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NLRP6 controls pulmonary inflammation to cigarette smoke in a gut microbiota dependent manner

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Running Title: NLRP6 in response to lung injury

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31

32 **Abstract**

33 Chronic obstructive pulmonary disease (COPD) is a major health issue primarily caused by
34 cigarette smoke (CS) and characterized by breathlessness and repeated airway inflammation.
35 NLRP6 is a cytosolic innate receptor controlling intestinal inflammation and orchestrating
36 colonic host-microbial interface. However, its roles in the lungs remain largely unexplored. Using
37 CS exposure models, our data show that airway inflammation is strongly impaired in Nlrp6-
38 deficient mice with drastically less recruited neutrophils, a key cell subset in inflammation and
39 COPD. We found that NLRP6 expression in lung epithelial cells is important to control airway
40 and lung tissue inflammation in an inflammasome-dependent manner. Since gut-derived
41 metabolites regulate NLRP6 inflammasome activation in intestinal epithelial cells, we
42 investigated the link between NLRP6, CS-driven lung inflammation and gut microbiota
43 composition. We report that acute CS exposure alters gut microbiota in both wild type (WT) and
44 Nlrp6-deficient mice and that antibiotic treatment decreases CS-induced lung inflammation. In
45 addition, gut microbiota transfer from dysbiotic Nlrp6-deficient to WT mice decreases airway
46 lung inflammation highlighting an NLRP6-dependent gut to lung axis controlling pulmonary
47 inflammation.

48

49 **Keywords:**

50 NLRP6, Lung inflammation, Cigarette smoke-exposure, Gut microbiota, Gut to lung axis

51

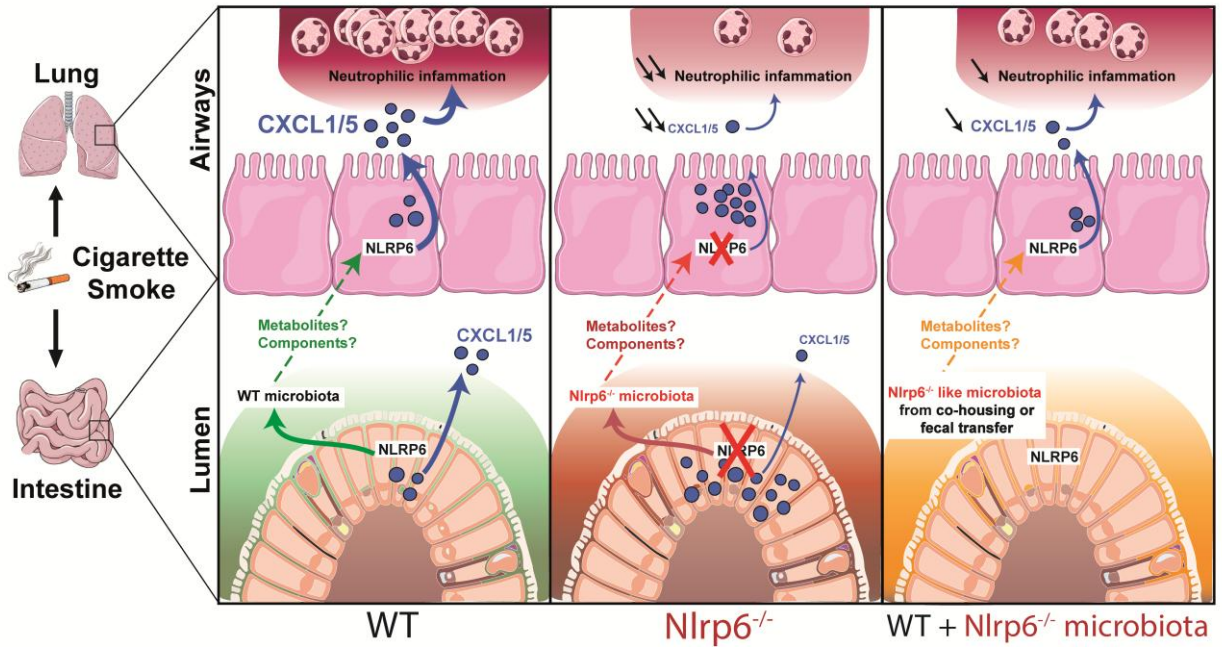
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56 **Graphical abstract**



