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Opposing roles of TLR2 and TLR4 in pathogenesis of COPD

Toll-like receptor 2 and 4 have Opposing Roles in the 
Pathogenesis of Cigarette Smoke-induced Chronic 
Obstructive Pulmonary Disease

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ABSTRACT

Chronic Obstructive Pulmonary Disease (COPD) is the third leading cause of morbidity and death and imposes major socioeconomic burdens globally. It is a progressive and disabling condition that severely impairs breathing and lung function. There is a lack of effective treatments for COPD, which is a direct consequence of the poor understanding of the underlying mechanisms involved in driving the pathogenesis of the disease. Toll-like receptor (TLR)2 and TLR4 are implicated in chronic respiratory diseases, including COPD, asthma and pulmonary fibrosis. However, their roles in the pathogenesis of COPD are controversial and conflicting evidence exists. In the current study, we investigated the role of TLR2 and TLR4 using a model of cigarette smoke (CS)-induced experimental COPD that recapitulates the hallmark features of human disease. TLR2, TLR4 and associated co-receptor mRNA expression were increased in the airways in both experimental and human COPD. Compared to WT mice, CS-induced pulmonary inflammation was unaltered in TLR2-deficient (Tlr2-/-), TLR4-deficient (Tlr4-/-) mice. CS-induced airway fibrosis, characterized by increased collagen deposition around small airways, was not altered in Tlr2-/- mice but was attenuated in Tlr4-/- mice compared to CS-exposed WT controls. However, Tlr2-/- mice had increased CS-induced emphysema-like alveolar enlargement, apoptosis and impaired lung function, whilst these features were reduced in Tlr4-/- mice compared to CS-exposed WT controls. Taken together, these data highlight the complex roles of TLRs in the pathogenesis of COPD and suggest that activation of TLR2 and/or inhibition of TLR4 may be novel therapeutic strategies for the treatment of COPD.

Key words: TLR2, TLR4, cigarette smoke, emphysema, COPD
INTRODUCTION

Chronic Obstructive Pulmonary Disease (COPD) is the third leading cause of morbidity and death and imposes significant socioeconomic burden worldwide (63). It is a complex, heterogeneous disease characterized by chronic pulmonary inflammation, emphysema and airway remodeling, which are associated with progressive lung function decline (39). Indeed, the major disease features are progressive and become more severe over time that is accelerated by infection-induced exacerbations. Cigarette smoke (CS) is a major risk factor for COPD (54). The current mainstay therapies for COPD are glucocorticoids, β2-adrenergic receptor agonists and long acting muscarinic antagonists (5, 93). However, these agents are largely ineffective and only provide symptomatic relief rather than modifying the causal factors or stopping disease progression (5). There is much current interest in increased microbial carriage and altered lung and gut microbiomes in COPD that could be modified for therapeutic gain and macrolide antibiotics are currently being tested as new treatments (14, 89, 92). Nevertheless, there is currently a lack of effective treatments for COPD that is largely due to the poor understanding of the underlying mechanisms.

Toll-like receptor (TLR)2 and TLR4 play vital roles in detecting and initiating immune responses to microbial membrane components (1, 36, 52). TLR2 and TLR4 are type I transmembrane receptors expressed on the cell surface (1, 36, 52). However, in some circumstances TLR4 can be internalized or expressed intracellularly in certain cells (1, 36, 52). TLR2 and TLR4 primarily signal through the adaptor protein myeloid differentiation primary response gene 88 (MyD88)-dependent or MyD88-adapter-like (Mal)-dependent pathways (1, 36, 52). Upon ligand (e.g. bacterial peptidoglycan) binding, TLR2 forms a heterodimer with either TLR1 or TLR6 and interacts with cluster of differentiation (CD)14 to form a functional complex (24, 48). In contrast, TLR4 forms a homodimer upon binding to its
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Ligand (e.g. bacterial lipopolysaccharide) and interacts with CD14 and/or MD2 (also known as lymphocyte antigen 96 [LY96] in humans) (57, 108). This initiates the recruitment of MyD88 to the intracellular Toll–interleukin 1 (IL-1) receptor (TIR) domain, that subsequently activates members of the IL-1 receptor-associated kinases (IRAKs) and tumor necrosis factor (TNF) receptor-associated factor 6 (TRAF6) (1, 52). Consequently, transcriptional factors of the mitogen-activated protein kinase (MAPK) family and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) are activated, leading to the expression of pro-inflammatory mediators (1, 36, 52).

TLR2 and TLR4 are widely implicated in chronic respiratory diseases, including asthma and pulmonary fibrosis (18, 25, 37, 42, 55, 56, 58, 59, 82, 86, 90, 106). Both are associated with increased susceptibility to asthma and allergies in children (25, 55). In adults, increased expression of TLR2, TLR4 and CD14 were observed in subjects with bronchiectasis and asthma (90). These observations were supported by findings from mouse models of allergic airway disease (18, 37, 59, 82, 106) and bleomycin-induced pulmonary fibrosis (42, 56, 58, 60, 86). We have also shown that TLR2 was essential in protecting against early-life respiratory infection and the development of subsequent chronic lung disease in later life (6, 27, 44, 96, 97). However, the role of TLR2 and TLR4 in the pathogenesis of COPD remains controversial and conflicting evidence exists in the literature. Some studies show that TLR2 and TLR4 expression are increased by CS exposure or in COPD patients (3, 21, 30, 33, 66, 69, 75, 80, 85, 91). However, others show that these TLRs are either not altered or are decreased by CS exposure or in COPD patients (22, 65, 80, 87).

Hence, the role of TLR2 and TLR4 in the pathogenesis of COPD remains unclear. Here, we investigated these roles using an established mouse model of CS-induced experimental COPD that recapitulates the critical features of human disease (7, 29, 31, 40, 41, 47, 62, 100) as well as gene expression analysis of lung tissues from human COPD
patients. TLR2, TLR4 and associated co-receptor mRNA were increased in the airways in both experimental and human COPD. Compared to WT controls, CS-induced pulmonary inflammation was largely unaltered in TLR2-deficient (Tlr2<sup>-/-</sup>) and TLR4-deficient (Tlr4<sup>-/-</sup>) mice. However, Tlr2<sup>-/-</sup> mice had increased CS-induced emphysema-like alveolar enlargement, apoptosis and impaired lung function whilst CS-induced airway fibrosis was not altered. In contrast, these features were reduced or completely attenuated in Tlr4<sup>-/-</sup> mice compared to WT controls, thus implicating this TLR in the pathogenesis of COPD.

**MATERIALS AND METHODS**

**Ethics statement.** This study was performed in accordance with the recommendations issued by the National Health and Medical Research Council of Australia. All experimental protocols were approved by the animal ethics committee of The University of Newcastle, Australia.

**Experimental COPD.** Female, 7-8-week-old, wild-type (WT), Tlr2<sup>-/-</sup> or Tlr4<sup>-/-</sup> BALB/c mice were purchased from the Australian Bioresource Facility, Moss Vale, NSW, Australia. Tlr2<sup>-/-</sup> and Tlr4<sup>-/-</sup> mice were generous gifts from Prof. Shizuo Akira, Osaka University, Japan and generated by using targeting vectors that introduce a targeted mutation in the mouse Tlr2 and Tlr4 genes as previously described (46, 99). Mice were housed under a 12-hour light/dark cycle and had free access to food (standard chow) and water. After period of acclimatization (up to 5 days), mice were randomly placed into experimental groups and exposed to either normal air or nose-only inhalation of CS for up to twelve weeks as described previously (7, 29, 31, 40, 41, 47, 62, 100). Recently, studies have shown that COPD prevalence and
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mortality are higher in females, and in the United States in 2009 women accounted for 53% of COPD deaths (78). It is for these and logistical reasons that female mice are used.

Isolation of RNA and qPCR. Total RNA was extracted and reversed transcribed from whole lung tissue, blunt-dissected airway and parenchyma and isolated lung macrophages (7, 41, 70, 101). mRNA transcripts were determined by real-time quantitative PCR (qPCR, ABIPrism7000, Applied Biosystems, Scoresby, Victoria, Australia) using custom designed primers (Integrated DNA Technologies, Baulkham Hills, New South Wales, Australia), normalized to the reference gene hypoxanthine-guanine phosphoribosyltransferase (HPRT) and expressed as relative abundance to WT air controls (Table 1) (7, 41, 70, 101).

