linked with swine confinement dust inhalation. Our findings justify worker protection through the most effective combination of administrative, engineering, and personal protection means that fit the Industry conditions. Together, these studies support a role for agricultural dust in SARS-CoV-2 infection and identify the mechanistic pathway of dust-enhanced virus entry to highlight the importance of studying how environmental dust exposures impact immunity against pathogens. Organic dusts in agricultural settings have complex compositions that can trigger the immune system in a variety of ways. Long-term exposure to such triggers can significantly deteriorate workers' respiratory health by not only predisposing them to chronic lung diseases, but also having a serious impact on their immunity against respiratory infections. During a global pandemic caused by a respiratory virus, these essential agricultural workers put themselves at considerable risk.

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Key Findings:

- Lung cells treated with swine barn-derived organic dust have the potential to increase SARS-CoV-2 infection under isolated laboratory conditions
- Specific enzymes in these lung cells are activated by the dust and lead to the expression of more of the cellular receptor for SARS-CoV-2
- Workers in the swine barn environment should comply with the best practices for Personal Protective Equipment (respirator masks), particularly under pandemic conditions

Keywords: SARS-CoV-2, ACE2, organic dust (ODE), PKCα, ADAM-17

Scientific Abstract:

SARS-CoV-2, the causative agent of the COVID-19 pandemic, has had a global impact, affecting millions over the last three years. The severity of COVID-19-associated symptoms can range from asymptomatic to death. The prognosis of hospitalized COVID-19 patients heavily depends on the presence of comorbidities such as pre-existing lung diseases. Agricultural workers inhale respiratory irritants regularly, substantially increasing their risk for developing chronic lung diseases with long-term exposures. This inherent risk associated with agricultural work puts the workers in danger of severe COVID-19. In previous studies, we characterized the protein kinase C (PKC)-dependent airway inflammation mediated by organic dust extract (ODE) derived from dust collected from swine confinement facilities in *in vitro* and *in vivo* models. Here, we studied the effect of ODE on SARS-CoV-2 pseudoviral infection in mice and BEAS-2B cells. In wild-type (WT) and humanized ACE2 mice, ODE increased ACE2 shedding by ADAM-17 in the lungs. After repeated ODE treatments, the increased soluble ACE2 correlated to higher pseudovirus titer in the mouse lungs. In the human bronchial epithelial cell line, BEAS-2B, ODE augmented PKCα activity in WT cells, and functional PKCα was needed for membrane ACE2 expression, as seen in PKCα-deficient cells. Unlike in the mice, increasing membrane ACE2 levels by treating with PKCα or ADAM-17 inhibitors and a low dose of ODE enhanced pseudoviral entry *in vitro*. Following viral entry, IL-8 secretion by the cells was diminished in a PKCα- and ADAM-17-independent manner. Together, the complex mechanisms involved in the synergistic effects of agricultural dust and SARS-CoV-2 highlight the importance of studying dust-mediated changes to immunity against circulating pathogens.

Introduction:

The coronavirus disease 2019 (COVID-19) pandemic caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has been one of the largest global pandemics in history. Due to its high transmission rate, SARS-CoV-2 spread to over 216 countries and territories in less than 8 months. As of September 2022, more than 610 million confirmed cases and over 6.5 million deaths have been reported worldwide [1]. Although most individuals are asymptomatic following infection, some develop symptoms ranging in severity. Compared to other common viral infections, COVID-19-related hospitalizations are substantially higher, with more patients in need of oxygen therapy and ventilatory support [2]. From fever to pneumonia to acute respiratory distress to multiorgan failure and death, the severity of symptoms and resulting prognosis depend heavily on pre-existing health conditions and exposure to risk factors [3]. COVID-19-associated morbidity and mortality markedly increase in the presence of comorbidities such as diabetes and liver, kidney, cardiovascular, and pulmonary disease [4].

