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

Tatt Jhong Haw[1,#], Malcolm R. Starkey[1,2,#], Stelios Pavlidis[3], Michael Fricker[1], Anya L. Arthurs[1], Prema M. Nair[1], Gang Liu[1], Irwan Hanish[4], Richard Y. Kim[1], Darryl A. Knight[1], Paul S. Foster[1], Jay C. Horvat[1], Ian M. Adcock[3] and Philip M. Hansbro[1*]

[1]Priority Research Centre for Healthy Lungs, School of Biomedical Sciences and Pharmacy, Hunter Medical Research Institute & University of Newcastle, Callaghan, New South Wales, Australia

[2]Priority Research Centre for Grow Up Well, School of Biomedical Sciences and Pharmacy, Hunter Medical Research Institute & University of Newcastle, University of Newcastle, Callaghan, New South Wales, Australia

[3]The Airways Disease Section, National Heart & Lung Institute, Imperial College London, London, UK

[4]Department of Microbiology, Faculty of Biotechnology and Biomolecular Sciences, Universiti Putra Malaysia, Serdang, Selangor, Malaysia

Running header: Opposing roles of TLR2 and TLR4 in pathogenesis of COPD

21 Author contributions: TJH, MRS and PMH designed the study. TJH and MRS performed the
22 experiments, collected and analyzed the data, generated the figures, and wrote the
23 manuscript. SP analyzed and generated the human data. MF and ALA analyzed and
24 generated isolated lung macrophage data. PMN, GL, IH and RYK assisted in performing the
25 experiments and collecting the data. JCH advised on experimental design and analysis and
26 edited the manuscript. IMA analyzed the human data and edited the manuscript. PMH
27 oversaw the project and advised on collection and analysis of data and edited the manuscript.

28

29 # TJH and MRS contributed equally to this work

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31 *Corresponding author: Philip M. Hansbro, PhD, Priority Research Centre for Healthy
32 Lungs, School of Biomedical Sciences and Pharmacy, Hunter Medical Research Institute &
33 University of Newcastle, Callaghan, New South Wales 2308, AUSTRALIA. Telephone:
34 (+61) 40420187 Email: Philip.Hansbro@newcastle.edu.au

35

36 # ABSTRACT

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

57

58 **Key words:** TLR2, TLR4, cigarette smoke, emphysema, COPD

59

60 # INTRODUCTION

61 Chronic Obstructive Pulmonary Disease (COPD) is the third leading cause of morbidity and
62 death and imposes significant socioeconomic burden worldwide (63). It is a complex,
63 heterogeneous disease characterized by chronic pulmonary inflammation, emphysema and
64 airway remodeling, which are associated with progressive lung function decline (39). Indeed,
65 the major disease features are progressive and become more severe over time that is
66 accelerated by infection-induced exacerbations. Cigarette smoke (CS) is a major risk factor
67 for COPD (54). The current mainstay therapies for COPD are glucocorticoids, β_2-adrenergic
68 receptor agonists and long acting muscarinic antagonists (5, 93). However, these agents are
69 largely ineffective and only provide symptomatic relief rather than modifying the causal
70 factors or stopping disease progression (5). There is much current interest in increased
71 microbial carriage and altered lung and gut microbiomes in COPD that could be modified for
72 therapeutic gain and macrolide antibiotics are currently being tested as new treatments (14,
73 89, 92). Nevertheless, there is currently a lack of effective treatments for COPD that is
74 largely due to the poor understanding of the underlying mechanisms.

75 Toll-like receptor (TLR)2 and TLR4 play vital roles in detecting and initiating
76 immune responses to microbial membrane components (1, 36, 52). TLR2 and TLR4 are type
77 I transmembrane receptors expressed on the cell surface (1, 36, 52). However, in some
78 circumstances TLR4 can be internalized or expressed intracellularly in certain cells (1, 36,
79 52). TLR2 and TLR4 primarily signal through the adaptor protein myeloid differentiation
80 primary response gene 88 (MyD88)-dependent or MyD88-adapter-like (Mal)-dependent
81 pathways (1, 36, 52). Upon ligand (e.g. bacterial peptidoglycan) binding TLR2 forms a
82 heterodimer with either TLR1 or TLR6 and interacts with cluster of differentiation (CD)14 to
83 form a functional complex (24, 48). In contrast, TLR4 forms a homodimer upon binding to its

84 ligand (e.g. bacterial lipopolysaccharide) and interacts with CD14 and/or MD2 (also known
85 as lymphocyte antigen 96 [LY96] in humans) (57, 108). This initiates the recruitment of
86 MyD88 to the intracellular Toll–interleukin 1 (IL-1) receptor (TIR) domain, that
87 subsequently activates members of the IL-1 receptor-associated kinases (IRAKs) and tumor
88 necrosis factor (TNF) receptor-associated factor 6 (TRAF6) (1, 52). Consequently,
89 transcriptional factors of the mitogen-activated protein kinase (MAPK) family and nuclear
90 factor kappa-light-chain-enhancer of activated B cells (NF-κB) are activated, leading to the
91 expression of pro-inflammatory mediators (1, 36, 52).

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

105 Hence, the role of TLR2 and TLR4 in the pathogenesis of COPD remains unclear.
106 Here, we investigated these roles using an established mouse model of CS-induced
107 experimental COPD that recapitulates the critical features of human disease (7, 29, 31, 40,
108 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*$^{-/-}$) and TLR4-deficient (*Tlr4*$^{-/-}$) mice~~ when compared to WT controls~~. However, *Tlr2*$^{-/-}$ mice had increased CS-induced emphysema-like alveolar enlargement, apoptosis and impaired lung function whilst CS-induced airway fibrosis was not altered~~ compared to WT controls~~. In contrast, these features were reduced or completely ablated in *Tlr4*$^{-/-}$ 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*$^{-/-}$ or *Tlr4*$^{-/-}$ BALB/c mice were purchased from the Australian Bioresource Facility, Moss Vale, NSW, Australia. *Tlr2*$^{-/-}$ and *Tlr4*$^{-/-}$ 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