Immunohistochemistry. Lungs were perfused, inflated, formalin-fixed, paraffin-embedded and sectioned (4-6μm). Longitudinal sections of the left lung were rehydrated through a series of xylene (2x) and ethanol gradient (2x absolute, 90%, 80%, 70%, 50%, 0.85% saline and phosphate-buffered saline [PBS]) washes followed by antigen retrieval with citrate buffer (10mM citric acid, 0.05% Tween 20, pH 6.0) at 100°C for 30 mins. Sections were blocked with casein blocker (Thermo Fisher Scientific, Pittsburgh, PA, USA) for 1 hour. Sections were then washed with PBS (5x, 5 mins each) and incubated with either anti-TLR2 (1:200 dilution, MABF84, clone 19B6.2, Merck Milipore, Bayswater, Victoria, Australia), anti-TLR4 (1:1000 dilution, ab47093, Abcam, Melbourne, Victoria, Australia) or anti-active caspase-3 (1:200 dilution, ab13847, Abcam) antibodies followed by either anti-rabbit (HAF008; R&D Systems, Gymea, New South Wales, Australia) or anti-mouse (ab6728; Abcam) secondary antibody conjugated with horseradish peroxidase and then 3,3′-Diaminobenzidine chromogen-substrate buffer (DAKO, North Sydney, New South Wales,
Australia) according to manufacturer’s instructions (40, 41). Sections were counterstained with hematoxylin, mounted and analyzed using a BX51 microscope (Olympus, Tokyo, Shinjuku, Japan) with Image-Pro Plus software (Media Cybernetics, Rockville, MD). The areas of active caspase-3 in lung parenchyma were determined (n=4 per group, 10 randomized parenchyma images per lung sections) using ImageJ software (Version 1.50, NIH, New York City, NY, USA), normalized to area of hematoxylin and represented as the percentage area of active caspase-3. Images with inflammation and airways were excluded from analysis.

Isolation of lung macrophages. Lungs were excised, washed and dissected into 1-2mm cubes in Dulbecco’s Modified Eagle Medium (DMEM, Sigma Aldrich, Castle Hill, New South Wales, Australia). Lung tissues were then transferred into Medicon cassettes (BD Biosciences, North Ryde, New South Wales, Australia) and disaggregated using a Medimachine (BD Biosciences) for 2 mins. Cell suspensions were collected, Histopaque 1083 (Sigma Aldrich) applied and centrifuged (825xg, 30 mins, 22.5 °C). The opaque interface cell layer where macrophages/monocytes were enriched was collected, washed with Hyclone™ Dulbecco’s PBS (GE Healthcare Life Sciences, South Logan, Utah, USA) and centrifuged (100xg, 5 mins, 22.5 °C). Cell pellets were resuspended in fresh DMEM (Sigma) and macrophages further enriched through adherence to plastic tissue culture flasks (3 hrs, 37 °C). Non-adherent contaminating cells were removed by gentle washing with PBS (GE Healthcare Life Sciences) prior to RNA isolation.

Flow Cytometry Analysis. The numbers of CD11b+ monocytes, neutrophils and myeloid dendritic cells (mDCs) in lung homogenates were determined based on surface marker
expression using flow cytometry (Table 2) (45, 53, 94, 97, 101). Flow cytometric analysis was performed using a FACSariaIII with FACSDiva software (BD Biosciences, North Ryde, Australia). Flow cytometry antibodies were purchased from Biolegend (Karrinyup, Western Australia, Australia) or BD Biosciences (Table 3). OneComp compensation beads (Thermo Fisher Scientific) were used to compensate for spectral overlap.

**Gene Expression in Human COPD Microarray Datasets.** Analysis of TLR2, TLR4 and co-receptors gene expression in published human array datasets (Affymetrix Human Genome U133 Plus 2.0 Array, Accession numbers: GSE5058 and GSE27597) (13, 16, 102) were performed using the Array Studio software (Omicsoft Corporation, Research Triangle Park, NC, USA) by applying a general linear model adjusting for age and gender and the Benjamini–Hochberg method for p-value adjustment (41). Data are expressed as log2 intensity robust multi-array average signals. The Benjamini–Hochberg method for adjusted P value/false discovery rate (FDR) was used to analyze differences between two groups. Statistical significance was set at FDR < 0.05.

**Pulmonary Inflammation.** Airway inflammation was assessed by differential enumeration of inflammatory cells in bronchoalveolar lavage fluid (BALF) (7, 27, 40, 41, 62, 70). Lung sections were stained with periodic acid-Schiff (PAS) and tissue inflammation assessed by enumeration of inflammatory cells (7, 41, 70). Histopathological score was determined in lung sections stained with hematoxylin and eosin (H&E) based on established custom-designed criteria (40, 44, 70).
Enzyme-linked immunosorbent assay (ELISA). Right lung lobes were homogenized on ice in 500μL of PBS supplemented with Complete mini protease inhibitor cocktail (Roche Diagnostic, Sydney, NSW, Australia) and PhosphoSTOP tablets (Roche Diagnostic). Lung homogenates were incubated on ice for 5 mins and subsequently centrifuged (8,000xg, 15 mins). Supernatants were collected, stored at -20°C overnight and total protein levels were determined using Pierce BCA assay kit (Thermo Fisher Scientific) prior to ELISA. TNFα protein levels were quantified with mouse TNFα Duoset ELISA kit as per manufacturer’s instructions (R&D systems). TNFα protein levels were normalized to total protein in lung homogenates. Hyaluronan protein levels in BALF were quantified with mouse hyaluronan Quantikine ELISA kits as per manufacturer’s instructions (R&D systems).

Airway Remodeling. Lung sections were stained with PAS or Masson’s Trichrome. Airway epithelial area (μm²), cell (nuclei) number and collagen deposition area (μm²) were assessed in a minimum of four small airways (basement membrane [BM] perimeter <1,000μm) per section (7, 40, 41, 62, 70). Data were quantified using ImageJ software (Version 1.50, NIH) and normalized to BM perimeter (μm).

Alveolar Enlargement. Lung sections were stained with H&E. Alveolar septal damage and diameter were assessed by using the destructive index technique (26) and mean linear intercept technique respectively (7, 41, 47, 62).

Apoptosis. Lung sections were stained with terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay kits (Promega, Sydney, New South Wales, Australia) according
to manufacturer’s instructions (41). Apoptosis in lung parenchyma was assessed by enumerating the numbers of TUNEL+ cells per high power fields (HPF; 100x) (41).

**Lung Function.** Mice were anaesthetized with ketamine (100mg/kg) and xylazine (10mg/kg, Troy Laboratories, Smithfield, Australia) prior to tracheostomy. Tracheas were then cannulated and attached to Buxco® Forced Maneuvers systems apparatus (DSI, St. Paul, Minnesota, USA) to assess total lung capacity (TLC) (7, 40). Mice were then attached to a FlexiVent apparatus (FX1 System; SCIREQ, Montreal, Canada) to assess transpulmonary resistance (tidal volume of 8mL/kg at a respiratory rate of 450 breaths/mins) (7). All assessments were performed at least three times and the average was calculated for each mouse.

**Statistical analyses.** Data are presented as means ± standard error of mean (SEM) and representative from two independent experiments with 6 mice per group. The two-tailed Mann-Whitney test was used to compare two groups. The one-way analysis of variance with Bonferroni post-test was used to compare 3 or more groups. Statistical significance was set at \( P < 0.05 \) and determined using GraphPad Prism Software version 6 (San Diego, CA, USA).

