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RAGE and TLR4 differentially regulate airway hyperresponsiveness: Implications for COPD

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Abstract

Background: The receptor for advanced glycation end products (RAGE) and Toll-like receptor 4 (TLR4) is implicated in COPD. Although these receptors share common ligands and signalling pathways, it is not known whether they act in concert to drive pathological processes in COPD. We examined the impact of RAGE and/or TLR4 gene deficiency in a mouse model of COPD and also determined whether expression of these receptors correlates with airway neutrophilia and airway hyperresponsiveness (AHR) in COPD patients.

Methods: We measured airway inflammation and AHR in wild-type, $RAGE^{-/-}$, $TLR4^{-/-}$ and $TLR4^{-/-}RAGE^{-/-}$ mice following acute exposure to cigarette smoke (CS). We also examined the impact of smoking status on *AGER* (encodes RAGE) and *TLR4* bronchial gene expression in patients with and without COPD. Finally, we determined whether expression of these receptors correlates with airway neutrophilia and AHR in COPD patients.

Results: $RAGE^{-/-}$ mice were protected against CS-induced neutrophilia and AHR. In contrast, $TLR4^{-/-}$ mice were not protected against CS-induced neutrophilia and had more severe CS-induced AHR. $TLR4^{-/-}RAGE^{-/-}$ mice were not protected against CS-induced neutrophilia but were partially protected against CS-induced mediator release and AHR. Current smoking was associated with significantly lower *AGER* and *TLR4* expression irrespective of COPD status, possibly reflecting negative feedback regulation. However, consistent with preclinical findings, *AGER* expression correlated with higher sputum neutrophil counts and more severe AHR in COPD patients. *TLR4* expression did not correlate with neutrophilic inflammation or AHR.

Conclusions: Inhibition of RAGE but not TLR4 signalling may protect against airway neutrophilia and AHR in COPD.

Venkata Sita Rama Raju Allam, Alen Faiz, Jane E Bourke and Maria B Sukkar contributed equally to this work.

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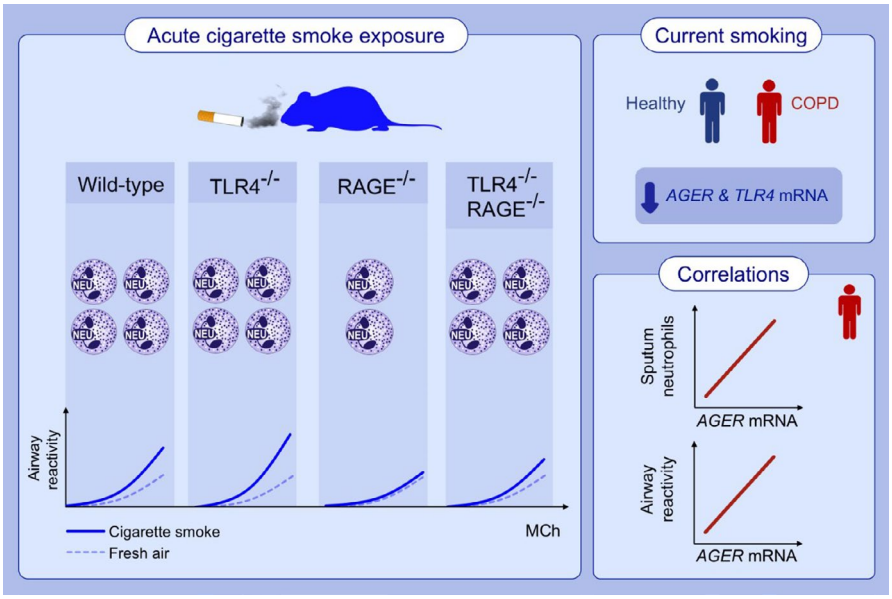
KEYWORDS
airway hyperresponsiveness, chronic obstructive pulmonary disease, cigarette smoke, receptor for advanced glycation end products, toll-like receptor 4



GRAPHICAL ABSTRACT

In mice, the absence of receptor for advanced glycation end products (RAGE) protects against neutrophilia and increased airway reactivity induced by acute smoke exposure, but this protection is largely lost when Toll-like receptor 4 (TLR4) is also absent. In humans, smoking is associated with lower advanced glycation end product receptor (*AGER*) (encodes RAGE) and *TLR4* expression irrespective of chronic obstructive pulmonary disease (COPD) status. *AGER* gene expression correlates with neutrophilic inflammation and more severe airway hyperresponsiveness in COPD patients. Abbreviations: AGER, advanced glycation end product receptor; COPD, chronic obstructive pulmonary disease; MCh, methacholine; RAGE, receptor for advanced glycation end products; TLR4, toll-like receptor 4.

1 | INTRODUCTION

Chronic obstructive pulmonary disease (COPD) is characterized by progressive loss of lung function and deterioration in health status. Cigarette smoking is one of the major risk factors for COPD, and however, several host factors including genetic background, low lung function at an early age and airway hyperresponsiveness (AHR) also contribute.[1] Currently, there is significant interest in the role of the receptor for advanced glycation end products (RAGE) in COPD pathogenesis as it is both a genetic determinant of low lung function and COPD susceptibility. It is also involved in the cellular and molecular response to cigarette smoke (CS) exposure.[1-4]

Increased RAGE protein expression is observed in bronchial biopsy tissue from smokers with COPD compared to smokers without COPD and never smokers.[5] In addition, studies using mouse models of COPD have demonstrated a role for RAGE in several pathological processes associated with COPD, particularly airway neutrophilia and emphysema.[6-10] AHR, defined by an exaggerated response of the airways to specific and nonspecific stimuli is a feature of COPD in some individuals.[11] However, despite considerable investigation of the ligand-RAGE axis in COPD, its functional role in AHR has not been investigated.

RAGE interacts with a broad repertoire of endogenous ligands such as HMGB1 and the heterodimeric complex S100A8/A9 which are also elevated in COPD.[5,12,13] While HMGB1 and S100A8/A9 are major RAGE ligands, they also signal via Toll-like receptor 4 (TLR4), another pattern recognition receptor implicated in COPD pathogenesis.[14,15] Increased levels of TLR4 protein are observed in the bronchial mucosa of patients with stable COPD, compared to nonsmoking control subjects,[16] while studies in mice have demonstrated a role for TLR4 in the acute neutrophilic response to CS exposure.[17-21] Moreover, HMGB1 facilitates LPS-mediated and TLR4-dependent inflammatory responses by engaging RAGE, thus suggesting functional interaction between RAGE and TLR4 signalling.[22] This notion is further supported by evidence that RAGE utilizes the TLR4 adaptor proteins TIRAP and MyD88 to mediate its biological effects.[23]

In the general population, the presence of asymptomatic AHR is a powerful predictor of respiratory symptoms and future risk of

developing COPD.[24,25] Moreover, among patients with COPD, AHR is associated with rapid decline in lung function, measures of gas trapping, airway inflammation and increased risk of respiratory mortality.[11,26-28] AHR is thought to occur as a result of variable and fixed components. The variable components largely derive from the acute release of pro-inflammatory mediators, while the persistent components result from structural changes in the airways and loss of elastic recoil due to emphysema.[1] Previous studies have shown that acute CS exposure for a period of three to 4 days leads to induction of AHR in mice.[29,30] Thus, acute CS exposure in mice is useful for the investigation of early signalling events that mediate AHR relevant to the nascent stages of COPD, as it removes the additional impact of structural changes which develop following chronic smoke exposure.[31]

In this study, we utilized a mouse model of acute CS exposure to test the hypothesis that RAGE, either alone or in co-operation with TLR4, promotes airway neutrophilia and AHR. We also examined the impact of smoking on *AGER* (which encodes RAGE) and *TLR4* bronchial gene expression in healthy control subjects and COPD patients. Finally, we determined whether expression of these receptors correlates with airway neutrophilia and AHR in COPD.

2 | METHODS

A full description of methods is provided in the Appendix S1.

3 | RESULTS

3.1 | RAGE but not TLR4 mediates acute CS-induced airway neutrophilia and AHR in mice

To investigate whether RAGE and TLR4 co-operate in the initial inflammatory response to acute CS exposure, we exposed wild-type (WT), $TLR4^{-/-}$, $RAGE^{-/-}$ and $TLR4^{-/-}RAGE^{-/-}$ mice to either fresh air (FA) or CS from three cigarettes three times a day for 4 days. This protocol elicited a twofold increase in total inflammatory cells in WT mice, that could be almost completely attributed to the increase in the number of neutrophils 24-hours post-CS exposure, similar to previous studies[32] (Figure 1A-C). It also led to a significant increase in S100A8 and CCL3 protein levels in BALF, as well as a trend towards increased levels of CXCL1 (Figure 1D-F). Other pro-neutrophilic mediators, including TNFα, IL-6, IL-17A and IL-17E, were not detected (data not shown).