Inhalation of aerosols for long periods of time can lead to development of lung diseases that predispose individuals to severe COVID-19. Animal feeding operations in the United States in large, confined spaces produce aerosols that pose a significant health risk to agricultural workers. Long-term exposures to these aerosols can have deleterious effects on pulmonary functions, increasing the risk of respiratory diseases including asthma, chronic bronchitis, rhinosinusitis, and chronic obstructive pulmonary disease [5]. These agricultural workers often report respiratory and flu-like symptoms mainly due to the highly inflammatory composition of the aerosols [6-8].

Swine dust is composed of gram-positive and gram-negative bacteria, fungal spores, and other particulates that can elicit pro-inflammatory responses in the lungs [9]. Indeed, exposure to complex swine confinement organic dust extracts (ODE) has been shown to induce an influx of neutrophils, macrophages, and lymphocytes, as well as the release of pro-inflammatory cytokines resulting in airway hyper-responsiveness in animal models, resembling human disease [9]. Cytokines, such as TNF-α and IL-6, and neutrophil-attracting chemokines, such as CXCL1 and CXCL2, are released following ODE exposure in animals [9].

In our previous studies, we demonstrated that ODE activates protein kinase C (PKC), mediating release of IL-6 and IL-8 from bronchial epithelial cells, a process shown to be dependent on TNF-α and TNF receptor [10,11]. Signals, including activation of PKC and toll-like receptors responding to pathogens, can activate ADAM-17 and its "sheddase" activity [12]. A disintegrin and metalloprotease 17 (ADAM-17), also called TACE (tumor necrosis factor-α-converting enzyme), is a membrane protease that, when activated, removes the ectodomains of membrane proteins like ACE2, an entry receptor for SARS-CoV-2. ADAM-17 is expressed in many tissues such as muscle, thymus, heart, small intestine, gonads, placenta, kidney, pancreas, and lung [13].

ACE2 is a type I transmembrane carboxymonopeptidase protein expressed in several organs ubiquitously, with highest levels of expression in the cardiovascular system, brain, testicles, kidneys, intestine, and lungs [14]. During infection, SARS-CoV-2 spike protein binds the ACE2 receptor on target host cells, allowing fusion of the viral envelope and host cell membrane, leading to subsequent viral entry [15,16]. Here, since ODE alters ADAM-17 function through PKC signaling, which can then affect ACE2, we studied the changes in SARS-CoV-2 entry following ODE exposure and its dependence on PKCα and ADAM-17 *in vivo* in mice and *in vitro* in a human bronchial epithelial cell line.

Objectives:

The objective of this research is the definitive identification of whether swine dust exposure enhances infection and severity of COVID-19. We also investigated whether a promising COVID-19 drug at the time, Remdesivir, has an impact on that mechanism (similar to subsequent reports replete in the literature, we observed no effects with Remdesivir). Our objectives are a peer-reviewed manuscript to document the findings (ready for submission), presentation of the findings at national conferences (presentations made at AgriSafe, ISASH, and ATS). The research team will work with NPB to disseminate the information to key media and stakeholders in the pork production industry. The Central States Center for Agricultural Safety and Health Outreach Core will disseminate the information directly to producers in the Center's seven-state region and indirectly through the national network of NIOSH agricultural health and safety centers.

Materials & Methods:

SARS-CoV-2 pseudovirus: 293T cells (ATCC: CRL-3216) grown in Dulbecco's Modified Eagle Medium (DMEM) with 10% fetal bovine serum were transfected with 7 µg of pLVx-tdTomato-N1 Vector (Takara Bio, Palo Alto, CA, USA) and one tube of Lenti-X™ SARS-CoV-2 Packaging Mix (WT Spike, Full

Length) (Takara Bio, Palo Alto, CA, USA) according to manufacturer's instructions. The supernatant from the cells containing pseudovirus was harvested 48 hours post-transfection, centrifuged, and stored at -80°C. The supernatant was sucrose-purified using ultracentrifugation for animal experiments and stored at -80°C.