**RESULTS**

**TLR2 and TLR4 mRNA Expression and Protein Levels are Increased in CS-induced Experimental COPD**
To determine whether TLR2 and TLR4 levels are altered in COPD, we first interrogated our mouse model of experimental COPD (7, 29, 31, 40, 41, 47, 62, 100). WT mice were exposed to CS for 4, 8 and 12 weeks and TLR2 and TLR4 mRNA expression were assessed. TLR2, but not TLR4 mRNA was significantly increased in whole lung homogenates after 4, 8 and 12 weeks of CS exposure compared to normal air-exposed mice (Figure 1A-B). We have previously shown that many of the hallmark features of COPD were established in mice after 8 weeks of CS exposure (7, 29, 31, 40, 41, 47, 62, 100). To determine the compartment of the lung in which altered TLR2 and TLR4 expression occurred following establishment of disease, blunt dissected airways and lung parenchyma were assessed at this time point. To confirm separation of blunt dissected airways from parenchymal tissue we assessed the mRNA expression of airway epithelial cell-specific mucin 5ac (Muc5ac) and the mouse type II alveolar epithelial cell-marker surfactant protein C (Sftpc) in normal air-exposed mice (Figure 1C). The levels of Muc5ac mRNA were increased in airways compared to lung parenchyma. Conversely, the levels of Sftpc mRNA were increased in lung parenchyma compared to airways. This confirms the tissue specific isolation and location of TLR2, TLR4 and co-receptors in these tissues. TLR2 mRNA was increased in both the airways and parenchyma of CS-exposed mice (Figure 1D-E). In contrast, TLR4 mRNA was increased in the airways, but not parenchyma (Figure 1F-G). These mRNA expression data were supported by increased TLR2 (Figure 1H-I) and TLR4 (Figure 1J-K) protein levels in small airway epithelial cells and increased infiltration of parenchyma-associated inflammatory cells that expressed TLR2 or TLR4 in lung histology sections detected by immunohistochemistry.

To identify the inflammatory cell source of TLR2 and TLR4, macrophages were isolated from whole lung homogenates for downstream mRNA analysis. Interestingly, the mRNA expression of TLR2 and TLR4 were not altered in lung macrophages isolated from CS-exposed mice (Figure 2A-B). CD11b⁺ monocytes, neutrophils and mDCs are known to...
express TLR2 and/or TLR4 (6, 10, 80, 81), and have roles in COPD pathogenesis (41, 91).

Thus, we next determined whether CS altered the numbers of these immune cells that expressed TLR2 and/or TLR4 in the lung using flow cytometry. CS-exposed mice had increased the numbers of CD11b+ monocytes that expressed TLR2 (TLR2+), but not those that expressed TLR4 (TLR4+) or co-expressed TLR2 and TLR4 (TLR2+TLR4+) (Figure 2C) compared to normal air-exposed controls. In contrast, CS-exposed mice had significantly increased numbers of neutrophils and mDCs that were either TLR2+, TLR4+ or TLR2+TLR4+ (Figure 2D-E) compared to normal air-exposed controls.

**TLR2 and TLR4 Co-receptor mRNA Expression are Increased in CS-induced Experimental COPD**

When activated, TLR2 and TLR4 interact with co-receptors TLR1, TLR6, CD14 and/or MD2/Ly96 to mediate inflammatory responses (1, 36, 52). Therefore, we next determined whether the mRNA expression of these co-receptors was altered by CS exposure. TLR1 mRNA was increased in blunt dissected lung parenchyma, but not airways compared to normal air-exposed controls (Figure 3A-B). In contrast, TLR6 and CD14 mRNA were increased in both airways and parenchyma (Figure 3C-F). MD2/Ly96 mRNA expression was not altered by CS exposure (Figure 3G-H).

**TLR2, TLR4 and Co-receptor mRNA Expression are Increased in the Airways in Human COPD**

We next sought to determine whether the mRNA expression of TLR2, TLR4 and their co-receptors were altered in humans with mild-to-moderate COPD (Global Initiative for Chronic
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Obstructive Lung Disease [GOLD] Stage I or II (103)). Pre-existing microarray data from non-COPD (healthy non-smokers and healthy smokers) and COPD patients were interrogated (13, 16, 102). TLR2, TLR4 and co-receptor (TLR1, TLR6, CD14 and LY96) mRNA expression were not significantly altered in airway epithelial brushings from healthy smokers compared to non-smokers (Accession: GSE5058 (102), Figure 4A-F). Importantly, however, TLR2 (~2.4-fold), TLR4 (~8.7-fold), TLR1 (~7.1-fold), TLR6 (~1.5-fold), CD14 (~3.9-fold) and LY96 (~12.9-fold) mRNA expression were increased in airway epithelial brushings from patients with mild-to-moderate COPD compared to non-smokers. Notably, TLR2 (~2.4-fold), TLR4 (~7.2-fold), TLR1 (~4.7-fold), CD14 (~3.6-fold) and LY96 (~6.8-fold) but not TLR6 (~0.8-fold) mRNA expression were also increased in airway brushings from COPD patients compared to healthy smokers (Figure 4A-F).

TLR2, TLR4 and Co-receptor mRNA Expression are Decreased in the Parenchyma in Human COPD

Similarly, we then assess the expression of TLR2, TLR4 and co-receptor expression in pre-existing microarray data from lung parenchyma cores from severe COPD patients (GOLD Stage IV (103)) compared to non-smokers without COPD (Accession: GSE27597 (13, 16)). In contrast to the data from the airways, TLR2 (~1.5-fold) and TLR4 (~2.0-fold) expression were reduced, whereas co-receptors TLR1, TLR6 and LY96 were not altered, in the parenchyma from severe COPD patients compared to non-smokers without COPD (Figure 4G-K). CD14 was not detectable in this dataset.
We next investigated whether TLR2 and TLR4 play a role in the pathophysiology of CS-induced experimental COPD. WT, *Tlr2*−/− and *Tlr4*−/− mice were exposed to normal air or CS for 8 weeks (7, 29, 31, 40, 41, 47, 62, 100). We first assessed pulmonary inflammation in BALF by staining and differential enumeration of inflammatory cells. As expected, CS-exposure of WT mice resulted in significantly increased total leukocytes, macrophages, neutrophils and lymphocytes in BALF compared to normal air-exposed WT controls (Figure 5A-D). CS-exposed *Tlr2*−/− mice had increased neutrophils and lymphocytes, but not total leukocytes and macrophages, compared to normal air-exposed *Tlr2*−/− controls. This was due to an increase in total leukocytes and macrophages in normal air-exposed *Tlr2*−/− controls compared to normal air-exposed WT controls. In contrast, CS-exposed *Tlr4*−/− mice had increased total leukocytes, macrophages, neutrophils and lymphocytes in BALF compared to normal air-exposed *Tlr4*−/− controls. Importantly, total leukocytes, macrophages, neutrophils and lymphocytes in BALF were not significantly altered in CS-exposed *Tlr2*−/− or *Tlr4*−/− mice compared to CS-exposed WT controls.

We then assessed inflammatory cell numbers in the parenchymal histology. CS exposure of WT mice significantly increased inflammatory cell numbers in the parenchyma compared to normal air-exposed WT controls (Figure 5E). CS-exposed *Tlr2*−/− and *Tlr4*−/− mice also had increased parenchymal inflammatory cells compared to their respective normal air-exposed controls and were not significantly different from CS-exposed WT controls.

Next, histopathology was scored according to a set of custom-designed criteria as described previously (40, 44). CS exposure of WT mice increased histopathology score (Figure 5F), which was characterized by increased airway, vascular and parenchymal
inflammation (Figure 5G-I). CS-exposed Tlr2^{-/-} and Tlr4^{-/-} mice also had increased histopathology, airway, vascular and parenchymal inflammation scores compared to their respective normal air-exposed controls. Representative images are shown in Figure 5J. Tlr2^{-/-}, but not Tlr4^{-/-} mice had a small but significantly increased total histopathology score compared to CS-exposed WT controls, which was characterized by increased parenchymal inflammation score. Normal air-exposed Tlr2^{-/-}, but not Tlr4^{-/-} mice also had a small increase in vascular and parenchymal inflammation scores compared to normal air-exposed WT mice.

We then profiled the mRNA expression of the pro-inflammatory cytokine TNFα, chemokine (C-X-C motif) ligand (CXCL)1, chemokine (C-C motif) ligands (CCL)2, CCL3, CCL4, CCL12, CCL22 and COPD-related factors matrix metalloproteinase (MMP)-12 and serum amyloid A3 (SAA3, Figure 6A-I). CS exposure induced this cytokine, and these chemokines and factors in WT, Tlr2^{-/-} and Tlr4^{-/-} mice. However, some were not altered in CS-exposed Tlr2^{-/-} and/or Tlr4^{-/-} mice compared to CS-exposed WT controls, whilst others showed differences (e.g. increased TNFα, CXCL1, CCL2, CCL12 and CCL22 in CS-exposed Tlr4^{-/-} mice compared to CS-exposed WT controls) in expression but were not consistent with the inflammatory cell profile (Figure 5) observed in CS-exposed Tlr2^{-/-} and Tlr4^{-/-} mice compared to CS-exposed WT controls. Notably, however, the mRNA expression of the pro-fibrotic and emphysema factor MMP-12 (Figure 6H) was significantly reduced in CS-exposed Tlr4^{-/-} mice compared to CS-exposed WT controls.