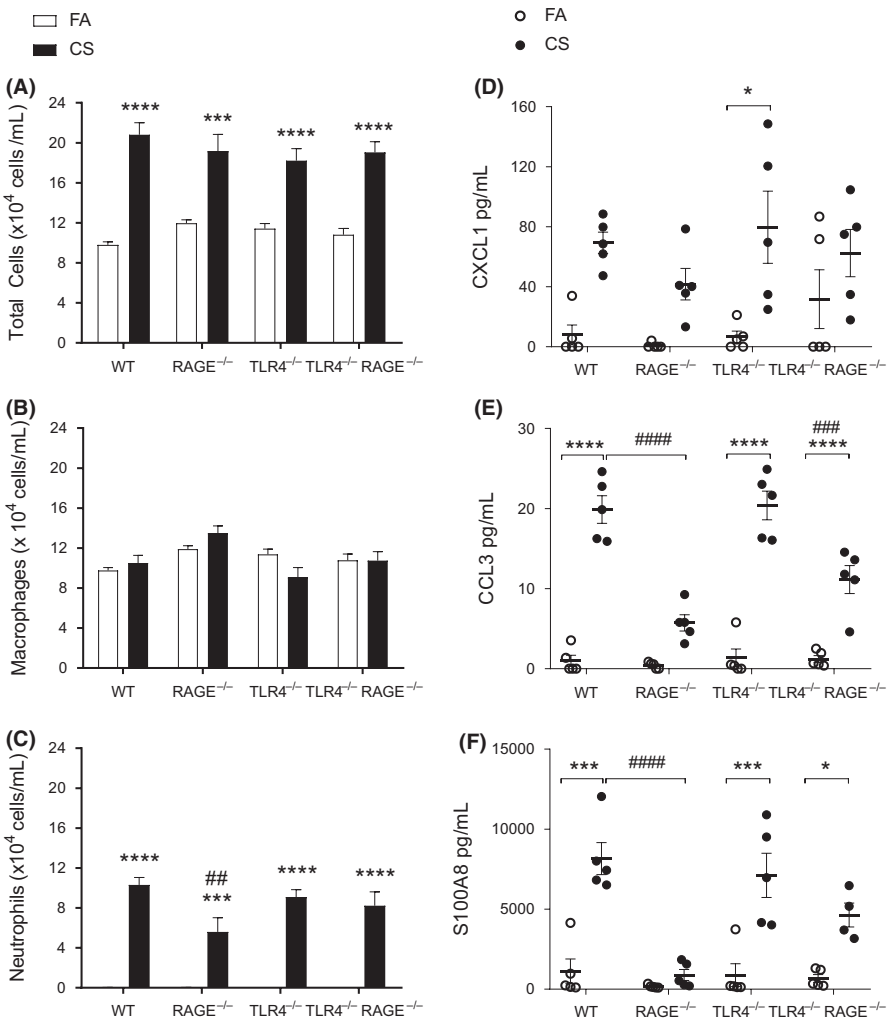


FIGURE 1 RAGE but not TLR4 mediates acute cigarette smoke-induced airway neutrophilia in mice. WT, $RAGE^{-/-}$, $TLR4^{-/-}$ and $TLR4^{-/-}RAGE^{-/-}$ mice were exposed to either fresh air (FA) or cigarette smoke (CS) from 3 cigarettes 3 times a day for 4 d. A, Total (B) macrophage and (C) neutrophil cell counts in BALF. (C) CXCL1 (D) CCL3 and (E) S100A8 protein concentrations in BALF. Data represent mean ± SEM $^{*}P < .05$, $^{***}P < .001$, $^{****}P < .0001$ vs respective fresh air-exposed mice. $\#P < .05$, $\#\#P < .01$, $\#\#\#P < .001$ and $\#\#\#\#P < .001$ vs cigarette smoke-exposed WT mice. N = 5-8 mice per group

RAGE$^{-/-}$ mice were protected against acute CS-induced inflammation. This was evidenced by significantly reduced numbers of infiltrating neutrophils (Figure 1C) and significant attenuation in BALF levels of both S100A8 and CCL3 compared to WT mice (Figure 1E and F). Reduced airway neutrophilia in RAGE$^{-/-}$ mice was not associated with attenuated CXCL1 expression. Notably, TLR4$^{-/-}$ mice were not protected against airway neutrophilia nor inflammatory mediator release in the BALF (Figure 1C-F). TLR4$^{-/-}$RAGE$^{-/-}$ mice were also not protected against airway neutrophilia, even though they had significantly reduced levels of CCL3 and a trend towards reduced levels of S100A8 in BALF.

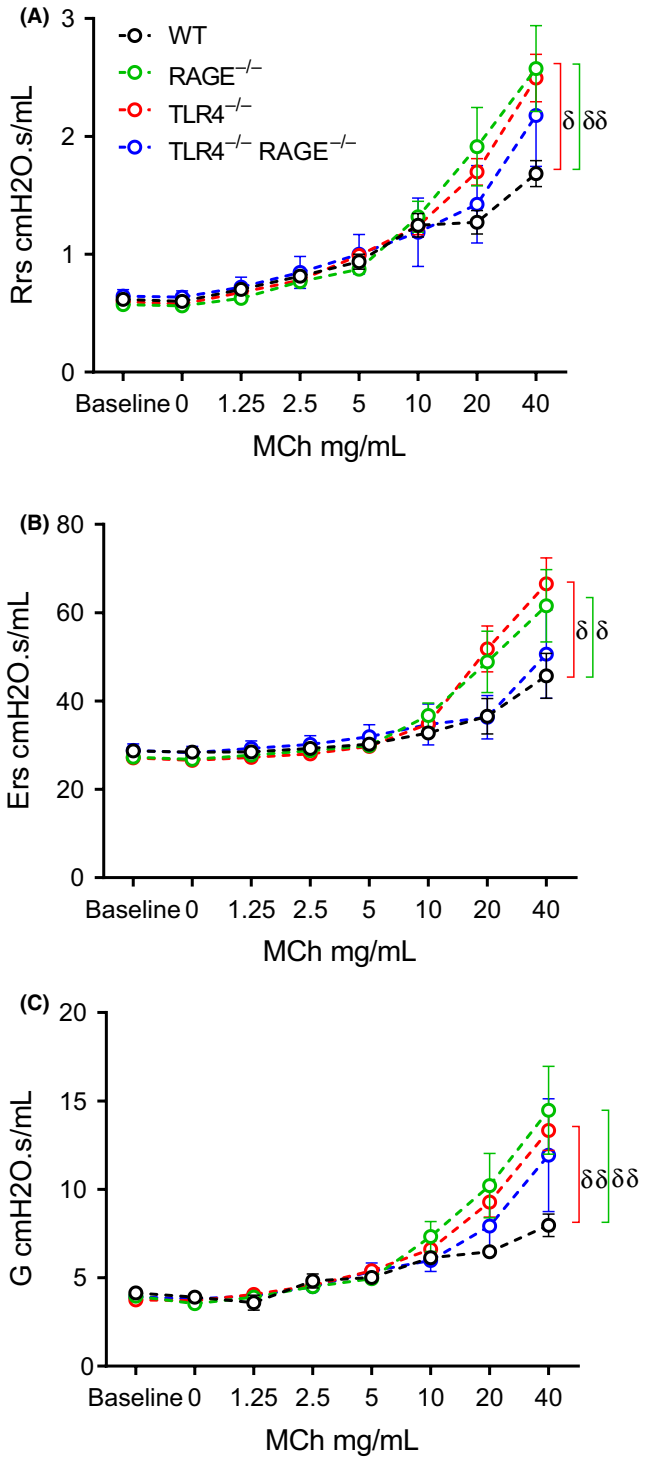


FIGURE 2 Impact of TLR4 and/or RAGE gene deficiency on in vivo airway reactivity in mice exposed to fresh air. WT, RAGE$^{-/-}$, TLR4$^{-/-}$ and TLR4$^{-/-}$RAGE$^{-/-}$ mice were exposed to fresh air (FA) 3 times a day for 4 d. Comparison of (A) total respiratory resistance (Rrs), (B) total elastance (Ers) and (C) tissue dampening (G) in all four strains. Data represent mean ± SEM. $\delta P < 0.05$, $\delta\delta P < 0.01$ vs fresh air-exposed WT mice. N = 6-10 mice per group

The contributions of RAGE and/or TLR4 to airway reactivity were also assessed. There was no difference in any of the in vivo lung function parameters measured at baseline prior to MCh administration in WT, RAGE$^{-/-}$, TLR4$^{-/-}$ or TLR4$^{-/-}$RAGE$^{-/-}$ mice. Notably, however, in the fresh air groups, the increases in total respiratory resistance (Rrs), total elastance (Ers) and distal airway dampening (G) in response to MCh were approximately 50% greater in RAGE$^{-/-}$ and TLR4$^{-/-}$ mice than in WT mice, indicating that RAGE and TLR4 inherently regulate airway reactivity (Figure 2A-C). Although the increases in Rrs, Ers and G in response to MCh tended to be greater in TLR4$^{-/-}$RAGE$^{-/-}$ mice than WT mice, they were not significantly increased (Figure 2A-C). Changes in total compliance (Crs), proximal airway resistance (Rn) and tissue elastance (H) with increasing MCh were similar between all groups (data not shown).