133 mortality are higher in females, and in the United States in 2009 women accounted for
134 53% of COPD deaths (78). It is for these and logistical reasons that female mice are used.
135
136 **Isolation of RNA and qPCR.** Total RNA was extracted and reversed transcribed from whole
137 lung tissue, blunt-dissected airway and parenchyma and isolated lung macrophages (7, 41, 70,
138 101). mRNA transcripts were determined by real-time quantitative PCR (qPCR,
139 ABIPrism7000, Applied Biosystems, Scoresby, Victoria, Australia) using custom designed
140 primers (Integrated DNA Technologies, Baulkham Hills, New South Wales, Australia),
141 normalized to the reference gene hypoxanthine-guanine phosphoribosyltransferase (HPRT)
142 and expressed as relative abundance to WT air controls (**Table 1**) (7, 41, 70, 101).
143
144 **Immunohistochemistry.** Lungs were perfused, inflated, formalin-fixed, paraffin-embedded
145 and sectioned (4-6µm). Longitudinal sections of the left lung were rehydrated through a
146 series of xylene (2x) and ethanol gradient (2x absolute, 90%, 80%, 70%, 50%, 0.85% saline
147 and phosphate-buffered saline [PBS]) washes followed by antigen retrieval with citrate buffer
148 (10mM citric acid, 0.05% Tween 20, pH 6.0) at 100°C for 30 mins. Sections were blocked
149 with casein blocker (Thermo Fisher Scientific, Pittsburgh, PA, USA) for 1 hour. Sections
150 were then washed with PBS (5x, 5 mins each) and incubated with either anti-TLR2 (1:200
151 dilution, MABF84, clone 19B6.2, Merck Milipore, Bayswater, Victoria, Australia), anti-
152 TLR4 (1:1000 dilution, ab47093, Abcam, Melbourne, Victoria, Australia) or anti-active
153 caspase-3 (1:200 dilution, ab13847, Abcam) antibodies followed by either anti-rabbit
154 (HAF008; R&D Systems, Gymea, New South Wales, Australia) or anti-mouse (ab6728;
155 Abcam) secondary antibody conjugated with horseradish peroxidase and then 3,3'-
156 Diaminobenzidine chromogen-substrate buffer (DAKO, North Sydney, New South Wales,

157 Australia) according to manufacturer's instructions (40, 41). Sections were counterstained
158 with hematoxylin, mounted and analyzed using a BX51 microscope (Olympus, Tokyo,
159 Shinjuku, Japan) with Image-Pro Plus software (Media Cybernetics, Rockville, MD). The
160 a~~A~~reas of active caspase-3 in lung parenchyma were determined (n=4 per group, 10
161 randomized parenchyma images per lung sections) using ImageJ software (Version 1.50,
162 NIH, New York City, NY, USA), normalized to area of hematoxylin and represented as the
163 percentage area of active caspase-3. Images with inflammation and airways were excluded
164 from analysis.

165

166 **Isolation of lung macrophages.** Lungs were excised, washed and dissected into 1-2mm
167 cubes in Dulbecco's Modified Eagle Medium (DMEM, Sigma Aldrich, Castle Hill, New
168 South Wales, Australia). Lung tissues were then transferred into Medicon cassettes (BD
169 Biosciences, North Ryde, New South Wales, Australia) and disaggregated using a
170 Medimachine (BD Biosciences) for 2 mins. Cell suspensions were collected, Histopaque
171 1083 (Sigma Aldrich) applied and centrifuged (825x*g*, 30 mins, 22.5 °C). The opaque
172 interface cell layer where macrophages/monocytes were enriched was collected, washed with
173 Hyclone™ Dulbecco's PBS (GE Healthcare Life Sciences, South Logan, Utah, USA) and
174 centrifuged (100x*g*, 5 mins, 22.5 °C). Cell pellets were resuspended in fresh DMEM (Sigma)
175 and macrophages further enriched through adherence to plastic tissue culture flasks (3 hrs, 37
176 °C). Non-adherent contaminating cells were removed by gentle washing with PBS (GE
177 Healthcare Life Sciences) prior to RNA isolation.

178

179 **Flow Cytometry Analysis.** The numbers of $CD11b^{+}$ monocytes, neutrophils and myeloid
180 dendritic cells (mDCs) in lung homogenates were determined based on surface marker

181 expression using flow cytometry (**Table 2**) (45, 53, 94, 97, 101). Flow cytometric analysis
182 was performed using a FACSAriaIII with FACSDiva software (BD Biosciences, North Ryde,
183 Australia). Flow cytometry antibodies were purchased from Biolegend (Karrinyup, Western
184 Australia, Australia) or BD Biosciences (**Table 3**). OneComp compensation beads (Thermo
185 Fisher Scientific) were used to compensate for spectral overlap.

186

187 **Gene Expression in Human COPD Microarray Datasets.** Analysis of TLR2, TLR4 and
188 co-receptors gene expression in published human array datasets (Affymetrix Human Genome
189 U133 Plus 2.0 Array, Accession numbers: GSE5058 and GSE27597) (13, 16, 102) were
190 performed using the Array Studio software (Omicsoft Corporation, Research Triangle Park,
191 NC, USA) by applying a general linear model adjusting for age and gender and the
192 Benjamini–Hochberg method for p-value adjustment (41). Data are expressed as $\log_2$
193 intensity robust multi-array average signals. The Benjamini–Hochberg method for adjusted P
194 value/false discovery rate (FDR) was used to analyze differences between two groups.
195 Statistical significance was set at FDR < 0.05.

196

197 **Pulmonary Inflammation.** Airway inflammation was assessed by differential enumeration
198 of inflammatory cells in bronchoalveolar lavage fluid (BALF) (7, 27, 40, 41, 62, 70). Lung
199 sections were stained with periodic acid-Schiff (PAS) and tissue inflammation assessed by
200 enumeration of inflammatory cells (7, 41, 70). Histopathological score was determined in
201 lung sections stained with hematoxylin and eosin (H&E) based on established custom-
202 designed criteria (40, 44, 70).

203

Enzyme-linked immunosorbent assay (ELISA). Right lung lobes were homogenized on ice in 500uL 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 (8,000x*g*, 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 ~~enzyme-linked immunosorbent assays (~~ELISAs~~)~~. 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 (μm^2), cell (nuclei) number and collagen deposition area (μm^2) 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

228 to manufacturer's instructions (41). Apoptosis in lung parenchyma was assessed by
229 enumerating the numbers of TUNEL^{+} cells per high power fields (HPF; 100x) (41).

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

238

239 **Statistical analyses.** Data are presented as means ± standard error of mean (SEM) and
240 representative from two independent experiments with 6 mice per group. The two-tailed
241 Mann-Whitney test was used to compare two groups. The one-way analysis of variance with
242 Bonferroni post-test was used to compare 3 or more groups. Statistical significance was set at
243 $P < 0.05$ and determined using GraphPad Prism Software version 6 (San Diego, CA, USA).