Given that TLR2 and TLR4 have been previously shown to play roles in mediating pulmonary oxidative stress (32, 61, 83, 109), we also profiled the mRNA expression of NADPH oxidase (Nox)1, Nox2, Nox3, Nox4, NAD(P)H quinone dehydrogenase (Nqo)1, nuclear factor (erythroid-derived 2)-like-2 factor (Nrf2), glutamate-cysteine ligase catalytic subunit (Gclc), glutathione peroxidase (Gpx)2, heme oxygenase (Hmox)1 and glutathione S-transferase pi (Gstp)1 (Figure 6J-S). CS exposure induced the expression of Nox2 (Figure
6K) and suppressed Gstp1 (Figure 6S), whilst other genes were not altered in \textit{Tlr}2\textsuperscript{-/-} or \textit{Tlr}4\textsuperscript{-/-} mice compared to WT controls. Interestingly, Nox3 (Figure 6L) was increased in normal air-exposed \textit{Tlr}4\textsuperscript{-/-} mice compared to normal air-exposed WT controls, but was reduced by CS exposure. Other oxidative stress-associated genes were, however, not significantly altered in normal air-exposed \textit{Tlr}2\textsuperscript{-/-} or \textit{Tlr}4\textsuperscript{-/-} mice compared to normal air-exposed WT controls.

To determine whether the TNF\(\alpha\) signaling pathway was altered in CS-exposed \textit{Tlr}4\textsuperscript{-/-} mice, we next assessed the levels of TNF\(\alpha\) protein in lung homogenates. As expected, TNF\(\alpha\) protein levels were increased in CS-exposed WT mice compared to normal air-exposed WT controls (Figure 6T). In contrast, TNF\(\alpha\) protein levels were not altered in CS-exposed \textit{Tlr}2\textsuperscript{-/-} and \textit{Tlr}4\textsuperscript{-/-} mice compared to normal air-exposed \textit{Tlr}2\textsuperscript{-/-} and \textit{Tlr}4\textsuperscript{-/-} mice, respectively. The lack of increase in CS-exposed \textit{Tlr}2\textsuperscript{-/-} mice was due to increased TNF\(\alpha\) levels in normal air-exposed \textit{Tlr}2\textsuperscript{-/-} mice compared normal air-exposed WT mice. Notably, TNF\(\alpha\) protein was significantly reduced in CS-exposed \textit{Tlr}4\textsuperscript{-/-} mice compared to CS-exposed WT controls.

\textbf{CS-induced Collagen Deposition is not Altered in TLR2-deficient Mice but Completely Attenuated in TLR4-deficient Mice with Experimental COPD}

We have previously shown that mice develop small airway remodeling and fibrosis in experimental COPD (7, 40, 41, 62). In agreement with this, CS exposure of WT mice increased small airway epithelial cell area (epithelial thickening) compared to normal air-exposed WT controls (Figure 7A). CS-exposed \textit{Tlr}2\textsuperscript{-/-} and \textit{Tlr}4\textsuperscript{-/-} mice also had increased small airway epithelial cell thickening compared to their respective normal air-exposed controls, but were not altered compared to CS-exposed WT controls. CS-induced small airway epithelial cell thickening in WT, \textit{Tlr}2\textsuperscript{-/-} and \textit{Tlr}4\textsuperscript{-/-} mice was associated with increased numbers of nuclei in the small airways, which is an indicator of increased numbers of
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epithelial cells (Figure 7B). The numbers of nuclei in CS-exposed \textit{Tlr2}^{-/-} and \textit{Tlr4}^{-/-} mice were not altered compared to CS-exposed WT controls.

We next assessed airway fibrosis in terms of collagen deposition around small airways. As expected, CS-exposed WT mice had increased collagen deposition compared to normal air-exposed WT controls (Figure 7C). However, CS-exposed \textit{Tlr2}^{-/-} and \textit{Tlr4}^{-/-} mice did not have increased collagen deposition compared to their respective normal air-exposed controls. The former was due to an increase in collagen deposition in normal air-exposed \textit{Tlr2}^{-/-} mice compared normal air-exposed WT controls. The latter was due to no increase in collagen deposition in CS-exposed \textit{Tlr4}^{-/-} mice compared to normal air-exposed \textit{Tlr4}^{-/-} controls. Notably, CS-induced collagen deposition was attenuated in CS-exposed \textit{Tlr4}^{-/-} mice compared to CS-exposed WT controls. This was associated with a concomitant increase in the levels of hyaluronan in BALF and decrease in fibronectin mRNA expression in lung homogenates of \textit{Tlr4}^{-/-} mice compared to WT mice, independent of CS exposure (Figure 7D-E). Notably, CS-induced expression of IL-33 mRNA was also attenuated in \textit{Tlr4}^{-/-} mice (Figure 7F).

CS-induced Emphysema-like Alveolar Enlargement, Apoptosis and Lung Function Impairment are Increased in TLR2-deficient Mice and Decreased in TLR4-deficient Mice with Experimental COPD

We have previously shown that CS-exposed WT mice developed emphysema-like alveolar enlargement and impaired lung function after 8 weeks of CS exposure (7, 40, 41, 62). Thus, we next sought to determine whether TLR2 and TLR4 contribute to these disease features. In agreement with our previous studies, CS-exposed WT mice had significantly increased alveolar septal damage and diameter, determined by destructive index and mean linear
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intercept techniques respectively, compared to normal air-exposed WT controls (Figure 8A-B). CS-exposed Tlr2−/− and Tlr4−/− mice also had increased alveolar septal damage and alveolar diameter compared to normal air-exposed Tlr2−/− and Tlr4−/− controls, respectively. However, CS-exposed Tlr2−/− mice had increased alveolar damage and diameter compared to CS-exposed WT controls. Conversely, CS-exposed Tlr4−/− mice had reduced alveolar septal damage and diameter compared to CS-exposed WT controls.

We have previously shown that increased CS-induced emphysema-like alveolar enlargement was associated with increased numbers of TUNEL + cells in the parenchyma, which indicates increased apoptosis (41). In agreement with this, CS-exposed WT mice had increased TUNEL + cells in the parenchyma compared to normal air-exposed WT controls (Figure 8C). CS-exposed Tlr2−/− mice had increased TUNEL + cells in the parenchyma compared to normal air-exposed Tlr2−/− controls. Importantly, and in agreement with the reduced emphysema-like alveolar enlargement, CS-exposed Tlr4−/− mice did not have increased apoptosis compared to normal air-exposed Tlr4−/− controls. Accordingly, the numbers of TUNEL + cells were increased in CS-exposed Tlr2−/− mice, but reduced significantly in CS-exposed Tlr4−/− mice compared to CS-exposed WT controls.

To provide further evidence for changes in apoptosis, we also assessed the levels of active caspase-3, a key molecular marker of apoptosis (19, 34, 79), in whole lung sections by immunohistochemistry. Caspase-3 levels were increased in the lung parenchyma of CS-exposed WT mice compared to normal air-exposed WT controls (Figure 8D). Caspase-3 was also increased in the lung parenchyma of CS-exposed Tlr2−/− mice compared to normal air-exposed Tlr2−/− controls. In contrast, caspase-3 levels were not altered in CS-exposed Tlr4−/− mice compared to normal air-exposed Tlr4−/− controls. Notably, the levels of caspase-3 were increased in CS-exposed Tlr2−/− mice but were significantly reduced in CS-exposed Tlr4−/− mice compared to CS-exposed WT mice.
We next assessed the roles of TLR2 and TLR4 in CS-induced impairment of lung function, measured in terms of TLC and transpulmonary resistance. As expected, CS-exposed WT mice had increased TLC (Figure 8E) and transpulmonary resistance (Figure 8F) compared to normal air-exposed WT controls. CS-exposed Tlr2\(^{-/-}\) mice did not have altered TLC compared to normal air-exposed Tlr2\(^{-/-}\) controls. This was due to increased TLC in normal air-exposed Tlr2\(^{-/-}\) mice compared to normal air-exposed WT controls. However, CS-exposed Tlr2\(^{-/-}\) mice did have increased transpulmonary resistance compared to normal air-exposed Tlr2\(^{-/-}\) mice. Notably, both TLC and transpulmonary resistance were significantly increased in CS-exposed Tlr2\(^{-/-}\) mice compared to CS-exposed WT mice. In contrast, Tlr4\(^{-/-}\) mice did not have increases in TLC and transpulmonary resistance compared to normal air-exposed Tlr4\(^{-/-}\) controls.