Acute CS exposure induced AHR to MCh, as indicated by significant increases in Rrs, Ers and G in WT mice relative to their fresh air controls (Figures 3A and 4A-C). Despite the increased responses of RAGE$^{-/-}$ mice to FA, RAGE$^{-/-}$ mice were protected from further increases in Rrs, Ers and G following acute CS exposure (Figures 3B and 4A-C). In contrast, MCh-induced increases in all these parameters were further elevated with CS exposure in TLR4$^{-/-}$ mice (Figures 3C and 4A-C), whereas only Rrs was elevated in TLR4$^{-/-}$RAGE$^{-/-}$ mice (Figures 3D and 4A-C).

3.2 | Impact of RAGE and TLR4 on small airway reactivity ex vivo

We have previously shown that acute CS exposure in vivo modulates small airway reactivity to contractile stimuli in mouse precision cut lung slices (PCLS) ex vivo.[33] Thus, we extended our studies to determine whether loss of RAGE and/or TLR4 also alters CS-induced changes in small airway reactivity ex vivo. Although air-exposed TLR4$^{-/-}$ and RAGE$^{-/-}$ mice exhibited enhanced airway reactivity to MCh relative to WT mice in vivo, this inherent AHR was not reflected in the small airways ex vivo. The contractile responses to MCh in PCLS from air-exposed RAGE$^{-/-}$, TLR4$^{-/-}$ and TLR4$^{-/-}$RAGE$^{-/-}$ mice were comparable to air-exposed WT mice, with maximum reductions in airway area of 40%-50% (Figure 5A).

In vitro responsiveness to MCh in PCLS from WT was significantly attenuated rather than increased following acute smoke exposure. The maximum reduction in airway area of 50% was reduced by approximately 20% in PCLS from CS-exposed WT mice (Figure 5B). However, there were no differences in small airway

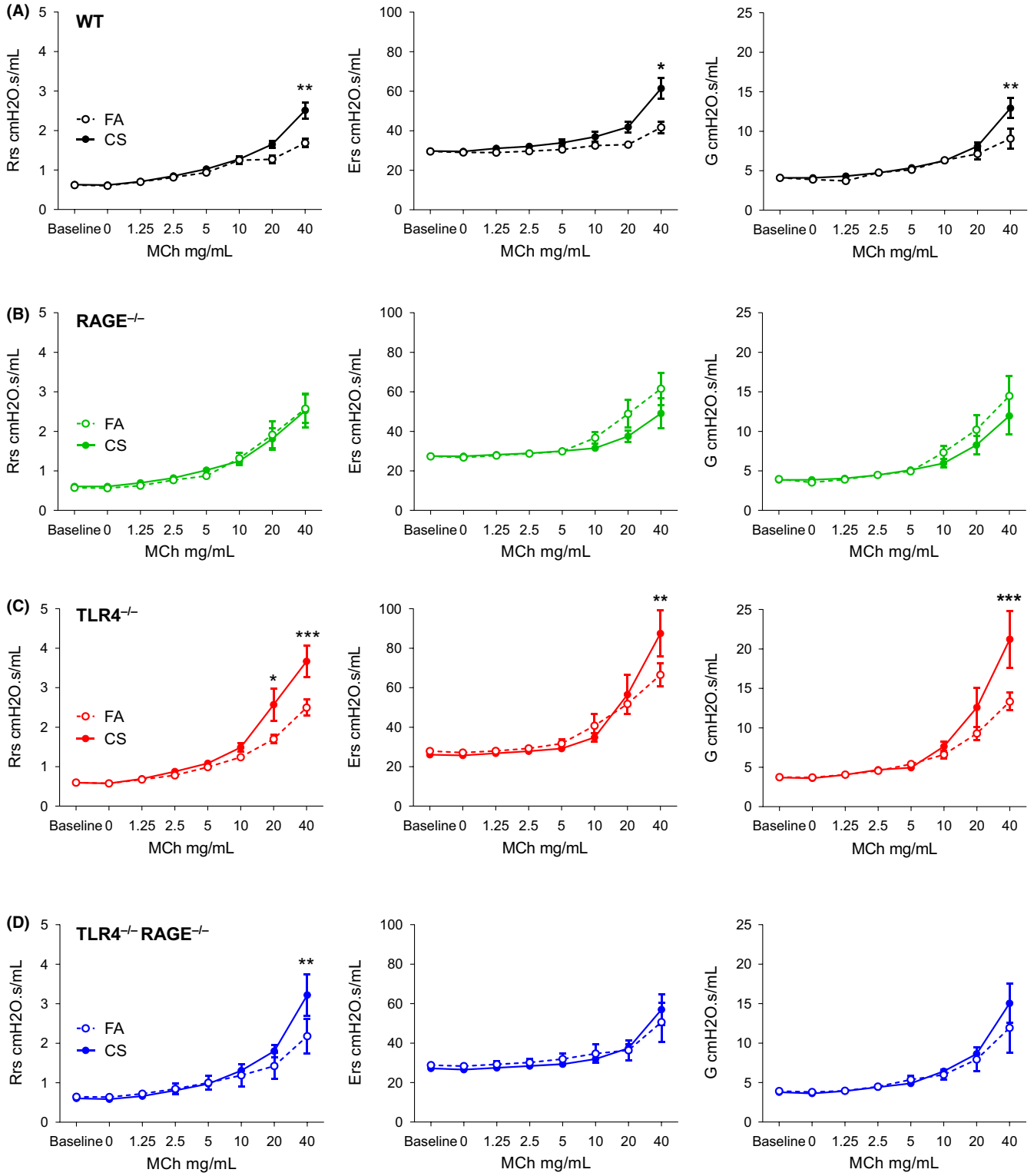


FIGURE 3 Impact of TLR4 and/or RAGE gene deficiency on in vivo airway reactivity in mice exposed to cigarette smoke. WT, RAGE$^{-/-}$, TLR4$^{-/-}$ and TLR4$^{-/-}$RAGE$^{-/-}$ mice were exposed to either fresh air (FA) or cigarette smoke (CS) from 3 cigarettes 3 times a day for 4 d. Total respiratory resistance (Rrs), total elastance (Ers) and tissue dampening (G) following exposure to FA or CS in (A) WT, (B) RAGE$^{-/-}$, (C) TLR4$^{-/-}$ and (D) TLR4$^{-/-}$RAGE$^{-/-}$ mice. Data represent mean ± SEM. *$P < .05$, **$P < .01$ ***$P < .001$ vs respective fresh air-exposed mice. N = 6-10 mice per group

contractility to MCh in PCLS from CS-exposed RAGE$^{-/-}$, TLR4$^{-/-}$ and TLR4$^{-/-}$RAGE$^{-/-}$ mice compared to their matched air-exposed groups (Figure 5C-E).

3.3 | Smoking lowers *AGER* and *TLR4* gene expression in bronchial biopsies from healthy and COPD patients

We have previously shown that current smoking is associated with significantly lower levels of *AGER* bronchial gene expression in healthy subjects[34] (Figure 6A; Table 1). Thus, we extended these studies to determine whether smoking impacts *TLR4* bronchial gene expression in healthy subjects and whether it impacts *AGER* and *TLR4* gene expression in COPD patients. Compared to healthy never smokers, healthy smokers had significantly lower levels of *TLR4* mRNA in bronchial biopsy tissue (Figure 6B). Healthy smokers also had significantly lower levels of other TLR family members, including *TLR5*, *TLR7* and *TLR10* mRNA, indicating this effect is not specific to TLR4 (Figure 6E, Table 1). Notably, we also found that current smokers with COPD had significantly lower levels of *AGER*, *TLR4*, *TLR5*, *TLR7* and TLR10 when compared to ex-smokers with COPD (Figure 6C, D, F; Table 1). These data indicate that smoking down-regulates the expression of multiple pattern recognition receptors and that this effect is not specific to COPD.