Mice experiments: Eight-week-old wild-type (WT) or humanized ACE2 mice (Jackson Labs, Bar Harbor, ME) were used for all animal experiments. WT mice were intranasally instilled with saline or 12.5% organic dust extract (ODE; characterized in Boissy [17]) once over 1-week or 13 times over 3-weeks. Lungs were then collected after the last ODE exposure, homogenized, and mouse ACE2 was quantified. Similarly, humanized ACE2 mice were intranasally treated with sterile saline, 12.5% ODE, or TAPI-1 (1 μM, Sigma-Aldrich, St. Louis, MO) and 12.5% ODE. After ODE exposure, lungs were collected, homogenized, and human ACE2 was quantified. For pseudovirus infection experiments, humanized ACE2 mice were intranasally treated once or 13 times with ODE and infected with purified pseudovirus (MyBioSource, San Diego, CA). Five days post-infection, lungs were collected for homogenization and RNA extraction to determine viral titer. All animal experiments were reviewed and approved by the Institutional Animal Care and Use Committee of University of Nebraska Medical Center and were conducted in accordance with the Institutional Animal Care and Use Committee guidelines and regulations.

Mouse lung ACE2 quantitation: Soluble ACE2 in the supernatant of homogenized and centrifuged mouse lungs was determined using Mouse ELISA kit in the WT mice and Human ELISA kit in the humanized ACE2 mice according to manufacturer's (R&D Systems, Minneapolis, MN) instructions. ACE2 is reported as pg/mL.

RNA extraction and Quantitative Polymerase Chain Reaction (qPCR): RNA was isolated from the mouse lungs using Direct-zol™ RNA Miniprep Plus (Zymo Research, Irvine, CA, USA). Lungs were collected in TRI Reagent® (Zymo Research, Irvine, CA, USA), homogenized, and centrifuged. The supernatant was then used for RNA extraction according to manufacturer's instructions.

Virus titer was determined using the Lenti-X^{TM} qRT-PCR Titration Kit (Takara Bio, Palo Alto, CA, USA) according to manufacturer's instructions. The Lenti-X^{TM} RNA Control Template was used alongside the samples to quantify RNA copies. QuantStudio 3 Real-Time PCR machine (Applied Biosystems, Waltham, MA, USA) was used with QuantStudio Design and Analysis software version 1.5.1 (Applied Biosystems, Waltham, MA, USA) for analysis. Results are expressed as RNA copies per lung (log).

BEAS-2B cells: Wild-type (WT) and PKCα-deficient (DN) cell lines were generated and cultured as previously described [18].

PKCα activity assay: WT and DN cells were treated with 5% ODE or media for 1 hour at 37°C and 5% CO_2. Calcium-dependent PKC alpha isoform-specific activity was measured using radiolabeled substrate phosphate transfer of P^{32}-ATP as previously described [18].

BEAS-2B cell treatments and infection: At 80-90% confluency, in a 96-well format, WT and DN BEAS-2B cells were treated with Gö-6976 (1 μM; Sigma-Aldrich), TAPI-1 (20 μM), or media for 1-hour at 37°C. Then, the treatments were removed, and 0.5% ODE or media was added. After incubating at 37°C for 1 hour, half the wells were used for flow cytometry, and the rest were infected with unpurified SARS-CoV-2 pseudovirus. The virus was diluted in cell growth media containing 6 μg/mL Polybrene (Millipore-Sigma, Darmstadt, Germany). The next day, media with virus and polybrene was removed, and fresh media was added. The infection was continued for another 24 hr (48 hr from the time of

addition of virus). Supernatant was collected for cytokine ELISAs, and the cells were used for immunofluorescence.

Flow cytometry: Treated BEAS-2B cells were trypsinized and collected for flow cytometry analysis. The cells were washed and stained with Alexa Fluor 647-conjugated anti-human ACE2 antibody (R&D Systems) for 30 min on ice. The stained cells were washed twice and fixed with 4% paraformaldehyde (Thermo Fisher Scientific, Waltham, MA, USA). Data was acquired using NovoCyte flow cytometer (ACEA Biosciences, San Diego, CA, USA). Unstained controls were used for gating. Data analysis was completed using NovoExpress 1.5.0 (Agilent Technologies, Santa Clara, CA, USA) software.