**DISCUSSION**

In this study, we demonstrate that both TLR2 and TLR4 play important, but opposing roles, in the pathogenesis of CS-induced experimental COPD. TLR2 and TLR4 mRNA were increased in airway epithelium and parenchyma of mice chronically exposed to CS and in human COPD patients. Expression of the co-receptors TLR1, TLR6, CD14 or MD2/Ly96 were also increased in CS-exposed mice and human COPD. CS-induced pulmonary inflammation was unaltered in Tlr2\(^{-/-}\) and Tlr4\(^{-/-}\) mice compared to WT controls. In contrast, Tlr2\(^{-/-}\) mice had increased CS-induced emphysema-like alveolar enlargement, apoptosis and impaired lung function, whilst importantly these features were reduced in Tlr4\(^{-/-}\) mice. CS-induced small airway epithelial thickening and fibrosis were not altered in Tlr2\(^{-/-}\) mice compared to CS-exposed WT controls. In contrast, CS-induced airway fibrosis, but not small airway epithelial thickening, was significantly attenuated in Tlr4\(^{-/-}\) mice compared to CS-
exposed WT controls. This study provides new insights into the role of TLR2 and TLR4 in the pathogenesis of COPD.

The roles of TLR2 and TLR4 in the pathogenesis of COPD are controversial with conflicting data in the literature (3, 21, 22, 30, 33, 65, 66, 69, 75, 80, 85, 87, 91). These conflicting data are likely due to differences between experimental analytes (e.g. peripheral blood monocytes vs. macrophages), cohorts of patients with varying medical backgrounds, potential tissue-specificity of TLR expression and the experimental models used (e.g. acute vs. chronic CS exposure). Notably, the experimental models utilized either in vitro, acute or whole-body CS exposure models, which do not replicate in vivo chronic inhaled mainstream CS exposure associated with the induction of COPD in humans (3, 21, 66, 75). Furthermore, these models did not report or demonstrate chronic CS-induced impairment of lung function, which is a key feature of human COPD (7, 31).

We aimed to address these discrepancies and delineate the roles of TLR2 and TLR4 in COPD by using an established mouse model of tightly controlled chronic nose-only CS-induced experimental COPD (7, 29, 31, 40, 41, 47, 100). Our models are representative of a pack-a-day smoker and 8 weeks of smoking in a mouse that lives for a year is equivalent to 10 years in a human smoker (31, 51). Importantly, we have consistently shown that 8 weeks of CS exposure in our models is sufficient to induce the hallmark features of human COPD: chronic inflammation, airway remodelling, emphysema and impaired lung function (7, 13, 16, 29, 31, 40, 41, 47, 100, 102). This 8-week timepoint was specifically chosen to investigate the underlying pathogenic mechanism(s) during the early stages (GOLD I/II) and identify potential therapeutic targets to halt the progression of COPD. Moreover, these shorter-term models may be relevant for testing of therapeutic interventions because new therapeutics such as targeting TLR signaling are likely to have little effect during more severe stages of disease, when invasive interventions, such as lung surgery/transplant, may be
required (103). Although there is a possibility that nose-only inhalation murine models of emphysema may introduce other variables such as stress-related to restraint, we observe that mice quickly acclimatize and grew accustomed to our purpose-built restraining tubes (7). This is confirmed by an initial increase in blood corticosterone levels (indicator of stress) in restrained mice during the first week of CS exposure, but these levels return to baseline after that (unpublished data).

Interestingly, 

\( Tlr2^{-/-} \) mice have not been assessed in the context of CS-induced COPD. 

\( Tlr4^{-/-} \) mice on a variety of backgrounds (e.g. C3H/HeJ, C57BL/10ScNJ or C57BL/6J) have been investigated in other CS-exposure models (21, 66), however, \( Tlr4^{-/-} \) mice on a BALB/c background have not been investigated. Given that MyD88 is the common downstream signaling molecule of all TLRs including both TLR2 and TLR4 (1, 52) we also subjected \( MyD88^{-/-} \) mice to CS-induced experimental COPD to determine if TLR2- and TLR4-mediated effects were MyD88-dependent. However, these mice became very ill and suffered significant weight loss (>15% body weight) after 3-4 weeks of CS exposure (data not shown). \( MyD88^{-/-} \) mice are known to be susceptible to opportunistic infections and this was the likely cause of illness in these mice (9, 98).

Hence, our study adds to the current literature by investigating the pathogenesis of COPD with previously uninvestigated \( Tlr2^{-/-} \) and \( Tlr4^{-/-} \) on a BALB/c background. Moreover, our study also used an established experimental COPD model that utilizes a more clinically relevant CS exposure protocol and is supported by gene expression analysis of published human microarray datasets from healthy non-smokers and COPD subjects (7, 13, 16, 29, 31, 40, 41, 47, 100, 102).

We showed that TLR2 mRNA was increased in blunt dissected airways and parenchyma, whereas TLR4 mRNA was increased only in the airways. This suggests that the
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expression of TLR2 and TLR4 may be tissue-specific within the lung, which may in part explain the discrepancies in the literature on the expression of TLR2 and TLR4 in COPD. Interestingly, the expression of TLR2 and TLR4 were not altered in lung-isolated macrophages from CS-exposed mice. This indicates that CS-induced increases in TLR expression occurs on small airway epithelial cells whilst influxing macrophages may contribute by having constitutive levels of TLR expression and by increasing in numbers. These observations were confirmed with immunohistochemistry that showed that TLR2 and TLR4 expressions were detected on small airway epithelial cells and parenchymal inflammatory cells, which were significantly increased in experimental COPD. Furthermore, subsequent flow cytometry analysis showed CS exposure increased the numbers of TLR2- and/or TLR4-expressing neutrophils and mDCs in the lungs. These results mirror previous studies, by us and others, that showed TLR2 and/or TLR4 are expressed on various cells, including macrophages, peripheral monocytes, neutrophils, mDCs and airway/bronchial epithelial cells in inflammatory disease setting including experimental and human COPD (3, 6, 10, 22, 33, 65, 69, 75, 80, 81, 85, 87, 91).

Importantly, using pre-existing microarray datasets, we also showed that the expression of TLR2 and TLR4 mRNA was increased in airway epithelial cells from bronchial brushings of patients with mild to moderate COPD. In contrast, TLR2 and TLR4 mRNA were reduced in lung parenchymal cores from patients with severe COPD. Interestingly, a previous human study also described similar observations whereby TLR4 expression was increased in the airway epithelium in mild-to-moderate COPD (FEV\textsubscript{1} >1L) but reduced in severe disease (FEV\textsubscript{1} <1L) (65). Collectively, these data suggest that TLR2 and TLR4 expression are increased in the lung parenchyma early in disease in response to CS-exposure, but are reduced in severe COPD patients, which may be due to greater tissue destruction that
results in the loss of tissues/cells expressing these TLRs. This also may explain the current
discrepancies in the literature on the expression of TLR2 and TLR4 in COPD.

Others have shown that TLR1 and TLR6 were increased on CD8^+ T cells from COPD
patients (30), and that CD14 levels were increased by CS and associated with impaired lung
function in smokers (17, 110). It has been reported that MD2 expression was unaltered in
small airway epithelium, but was reduced in the large airways of smokers and patients with
stable COPD (74). These data suggest that the effects of CS on the expression of TLR2 and
TLR4 co-receptors may also be tissue- or cell-specific (e.g. airway epithelium vs.
inflammatory cells) and dependent on the severity of the disease. Thus, ligation of TLR2 and
TLR4 with their co-receptors may also partially explain the discrepancies in the current
literature.