3.4 | *AGER* gene expression correlates with sputum neutrophils and AHR in COPD

We determined whether *TLR4* and *AGER* gene expression correlates with airway neutrophilia or AHR in patients with COPD. We observed a significant albeit weak correlation between *AGER* gene expression and sputum neutrophil counts in COPD patients ($\rho = 0.330$, n = 46, $P = .025$, Figure 7A). Furthermore, there was also a significant weak correlation between *AGER* gene expression and AHR severity as determined by the provocative concentration of methacholine that results in a 20% drop in FEV_1 (PC_{20}) ($\rho = -0.285$, n = 50, $P = .045$) (Figure 7B). There was no correlation between *TLR4* gene expression, sputum neutrophils ($\rho = 0.027$, n = 46, $P = .861$) or AHR ($\rho = -0.266$, n = 50, $P = .062$) in these patients (Figure 7C and D). Correlations of *AGER* gene expression with PC_{20} and sputum neutrophil counts remained significant after correcting for *TLR4* (data not shown).

4 | DISCUSSION

In this study, we demonstrated that the pattern recognition receptors TLR4 and RAGE regulate AHR in mice. Intriguingly, however, although RAGE and TLR4 share a number of common ligands and signalling pathways, these receptors differentially regulate the

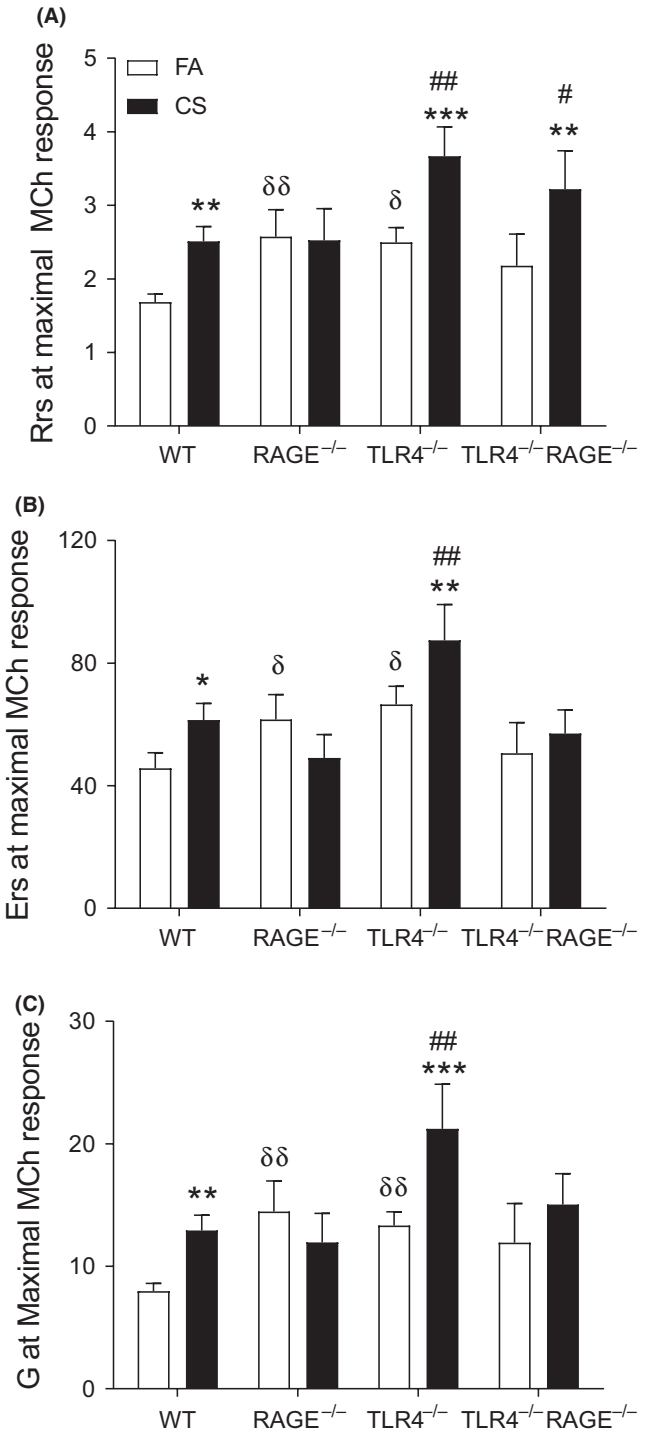


FIGURE 4 RAGE and TLR4 differentially regulate in vivo airway reactivity induced by acute cigarette smoke exposure in mice. WT, RAGE$^{-/-}$, TLR4$^{-/-}$ and TLR4$^{-/-}$RAGE$^{-/-}$ mice were exposed to either fresh air (FA) or cigarette smoke (CS) from 3 cigarettes 3 times a day for 4 d. Maximal increase in (A) total respiratory resistance (Rrs), (B) total elastance (Ers) and (C) tissue dampening (G) following exposure to FA or CS in all four strains. Data represent mean ± SEM. *$P < .05$, **$P < .01$, ***$P < .001$ vs respective FA groups. δ$P < 0.05$, δδ$P < 0.01$ vs fresh air-exposed WT mice. #$P < .05$, ##$P < .01$ vs cigarette smoke-exposed WT mice. N = 6-10 mice per group

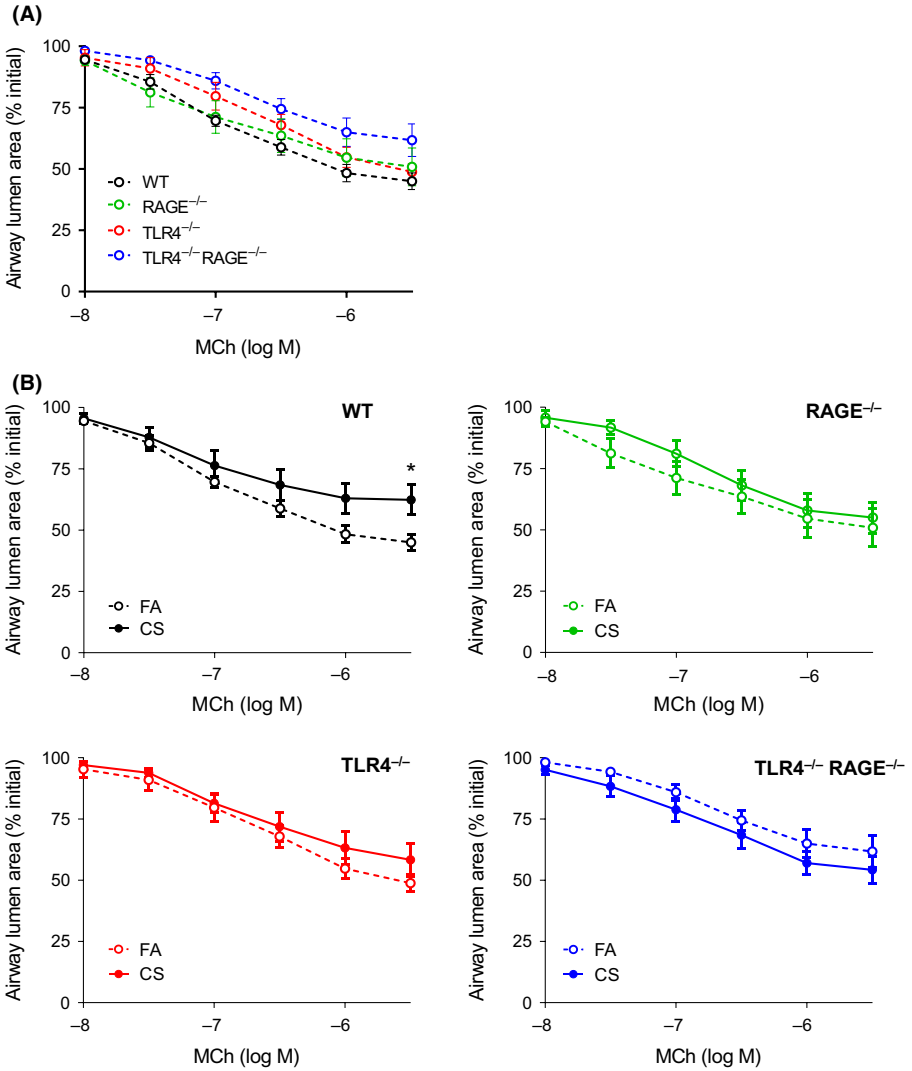


FIGURE 5 RAGE and TLR4 do not mediate cigarette smoke-induced changes in small airway reactivity ex vivo. WT, RAGE$^{-/-}$, TLR4$^{-/-}$ and TLR4$^{-/-}$RAGE$^{-/-}$ mice were exposed to either fresh air (FA) or cigarette smoke (CS) from 3 cigarettes 3 times a day for 4 d. A, Airway contraction expressed as % initial airway lumen area in all four strains w exposure to fresh air only. B, Airway contraction expressed as % initial airway lumen area in individual strains exposed to fresh air or cigarette smoke

airway response to acute CS exposure. We demonstrated that RAGE signalling augments AHR induced by acute CS exposure in mice and that this effect was associated with RAGE-dependent neutrophil infiltration into the airway lumen. Consistent with this, *AGER* gene expression was associated with neutrophilic inflammation in sputum and more severe AHR in patients with COPD. In contrast, we demonstrated that TLR4 signalling protects against acute CS-induced AHR in mice without impacting the neutrophilic response. We did not observe any correlation between *TLR4* gene expression and sputum neutrophils or AHR in patients with COPD. Our findings suggest that inhibition of RAGE but not TLR4 signalling is likely to afford protection against airway neutrophilia and AHR in COPD.

