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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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Enzymatic activity of ACE2 regulates type 2 airway inflammation in mice

To the Editor,

Coronavirus disease 2019 (COVID-19), caused by novel severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), has led to a global pandemic. SARS-CoV-2 spike protein binds to angiotensin-converting enzyme 2 (ACE2), a transmembrane endopeptidase on host cells of the airway epithelium surface for invasion and infection[1]; therefore, most COVID-19 research has focused on ACE2. Patients with chronic obstructive pulmonary disease and idiopathic pulmonary fibrosis are reportedly at a high risk of COVID-19 morbidity and mortality,[2,3] regardless of whether they have asthma.[4] Asthma is a heterogeneous disease triggered by environmental factors such as house dust mites (HDM) and viruses that cause chronic airway inflammation.[5] These factors promote epithelial cell damage, leading to the release of cytokines that provoke a type 2 (T2) inflammatory response.[6] Kimura et al[7] reported that interleukin (IL)-13 exposure reduces ACE2 expression in the airway epithelium of patients with asthma, whereas interferons enhance ACE2 expression.[3,8] Camiolo et al[9] also indicated that ACE2 expression is linked to up-regulation of viral response genes, such as IFNs and T-cell-activating factors, in a subset of patients with T2 inflammation-low asthma with characteristics corresponding to risk factors for severe COVID-19. Overall, low ACE2 expression in epithelial cells may protect patients with asthma from COVID-19.

ACE2 is not only a receptor for SARS-CoV-2 but also the main enzyme for catalyzing the conversion of angiotensin II into angiotensin (1–7) and exerts anti-inflammatory effects in cardiovascular diseases.[10] However, the relationship between asthma-related allergic inflammation and ACE2 enzymatic activity, but not its expression in the airway, has not been assessed in vivo.

To explore these associations, we used an HDM-induced asthma mouse model, which is T2 dominant asthma model. C57BL/6 J mice were intratracheally exposed or not to HDM (10–15 mice/group) at days 0, 7, and 14. Bronchoalveolar lavage fluid (BALF) and lung tissue samples were harvested at days 3, 10, and 17 to count eosinophils and measure *Ace2* expression (Figure 1A, Appendix S1).

The eosinophil counts in BALF samples increased from day 3 to 17 concomitantly with repeated HDM exposure in a stepwise