In contrast to our observations in Tlr2^-/- mice, previous studies showed that inhibition
of TLR2 reduced pulmonary inflammation, apoptosis and lung function impairment in other
chronic lung diseases, including pulmonary fibrosis and asthma (59, 106). Mice deficient in
TLR2 or treated with a neutralizing antibody had reduced bleomycin-induced pulmonary
inflammation, collagen deposition and hydroxyproline levels in the lungs (106). Moreover,
Tlr2^-/- mice had reduced airway inflammation and peri-bronchial collagen deposition in an
OVA-induced model of experimental allergic asthma (59). In addition, TLR2 was shown to
promote apoptosis in human kidney epithelial (HEK)-293, human monocytic (THP-1) cells
and endothelial cells in vitro (2, 81). Ovalbumin-induced airway hyperresponsiveness (AHR)
were also previously shown to be reduced in Tlr2^-/- mice (38, 59). This may be due to
differences in pathogenesis, mechanisms and inflammatory cells/responses that may dictate
the role of TLR2 in various chronic lung diseases. For example, allergic asthma is dominated
by aberrant Th2-type responses typified by increased infiltration of eosinophils and activated
mast cells into the airways and increased levels of Th2-associated cytokines such as IL-5 and
IL-13 (44, 45, 95, 96). In contrast, COPD is typically associated with Th1/Th17-type inflammatory responses characterized by infiltration of macrophages, neutrophils and cytotoxic T cells and the production of Th1/Th17-associated cytokines such as IFN-γ and IL-17A (7, 30, 69, 80, 91). Hence, the role of TLR2 in different chronic lung diseases may largely depend on the presence and type of specific TLR2-expressing cells. Nevertheless, our studies suggest that TLR2 may potentially have a protective role in CS-induced COPD. The underlying mechanism remained unclear, but appeared to be independent of oxidative stress in the lung.

Interestingly, inflammatory infiltrates in the airway lumen and around blood vessels, TNFα protein and TLC were increased in normal air-exposed Tlr2-/- mice compared to WT controls. Previous studies showed that airway inflammation is negatively associated with lung function (4, 8) and may cause lung hyperinflation (increased in TLC) (28, 49, 76). Moreover, increased sputum inflammatory cells (e.g. neutrophils) have been shown to significantly correlate with air trapping in COPD patients (71, 72). Another study showed that TLR2 was highly expressed in human fetal lungs and may be important in regulating the development of this organ (77). Recent studies also have emerged that highlight the importance and interactions of host microbiome, commensal bacteria, infectious exacerbations and TLRs in shaping and regulating immune responses in COPD and other chronic respiratory diseases (11, 14, 15, 43, 68, 89). Taken together, the increased inflammation and TLC observed in normal air-exposed Tlr2-/- mice may be a consequence of altered host immune responses, lung development and/or microbiome composition. It is likely that TLR2 and TLR4 will be important in these interactions and during lung development. However, this is outside the scope of this study and will require further work to delineate the relationships.
TLR4 has been implicated in CS-induced pulmonary inflammation (21, 66). In other studies, acute (3 days) CS exposure of Tlr4<sup>-/-</sup> mice on a C57BL/6 background resulted in reductions in total inflammatory cells in BALF and lung tissue (21). Sub-acute (5 weeks) exposure of Tlr4<sup>defective</sup> mice also resulted in reduced numbers of BALF total inflammatory cells compared to WT C3H/HeJ controls (66). However, in support of our observations, the numbers of BALF total inflammatory cells were not significantly different in these Tlr4<sup>defective</sup> mice compared to WT C3H/HeJ controls following chronic CS exposure (26 weeks) (66). Collectively, these suggest TLR4 may play only a minor role at the chronic and severe stages of the disease. Whilst CS-exposed Tlr4<sup>-/-</sup> mice had increased pro-inflammatory cytokine TNFα mRNA expression in the lung compared to CS-exposed WT controls, this increase in mRNA expression was in contrast to the significant reduction in TNFα protein levels in CS-exposed Tlr4<sup>-/-</sup> mice. Taken together, these results suggest that there are alterations in the regulation of gene transcriptional and post-translational protein production in CS-exposed Tlr4<sup>-/-</sup> mice, which collectively does not affect airway or parenchymal inflammation. The biology of these changes is not understood.

We previously showed that parenchymal inflammatory cells and alveolar destruction were further increased in WT mice after 12 weeks of CS compared to those exposed to CS for 8 weeks (7). Hence, increasing CS exposure time in mice (e.g. from 8 to 12 weeks) may lead to further alterations in inflammatory gene expression and inflammation. However, given that TLR2 and TLR4 expression were reduced in lung parenchymal cores from severe (GOLD III/IV) COPD patients (Figure 2), we speculate that increasing CS exposure time to induce more severe disease in our model may have similar effects and promote further loss of tissue/cells expressing TLR2 and/or TLR4. In support of this, a previous human study also found that TLR4 expression was inversely proportional to COPD severity (65). Moreover, we also showed that the expression of pro-inflammatory TNFα and chemokine CXCL1 were not
increased further with longer exposures, despite increases in parenchymal inflammatory cells, in WT mice exposed to CS for 12 weeks compared to 8 weeks (7). This may be due to some of the functional/molecular changes being restricted to specific cell types (e.g. structural versus immune cells) that express TLR2 and/or TLR4 in the lung. Hence, profiling changes in whole lung tissue may have concealed small but potentially critical functional/molecular changes. The roles of TLRs (TLR2 or TLR4) on specific cells in the lungs also remain unclear. This could be addressed in future studies using cell-specific TLR\(^{-/-}\) mice or bone marrow chimera studies using \(Tlr2^{-/-}\) or \(Tlr4^{-/-}\) mice to delineate the role of TLRs on inflammatory versus structural cells.

CS-induced small airway fibrosis was significantly reduced in lungs of \(Tlr4^{-/-}\) mice compared to WT controls. Others have proposed that hyaluronan plays a role in modulating the expression of fibronectin and pulmonary fibrosis in a TLR4-dependent manner (50, 107). Moreover, IL-33 is known to be a pro-fibrotic factor and has been shown to be important in chronic lung diseases, including COPD and asthma (20, 84, 105). Thus, the reduction in small airway fibrosis in \(Tlr4^{-/-}\) mice may result from the impairment of the effects of hyaluronan in BALF and IL-33 and fibronectin in the lungs. TLR4 also plays a critical role in bleomycin-induced pulmonary fibrosis (42, 58, 60, 86). TLR4 mRNA expression was increased in mice administered with bleomycin (58), and \(Tlr4^{-/-}\) mice were protected against bleomycin-induced pulmonary fibrosis (increased lung collagen levels) and mortality (60). Inhibition of TLR4 with an antagonist (E5564) or an inhibitory small hairpin RNA also reduced collagen synthesis and secretion in the lungs (42, 86). Our data further these observations by showing that TLR4 is a mediator of small airway fibrosis induced by CS.

Previous studies suggest that mice deficient in, or with mutations of, TLR4 on other genetic backgrounds (e.g. C3H/HeJ and C57BL/10ScNJ) developed spontaneous emphysema after 3 months of age in the absence of noxious challenges such as CS (104, 109). This was
associated with increased Nox3 expression in lungs of these mice (104, 109). We also observed an increase in Nox3 mRNA in normal air-exposed Tlr4−/− BALB/c mice, however, these mice did not develop spontaneous emphysema even at 15-16 weeks of age. In fact, Tlr4−/− mice were protected from CS-induced emphysema-like alveolar enlargement, which was associated with reduced apoptosis in the lungs. These findings were supported by the observation of reduced expression of MMP-12, which has been linked to the induction of emphysema (7, 41), and improved lung function in CS-exposed Tlr4−/− mice. Moreover, CS exposure appeared to reduce Nox3 mRNA expression in Tlr4−/− BALB/c mice back to levels observed in normal air-exposed WT BALB/c mice.

The differences in genetic background may account for the conflicting results. For example, spontaneous chronic lung disease (severe lung inflammation, increased collagen deposition and alveolar wall thickening) only manifest in Src homology 2 domain–containing inositol-5-phosphatase 1 (SHIP-1)-deficient mice on C57BL/6, but not BALB/c, background (23, 67). In the context of human COPD, this may be important as the severity of the disease in humans often varies between individuals and genetic make-up in combination with environmental exposures are critical. These observations highlight the potential importance of genetic factors in predisposing certain individuals to COPD. This is clinically relevant as only 50% of life-long smokers developed COPD (64). Importantly, this may also indicate that certain individuals may respond better to TLR-based interventions such as Eritoran (currently in phase 3 clinical trial (73)). Eritoran is a synthetic TLR4 antagonist and was shown to protect mice against acute lethal influenza infection (88).