244

245 RESULTS

246 TLR2 and TLR4 mRNA Expression and Protein Levels are Increased in 247 CS-induced Experimental COPD

248 To determine whether TLR2 and TLR4 levels are altered in COPD, we first interrogated our
249 mouse model of experimental COPD (7, 29, 31, 40, 41, 47, 62, 100). WT mice were exposed

250 to CS for 4, 8 and 12 weeks and TLR2 and TLR4 mRNA expression were assessed. TLR2,
251 but not TLR4 mRNA was significantly increased in whole lung homogenates after 4, 8 and
252 12 weeks of CS exposure compared to normal air-exposed mice (Figure 1A-B). We have
253 previously shown that many of the hallmark features of COPD were established in mice after
254 8 weeks of CS exposure (7, 29, 31, 40, 41, 47, 62, 100). To determine the compartment of the
255 lung in which altered TLR2 and TLR4 expression occurred following establishment of
256 disease, blunt dissected airways and lung parenchyma were assessed at this time point. To
257 confirm separation of blunt~~ly-~~-dissected airways from parenchymal tissue we assessed the
258 mRNA expression of airway epithelial cell-specific mucin 5ac (Muc5ac) and the mouse type
259 II alveolar epithelial cell-marker surfactant protein C (Sftpc) in normal air-exposed mice
260 (Figure 1C). T~~Accordingly, t~~he levels of Muc5ac mRNA were~~as~~ increased in airways
261 compared to lung parenchyma~~ (Figure 1C)~~. Conversely, the levels of Sftpc mRNA were~~as~~
262 increased in lung parenchyma compared to airways~~ (Figure 1C)~~. This confirms the tissue
263 specific isolation and location of TLR2, TLR4 and co-receptors in these tissues. TLR2
264 mRNA was increased in both the airways and parenchyma of CS-exposed mice (Figure 1D-
265 E). In contrast, TLR4 mRNA was increased in the airways, but not parenchyma (Figure 1F-
266 G). These mRNA expression data were supported by increased TLR2 (Figure 1H-I) and
267 TLR4 (Figure 1J-K) protein levels in small airway epithelial cells and increased infiltration of
268 parenchyma-associated inflammatory cells that expressed TLR2 or TLR4 in lung histology
269 sections detected by immunohistochemistry.

270 To identify the inflammatory cell source of TLR2 and TLR4, macrophages were
271 isolated from whole lung homogenates for downstream mRNA analysis. Interestingly, the
272 mRNA expression of TLR2 and TLR4 were not altered in lung macrophages isolated from
273 CS-exposed mice (Figure 2A-B). CD11b^{+} monocytes, neutrophils and mDCs are known to
274 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 usingby 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 the 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 Obstructive Lung Disease [GOLD] Stage I or II (103)). Pre-existing microarray data from

298 non-COPD (healthy non-smokers and healthy smokers) and COPD patients were interrogated
299 (13, 16, 102). TLR2, TLR4 and co-receptor (TLR1, TLR6, CD14 and LY96) mRNA
300 expression were not significantly altered in airway epithelial brushings from healthy smokers
301 compared to non-smokers (Accession: GSE5058 (102), Figure 4A-F). Importantly, however,
302 TLR2 (~2.4-fold), TLR4 (~8.7-fold), TLR1 (~7.1-fold), TLR6 (~1.5-fold), CD14 (~3.9-fold)
303 and LY96 (~12.9-fold) mRNA expression were increased in airway epithelial brushings from
304 patients with mild-to-moderate COPD compared to non-smokers. Notably, TLR2 (~2.4-fold),
305 TLR4 (~7.2-fold), TLR1 (~4.7-fold), CD14 (~3.6-fold) and LY96 (~6.8-fold) but not TLR6
306 (~0.8-fold) mRNA expression were also increased in airway brushings from COPD patients
307 compared to healthy smokers (Figure 4A-F).

308

309 TLR2, TLR4 and Co-receptor mRNA Expression are Decreased in the 310 Parenchyma in Human COPD

311 Similarly, we then assess the expression of TLR2, TLR4 and co-receptor expression in pre-
312 existing microarray data from lung parenchyma cores from severe COPD patients (GOLD
313 Stage IV (103)) compared to non-smokers without COPD (Accession: GSE27597) (13, 16).
314 In contrast to the data from the airways, TLR2 (~1.5-fold) and TLR4 (~2.0-fold) expression
315 were reduced, whereas co-receptors TLR1, TLR6 and LY96 were not altered, in the
316 parenchyma from severe COPD patients compared to non-smokers without COPD (Figure
317 4G-K). CD14 was not detectable in this dataset.

318

319 CS-induced Pulmonary Inflammation was Largely Unaltered in TLR2- 320 deficeint and TLR4-deficient mice with Experimental COPD

321 We next investigated whether TLR2 and TLR4 play a role in the pathophysiology of CS-
322 induced experimental COPD. WT, *Tlr2*$^{-/-}$ and *Tlr4*$^{-/-}$ mice were exposed to normal air or CS
323 for 8 weeks (7, 29, 31, 40, 41, 47, 62, 100). We first assessed pulmonary inflammation in
324 BALF by staining and differential enumeration of inflammatory cells. As expected, CS-
325 exposure of WT mice resulted in significantly increased total leukocytes, macrophages,
326 neutrophils and lymphocytes in BALF compared to normal air-exposed WT controls (Figure
327 5A-D). CS-exposed *Tlr2*$^{-/-}$ mice had increased neutrophils and lymphocytes, but not total
328 leukocytes and macrophages, compared to normal air-exposed *Tlr2*$^{-/-}$ controls. This was due
329 to an increase in total leukocytes and macrophages in normal air-exposed *Tlr2*$^{-/-}$ controls
330 compared to normal air-exposed WT controls. In contrast, CS-exposed *Tlr4*$^{-/-}$ mice had
331 increased total leukocytes, macrophages, neutrophils and lymphocytes in BALF compared to
332 normal air-exposed *Tlr4*$^{-/-}$ controls. Importantly, total leukocytes, macrophages, neutrophils
333 and lymphocytes in BALF were not significantly altered in CS-exposed *Tlr2*$^{-/-}$ or *Tlr4*$^{-/-}$ mice
334 compared to CS-exposed WT controls.

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

340 Next, histopathology was scored according to a set of custom-designed criteria as
341 described previously (40, 44). CS exposure of WT mice increased histopathology score
342 (Figure 5F), which was characterized by increased airway, vascular and parenchymal
343 inflammation (Figure 5G-I). CS-exposed *Tlr2*$^{-/-}$ and *Tlr4*$^{-/-}$ mice also had increased
344 histopathology, airway, vascular and parenchymal inflammation scores compared to their
345 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 TNF-α, 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 TNF-α, 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 *Tlr2*$^{-/-}$ or *Tlr4*$^{-/-}$ mice compared to WT controls. Interestingly, Nox3 (Figure 6L) was increased in normal air-

371 exposed *Tlr4*$^{-/-}$ mice compared to normal air-exposed WT controls, but was reduced by CS
372 exposure. Other oxidative stress-associated genes were, however, not significantly altered in
373 normal air-exposed *Tlr2*$^{-/-}$ or *Tlr4*$^{-/-}$ mice compared to normal air-exposed WT controls.