Immunofluorescence: Treated and infected BEAS-2B cells were fixed with 4% paraformaldehyde (Thermo Fisher Scientific, Waltham, MA, USA) for 10 min at room temperature. The fixative was then removed, and Hoechst 33342 nuclear stain (Invitrogen, Waltham, MA, USA) was added at 1/20,000 dilution for 15 min at room temperature. Stained cells were visualized using the Operetta CLS™ system (Perkin Elmer, Waltham, MA, USA). dTomato-positive cells (infected with pseudovirus) were identified under 20x air objective and 9 fields/well were analyzed using Harmony 4.9 (Perkin Elmer, Waltham, MA, USA) software.

Interleukin-8 (IL-8) ELISA: Secreted IL-8 in the treated/infected BEAS-2B cells was determined using Human IL-8/CXCL8 DuoSet ELISA kit (R&D Systems) according to manufacturer's instructions. IL-8 is reported as ng/mL.

Statistical analysis: Statistical analysis was conducted using Student's t-test, one-way analysis of variance (ANOVA), or two-way ANOVA. Tukey's post hoc test was used to adjust for multiple comparisons between different test groups. Tests were performed at a 5% significance level. All statistical analyses were performed using GraphPad Prism 9 (San Diego, CA, USA) software.

Results:

ODE augments ADAM-17 activity increasing soluble ACE2 levels in mouse lungs: First, we aimed to determine if levels of ACE2, a SARS-CoV-2 entry receptor, shed from the cell membrane are affected following administration of organic dust extract (ODE) in mice. To that end, WT mice were exposed to ODE once or 15 times, and their lungs were collected for homogenization and ACE2 quantitation. Compared to saline controls, ODE treatment significantly increased soluble ACE2 levels. Repetitive ODE treatment further enhanced ACE2 amounts compared to single ODE exposure. This effect of ODE on ACE2 was then confirmed in humanized ACE2 transgenic mice where, compared to media control, ODE treatment significantly elevated ACE2 levels. As expected, this ODE-mediated increase was reversed by the addition of TAPI-1, an ADAM-17 inhibitor, reiterating the role of ADAM-17 in cleaving ACE2 from the cell membrane [19,20]. Finally, higher levels of soluble ACE2 led to an increase in SARS-CoV-2 pseudovirus entry in humanized ACE2 mice. Although no difference in pseudovirus titer was observed in mice following single ODE exposure compared to virus only and naïve controls, repetitive exposure significantly augmented lung viral titer. *Ex vivo* pseudoviral infection of mouse tracheal epithelial cells collected from humanized ACE2 mice following 0.5% ODE treatment also resulted in higher viral titer compared to untreated cells. Together, ODE enhances ADAM-17-dependent ACE2 shedding in mice, augmenting SARS-CoV-2 spike protein-mediated viral entry.

ODE treatment of an immortalized human epithelial cell line, BEAS-2B, increases PKCα activity in vitro*:* PKCα is a known activator of ADAM-17 [21]. Thus, we sought to deduce the effect of ODE on PKCα activity in a human cell line *in vitro*. Wild-type (WT) and PKCα-deficient (DN) BEAS-2B cells, an immortalized epithelial cell line from human bronchial epithelium, were treated with 5% ODE. Following

one hr of treatment, PKCα activity was quantified in the WT and DN cells. DN cells had minimal PKCα activity that did not change with ODE treatment, whereas WT cells had a significant increase in PKCα activity when exposed to ODE. Therefore, in the presence of functional PKCα, ODE enhances its activity, which could then promote ADAM-17-mediated ACE2 shedding.