Our study demonstrates a previously unrecognized protective role for TLR2 in the pathogenesis of COPD. This supports current evidence in the literature that shows a loss-of-function polymorphism in the TLR2, but not TLR4, gene is associated with accelerated lung function declines in COPD patients (12). TLR2 polymorphisms also predispose patients with
other chronic lung diseases (e.g. cystic fibrosis) to rapid lung function decline (35). This further highlights the potential protective role of TLR2 in chronic lung diseases, and screening for TLR2 polymorphisms may be useful in the prognosis of COPD patients. Furthermore, using a clinically-relevant and established model of CS-induced COPD, our study demonstrates that TLR4 promotes CS-induced airway fibrosis, apoptosis, emphysema-like alveolar enlargement and lung function impairment. Hence, activating TLR2 and/or inhibiting TLR4 may be potential therapeutic strategies in COPD.

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COMPETING INTEREST

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FIGURE LEGENDS

Figure 1. TLR2 and TLR4 mRNA expression and protein levels are increased in CS-induced experimental COPD. Wild-type (WT) BALB/c mice were exposed to normal air or CS and sacrificed after 4, 8 and 12 weeks. (A) TLR2 and (B) TLR4 mRNA expression in whole lung tissues. (C) Muc5ac and Sftpc mRNA expression in blunt dissected airways and parenchyma in normal air-exposed WT mice. (D-E) TLR2 and (F-G) TLR4 mRNA expression in blunt dissected airways and parenchyma after 8 weeks CS exposure. Muc5ac, Sftpc, TLR2 and TLR4 mRNA expression were normalized to reference gene HPRT and expressed as relative abundance compared to either blunt dissected airways (for Muc5ac and Sftpc mRNA) or normal air-exposed WT controls (for TLR2 and TLR4 mRNA). Immunohistochemistry for (H-I) TLR2 and (J-K) TLR4 protein on small airway epithelium and lung parenchyma after 8 weeks of CS exposure; scale bars equal 50µm. Arrowheads indicate TLR2 or TLR4 expressing inflammatory cells. Graphs were presented as mean ± SEM and representative from two independent experiments of 6 mice per group. Two-tailed Mann-Whitney t-test analyzed differences between two groups, whereby *p<0.05; **p<0.01; ***p<0.001 compared to normal air-exposed WT controls and #p<0.05; ###p<0.001 compared to blunt dissected airway.

Figure 2. TLR2- and/or TLR4-expressing neutrophils and mDCs cells were increased in CS-induced experimental COPD. Wild-type (WT) BALB/c mice were exposed to cigarette smoke (CS) or normal air for 8 weeks. (A) TLR2 and (B) TLR4 mRNA expressions were determined in macrophage isolated from lungs by qPCR. The number of (C) CD11b+ monocytes, (D) neutrophils and (E) myeloid dendritic cells (mDCs) expressing TLR2 and/or TLR4 in lungs. mRNA expressions were normalized to reference gene and expressed as
relative abundance compared to normal air-exposed WT controls. Graphs were presented as
mean ± SEM and representative from two independent experiments of 6 mice per group.
Two-tailed Mann-Whitney t-test analyzed differences between two groups, whereby *p<0.05;
**p<0.01 compared to normal air-exposed WT controls.

**Figure 3. TLR2 and TLR4 co-receptor expression are increased in CS-induced experimental COPD.** Wild-type (WT) BALB/c mice were exposed to normal air or CS for 8 weeks to induce experimental COPD. (A-B) TLR1, (C-D) TLR6, (E-F) CD14 and (G-H) MD2/Ly96 mRNA expressions in blunt dissected airways and parenchyma. mRNA expressions were normalized to reference gene HPRT and expressed as relative abundance compared to normal air-exposed WT controls. Graphs were presented as mean ± SEM and representative from two independent experiments of 6 mice per group. Two-tailed Mann-Whitney t-test analyzed differences between two groups, whereby *p<0.05; **p<0.01; ***p<0.001 compared to normal air-exposed WT controls.

**Figure 4. TLR2, TLR4 and co-receptor mRNA expressions are increased in the airways in humans with mild to moderate COPD.** Airway epithelial cells were collected from human healthy non-smokers (NS), healthy smokers without COPD (Smoker) and COPD patients with Global Initiative for Chronic Obstructive Lung Disease (GOLD) stage I (Mild) or II (Moderate) disease. (A) TLR2, (B) TLR4, (C) TLR1, (D) TLR6, (E) CD14 and (F) LY96 mRNA expression were assessed by microarray profiling. Lung parenchymal cores were collected from human healthy non-smokers (NS) and COPD patients with Global Initiative for Chronic Obstructive Lung Disease (GOLD) stage IV (severe) disease. (G) TLR2, (H) TLR4, (I) TLR1, (J) TLR6 and (K) LY96 mRNA expression were assessed by
microarray profiling. Graphs are expressed as log$_2$ intensity robust multi-array average signals. The Benjamini–Hochberg method for adjusted P value/false discovery rate (FDR) analyzed differences between NS or Smokers and COPD patients.

**Figure 5. CS-induced pulmonary inflammation is unaltered in TLR2-deficient and TLR4-deficient mice with experimental COPD.** Wild-type (WT), TLR2-deficient (Tlr2$^{-/-}$) or TLR4-deficient (Tlr4$^{-/-}$) BALB/c mice were exposed to normal air or CS for 8 weeks to induce experimental COPD. (A) Total leukocytes, (B) macrophages, (C) neutrophils and (D) lymphocytes were enumerated in May-Grunwald Giemsa stained bronchoalveolar lavage fluid (BALF) cytopsin slides. (E) The numbers of parenchymal inflammatory cells (arrowheads) were enumerated in periodic acid-Schiff (PAS)-stained lung sections; scale bars equal 20µm. (F) Total histopathology score in lung sections and inflammation scores specifically in the (G) airway, (H) vascular and (I) parenchymal regions. (J) Representative images of lung histopathology scoring; scale bars equal 50µm. Graphs were presented as mean ± SEM and representative from two independent experiments of 6 mice per group. The one-way analysis of variance with Bonferroni post-test analyzed differences between 3 or more groups, whereby *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001 compared to normal air-exposed WT, Tlr2$^{-/-}$ or Tlr4$^{-/-}$ controls, #p<0.05; ##p<0.01 compared to CS-exposed WT controls and ϕp<0.05; ϕϕp<0.01; ϕϕϕp<0.001 compared to normal air-exposed WT controls.

**Figure 6. Pro-inflammatory cytokine, chemokine, COPD-related factors and oxidative stress-associated gene expressions in CS-induced experimental COPD.** Wild-type (WT), TLR2-deficient (Tlr2$^{-/-}$) or TLR4-deficient (Tlr4$^{-/-}$) BALB/c mice were exposed to normal air or cigarette smoke (CS) for 8 weeks. Pro-inflammatory cytokine (A) tumor necrosis factor-α
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(TNFα), (B) chemokine (C-X-C motif) ligand (CXCL)1, (C) chemokine (C-C motif) ligand (CCL)2, (D) CCL3, (E) CCL4, (F) CCL12, (G) CCL22, other COPD-related factors; (H) matrix metalloproteinase (MMP)-12 and (I) serum amyloid A3 (SAA3) mRNA expression were determined in whole lung homogenates by qPCR. Oxidative stress-associated genes (J) NADPH oxidase (Nox)1, (K) Nox2, (L) Nox3, (M) Nox4, (N) NAD(P)H quinone dehydrogenase (Nqo)1, (O) nuclear factor, erythroid 2 like 2 (Nrf2), (P) glutamate-cysteine ligase catalytic subunit (Gclc), (Q) glutathione peroxidase (Gpx)2, (R) heme oxygenase (Hmox)1 and (S) glutathione S-transferase pi (Gstp)1 expression were determined in whole lung homogenates by qPCR. mRNA expression was normalized to the reference gene HPRT and expressed as relative abundance compared to normal air-exposed WT controls. (T) The levels of TNFα protein in lung homogenates were determined by ELISA and normalized to total protein. Graphs were presented as mean ± SEM and representative from two independent experiments of 6 mice per group. The one-way analysis of variance with Bonferroni post-test was used to analyze differences between 3 or more groups, whereby *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001 compared to normal air-exposed WT, Tlr2−/− or Tlr4−/− controls, #p<0.05; ##p<0.01; ###p<0.001; ####p<0.0001 compared to CS-exposed WT controls, ϕp<0.05; ϕϕϕϕp<0.0001 compared normal air-exposed WT controls and ns denotes not significant.