57

58

59 **Highlights**

- 60 • NLRP6 controls lung inflammation to cigarette smoke through CXCL1/CXCL5-mediated
- 61 neutrophilic influx
- 62 • NLRP6-mediated lung inflammation is dependent on the inflammasome
- 63 • NLRP6 expression in airway epithelial cells controls lung inflammation to cigarette
- 64 smoke
- 65 • Cigarette smoke exposure alters gut microbiota

- 66 • Impaired lung inflammation in cigarette smoke-exposed Nlrp6-deficient mice is
67 transferable to wild type mice by cohousing experiments or fecal microbiota
68 transplantation
- 69 • Gut microbiota from Nlrp6-deficient mice limits neutrophilic lung inflammation

70
71

72 **Introduction**

73 Chronic obstructive pulmonary disease (COPD) is a severe inflammatory disease characterized
74 by airway obstruction due to bronchial immune cell recruitment and mucus secretion and
75 resulting in impaired lung functions. In a fraction of patients, chronic bronchial inflammation
76 evolve to alveolar disease with alveolar wall destruction, leading to enlargement of airway, that
77 causes shortness of breath and called emphysema [1]. COPD is a major health issue affecting 4 to
78 10% of the worldwide population and is the third cause of death in the world and 600 million
79 people will suffer from COPD in 2050 if nothing changes [2]. Cigarette smoking is a major
80 societal cause of COPD, counting for more than 95% of cases in industrialized countries [3; 4].
81 Inflammation can persist even when smoking cessation. Current therapeutics target symptoms,
82 attempting to alleviate chronic inflammation and prevent infection-driven exacerbations however
83 there is no disease-modifying treatment available [5]. Chronic inflammation is characterized by
84 recruitment and activation of innate immune cells in particular neutrophils and macrophages as well
85 as adaptive immune cells such as lymphocytes that produce inflammatory and tissue damaging
86 mediators [6; 7; 8]. Inflammatory neutrophils display increased degranulation and reactive oxygen
87 species release and have been associated with COPD severity [9]. Neutrophils targeting to lung
88 tissue through ELR⁺ CXC chemokines (i.e. containing a tripeptide motif glutamic acid-leucine-

89 arginine and one amino acid between the first two cysteines), essential for both neutrophil influx
90 and activation [10] through signaling on the transmembrane receptors CXCR1 and CXCR2. In
91 particular, CXCL5/LIX (LPS-induced CXC chemokine) is mainly produced by epithelial cells
92 and promote airway neutrophil inflammation [11; 12; 13; 14; 15]. In contrast, alveolar
93 macrophages secrete CXCL1/KC and CXCL2/MIP-2 but not CXCL5/LIX [11]. The important
94 contributions of epithelial cells-derived chemokines to immunity were recently highlighted [16;
95 17].

96 Using mouse models of cigarette smoke (CS) exposure and elastase-induced injury, we
97 previously showed that interleukin (IL-) 1 β and the adaptor molecule apoptosis-associated speck-
98 like protein containing a CARD (ASC) are essential to promote inflammation, remodeling and
99 emphysema suggesting that inflammasome pathways are involved in the establishment of COPD
100 [18; 19]. Inflammasomes are cytoplasmic multiprotein complexes orchestrating diverse functions
101 during homeostasis and inflammation [20; 21; 22]. These complexes are generally composed of a
102 Nod-like receptor (NLR), ASC adaptor molecule and caspase-1 (Casp-1) or caspase-11 (Casp-11)
103 proteases which activation leads to pro-IL-1 β and pro-IL-18 maturation into biologically active
104 IL-1 β and IL-18 pro-inflammatory cytokines [20; 21; 22].