374 To determine whether the TNFα signaling pathway was altered in CS-exposed *Tlr4*$^{-/-}$
375 mice, we next assessed the levels of TNFα protein in lung homogenates. As expected, TNFα
376 protein levels were increased in CS-exposed WT mice compared to normal air-exposed WT
377 controls (Figure 6T). In contrast, TNFα protein levels were not altered in CS-exposed *Tlr2*$^{-/-}$
378 and *Tlr4*$^{-/-}$ mice when compared to normal air-exposed *Tlr2*$^{-/-}$ and *Tlr4*$^{-/-}$ mice, respectively.
379 The lack of increase in CS-exposed *Tlr2*$^{-/-}$ mice was due to increases in levels in normal air-
380 exposed *Tlr2*$^{-/-}$ mice compared normal air-exposed WT mice. Notably, TNFα protein was
381 significantly reduced in CS-exposed *Tlr4*$^{-/-}$ mice compared to CS-exposed WT controls.

382

383 CS-induced Collagen Deposition is not Altered in TLR2-deficeint Mice but 384 Completely Ablated in TLR4-deficient Mice with Experimental COPD

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

395 We next assessed airway fibrosis in terms of collagen deposition around small
396 airways. As expected, CS-exposed WT mice had increased collagen deposition compared to
397 normal air-exposed WT controls (Figure 7C). However, CS-exposed *Tlr2*$^{-/-}$ and *Tlr4*$^{-/-}$ mice
398 did not have increased collagen deposition compared to their respective normal air-exposed
399 controls. The former was due to an increase in collagen deposition in normal air-exposed
400 *Tlr2*$^{-/-}$ mice compared normal air-exposed WT controls. The latter was due to no increase in
401 collagen deposition in CS-exposed *Tlr4*$^{-/-}$ mice compared to normal air-exposed *Tlr4*$^{-/-}$
402 controls. Notably, CS-induced collagen deposition was attenuated in CS-exposed *Tlr4*$^{-/-}$ mice
403 compared to CS-exposed WT controls. This was associated with a concomitant increase in
404 the levels of hyaluronan in BALF and fibronectin mRNA expression in lung homogenates in
405 *Tlr4*$^{-/-}$ compared to WT mice, independent of CS exposure (Figure 7D-E). Notably, CS-
406 induced expression of IL-33 mRNA was also attenuated in *Tlr4*$^{-/-}$ mice (Figure 7F).
407

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

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

CS-exposed *Tlr2*$^{-/-}$ mice had increased alveolar damage and diameter compared CS-exposed WT controls. Conversely, CS-exposed *Tlr4*$^{-/-}$ mice had reduced alveolar septal damage and diameter compared 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 ~~lung parenchyma of~~ 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 significantly reduced in CS-exposed *Tlr4*$^{-/-}$ mice ~~compared to CS-exposed WT mice, whilst they were significantly reduced in CS-exposed *Tlr4*$^{-/-}$ mice~~ compared to CS-exposed WT mice.

442 We next assessed the roles of TLR2 and TLR4 in CS-induced impairment of lung
443 function, measured in terms of TLC and transpulmonary resistance. As expected, CS-exposed
444 WT mice had increased TLC (Figure 8E) and transpulmonary resistance (Figure 8F)
445 compared with normal air-exposed WT controls. CS-exposed *Tlr2*$^{-/-}$ mice did not have altered
446 TLC compared to normal air-exposed *Tlr2*$^{-/-}$ controls. This was due to increased TLC in
447 normal air-exposed *Tlr2*$^{-/-}$ mice compared to normal air-exposed WT controls. However, CS-
448 exposed *Tlr2*$^{-/-}$ mice did have increased transpulmonary resistance compared to normal air-
449 exposed *Tlr2*$^{-/-}$ mice. Notably, both TLC and transpulmonary resistance were significantly
450 increased in CS-exposed *Tlr2*$^{-/-}$ mice compared to CS-exposed WT mice. In contrast, *Tlr4*$^{-/-}$
451 mice did not have increases in TLC and transpulmonary resistance compared to normal air-
452 exposed *Tlr4*$^{-/-}$ controls.

453

454 # DISCUSSION

455 In this study, we demonstrate that both TLR2 and TLR4 play important, but opposing
456 roles, in the pathogenesis of CS-induced experimental COPD. TLR2 and TLR4 mRNA were
457 increased in airway epithelium and parenchyma of mice chronically exposed to CS and in
458 human COPD patients. Expression of the co-receptors TLR1, TLR6, CD14 or MD2/Ly96
459 were also increased in CS-exposed mice and human COPD. CS-induced pulmonary
460 inflammation was unaltered in *Tlr2*$^{-/-}$ and *Tlr4*$^{-/-}$ mice compared to WT controls. In contrast,
461 *Tlr2*$^{-/-}$ mice had increased CS-induced emphysema-like alveolar enlargement, apoptosis and
462 impaired lung function, whilst importantly these features were reduced in *Tlr4*$^{-/-}$ mice. CS-
463 induced small airway epithelial thickening and fibrosis were not altered in *Tlr2*$^{-/-}$ mice ~~when~~
464 compared to CS-exposed WT controls. In contrast, CS-induced airway fibrosis, but not small
465 airway epithelial thickening, was significantly attenuated in *Tlr4*$^{-/-}$ mice compared to CS-

466 exposed WT controls. This study provides new insights into the role of TLR2 and TLR4 in
467 the pathogenesis of COPD.

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

478 We aimed to address these discrepancies and delineate the roles of TLR2 and TLR4 in
479 COPD by using an established mouse model of tightly controlled chronic nose-only CS-
480 induced experimental COPD (7, 29, 31, 40, 41, 47, 100). Our models are representative of a
481 pack-a-day smoker and 8 weeks of smoking in a mouse that lives for a year is equivalent to
482 10 years in a human smoker (31, 51). Importantly, we have consistently shown that 8 weeks
483 of CS exposure in our models is sufficient to induce the hallmark features of human COPD:
484 chronic inflammation, airway remodelling, emphysema and impaired lung function (7, 13,
485 16, 29, 31, 40, 41, 47, 100, 102). This 8-week timepoint was specifically chosen to
486 investigate the underlying pathogenic mechanism(s) during the early stages (GOLD I/II) and
487 identify potential therapeutic targets to halt the progression of COPD. Moreover, these
488 shorter term models may be relevant for testing of therapeutic interventions because new
489 therapeutics such as targeting TLR signaling are likely to have little effect during more severe
490 stages of disease, when invasive interventions, such as lung surgery/transplant, may be

required (103). Although ~~it is possible~~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 grow accustomed to our purpose-built restraining tubes (7). This is ~~reflected~~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 returne~~ed~~ to baseline after that (unpublished data).