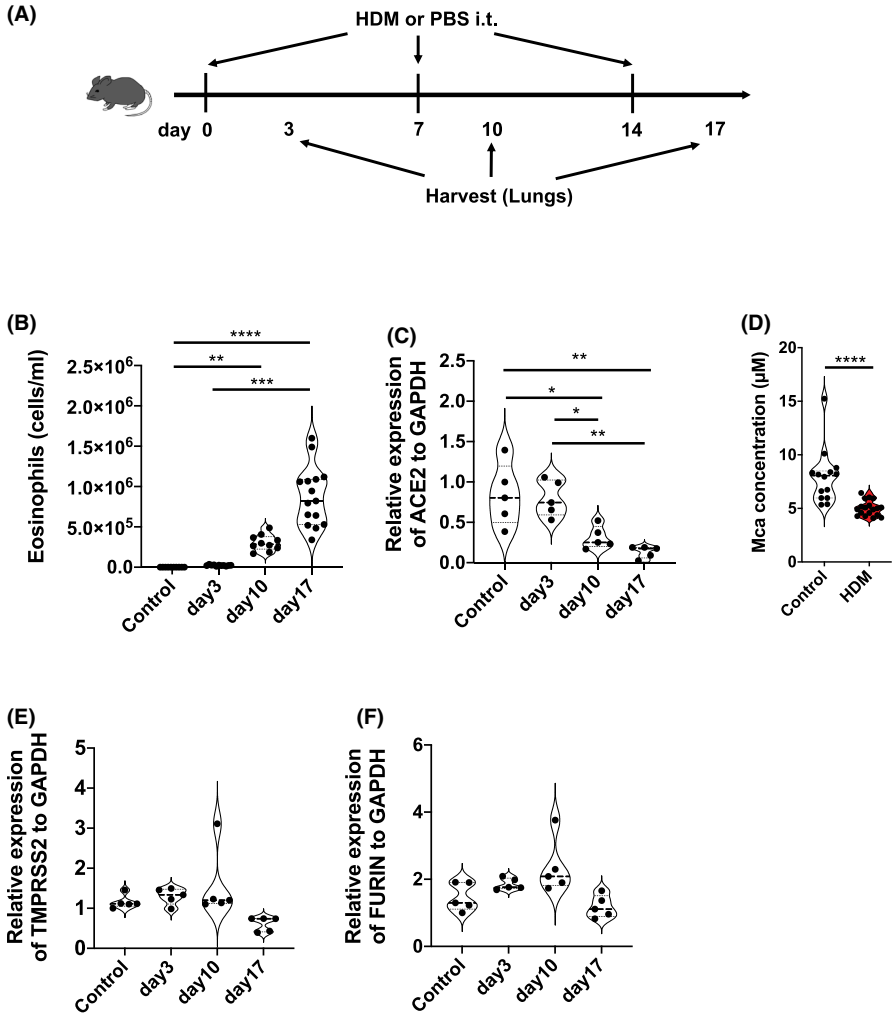


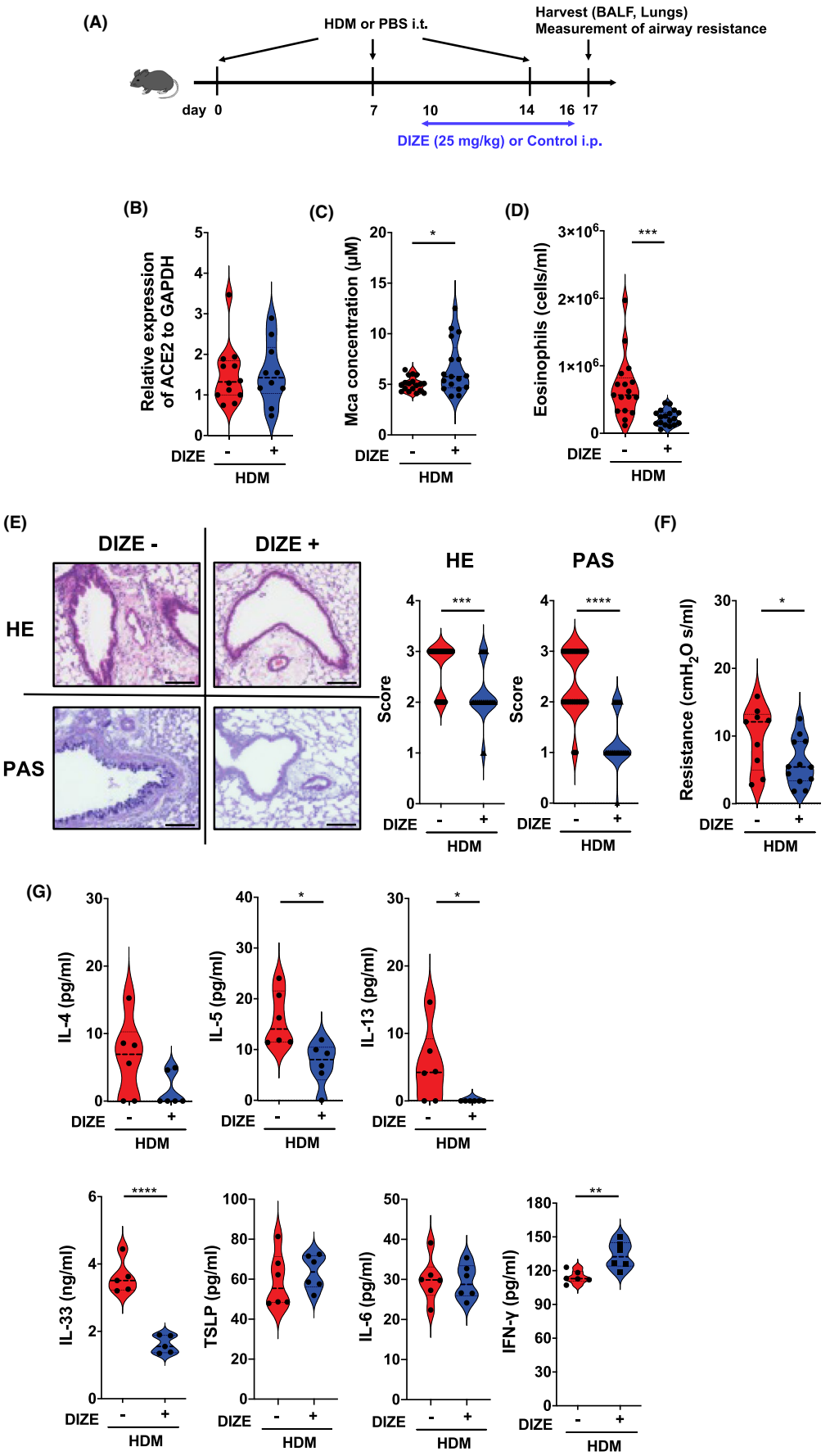
FIGURE 1 Eosinophils counts in the bronchoalveolar fluid (BALF) and *Ace2* expression in lung tissues from house dust mite (HDM)-exposed mice. (A) Experimental scheme. (B) Number of eosinophils in BALF samples. (C) Relative *Ace2* expression in lung tissues analyzed using quantitative real-time polymerase chain reaction (qRT-PCR). (D) ACE2 enzymatic activity in BALF was measured on day 14 after 6 h (14–18 mice/group from three independent experiments). (E and F) Relative *TMPRSS2* (E) and *FURIN* (F) expression in lung tissues. Results are shown as the medians (solid lines) and interquartile ranges (dot plot). Significance was determined using Kruskal-Wallis test and Dunn's multiple comparisons test (B and E), one-way analysis of variance and Tukey's multiple comparisons test (C and F), and Mann-Whitney U test (D). *$p < .05$, **$p < .01$, and ****$p < .0001$