In the absence of CS exposure, RAGE$^{-/-}$ and TLR4$^{-/-}$ mice exhibited a greater degree of airway reactivity to methacholine in vivo compared to their WT counterparts, without any evidence of increased airway inflammation. Increased airway reactivity to methacholine in the absence of any environmental insult has previously been reported in RAGE$^{-/-}$ mice[35,36] but not in TLR4$^{-/-}$ mice.[37] Aberrant expression of RAGE in the lung, irrespective of whether it is increased or decreased, is associated with abnormal lung morphogenesis, airspace enlargement and the development of emphysema-like pathology.[7,38-42] Moreover, TLR4$^{-/-}$ mice develop emphysema as they age, largely as a result of increased oxidant generation and elastolytic activity.[43,44] Thus, collectively, the current evidence indicates important roles for RAGE and TLR4 in maintaining lung homeostasis, structure and function and further emphasizes the need to better understand the role of these receptors in the lung, both in health and disease.

Accordingly, the studies reported here are the first to examine both the individual and combined impact of TLR4 and RAGE gene deficiency on the airway response to acute CS exposure. Consistent with previous studies, we have shown that RAGE mediates lung neutrophil recruitment following acute CS exposure in mice.[6-10] In

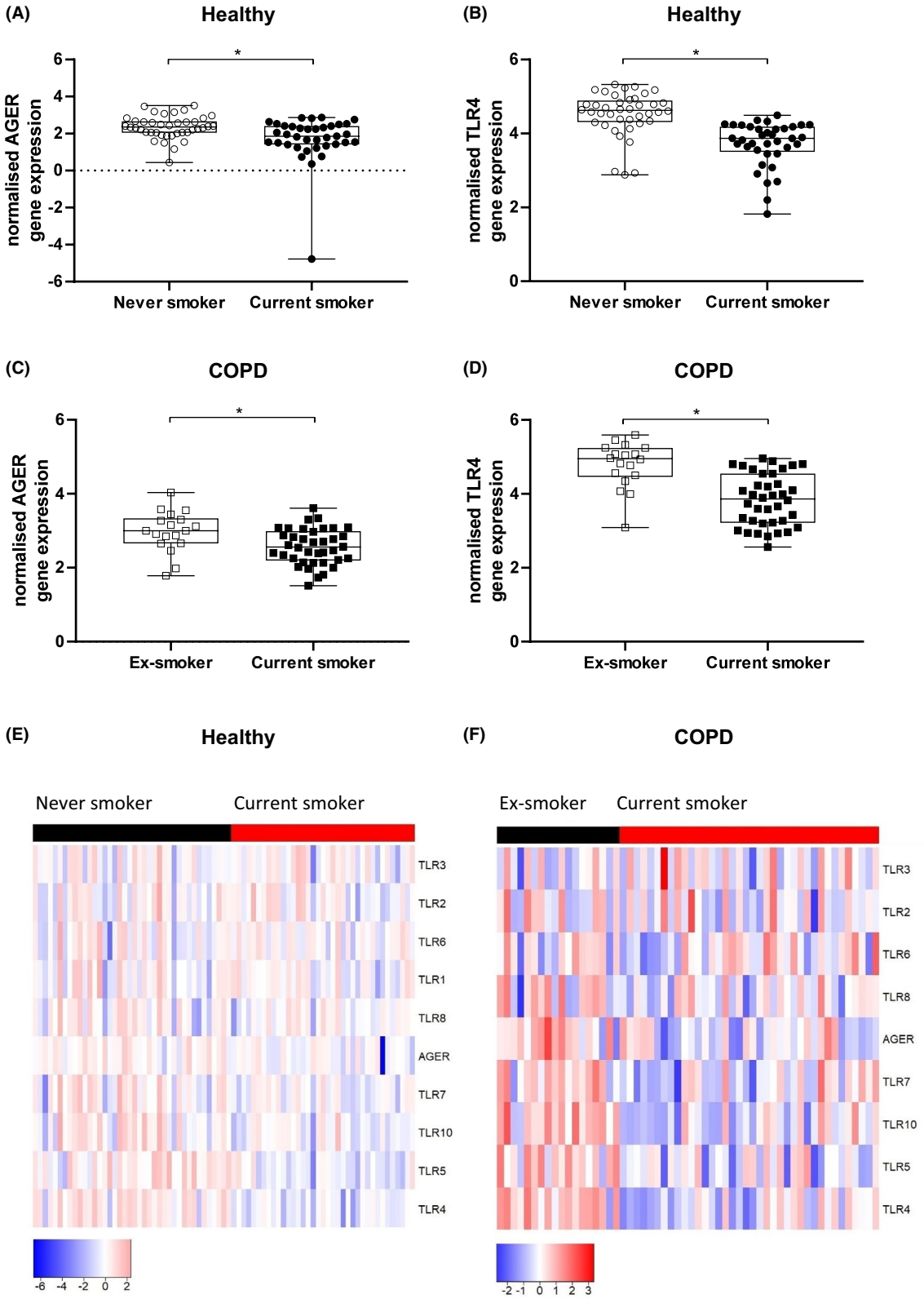


FIGURE 6 Smoking lowers *AGER* and *TLR4* gene expression in bronchial biopsies from healthy and COPD patients. Bronchial biopsy gene expression of (A) *AGER* and (B) *TLR4* in healthy (asymptomatic) smokers (n = 37) and never smokers (n = 40). Bronchial biopsy gene expression of (C) *AGER* and (D) *TLR4* in COPD current smokers (n = 38) and ex-smokers (n = 18). Heatmap of *AGER* and TLR family bronchial biopsy gene expression of (E) healthy smokers (n = 37) and never smokers (n = 40) and (F) COPD current smokers (n = 38) and ex-smokers (n = 18)

addition, we demonstrated that this effect is associated with concomitant induction of AHR since RAGE deficiency protected against both of these outcomes. However, in contrast to a similar acute CS exposure study,[18] our data suggest that TLR4 does not directly regulate CS-induced lung neutrophil recruitment. While the reason for this difference in findings is not clear, our data are consistent with a chronic CS exposure study which also found that TLR4 deficiency does not protect against CS-induced airway inflammation in mice.[45] Intriguingly, however, the protective effects of RAGE gene deficiency were largely lost in TLR4$^{-/-}$RAGE$^{-/-}$ mice, as airway neutrophil numbers in these mice were similar to those in WT mice, despite some reduction in inflammatory mediator release. Protection observed in the absence of RAGE might therefore be partially dependent on TLR4 or, alternatively, loss of both TLR4 and RAGE may lead to the activation of compensatory pathways that operate independently of these receptor pathways. These possibilities raise an added level of complexity that requires further investigation.

We have demonstrated for the first time that RAGE and TLR4 differentially regulate AHR in the context of acute CS exposure. Indeed, while our findings show that RAGE$^{-/-}$ mice were protected against AHR, possibly as a consequence of reduced neutrophil infiltration, TLR4$^{-/-}$ mice had worse CS-induced AHR than WT controls, despite a similar increase in neutrophils. Since TLR4 acts as a tonic suppressor of the NADPH oxidase enzyme Nox3 in lung endothelial cells and loss of TLR4 leads to a profound increase in lung oxidant generation in the absence of overt lung inflammation,[43] this may underpin the phenotype observed in TLR4$^{-/-}$ mice. In contrast to TLR4, RAGE signalling leads to the activation of the NADPH oxidase system in endothelial cells[46] and neutrophils[47]; thus, protection against AHR in RAGE$^{-/-}$ mice may be due to reduced neutrophil infiltration and an overall decrease in lung oxidant generation. Mice deficient in both TLR4 and RAGE were partially but not completely protected against CS-induced AHR as some but not all measures of airway function were normalized. This finding is consistent with opposing outcomes observed in single-gene-deficient strains and further substantiates the differential effects of TLR4 and RAGE signalling on AHR.