Figure 7. CS-induced airway remodeling and fibrosis is not altered in TLR2-deficient mice whilst CS-induced airway fibrosis, but not remodeling, is completely attenuated in TLR4-deficient mice with experimental COPD. Wild-type (WT), TLR2-deficient (Tlr2−/−) or TLR4-deficient (Tlr4−/−) BALB/c mice were exposed to normal air or CS for 8 weeks to induce experimental COPD. (A) Small airway epithelial thickness in terms of epithelial cell area (µm²) per basement membrane (BM) perimeter (µm) was determined in periodic acid-
Schiff (PAS)-stained whole lung sections; scale bars equal 50µm. (B) The number of epithelial cells in PAS-stained lung sections was assessed by enumerating the number of nuclei per 100µm of BM perimeter; scale bars equal 20µm. (C) Area of collagen deposition (µm²) per BM perimeter (µm) was determined in Masson’s Trichrome-stained lung sections; scale bars equal 200µm. Inserts show expanded images of indicated regions; scale bars equal 200µm. (D) The level of hyaluronan in BALF was determined by ELISA. The mRNA expression of (E) fibronectin and (F) interleukin (IL)-33 were determined in whole lung homogenates by qPCR. mRNA expression was normalized to the reference gene HPRT and expressed as relative abundance compared to normal air-exposed WT controls. Graphs were presented as mean ± SEM and representative from two independent experiments of 6 mice per group. The one-way analysis of variance with Bonferroni post-test analyzed differences between 3 or more groups, whereby *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001 compared to normal air-exposed WT, Tlr2⁻/⁻ or Tlr4⁻/⁻ controls, #p<0.05; ##p<0.01; ####p<0.0001 compared to CS-exposed WT controls, ϕp<0.05; ϕϕϕϕp<0.0001 compared to normal air-exposed WT controls and ns denotes not significant.

Figure 8. CS-induced emphysema-like alveolar enlargement, apoptosis and impaired lung function are increased in TLR2-deficient mice and decreased in TLR4-deficient mice with experimental COPD. Wild-type (WT), TLR2-deficient (Tlr2⁻/⁻) or TLR4-deficient (Tlr4⁻/⁻) BALB/c mice were exposed to normal air or CS for 8 weeks to induce experimental COPD. (A) Alveolar septal damage and (B) diameter (µm) were determined in H&E-stained lung sections using destructive index and mean linear intercept techniques respectively; scale bars equal 50µm. (C) The numbers of TUNEL⁺ cells (arrowheads) were enumerated in whole lung sections at high power field (HPF; 100x); scale bars equal 20µm. Immunohistochemistry for (D) active caspase-3 protein on lung parenchyma after 8 weeks of
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CS exposure; scale bars equal 50µm. Arrowheads indicate caspase-3-expressing alveolar septa cells. Lung function was assessed in terms of (E) total lung capacity (TLC) and (F) transpulmonary resistance. Graphs were presented as mean ± SEM and representative from two independent experiments of 6 mice per group. The one-way analysis of variance with Bonferroni post-test analyzed differences between 3 or more groups, whereby **p<0.01; ***p<0.001; ****p<0.0001 compared to normal air-exposed WT, Tlr2−/− or Tlr4−/− controls, #p<0.05; ##p<0.01; ####p<0.0001 compared to CS-exposed WT controls, ϕϕϕϕp<0.0001 compared to normal air-exposed WT controls and ns denotes not significant.
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<td>GGAACTACCTCTATGCAGGGAT</td>
</tr>
<tr>
<td>Muc5ac forward</td>
<td>GCAGTTGTGTACCCATCATCTGTG</td>
</tr>
<tr>
<td>Muc5ac reverse</td>
<td>GGGGCAGTCTTGACTAACCCTCTT</td>
</tr>
<tr>
<td>SftpC forward</td>
<td>TGTATGACTACCAGCGGCTC</td>
</tr>
<tr>
<td>SftpC reverse</td>
<td>AGCGAAGCCTCAAGACTAGG</td>
</tr>
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<td>TNFα forward</td>
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</tr>
<tr>
<td>TNFα reverse</td>
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<tr>
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<td>GCTGGGATTCACCTCAAGAA</td>
</tr>
<tr>
<td>CXCL1 reverse</td>
<td>CTTGGGAGACACCTTTTAGCA</td>
</tr>
<tr>
<td>CCL2 forward</td>
<td>TGAGTAGCAGCAGGTGAGTGAGG</td>
</tr>
<tr>
<td>CCL2 reverse</td>
<td>TGTTCAAGGTTGCCGGCTGGAG</td>
</tr>
<tr>
<td>CCL3 forward</td>
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<td>CCL3 reverse</td>
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<tr>
<td>CCL4 forward</td>
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<tr>
<td>CCL4 reverse</td>
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<td>CCL12 forward</td>
<td>CCGGGAGCTGTGATCTTCA</td>
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<tr>
<td>CCL12 reverse</td>
<td>AACCCACTTTCGGGGGT</td>
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<tr>
<td>CCL22 forward</td>
<td>TGCTACCTGCCGCTGCTGCTCCCA</td>
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<tr>
<td>MMP-12 forward</td>
<td>CGTGATGCGCAGGGGTCGACGG</td>
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<tr>
<td>MMP-12 reverse</td>
<td>CCTCAGGCTTTGCTGACCCGA</td>
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<tr>
<td>SAA3 forward</td>
<td>TGATCCTGGGAGGTGACAGCCAA</td>
</tr>
<tr>
<td>SAA3 reverse</td>
<td>ACCGCTCGGGCAGCAGCATCATA</td>
</tr>
<tr>
<td>Nox1 forward</td>
<td>CCCCTGAGTCTTGAGAAGTTGG</td>
</tr>
<tr>
<td>Nox1 reverse</td>
<td>GGGTGCAATGCAACCTTGGTA</td>
</tr>
<tr>
<td>Nox2 forward</td>
<td>AACTGGGCTGTGAATGAAGG</td>
</tr>
<tr>
<td>Nox2 reverse</td>
<td>CAGCAGGATCAGCATACTAGTGTG</td>
</tr>
<tr>
<td>Gene</td>
<td>Forward</td>
</tr>
<tr>
<td>--------</td>
<td>---------</td>
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<tr>
<td>Nox3</td>
<td>CTCGTTGCCTACGGGATAGC</td>
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<tr>
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<td>ACAACCAAGGGCCAGAATACTACTAC</td>
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<td>Hmox1</td>
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<td>Gstp1</td>
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<tr>
<td>Fibronectin</td>
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<tr>
<td>IL-33</td>
<td>CCTCCCTGAGTACATACAATGACC</td>
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<td>HPRT</td>
<td>AGGCCAGACTTTGTGGATTTGAA</td>
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</table>

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1217
Table 2. Surface antigens used to characterize mouse lung cell subsets by flow cytometry

<table>
<thead>
<tr>
<th>Cell subset</th>
<th>Cell surface antigens</th>
</tr>
</thead>
<tbody>
<tr>
<td>Myeloid dendritic cell</td>
<td>CD45⁺ F4/80⁻ CD11c⁺ CD11b⁺ MHCII⁺ TLR2⁻/⁺ TLR4⁻/⁺</td>
</tr>
</tbody>
</table>
Table 3. Antibodies used in flow cytometry analysis

<table>
<thead>
<tr>
<th>Cell surface antigens</th>
<th>Clone</th>
<th>Fluorophore</th>
<th>Company</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD45</td>
<td>30-F11</td>
<td>AF-700</td>
<td>Biolegend</td>
</tr>
<tr>
<td>F4/80</td>
<td>T45-2342</td>
<td>BV711</td>
<td>BD Biosciences</td>
</tr>
<tr>
<td>CD11c</td>
<td>HL3</td>
<td>BV421</td>
<td>BD Biosciences</td>
</tr>
<tr>
<td>CD11b</td>
<td>M1/70</td>
<td>PerCPCy5.5</td>
<td>Biolegend</td>
</tr>
<tr>
<td>Ly6G</td>
<td>1A8</td>
<td>BV510</td>
<td>Biolegend</td>
</tr>
<tr>
<td>MHCII</td>
<td>M5/114.15.2</td>
<td>APC</td>
<td>Biolegend</td>
</tr>
<tr>
<td>TLR2</td>
<td>T2.5</td>
<td>FITC</td>
<td>Biolegend</td>
</tr>
<tr>
<td>TLR4</td>
<td>MTS510</td>
<td>PE</td>
<td>BD Biosciences</td>
</tr>
</tbody>
</table>
Figure 1

A

B

C

D

E

F

G

H

I

J

K

Normal air

8 weeks CS

Normal air

8 weeks CS

Normal air

8 weeks CS