manner (Figure 1B; control vs day 10 [$p = .002$], control vs day 17 [$p < .0001$], day 3 vs 17 [$p = .0001$]), whereas lung tissue *Ace2* expression decreased (Figure 1C, Appendix S1; control vs day 10 [$p = .013$ control] vs day 17 [$p = .0012$], day 3 vs 10 [$p = .0233$] and day 3 vs 17 [$p = .0022$]). These findings corroborate with those of Jackson et al[11] showing that *ACE2* was significantly reduced in human bronchial epithelial brush samples after segmental bronchial allergen challenge. ACE2 activity was also significantly suppressed by HDM injection (Figure 1D, $p < .0001$). In contrast, expression levels of the SARS-CoV-2 associated proteases *TMPRSS2* and *FURIN* in the lungs of HDM-treated mice did not significantly differ compared to those in control mice (Figure 1E and F, Appendix S1). These results indicate that ACE2 expression and/or function is associated with the pathogenesis of allergic airway inflammation.

We next investigated whether ACE2 enzymatic activity can modulate T2 inflammation in asthma. We used diminazene aceturate (DIZE), an activator of ACE2, which was reported by Dhawale et al[12] to attenuate OVA-induced allergic airway inflammation in rats. We injected HDM-induced asthma mice and examined its impact on airway inflammation (Figure 2A).

DIZE treatment did not impact *Ace2* expression (Figure 2B, Appendix S1), whereas its enzymatic activity was significantly elevated(Figure 2C, Appendix S1, $p = .0418$). DIZE also significantly prevented eosinophilia induced by HDM exposure (Figure 2D,