Interestingly, *Tlr2*$^{-/-}$ mice have not been assessed in the context of CS-induced ~~pathogenesis of~~ COPD. ~~whilst~~ *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), h~~. H~~owever, *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

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~~were in line with~~ previous studies, by us and others, that showed TLR2 and/or TLR4 a~~we~~re 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 ~~nasal~~airway epithelium in mild-to-moderate COPD (FEV$_1$ >1L) but reduced in severe disease~~COPD~~ (FEV$_1$ <1L) (65). Collectively, these data suggest that TLR2 and TLR4 expression is increased in the lung parenchyma early in disease in response to CS-exposure, but are reduced in of severe COPD patients, which may be due to ~~increased~~greater tissue destruction that results in the loss of tissues/cells expressing these TLRs. This also may

540 explain the current discrepancies in the literature on the expression of TLR2 and TLR4 in
541 COPD.

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

551 In contrast to our observations in *Tlr2*$^{-/-}$ mice, previous studies showed that inhibition
552 of TLR2 reduced pulmonary inflammation, apoptosis and lung function impairment in other
553 chronic lung diseases, including pulmonary fibrosis and asthma (59, 106). Mice deficient in
554 TLR2 or treated with a neutralizing antibody had reduced bleomycin-induced pulmonary
555 inflammation, collagen deposition and hydroxyproline levels in the lungs (106). Moreover,
556 *Tlr2*$^{-/-}$ mice had reduced airway inflammation and peri-bronchial collagen deposition in an
557 OVA-induced model of experimental allergic asthma (59). In addition, TLR2 was shown to
558 promote apoptosis in human kidney epithelial (HEK)-293, human monocytic (THP-1) cells
559 and endothelial cells *in vitro* (2, 81). Ovalbumin-induced airway hyperresponsiveness (AHR)
560 were also previously shown to be reduced in *Tlr2*$^{-/-}$ mice (38, 59). This may be due to
561 differences in pathogenesis, mechanisms and inflammatory cells/responses that may dictate
562 the role of TLR2 in various chronic lung diseases. For example, allergic asthma is dominated
563 by aberrant Th2-type responses typified by increased infiltration of eosinophils and activated
564 mast cells into the airways and increased levels of Th2-associated cytokines such as IL-5 and

565 IL-13 (44, 45, 95, 96). In contrast, COPD is typically associated with Th1/Th17-type
566 inflammatory responses characterized by infiltration of macrophages, neutrophils and
567 cytotoxic T cells and the production of Th1/Th17-associated cytokines such as IFN-γ and IL-
568 17A (7, 30, 69, 80, 91). Hence, the role of TLR2 in different chronic lung diseases may
569 largely depend on the presence and type of specific TLR2-expressing cells. Nevertheless, our
570 studies suggest that TLR2 may potentially have a protective role in CS-induced COPD. The
571 underlying mechanism remained unclear, but appeared to be independent of oxidative stress
572 in the lung.

573 Interestingly, inflammatory infiltrates in the airway lumen and around blood vessels,
574 TNFα protein and TLC were increased in normal air-exposed *Tlr2*$^{-/-}$ mice compared WT
575 controls. Previous studies showed that airway inflammation is negatively associated with
576 lung function (4, 8) and may cause lung hyperinflation (increased in TLC) (28, 49, 76).
577 Moreover, increased sputum inflammatory cells (e.g. neutrophils) have been shown to
578 significantly correlate with air trapping in COPD patients (71, 72). Another study showed
579 that TLR2 was highly expressed in human fetal lungs and may be important in regulating the
580 development of this organ (77). Recent studies also have emerged that highlight the
581 importance and interactions of host microbiome, commensal bacteria, infectious
582 exacerbations and TLRs in shaping and regulating immune responses in COPD and other
583 chronic respiratory diseases (11, 14, 15, 43, 68, 89). Taken together, the increased
584 inflammation and TLC observed in normal air-exposed *Tlr2*$^{-/-}$ mice may be a consequence of
585 altered host immune responses, lung development and/or microbiome composition. It is
586 likely that TLR2 and TLR4 will be important in these interactions and during lung
587 development. However, this is outside the scope of this study and will require further work to
588 delineate the relationships.

589 TLR4 has been implicated in CS-induced pulmonary inflammation (21, 66). In other
590 studies, acute (3 days) CS exposure of *Tlr4*$^{-/-}$ mice on a C57BL/6 background resulted in
591 reductions in total inflammatory cells in BALF and lung tissue (21). Sub-acute (5 weeks)
592 exposure of *Tlr4*^{defective} mice also resulted in reduced numbers of BALF total inflammatory
593 cells compared to WT C3H/HeJ controls (66). However, in support of our observations, the
594 numbers of BALF total inflammatory cells were not significantly different in these
595 *Tlr4*^{defective} mice compared to WT C3H/HeJ controls following chronic CS exposure (26
596 weeks) (66). Collectively, these suggest TLR4 may play only a minor role at the chronic and
597 severe stages of the disease. Whilst CS-exposed *Tlr4*$^{-/-}$ mice had increased pro-inflammatory
598 cytokine TNFα mRNA expression in the lung compared to CS-exposed WT controls, this
599 increase in mRNA expression was in contrast to the significant reduction in TNFα protein
600 levels in CS-exposed *Tlr4*$^{-/-}$ mice. Taken together, these results suggest that there are
601 alterations in the regulation of gene transcriptional ~~regulation~~ and post-translational protein
602 ~~modifications~~production in CS-exposed *Tlr4*$^{-/-}$ mice, which collectively does not affect
603 airway or parenchymal inflammation. The biology of these changes is not understood.