TABLE 1 Change in *AGER* and *TLR* gene expression in bronchial biopsy tissue from healthy smokers relative to never smokers and COPD current smokers relative to ex-smokers

	Healthy		COPD	
Gene	log2 fold change	*P* value	log2 fold change	*P* value
TLR1	−0.043	7.213E-01	NA	NA
TLR10	−0.709	5.830E-04	−1.502	4.160E-06
TLR2	0.071	4.403E-01	−0.050	7.256E-01
TLR3	0.132	2.076E-01	0.117	3.059E-01
TLR4	−0.676	8.040E-10	−0.839	4.324E-07
TLR5	−0.323	3.390E-06	−0.292	2.489E-03
TLR6	0.032	8.167E-01	−0.009	9.552E-01
TLR7	−0.505	5.908E-03	−0.732	1.133E-02
TLR8	−0.314	1.292E-01	−0.629	3.464E-02
AGER	−0.360	6.209E-03	−0.404	8.193E-03

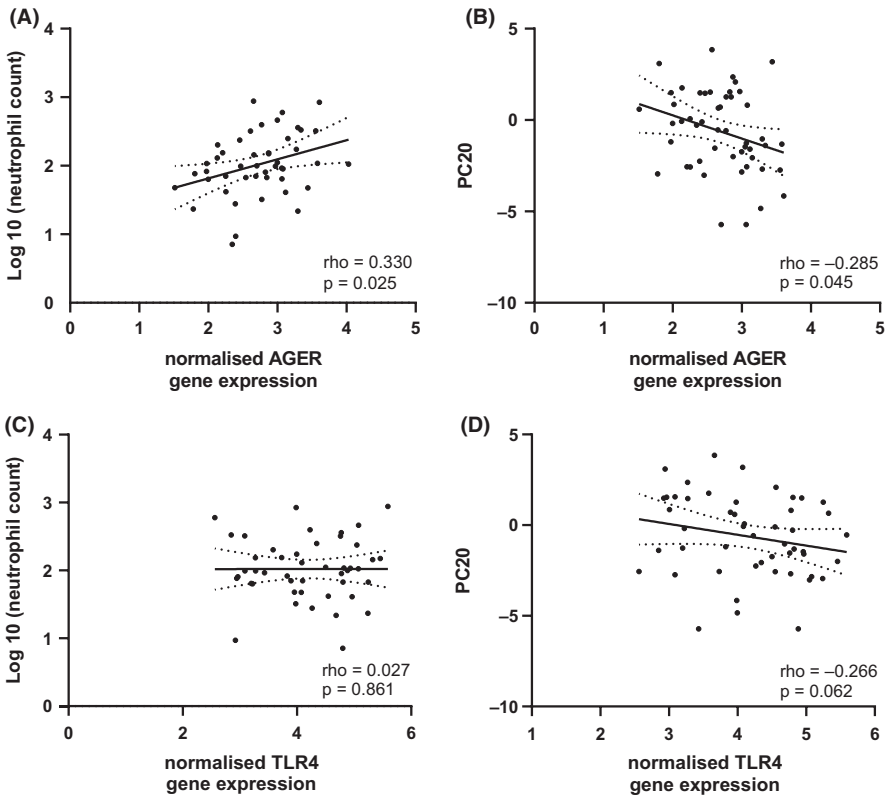


FIGURE 7 *AGER* gene expression correlates with sputum neutrophils and more severe AHR in COPD patients. Correlation between *AGER* normalized gene expression and (A) log sputum neutrophil counts and (B) the provocative concentration of methacholine that results in a 20% drop in FEV_1 (PC_{20}). *$P < .05$

To gain further insight into the relative contributions of RAGE and TLR4 in the disease process in COPD, we examined the impact of current smoking status on AGER and TLR gene expression in two independent data sets consisting of healthy control and COPD patients. As such, it was not possible to determine whether AGER and TLRs were differentially expressed between these populations. However, current smoking was associated with significant repression of AGER, TLR4, TLR5, TLR7 and TLR10 bronchial gene expression in both populations, indicating a broad inhibitory effect of current smoking on pattern recognition receptor expression, irrespective of disease status. We are aware of only one other study which examined the impact of current smoking status on TLR family gene expression in airway tissue samples ex vivo as we have done here.[48] Consistent with our findings, current smoking was associated with lower levels of TLR4 and TLR5 mRNA expression in small airway epithelium samples from healthy subjects. Moreover, although the impact of current smoking on TLR expression in tissue samples from COPD subjects was not examined, there was further repression of TLR5 but not TLR4 in smokers with COPD compared to healthy smokers. Experimental CS exposure in mice is associated with increased TLR4 and RAGE expression in lung immune and structural cells which appears contradictory to our findings here.[20,21,45,49] However, reduced TLR and AGER expression in current smokers most likely reflects a host protective response that acts to counteract continuous activation of the immune response by TLR4 and RAGE ligands which may be derived exogenously from cigarette smoke, such as the potent RAGE ligand methylglyoxal or produced endogenously by airway epithelial cells in response to CS.[20,50-53] Consistent with this idea, Goldklang and colleagues showed that while lung RAGE protein expression was increased after 4 weeks of CS exposure in mice, it returned to baseline levels after an extended CS exposure period of 16 weeks.[49]

Despite the repressive effects of current smoking, *AGER* bronchial gene expression was weakly but significantly associated with higher sputum neutrophil counts and more severe AHR in COPD patients. It was not possible to stratify our analysis according to smoking status due to the small sample size, although given smoking was associated with lower levels of *AGER* mRNA while airway neutrophilia and more severe AHR were associated with higher levels, it is unlikely that the observed correlations were confounded by smoking but this needs to be clarified in future studies. Together with the preclinical findings, these data suggest that RAGE might promote the development of airway neutrophilia and AHR in COPD. Indeed, we and others have previously shown that RAGE is critically required for type 2 cytokine-driven airway inflammation and AHR in mouse models of allergic asthma.[35,36,54-56] Moreover, Oczypok and colleagues identified a critical role for RAGE in IL-33 secretion and IL-33-dependent accumulation and activation of group 2 innate lymphoid cells (ILCs) in asthma which may potentially be relevant to mechanisms in COPD.[55] In a seminal paper, Kearley and colleagues reported increased IL-33 expression in the bronchial epithelium of patients with severe COPD and further showed that acute and chronic CS exposure in mice leads to increased expression of IL-33 in airway epithelial cells. Notably, however, they showed that CS alters the cellular distribution of the IL-33 receptor ST2 in the lung, decreasing its expression in ILC2s while at the same time increasing its expression in macrophages and natural killer (NK) cells. Hence, in this way CS silences ILC2 type 2 cytokine production in response to IL-33 and amplifies IL-33-mediated type 1 cytokine production in macrophages and NK cells. Although CS enhances IL-33 expression in epithelial cells it does not promote IL-33 secretion into the extracellular space and indeed Kearley showed that a second signal such as viral-induced epithelial cell damage was required for IL-33 secretion.[57] Thus, future studies should investigate whether RAGE is involved in CS-induced epithelial IL-33 expression and whether it regulates IL-33 secretion and the immune response to IL-33 in COPD as this may potentially identify a common pathway that leads to abnormal airway function in chronic airways disease.

Studies using irradiated, bone marrow chimeric mice have shown that loss of RAGE in structural but not hematopoietic cells significantly inhibits neutrophilic inflammation and lung emphysematous changes in a mouse model of emphysema.[6] While this suggests that RAGE activity in structural cells is likely to be of critical importance in COPD pathogenesis, it does not exclude the involvement of immune cells or crosstalk between immune and structural cells. Indeed, in patients with COPD, increased expression of RAGE and its major ligand HMGB1 are detected in alveolar macrophages, bronchial epithelial and smooth muscle cells,[5] indicating that RAGE activity in both immune and structural cells contributes to pathological processes in COPD. Similarly, in mouse models of asthma, loss of RAGE in structural tissue cells but not haematopoietic cells impacts airway inflammatory responses and AHR, further emphasising this point.[36,55] However, there still remains little understanding of the role of RAGE in cellular and molecular mechanisms of chronic airways disease and certainly this should be the focus of future studies.