FIGURE 2 ACE2 activation by diminazene aceturate (DIZE) reduces type 2 inflammation in house dust mite (HDM)-exposed mice. (A) Experimental scheme. (B), *Ace2* expression in the lung determined using qRT-PCR (10–12 mice/group from two independent experiments). (C) ACE2 enzymatic activity in bronchoalveolar lavage fluid (BALF) was measured on day 14 after 6 h (14–18 mice/group from three independent experiments). (D) Eosinophils count in BALF (5–6 mice/group from three independent experiments). (E) Hematoxylin and eosin (HE) and periodic acid-Schiff (PAS) staining in the lung were scored by five individuals who were blinded on a scale of 0–3 for peribronchial inflammation (6 mice/group). (F) Bronchial hyperresponsiveness to 50 mg/mL methacholine (4–6 mice/group from two independent experiments). (G) IL-4, IL-5, and IL-13 protein levels in BALF and IL-33, TSLP, IL-6, and IFN-γ expression in lung tissue were measured using enzyme-linked immunosorbent assay (5–6 mice/group). The results are shown as the medians (solid lines) and interquartile ranges (dot plot) (B–G). Significance was determined using unpaired Student's *t* test (B, D, F, and G [IL-5, IL-33, TSLP, IL-6, and IFN-γ]) and Mann-Whitney U test (C, E, and G [IL-4 and IL-13]). *$p < .05$, **$p < .01$,***$p < .001$, and ****$p < .0001$


(A)
HDM or PBS i.t.
Harvest (BALF, Lungs)
Measurement of airway resistance
day 0
7
10
14
16
17
DIZE (25 mg/kg) or Control i.p.
(B)
Relative expression of ACE2 to GAPDH
DIZE
HDM
(C)
Mca concentration (μM)
(D)
Eosinophils (cells/ml)
(E)
DIZE -
DIZE +
HE
PAS
Score
(F)
Resistance (cmH2O s/ml)
(G)
IL-4 (pg/ml)
IL-5 (pg/ml)
IL-13 (pg/ml)
IL-33 (ng/ml)
TSLP (pg/ml)
IL-6 (pg/ml)
IFN-γ (pg/ml)

p = .0003). Hematoxylin and eosin (HE) and periodic acid-Schiff (PAS) staining showed that DIZE markedly inhibited HDM-induced infiltration of inflammatory cells and goblet cell hyperplasia (Figure 2E, Appendix S1; HE [p = .0007], PAS [p < .0001]). DIZE treatment also suppressed HDM-induced airway hyperresponsiveness in response to 50 mg/mL methacholine challenge (Figure 2F, p = .0483). Furthermore, DIZE treatment resulted in significantly lower levels of IL-5 and IL-13 in the BALF and of IL-33 in the lungs, whereas levels of IL-4 in the BALF and TSLP and IL-6 in the lung remained unchanged (Figure 2G, Appendix S1; IL-5 [p = .0108], IL-13 [p = .0303] and IL-33 [p < .0001]). The IFN-γ concentration in the lung was significantly increased by DIZE treatment (Figure 2G, Appendix S1, p = .0047). These results suggest that ACE2 expression and its enzymatic activity can regulate T2 allergic airway inflammation in asthma. Interestingly, reduced IL-13 levels in the BALF did not impact *Ace2* expression in the lungs of this asthma model. IFN-γ reportedly enhances ACE2 expression in human nasal epithelial cells.[8] IFNs and T-cell-activating factors linked with ACE2 expression have been reported as therapeutic targets for patients with T2 inflammation-low asthma suffering from COVID-19.[9] In contrast, IFNs (eg, IFN-β, IFN-γ, and IFN-λ1) did not influence ACE2 expression in human bronchial epithelial cells.[13] In our animal model of asthma using HDM, the elevated IFN-γ concentration in the lungs of DIZE-treated asthma mice may not up-regulate ACE2 expression in lung epithelial cells.