604 We previously showed that parenchymal inflammatory cells and alveolar destruction
605 were further increased in WT mice after 12 weeks of CS compared to those exposed to CS
606 for 8 weeks (7). Hence, increasing CS exposure time in mice (e.g. from 8 to 12 weeks) may
607 lead to further alterations in inflammatory gene expression and inflammation. However,
608 given that TLR2 and TLR4 expression were reduced in lung parenchymal cores from severe
609 (GOLD III/IV) COPD patients (Figure 2), we speculate that increasing CS exposure time to
610 induce more severe disease in our model may have similar effects and promote further loss of
611 tissue/cells expressing TLR2 and/or TLR4. In support of this, a previous human study also
612 found that TLR4 expression was inversely proportional to COPD severity (65). Moreover, we
613 also showed that the expression of pro-inflammatory TNF-α and chemokine CXCL1 were not

increased further with longer exposures, despite ~~an~~ increases~~d~~ in parenchymal inflammatory cells, in WT mice exposed to CS for 12 weeks ~~of CS~~ compared to ~~those exposed for~~ 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~~ed~~ TLR2 and/or TLR4 in the lung. Hence, profiling ~~functional/molecular~~ changes in whole lung tissue may have concealed small but potentially critical functional/molecular changes. T~~In addition, t~~he roles of TLRs (TLR2 or TLR4) on specific cells in the lungs also remains 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

639 associated with increased Nox3 expression in lungs of these mice (104, 109). We also
640 observed an increase in Nox3 mRNA in normal air-exposed *Tlr4*$^{-/-}$ BALB/c mice, however,
641 these mice did not develop spontaneous emphysema even at 15-16 weeks of age. In fact,
642 *Tlr4*$^{-/-}$ mice were protected from CS-induced emphysema-like alveolar enlargement, which
643 was associated with reduced apoptosis in the lungs. These findings were supported by the
644 observation of reduced expression of MMP-12, which has been linked to the induction of
645 emphysema (7, 41), and improved lung function in CS-exposed *Tlr4*$^{-/-}$ mice. Moreover, CS
646 exposure appeared to reduce Nox3 mRNA expression in *Tlr4*$^{-/-}$ BALB/c mice back to levels
647 observed in WT BALB/c mice.

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

660 Our study demonstrates a previously unrecognized protective role for TLR2 in the
661 pathogenesis of COPD. This supports current evidence in the literature that shows a loss-of-
662 function polymorphism in the TLR2, but not TLR4, gene is associated with accelerated lung
663 function declines in COPD patients (12). TLR2 polymorphisms also predispose patients with

664 other chronic lung diseases (e.g. cystic fibrosis) to rapid lung function decline (35). This
665 further highlights the potential protective role of TLR2 in chronic lung diseases, and
666 screening for TLR2 polymorphisms may be useful in the prognosis of COPD patients.
667 Furthermore, using a clinically-relevant and established model of CS-induced COPD, our
668 study demonstrates that TLR4 promotes CS-induced airway fibrosis, apoptosis, emphysema-
669 like alveolar enlargement and lung function impairment. Hence, activating TLR2 and/or
670 inhibiting TLR4 may be potential therapeutic strategies in COPD.

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680

681 # COMPETING INTEREST

682 No conflict of interest, financial or otherwise are declared by the authors.

683

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1091

1092 # FIGURE LEGENDS

1093 **Figure 1. TLR2 and TLR4 mRNA expression and protein levels are increased in CS-**
1094 **induced experimental COPD.** Wild-type (WT) BALB/c mice were exposed to normal air or
1095 CS and sacrificed after 4, 8 and 12 weeks. (**A**) TLR2 and (**B**) TLR4 mRNA expression in
1096 whole lung tissues. (C) Muc5ac and Sftpc mRNA expressions in blunt dissected airways and
1097 parenchyma from in normal air-exposed mice. (**D**-**E**) TLR2 and (**F**-**G**) TLR4 mRNA
1098 expression in blunt dissected airways and parenchyma after 8 weeks CS exposure.
1099 Immunohistochemistry for (**H**-**I**) TLR2 and (**J**-**K**) TLR4 protein on small airway epithelium
1100 and lung parenchyma after 8 weeks of CS exposure; scale bars equal 50µm. Arrowheads
1101 indicate TLR2 or TLR4 expressing inflammatory cells. TLR2 and TLR4 mRNA expression
1102 were normalized to reference gene and expressed as relative abundance compared to normal
1103 air-exposed controls. Graphs were presented as mean ± SEM and representative from two
1104 independent experiments of 6 mice per group. Two-tailed Mann-Whitney t-test analyzed
1105 differences between two groups, whereby *p<0.05; **p<0.01; ***p<0.001 compared to
1106 normal air-exposed controls and #p<0.05; ###p<0.001 compared to bluntly dissected airway.

1107

1108 **Figure 2. TLR2- and/or TLR4-expressing neutrophils and mDCs cells were increased in**
1109 **CS-induced experimental COPD.** Wild-type (WT) BALB/c mice were exposed to cigarette
1110 smoke (CS) or normal air for 8 weeks. (**A**) TLR2 and (**B**) TLR4 mRNA expressions was
1111 determined in macrophage isolated from lungs by qPCR. The number of (**C**) $CD11b^{+}$
1112 monocytes, (**D**) neutrophils and (**E**) myeloid dendritic cells (mDCs) expressing TLR2 and/or
1113 TLR4 in lungs. mRNA expressions were normalized to reference gene and expressed as
1114 relative abundance compared to normal air-exposed WT controls. Graphs were presented as
1115 mean ± SEM and representative from two independent experiments of 6 mice per group.

1116 Two-tailed Mann-Whitney t-test analyzed differences between two groups in Figure A and B.
1117 The one-way analysis of variance with Bonferroni post-test analyzed differences between 3
1118 or more groups for Figure C, D and E, whereby *p<0.05; **p<0.01 compared to normal air-
1119 exposed WT.

1120

1121 **Figure 3. TLR2 and TLR4 co-receptor expression are increased in CS-induced**
1122 **experimental COPD.** Wild-type (WT) BALB/c mice were exposed to normal air or CS for 8
1123 weeks to induce experimental COPD. (**A-B**) TLR1, (**C-D**) TLR6, (**E-F**) CD14 and (**G-H**)
1124 MD2/Ly96 mRNA expressions in blunt dissected airways and parenchyma. mRNA
1125 expressions were normalized to reference gene and expressed as relative abundance
1126 compared to normal air-exposed controls. Graphs were presented as mean ± SEM and
1127 representative from two independent experiments of 6 mice per group. Two-tailed Mann-
1128 Whitney t-test analyzed differences between two groups, whereby *p<0.05; **p<0.01;
1129 ***p<0.001 compared to normal air-exposed controls.