In contrast to our studies in mice, we did not observe any relationship between TLR4 bronchial gene expression and AHR in patients with COPD. These findings are also in contrast to a study by Di Stefano and colleagues which reported significant correlations between epithelial TLR4 protein expression and measures of airflow obstruction in patients with COPD,[16] indicating that TLR4 activity in airway epithelial cells adversely impacts lung function. The lack of association between TLR4 gene expression and clinical features of COPD in our study suggests that post-translational events that regulate TLR4 protein expression and other components of the TLR4 signalling complex in airway epithelia and other cell types are likely to be critical determinants of the functional response to TLR4.[58]

Given the small airways are a major site of disease pathology in COPD, we extended our studies to determine whether the differences in reactivity to MCh in vivo associated with RAGE and/or TLR4 deficiency were also evident in small airways ex vivo. We initially showed that contraction to MCh was similar in PCLS from all air-exposed wild-type and gene-deficient mice. This suggests that any structural changes in the airways or surrounding parenchyma associated with RAGE and/or TLR4 deficiency that might contribute to in vivo AHR in the absence of inflammation may be too subtle

to be detectable ex vivo in individual small airways. Despite causing in vivo AHR, acute CS exposure was associated with a significant reduction in small airway reactivity in PCLS from wild-type mice but not RAGE$^{-/-}$, TLR4$^{-/-}$ or RAGE$^{-/-}$TLR4$^{-/-}$ mice relative to matched air-exposed groups. Since RAGE$^{-/-}$ mice were protected against both CS-induced neutrophilia and in vivo AHR, and ex vivo contraction to MCh was unchanged, the persistent presence of the inflammatory milieu due to CS itself or the specific influence of neutrophilic inflammation, as occurs in vivo, may be required for increased airway contraction. We have yet to define the mechanism for the unexpected decrease in contraction in PCLS from wild-type mice and why it is abrogated in TLR4$^{-/-}$ and TLR4$^{-/-}$/RAGE$^{-/-}$ mice which had similar neutrophilia but relatively higher in vivo AHR after acute CS exposure.

In summary, the current study has increased our understanding of relative contributions of RAGE and TLR4 signalling to acute neutrophilic inflammation and AHR that might be relevant to the initiation of COPD. Collectively, our findings further substantiate a possible role for RAGE as a therapeutic target in COPD and provide further impetus for the investigation of this receptor in COPD and related airway diseases, taking into account the complicated effects of (current) smoking.

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CONFLICTS OF INTEREST

W.T reports personal fees from Pfizer, GlaxoSmithKline, Roche Diagnostics/Ventana, Merck Sharp & Dohme, Novartis, Lilly Oncology, Boehringer Ingelheim, Astra Zeneca, Bristol Myers Squibb and AbbVie outside the submitted work. PSH reports grants from Boehringer Ingelheim and Galapagos outside the submitted work. GWT is a full-time employee of Genentech Inc and a member of the Roche Group. M.N is a full-time employee of Genentech Inc and holds stock and options in the Roche Group. M.G is a full-time employee of Genentech Inc and holds stock and options in the Roche Group. MVBD reports a research grant to his institution from Genentech during the conduct of the study and research grants to his institution from GlaxoSmithKline, Astra Zeneca, TEVA and Chiesi outside the submitted work.

AUTHOR CONTRIBUTIONS

VSRRA, AF, JB, MBS involved in concept and design. VSRRA, AF, ML, SNHR, BD, SDP, CAB, WT, PSH, MG, GTW, MN, MVDB, SD, SP, JEB, MBS involved in acquisition of data, analysis and interpretation. VSRRA, AF, JB, MBS drafted the manuscript. All authors revised the manuscript critically.

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REFERENCES

1. Postma DS, Bush A, van den Berge M. Risk factors and early origins of chronic obstructive pulmonary disease. *Lancet*. 2015;385(9971):899-909.
2. Sukkar M, Ullah M, Gan WJ, et al. RAGE: a new frontier in chronic airways disease. *Br J Pharmacol*. 2012;167(6):1161-1176.
3. Yonchuk JG, Silverman EK, Bowler RP, et al. Circulating soluble receptor for advanced glycation end products (sRAGE) as a biomarker of emphysema and the RAGE axis in the lung. *Am J Respir Crit Care Med*. 2015;192(7):785-792.
4. Haider SH, Oskuei A, Crowley G, et al. Receptor for advanced glycation end-products and environmental exposure related obstructive airways disease: a systematic review. *Eur Respir Rev*. 2019;28(151):180096.
5. Ferhani N, Letuve S, Kozhich A, et al. Expression of high-mobility group box 1 and of receptor for advanced glycation end products in COPD. *Am J Respir Crit Care Med*. 2010;181(9):917-927.
6. Waseda K, Miyahara N, Taniguchi A, et al. Emphysema requires the receptor for advanced glycation end-products triggering on structural cells. *Am J Respir Cell Mol Biol*. 2014;52(4):482-491.
7. Sambamurthy N, Leme AS, Oury TD, Shapiro SD. The receptor for advanced glycation end products (RAGE) contributes to the progression of emphysema in mice. *PLoS One*. 2015;10(3):e0118979.
8. Lee H, Park J-R, Kim WJ, et al. Blockade of RAGE ameliorates elastase-induced emphysema development and progression via RAGE-DAMP signaling. *FASEB J*. 2017;31(5):2076-2089.
9. Chen M, Wang T, Shen Y, et al. Knockout of RAGE ameliorates mainstream cigarette smoke-induced airway inflammation in mice. *Int Immunopharmacol*. 2017;50:230-235.
10. Sanders KA, Delker DA, Hueckstead T, et al. RAGE is a critical mediator of pulmonary oxidative stress, alveolar macrophage activation and emphysema in response to cigarette smoke. *Sci Rep*. 2019;9(1):231.
11. van den Berge M, Vonk JM, Gosman M, et al. Clinical and inflammatory determinants of bronchial hyperresponsiveness in COPD. *Eur Respir J*. 2012;40(5):1098-1105.
12. Hou C, Zhao H, Liu L, et al. High mobility group protein B1 (HMGB1) in asthma: comparison of patients with chronic obstructive pulmonary disease and healthy controls. *Mol Med*. 2011;17:807-815.
13. Pouwels SD, Nawijn MC, Bathoorn E, et al. Increased serum levels of LL37, HMGB1 and S100A9 during exacerbation in COPD patients. *Eur Respir J*. 2015;45(5):1482-1485.
14. Ibrahim ZA, Armour CL, Phipps S, Sukkar MB. RAGE and TLRs: Relatives, friends or neighbours? *Mol Immunol*. 2013;56(4):739-744.
15. Pouwels SD, Heijink IH, ten Hacken NH, et al. DAMPs activating innate and adaptive immune responses in COPD. *Mucosal Immunol*. 2013;7:215.