IL-33 is released by the lung epithelia and evokes T2 cytokine production by group 2 innate lymphoid cells (ILC2).[6] As DIZE treatment significantly reduced the IL-33 concentration in the lung, DIZE may inhibit IL-33 production of lung epithelial cells, leading to suppression of ILC2-mediated cytokine release. We speculate that suppression of IL-13 by asthma treatment cancels any resistance against SARS-CoV-2 infection by reducing ACE2 enzymatic activity. Our findings suggest that potentiation of ACE2 enzymatic activity can prevent asthma-associated T2 allergic airway inflammation without affecting *Ace2* expression in the lungs. This effect provides new possibilities for treating patients with asthma and COVID-19. However, treatment with DIZE has been shown to cause brain damage in dogs and camels, but not in cattle, rats, or mice. Patients with trypanosomiasis treated with DIZE showed no adverse effects.[14] Further investigations are required to clarify whether DIZE is suitable for treating asthma in humans based on its adverse effect, asthmatic phenotype, and molecular targets of HDM-induced T2 allergic inflammation regulated by ACE2 enzymatic activity.

CONFLICT OF INTEREST

The authors have no relevant conflicts of interest to declare.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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Association of endopeptidases, involved in SARS-CoV-2 infection, with microbial aggravation in sputum of severe asthma

To the Editor,

COVID-19 can be a serious multisystem disease caused by the SARS-CoV-2 coronavirus, and the current pandemic has affected more than 80 million people and caused nearly two million deaths worldwide. The SARS-CoV-2 virus attaches to angiotensin-converting enzyme 2 (ACE2) receptors on the host cell membrane, with the help of dipeptidyl peptidase 4 (DPP4), both exopeptidases.[1] Cleavage of the virus spike protein (S-protein) by endopeptidases, such as transmembrane protease, serine 2 (TMPRSS2) and furin, occurs following which the virus enters the host cell leading to virus replication.[1] Other enzymes, such as the sialyltransferases, ST6GAL1 and ST3GAL4, play a role for the synthesis of influenza A virus entry receptors [2]; however, their role in SARS-CoV-2 infection has not been elucidated.

Asthma is a chronic inflammatory airway disease affecting 350 million people worldwide. It has not been linked to serious outcomes when presenting with COVID-19 infection, although a higher risk of death has been reported in severe asthma populations.[3] The heterogeneous inflammatory nature of asthma raises the possibility that the type of asthmatic inflammation might determine the outcome of SARS-CoV-2 infection in asthma. Type 2 (T2) inflammatory markers have been associated with decreased ACE2 expression in asthma[4,5] that could underlie the reduced risk of SARS-CoV-2 infection in asthmatics. In contrast, non-T2 asthma, particularly neutrophilic asthma, has been associated with higher ACE2 and endopeptidases (TMPRSS2 and furin) expression as compared with the T2-high phenotype[4,5] that might imply a worse outcome with COVID-19 infection.

Airway microbial imbalances have been reported in asthma, particularly in severe non-T2 asthma, and are characterized by decreased microbial α-diversity with increased pathogenic bacterial abundances in association with neutrophilia.[6] Endopeptidases involved in S-protein cleavage such as furin may also play a role in the cleavage of pathogenic bacteria such *Streptococcus pneumoniae* and *Pseudomonas aeruginosa* or bacterial toxins.[7,8] High expression of such endopeptidases may be associated not only with a higher risk of SARS-CoV-2 infection but also with microbial imbalances in severe asthma. Therefore, the aim of this study was to investigate associations of sputum endopeptidases gene expression with metagenomics composition and whether they could be used to stratify asthma patients according to risk of SARS-CoV-2 infection.

We examined the relation of SARS-CoV-2-associated endopeptidases with the airway bacterial composition, SARS-CoV-2-associated exopeptidases and sialyltransferases, and inflammatory profile (cells and proteins) in 120 sputum samples collected from severe nonsmoking asthmatics, severe smoking asthmatics, mild-moderate asthmatics, and healthy controls of the Unbiased BIOmarkers in PREDiction of respiratory disease outcomes (U-BIOPRED) adult cohort.[9] Definition of asthma severity within the U-BIOPRED cohort has been presented in details elsewhere.[9] Sputum transcriptomics, SomaScan® proteomics, and metagenomics were assayed as described previously.[5,6] Gene set variation analysis (GSVA) was performed to obtain the enrichment score (ES) of the endopeptidase genes (TMPRSS2 and furin). Spearman correlation coefficients were computed between endopeptidases ES and sputum inflammatory markers and metagenomics α-diversity measures. The median ES