Inhibition of PKCα or ADAM-17 along with ODE treatment synergistically increases membrane ACE2 levels, enhancing SARS-CoV-2 pseudovirus entry in BEAS-2B cells in vitro*:* Next, we aimed to determine the importance of functional PKCα and ADAM-17 in affecting membrane ACE2 expression and, in turn, SARS-CoV-2 pseudovirus entry in a human cell line. To that end, WT and DN BEAS-2B cells were treated with ODE, Gö-6976 (a PKCα inhibitor), TAPI-1 (an ADAM-17 inhibitor) or a combination of inhibitor and ODE for 1 hr *in vitro*. The cells were then analyzed by flow cytometry for membrane ACE2 expression. DN cells had baseline ACE2 expression which did not change following any of the treatments. However, WT cells had significantly higher ACE2 expression when treated with Gö-6976 or TAPI-1 along with ODE compared to media- and ODE-treated cells. This translated to higher infection by SARS-CoV-2 pseudovirus in WT cells treated with Gö-6976 and ODE as well as TAPI-1 and ODE. Notably, ODE treatment alone did not result in higher membrane ACE2 expression or pseudovirus entry compared to the untreated group. This could be because we used sub-stimulatory doses of ODE (0.5%) for these BEAS-2B treatment/infection experiments to ensure that cell viability is not affected due to ODE during the 48-hour pseudovirus infection. The lack of differences in lactate dehydrogenase among all treatment groups confirmed that the changes observed were not due to cell toxicity. Together, unlike in mice, PKCα and ADAM-17 signaling helps prevent SARS-CoV-2 spike protein-mediated viral entry into cells *in vitro* by decreasing ACE2 expression on the cell membrane.

SARS-CoV-2 pseudovirus infection reduces IL-8 secretion in a PKCα-independent manner in low-dose ODE-treated BEAS-2B cells: Following a 48 hr transduction with SARS-CoV-2 pseudovirus of Gö 6976, TAPI-1, and/or ODE-treated WT and DN BEAS-2B cells, we quantified cytokine levels in the supernatant. Since a sub-stimulatory dose of ODE was used, neither the treatments nor the pseudovirus had any effect on IL-6 secretion, as expected in BEAS-2B cells. However, IL-8 secretion was significantly decreased after pseudovirus infection in both WT and DN cells. The overall quantity of IL-8 detected in DN cells was much lower than in WT cells, emphasizing the importance of PKCα signaling for IL-8 production. Interestingly, IL-8 secretion was not altered by inhibition of PKCα or ADAM-17 or by ODE. Therefore, engaging membrane ACE2 by SARS-CoV-2 spike protein diminishes IL-8 secretion in a PKCα- and ADAM-17-independent manner *in vitro*.

Discussion:

Agricultural workers are exposed to a wide range of respiratory irritants every day they work. These aerosolized irritants have complex compositions that can trigger the immune system in a variety of ways. Long-term exposure to such triggers can significantly deteriorate workers' respiratory health by not only predisposing them to chronic lung diseases, but also having a serious impact on their immunity against respiratory infections. During a global pandemic caused by a respiratory virus, these essential agricultural workers put themselves at considerable risk. Indeed, studies conducted during the early stages of the COVID-19 pandemic showed high prevalence of SARS-CoV-2 among agricultural field workers, with about half the workers testing positive [22-24]. Therefore, it is crucial to understand the mechanisms underlying dust-mediated changes to immune responses against pathogens, especially SARS-CoV-2.

In this study, we focused on exposure to dust collected from swine confinement facilities (ODE) in the United States. In our previous works, the mechanisms underlying ODE-mediated airway inflammation have been well characterized in *in vitro* and *in vivo* models [25]. We have shown the sequential activation

of PKCα and PKCε leading to pro-inflammatory cytokine and chemokine release in response to ODE treatment of bronchial epithelial cells *in vitro* [26]. *In vivo*, mice exposed to a single dose of ODE had pronounced neutrophil influx and high levels of inflammatory mediators in bronchoalveolar lavage fluid. Interestingly, repeated ODE exposure exacerbated lung histopathology even though inflammatory cytokine levels and cell influx were not as striking as single exposure [27]. In this study, ODE treatment resulted in higher levels of soluble ACE2 in mouse lungs in both wild-type and humanized ACE mice in an ADAM-17-dependent manner, with repeated treatment further increasing ACE2 shedding in wild-type mice. This increase in soluble ACE2 directly correlated to SARS-CoV-2 pseudovirus entry in the repeated exposure model in humanized ACE mice.