1130

1131 **Figure 4. TLR2, TLR4 and co-receptor mRNA expressions are increased in the airways**
1132 **in humans with mild to moderate COPD.** Airway epithelial cells were collected from
1133 human healthy non-smokers (NS), healthy smokers without COPD (Smoker) and COPD
1134 patients with Global Initiative for Chronic Obstructive Lung Disease (GOLD) stage I (Mild)
1135 or II (Moderate) disease. (**A**) TLR2, (**B**) TLR4, (**C**) TLR1, (**D**) TLR6, (**E**) CD14 and (**F**)
1136 LY96 mRNA expression were assessed by microarray profiling. Lung parenchymal cores
1137 were collected from human healthy non-smokers (NS) and COPD patients with Global
1138 Initiative for Chronic Obstructive Lung Disease (GOLD) stage IV (severe) disease. (**G**)
1139 TLR2, (**H**) TLR4, (**I**) TLR1, (**J**) TLR6 and (**K**) LY96 mRNA expression were assessed by

1140 microarray profiling. Graphs are expressed as log_2 intensity robust multi-array average
1141 signals. The Benjamini–Hochberg method for adjusted P value/false discovery rate (FDR)
1142 analyzed differences between NS or Smoker and COPD patients.

1143

1144 **Figure 5. CS-induced pulmonary inflammation is unaltered in TLR2-deficient and**
1145 **TLR4-deficient mice with experimental COPD.** Wild-type (WT), TLR2-deficient ($Tlr2^{-/-}$)
1146 or TLR4-deficient ($Tlr4^{-/-}$) BALB/c mice were exposed to normal air or CS for 8 weeks to
1147 induce experimental COPD. (**A**) Total leukocytes, (**B**) macrophages, (**C**) neutrophils and (**D**)
1148 lymphocytes were enumerated in May-Grunwald Giemsa stained bronchoalveolar lavage
1149 fluid (BALF) cytospin slides. (**E**) The numbers of parenchymal inflammatory cells
1150 (arrowheads) were enumerated in periodic acid-Schiff (PAS)-stained lung sections; scale bars
1151 equal 20µm. (**G-I**) Histopathology score in lung sections; specifically, in the airway, vascular
1152 and parenchymal regions. **(J)** Representative images of lung histopathology scoring; scale
1153 bars equal 50µm. Graphs were presented as mean ± SEM and representative from two
1154 independent experiments of 6 mice per group. The one-way analysis of variance with
1155 Bonferroni post-test analyzed differences between 3 or more groups, whereby *$p<0.05$;
1156 **$p<0.01$; ***$p<0.001$; ****$p<0.0001$ compared to normal air-exposed WT, $Tlr2^{-/-}$ or $Tlr4^{-/-}$
1157 controls, #$p<0.05$; ##$p<0.01$ compared to CS-exposed WT controls and ϕ$p<0.05$; ϕϕ$p<0.01$;
1158 ϕϕϕ$p<0.001$ compared to normal air-exposed WT controls.

1159

1160 **Figure 6. Pro-inflammatory cytokine, chemokine, COPD-related factors and oxidative**
1161 **stress-associated gene expressions in CS-induced experimental COPD.** Wild-type (WT),
1162 TLR2-deficient ($Tlr2^{-/-}$) or TLR4-deficient ($Tlr4^{-/-}$) BALB/c mice were exposed to normal air
1163 or cigarette smoke (CS) for 8 weeks. Pro-inflammatory cytokine (**A**) tumor necrosis factor

1164 (TNF)-α, (**B**) chemokine (C-X-C motif) ligand (CXCL)1, (**C**) chemokine (C-C motif) ligand
1165 (CCL)2, (**D**) CCL3, (**E**) CCL4, (**F**) CCL12, (**G**) CCL22, other COPD-related factors; (**H**)
1166 matrix metalloproteinase (MMP)-12 and (**I**) serum amyloid A3 (SAA3) mRNA expression
1167 was determined in whole lung homogenates by qPCR. Oxidative stress-associated genes (**J**)
1168 NADPH oxidase (Nox)1, (**K**) Nox2, (**L**) Nox3, (**M**) Nox4, (**N**) NAD(P)H quinone
1169 dehydrogenase (Nqo)1, (**O**) nuclear factor, erythroid 2 like 2 (Nrf2), (**P**) glutamate-cysteine
1170 ligase catalytic subunit (Gclc), (**Q**) glutathione peroxidase (Gpx)2, (**R**) heme oxygenase
1171 (Hmox)1 and (**S**) glutathione S-transferase pi (Gstp)1 expression was determined in whole
1172 lung homogenates by qPCR. mRNA expression was normalized to the reference gene HPRT
1173 and expressed as relative abundance compared to normal air-exposed WT controls. (T) The
1174 levels of TNFα protein in lung homogenates were determined by ELISA and normalized to
1175 total protein. Graphs were presented as mean ± SEM and representative from two
1176 independent experiments of 6 mice per group. The one-way analysis of variance with
1177 Bonferroni post-test was used to analyze differences between 3 or more groups, whereby
1178 *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001 compared to normal air-exposed WT, *Tlr2*$^{-/-}$
1179 or *Tlr4*$^{-/-}$ controls, #p<0.05; ##p<0.01; ###p<0.001; ####p<0.0001 compared to CS-exposed
1180 WT controls, ϕp<0.05; ϕϕϕϕp<0.0001 compared WT air controls and *ns* denotes not
1181 significant.

1182

1183 **Figure 7. CS-induced airway remodeling and fibrosis is not altered in TLR2-deficient**
1184 **mice whilst CS-induced airway fibrosis, but not remodeling, is completely ablated in**
1185 **TLR4-deficient mice with experimental COPD.** Wild-type (WT), TLR2-deficient (*Tlr2*$^{-/-}$)
1186 or TLR4-deficient (*Tlr4*$^{-/-}$) BALB/c mice were exposed to normal air or CS for 8 weeks to
1187 induce experimental COPD. (**A**) Small airway epithelial thickness in terms of epithelial cell
1188 area (μm^2) per basement membrane (BM) perimeter (μm) was determined in periodic acid-

1189 Schiff (PAS)-stained whole lung sections; scale bars equal 50μm. (**B**) The number of
1190 epithelial cells in PAS-stained lung sections was assessed by enumerating the number of
1191 nuclei per 100μm of BM perimeter; scale bars equal 20μm. (**C**) Area of collagen deposition
1192 (μm^2) per BM perimeter (μm) was determined in Masson's Trichrome-stained lung sections;
1193 scale bars equal 200μm. Inserts show expanded images of indicated regions; scale bars equal
1194 200μm). (**D**) The level of hyaluronan in BALF was determined by ELISA. The mRNA
1195 expression of (**E**) fibronectin and (**F**) interleukin (IL)-33 were determined in whole lung
1196 homogenates by qPCR. mRNA expression was normalized to the reference gene HPRT and
1197 expressed as relative abundance compared to normal air-exposed WT controls. Graphs were
1198 presented as mean ± SEM and representative from two independent experiments of 6 mice
1199 per group. The one-way analysis of variance with Bonferroni post-test analyzed differences
1200 between 3 or more groups, whereby *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001
1201 compared to normal air-exposed WT, *Tlr2*$^{-/-}$ or *Tlr4*$^{-/-}$ controls, #p<0.05; ## p<0.01;
1202 ####p<0.0001 compared to CS-exposed WT controls, ϕp<0.05; ϕϕϕϕp<0.0001 compared to
1203 normal air-exposed WT controls and *ns* denotes not significant.