16. Di Stefano A, Ricciardolo FLM, Caramori G, et al. Bronchial inflammation and bacterial load in stable COPD is associated with TLR4 overexpression. *Eur Respir J*. 2017;49(5):1602006.
17. Maes T, Bracke KR, Vermaelen KY, et al. Murine TLR4 is implicated in cigarette smoke-induced pulmonary inflammation. *Int Arch Allergy Immunol*. 2006;141:354-368.
18. Doz E, Noulin N, Boichot E, et al. Cigarette smoke-induced pulmonary inflammation is TLR4/MyD88 and IL-1R1/MyD88 signaling dependent. *J Immunol*. 2008;180(2):1169-1178.
19. Geraghty P, Dabo AJ, D'Armiento J. TLR4 protein contributes to cigarette smoke-induced matrix metalloproteinase-1 (MMP-1) expression in chronic obstructive pulmonary disease. *J Biol Chem*. 2011;286(34):30211-30218.
20. Cheng Y, Wang D, Wang B, et al. HMGB1 translocation and release mediate cigarette smoke–induced pulmonary inflammation in mice through a TLR4/MyD88-dependent signaling pathway. *Mol Biol Cell*. 2017;28(1):201-209.
21. Wang D, Tao K, Xion J, et al. TAK-242 attenuates acute cigarette smoke-induced pulmonary inflammation in mouse via the TLR4/NF-κB signaling pathway. *Biochem Biophys Res Commun*. 2016;472(3):508-515.
22. Qin Y-H, Dai S-M, Tang G-S, et al. HMGB1 enhances the proinflammatory activity of lipopolysaccharide by promoting the phosphorylation of MAPK p38 through receptor for advanced glycation end products. *J Immunol*. 2009;183(10):6244-6250.
23. Sakaguchi M, Murata H, Yamamoto K-I, et al. TIRAP, an adaptor protein for TLR2/4, transduces a signal from RAGE phosphorylated upon ligand binding. *PLoS One*. 2011;6(8):e23132.
24. Xu X, Rijcken B, Schouten JP, Weiss ST. Airways responsiveness and development and remission of chronic respiratory symptoms in adults. *Lancet*. 1997;350(9089):1431-1434.
25. Brutsche MH, Downs SH, Schindler C, et al. Bronchial hyperresponsiveness and the development of asthma and COPD in asymptomatic individuals: SAPALDIA cohort study. *Thorax*. 2006;61(8):671-677.
26. Tashkin DP, Altose MD, Connett JE, Kanner RE, Lee WW, Wise RA. Methacholine reactivity predicts changes in lung function over time in smokers with early chronic obstructive pulmonary disease. The lung health study research group. *Am J Respir Crit Care Med*. 1996;153(6):1802-1811.
27. Tkacova R, Dai DLY, Vonk JM, et al. Airway hyperresponsiveness in chronic obstructive pulmonary disease: a marker of asthma-chronic obstructive pulmonary disease overlap syndrome? *J Allergy Clin Immunol*. 2016;138(6):1571-1579.
28. Teferra AA, Vonk JM, Boezen HM. Longitudinal changes in airway hyperresponsiveness and COPD mortality. *Eur Respir J*. 2020;55(2):1901378.
29. Nemmar A, Raza H, Subramaniyan D, et al. Evaluation of the pulmonary effects of short-term nose-only cigarette smoke exposure in mice. *Exp Biol Med*. 2012;237(12):1449-1456.
30. Dupont LL, Bracke KR, De Maeyer JH, et al. Investigation of 5-HT4 receptors in bronchial hyperresponsiveness in cigarette smoke-exposed mice. *Pulm Pharmacol Ther*. 2014;28(1):60-67.
31. Beckett EL, Stevens RL, Jarnicki AG, et al. A new short-term mouse model of chronic obstructive pulmonary disease identifies a role for mast cell tryptase in pathogenesis. *J Allergy Clin Immunol*. 2013;131(3):752-762.
32. Vlahos R, Bozinovski S, Jones JE, et al. Differential protease, innate immunity, and NF-κB induction profiles during lung inflammation induced by subchronic cigarette smoke exposure in mice. *Am J Physiol Lung Cell Mol Physiol*. 2006;290(5):L931-L945.
33. Donovan C, Seow HJ, Royce SG, Bourke JE, Vlahos R. Alteration of airway reactivity and reduction of ryanodine receptor expression by cigarette smoke in mice. *Am J RespirCell Mol Biol*. 2015;53(4):471-478.
34. Faiz A, van den Berge M, Vermeulen CJ, ten Hacken NHT, Guryev V, Pouwels SD. AGER expression and alternative splicing in bronchial biopsies of smokers and never smokers. *Respir Res*. 2019;20(1):70.
35. Milutinovic PS, Alcorn JF, Englert JM, Crum LT, Oury TD. The receptor for advanced glycation end products is a central mediator of asthma pathogenesis. *Amer J Pathol*. 2012;181(4):1215-1225.
36. Taniguchi A, Miyahara N, Waseda K, et al. Contrasting roles for the receptor for advanced glycation end-products on structural cells in allergic airway inflammation vs. airway hyperresponsiveness. *Am J Physiol Lung Cell Mol Physiol*. 2015;309(8):L789-L800.
37. Phipps S, Lam CE, Kaiko GE, et al. Toll/IL-1 signaling is critical for house dust mite-specific Th1 and Th17 responses. *Am J Respir Crit Care Med*. 2009;179(10):883-893.
38. Reynolds PR, Stogsdill JA, Stogsdill MP, Heimann NB. Up-regulation of RAGE by alveolar epithelium influences cytodifferentiation and causes severe lung hypoplasia. *Am J Respir Cell Mol Biol*. 2011;45(6):1195-1202.
39. Stogsdill JA, Stogsdill MP, Porter JL, Hancock JM, Robinson AB, Reynolds PR. Embryonic overexpression of receptors for advanced glycation end-products by alveolar epithelium induces an imbalance between proliferation and apoptosis. *Am J Respir Cell Mol Biol*. 2012;47(1):60-66.
40. Stogsdill MP, Stogsdill JA, Bodine BG, et al. Conditional overexpression of receptors for advanced glycation end-products in the adult murine lung causes airspace enlargement and induces inflammation. *Am J Respir Cell Mol Biol*. 2013;49(1):128-134.
41. Fineschi S, De Cunto G, Facchinetti F, et al. Receptor for advanced glycation end products contributes to postnatal pulmonary development and adult lung maintenance program in mice. *Am J Respir Cell Mol Biol*. 2013;48(2):164-171.
42. Wolf L, Herr C, Niederstraßer J, Beisswenger C, Bals R. Receptor for advanced glycation endproducts (RAGE) maintains pulmonary structure and regulates the response to cigarette smoke. *PLoS One*. 2017;12(7):e0180092.
43. Zhang X, Shan P, Jiang G, Cohn L, Lee PJ. Toll-like receptor 4 deficiency causes pulmonary emphysema. *Journal Clin Invest*. 2006;116(11):3050-3059.
44. Ruwanpura SM, McLeod L, Lilja AR, et al. Non-essential role for TLR2 and its signaling adaptor Mal/TIRAP in preserving normal lung architecture in mice. *PLoS One*. 2013;8(10):e78095.
45. Haw TJ, Starkey MR, Pavlidis S, et al. Toll-like receptor 2 and 4 have opposing roles in the pathogenesis of cigarette smoke-induced chronic obstructive pulmonary disease. *Am J Physiol Lung Cell Mol Physiol*. 2018;314(2):L298-L317.
46. Cai W, Torreggiani M, Zhu L, et al. AGER1 regulates endothelial cell NADPH oxidase-dependent oxidant stress via PKC-δ: implications for vascular disease. *Am J Physiol Lung Cell Mol Physiol*. 2010;298(3):C624-C634.
47. Bansal S, Siddarth M, Chawla D, Banerjee BD, Madhu SV, Tripathi AK. Advanced glycation end products enhance reactive oxygen and nitrogen species generation in neutrophils in vitro. *Mol Cell Biochem*. 2012;361(1):289-296.
48. Wang R, Ahmed J, Wang G, et al. Airway epithelial expression of TLR5 is downregulated in healthy smokers and smokers with chronic obstructive pulmonary disease. *J Immunol*. 2012;189(5):2217-2225.
49. Goldklang MP, Tekabe Y, Zelonina T, et al. Single-photon emission computed tomography/computed tomography imaging of RAGE in smoking-induced lung injury. *Respir Res*. 2019;20(1):116.
50. Pouwels SD, Zijlstra GJ, van der Toorn M, et al. Cigarette smoke-induced necroptosis and DAMP release trigger neutrophilic airway inflammation in mice. *Am J Physiol Lung Cell Mol Physiol*. 2016;310(4):L377-L386.
51. Pouwels SD, Hesse L, Faiz A, et al. Susceptibility for cigarette smoke-induced DAMP release and DAMP-induced inflammation in COPD. *Am J Physiol Lung Cell Mol Physiol*. 2016;311(5):L881-L1892.

52. Fujioka K, Shibamoto T. Determination of toxic carbonyl compounds in cigarette smoke. *Environ Toxicology*. 2006;21(1):47-54.
53. Xue J, Ray R, Singer D, et al. The receptor for advanced glycation end products (RAGE) specifically recognizes methylglyoxal-derived AGEs. *Biochem*. 2014;53(20):3327-3335.
54. Ullah MA, Loh Z, Gan WJ, et al. Receptor for advanced glycation end products and its ligand high-mobility group box-1 mediate allergic airway sensitization and airway inflammation. *J Allergy Clin Immunol*. 2014;134(2):440-450.
55. Oczypok EA, Milutinovic PS, Alcorn JF, et al. Pulmonary receptor for advanced glycation end-products promotes asthma pathogenesis through IL-33 and accumulation of group 2 innate lymphoid cells. *J Allergy Clin Immunol*. 2015;136(3):747-756.
56. Perkins TN, Oczypok EA, Dutz RE, Donnell ML, Myerburg MM, Oury TD. The receptor for advanced glycation end products is a critical mediator of type 2 cytokine signaling in the lungs. *J Allergy Clin Immunol*. 2019;144(3):796-808.
57. Kearley J, Silver JS, Sanden C, et al. Cigarette smoke silences innate lymphoid cell function and facilitates an exacerbated type I interleukin-33-dependent response to infection. *Immunity*. 2015;42(3):566-579.
58. Jia HP, Kline JN, Penisten A, et al. Endotoxin responsiveness of human airway epithelia is limited by low expression of MD-2. *Am J Physiol Lung Cell Mol Physiol*. 2004;287(2):L428-L437.

SUPPORTING INFORMATION

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

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