However, *in vitro*, ODE-mediated upregulation of membrane ACE2 following PKCα and ADAM-17 inhibition was important for increased SARS-CoV-2 entry. This suggests that, in a human bronchial epithelial cell line (BEAS-2B), PKCα and ADAM-17 signaling induced by ODE helps prevent SARS-CoV-2 spike protein-mediated viral entry into cells by decreasing ACE2 expression on the cell membrane. This points to the limitations associated with the *in vitro* model of ODE exposure. Apart from the direct ACE2 receptor and viral spike protein interaction, soluble ACE2 can also facilitate SARS-CoV-2 entry through receptor-mediated endocytosis [28] as seen in the mouse model. Changes in cell culture conditions, such as temperature, can lower the rate of receptor-mediated endocytosis, which can then affect infection efficiency of the cells. However, the use of transgenic *in vitro* models does allow for better understanding of the mechanisms involved.

PKCα-deficient (DN) BEAS-2B cells used in this study had significantly lower levels of membrane ACE2 in untreated and treated samples compared to WT cells, suggesting that functional PKCα may play a role in ACE2 expression. Even though low membrane ACE2 correlated to low pseudovirus infection in the DN cells suggesting a protective role, infection with pathogenic SARS-CoV-2 would have resulted in severe pathology in the absence of ACE2. Indeed, studies with influenza and respiratory syncytial virus have shown that administering recombinant ACE2 had beneficial effects on virus-induced lung lesions [29,30].

Downregulation of membrane ACE2 by SARS-CoV-2 can also lead to pulmonary injury as severe as lethal lung failure [31-33]. The high expression of ACE2 in the lungs may, in fact, be related to the protective effects the receptor provides [34]. Indeed, ACE2 protected mice infected with SARS-CoV-1 from acute lung disease since spike protein binding reduced ACE2 surface expression due to internalization of ACE2-virus complex [35]. Similarly, SARS-CoV-2-mediated downregulation of ACE2 also decreases the protective effects of ACE2 in kidneys, heart, gut, and lungs [36-38].

In addition, ACE2-mediated SARS-CoV-2 entry may have an anti-inflammatory effect. In BEAS-2B cells, SARS-CoV-2 pseudovirus infection significantly diminished IL-8 release under all treatment conditions in a PKCα- and ADAM-17-independent manner. IL-8, a neutrophil chemoattractant, plays a key role in inducing inflammation following ODE exposure [39]. In our previous studies, we showed that ODE activates PKC, which can then activate ADAM-17, mediating release of IL-6 and IL-8 in a TNF-α and TNF receptor-dependent pathway in bronchial epithelial cells [10-12]. Activation of ADAM-17 can subsequently induce ACE2 receptor shedding from the cell membrane. On the other hand, ADAM-17 activation also affects pro-inflammatory responses mediated by IL-8 and anti-inflammatory responses by TNF receptor shedding [12]. Thus, virus-induced reduction in pro-inflammatory responses elicited by ODE may delay immune cell recruitment, impeding viral clearance and prolonging infection. Studies are ongoing to determine the mechanisms underlying the inhibition of IL-8 production by membrane ACE2-activation.

Although short exposures to low doses of ODE could create a pro-inflammatory lung environment that may help enhance viral clearance, long-term ODE exposure-induced inflammatory responses may predispose individuals to severe COVID-19. Compounded with respiratory conditions associated with chronic ODE exposure, increased viral entry into cells through soluble ACE2 and delayed viral clearance due to virus-mediated suppression of inflammatory responses may lead to a persistent infection, further exacerbating pulmonary disease in agricultural workers.

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