1204

1205 **Figure 8. CS-induced emphysema-like alveolar enlargement, apoptosis and impaired**
1206 **lung function are increased in TLR2-deficient mice and decreased in TLR4-deficient**
1207 **mice with experimental COPD.** Wild-type (WT), TLR2-deficient (*Tlr2*$^{-/-}$) or TLR4-
1208 deficient (*Tlr4*$^{-/-}$) BALB/c mice were exposed to normal air or CS for 8 weeks to induce
1209 experimental COPD. (**A**) Alveolar septal damage and (**B**) diameter (μm) were determined in
1210 H&E-stained lung sections using destructive index and mean linear intercept techniques
1211 respectively; scale bars equal 50μm. (**C**) The numbers of TUNEL$^+$ cells (arrowheads) were
1212 enumerated in whole lung sections at high power field (HPF; 100x); scale bars equal 20μm.
1213 Immunohistochemistry for (D) active cCaspase--3 protein on lung parenchyma after 8 weeks

of CS exposure; scale bars equal 50µm. Arrowheads indicate cCaspase--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.

1223 **Table 1.** Custom-designed primers used in qPCR analysis

Primer	Primer sequence (5' → 3')
TLR2 forward	TGTAGGGGCTTCACTTCTCTGCTT
TLR2 reverse	AGACTCCTGAGCAGAACAGCGTTT
TLR4 forward	TGGTTGCAGAAAATGCCAGG
TLR4 reverse	GGAACTACCTCTATGCAGGGAT
TNF-α forward	TCTGTCTACTGAACTTCGGGGTGA
Muc5ac forward	GCAGTTGTGTCACCATCATCTGTG
Muc5ac reverse	GGGGCAGTCTTGACTAACCCTCTT
Sftpc forward	TGTATGACTACCAGCGGCTC
Sftpc reverse	AGCGAAAGCCTCAAGACTAGG
TNF-α reverse	TTGTCTTTGAGATCCATGCCGTT
CXCL1 forward	GCTGGGATTCACCTCAAGAA
CXCL1 reverse	CTTGGGGACACCTTTTAGCA
CCL2 forward	TGAGTAGCAGCAGGTGAGTGGGG
CCL2 reverse	TGTTCACAGTTGCCGGCTGGAG
CCL3 forward	CTCCCAGCCAGGTGTCATTTT
CCL3 reverse	CTTGGACCCAGGTCTCTTTGG
CCL4 forward	GTGGCTGCCTTCTGTGCTCCA
CCL4 reverse	AGCTGCCGGGAGGTGTAAGAGAA
CCL12 forward	CCGGGAGCTGTGATCTTCA
CCL12 reverse	AACCCACTTCTCGGGGT
CCL22 forward	TGGCTACCCTGCGTCGTGTCCCA
CCL22 reverse	CGTGATGGCAGAGGGTGACGG
MMP-12 forward	CCTCGATGTGGAGTGCCCGA
MMP-12 reverse	CCTCACGCTTCATGTCCGGAG
SAA3 forward	TGATCCTGGGAGTTGACAGCCAA
SAA3 reverse	ACCCCTCCGGGCAGCATCATA
Nox1 forward	CCCCTGAGTCTTGGAAGTGG
Nox1 reverse	GGGTGCATGACAACCTTGGTA
Nox2 forward	AACTGGGCTGTGAATGAAGG
Nox2 reverse	CAGCAGGATCAGCATACAGTTG

Nox3 forward	CTCGTTGCCTACGGGATAGC
Nox3 reverse	CCTTCAGCATCCTTGGCCT
Nox4 forward	ACAACCAAGGGCCAGAATACTACTAC
Nox4 reverse	GGATGAGGCTGCAGTTGAGG
Nqo1 forward	GTAGCGGCTCCATGTACTCTC
Nqo1 reverse	AGGATGCCACTCTGAATCGG
Nrf2 forward	CTTTAGTCAGCGACAGAAGGAC
Nrf2 reverse	AGGCATCTTGTTTGGGAATGTG
Gclc foward	CGACCAATGGAGGTGCAGTTA
Gclc reverse	AACCTTGGACAGCGGAATGA
Gpx2 forward	ACCAGTTCGGACATCAGGAG
Gpx2 reverse	CCCAGGTCGGACATACTTGA
Hmox1 forward	GGTGCAAGATACTGCCCCTG
Hmox1 reverse	TGAGGACCCACTGGAGGAG
Gstp1 forward	GGCATGCCACCATACACCAT
Gstp1 reverse	ATTCGCATGGCCTCACACC
Fibronectin forward	TGTGGTTGCCTTGCACGAT
Fibronectin reverse	GCTATCCACTGGGCAGTAAAGC
IL-33 forward	CCTCCCTGAGTACATACAATGACC
IL-33 reverse	GTAGTAGCACCTGGTCTTGCTCTT
HPRT forward	AGGCCAGACTTTGTTGGATTTGAA
HPRT reverse	CAACTTGCGCTCATCTTAGGATTT

1224

1225

1226 **Table 2.** Surface antigens used to characterize mouse lung cell subsets by flow cytometry

Cell subset	**Cell surface antigens**
$CD11b^{+}$ monocyte	$CD45^{+}$ $F4/80^{+}$ $CD11c^{-}$ $CD11b^{+}$ $Ly6C^{+}$$TLR2^{+/-}$ $TLR4^{+/-}$
Neutrophil	$CD45^{+}$ $F4/80^{-}$ $CD11b^{+}$$Ly6G^{+}$$TLR2^{+/-}$ $TLR4^{+/-}$
Myeloid dendritic cell	$CD45^{+}$ $F4/80^{-}$ $CD11c^{+}$ $CD11b^{+}$ $MHCII^{+}$$TLR2^{+/-}$ $TLR4^{+/-}$

1227

1228

1229 **Table 3.** Antibodies used in flow cytometry analysis

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

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