105 NOD-like receptor family pyrin domain containing (NLRP)6 inflammasome composed of
106 NLRP6, ASC and Casp-1 has been shown to regulate microbiota homeostasis and antibacterial
107 immunity in the intestine [23; 24; 25]. NLRP6 inflammasome orchestrates goblet cell mucin
108 granule exocytosis, as mucus accumulation and impaired secretion were observed in Nlrp6-
109 deficient goblet cells [25]. Moreover, recently identified intestinal sentinel goblet cells were
110 shown to non-specifically recognize bacterial compounds leading to NLRP6 inflammasome
111 activation and subsequent calcium-dependent mucin exocytosis from sentinel and adjacent goblet

112 cells [26]. In addition, NLRP6 expression in inflammatory monocytes reduces susceptibility to
113 chemically-induced intestinal injury [27]. An inflammasome-independent NLRP6 role in the
114 control of enteric virus infection was reported, where NLRP6 interacts with an RNA sensor
115 triggering type I and type III interferon-mediated antiviral responses [28]. In contrast to the gut,
116 NLRP6 role in lung inflammation and repair has been poorly evaluated. A few recent studies
117 showed that this multifaceted innate immune sensor controls neutrophil recruitment and function
118 during pulmonary gram-positive and gram-negative bacterial infections [29; 30; 31].
119 Here, we demonstrate for the first time that NLRP6 inflammasome governs lung inflammation to
120 CS by controlling neutrophilic inflammation. Impaired lung inflammation in CS-exposed *Nlrp6*-
121 deficient mice is transferable to WT mice by cohousing experiments or fecal microbiota
122 transplantation. Mechanistically, we show that gut microbiota from *Nlrp6*-deficient mice
123 regulates lung CXCL5 production and neutrophil influx. Our data also indicate that CS exposure
124 modulates gut microbiota composition in both WT and *Nlrp6*-deficient mice. In conclusion, we
125 report that smoking induces an unexpected gut to lung axis modulating pulmonary inflammation,
126 which depends on NLRP6 expression in lung and intestinal epithelial cells.

127

128 **Methods**

129 **Mice**

130 Wild type C57BL/6J (WT) mice were purchased from Janvier Labs or bred in our animal facility
131 for microbiota experiments together with gene deficient or conditional mouse strains. *Asc*^{-/-} mice
132 were given by Francis Derouet at Lausanne University [32], *Casp-1/11*^{-/-} by Seshadri Tara at
133 BASF Bioresearch corporation [33] and *Nlrp6*^{-/-} by Mathias Chamaillard at Lille Pasteur Institute
134 [24]. *Nlrp6* flox/flox mice are from Philip Rosenstiel Institute of clinical Molecular Biology,

135 Kiel, Germany. Nlrp6 tissue specific knockouts were obtained by breeding Nlrp6 flox/flox with
136 Aqp5Cre knock-in (Acid or Aqp5tm1.1(cre,DsRed)Pfl), allowing deletion in alveolar type I
137 (AT1) cells in adult lung [34]. All mouse strains were backcrossed 10 times or made on C57Bl/6J
138 background and housed at the animal facility at Transgenose Institute (UPS-TAAM) in CNRS
139 Orleans, France. Except for cohousing experiments, 6-10 weeks old mice were kept in sterile,
140 isolated and ventilated cages. All animal experiments followed the French government's ethical
141 and animal experiment regulations (CLE CCO 2015-1088).

142 **Cigarette smoke exposition**

143 3R4F cigarettes (University of Kentucky) were used without filter. Mice were placed in an
144 InExpose smoke chamber (EMKA Technologies) and inhaled smoke from 4 cigarettes, 3 times a
145 day for 4 days for acute inflammation model or 4 cigarettes, 3 times a day, 5 days a week for 6
146 weeks for subchronic inflammation model. Bronchoalveolar lavage (BAL) and lung tissue were
147 collected about 16 hours after the last CS exposure.

148

149 **Broncho-alveolar lavage (BAL)**

150 BAL was performed as previously described [19]. Differential cell counts were performed by
151 counting an average of 250 cells on Cytospin preparations (Shandon CytoSpin 3, ThermoFisher
152 Scientific) after May-Grünwald-Giemsa staining (RAL-Diff Quick, Siemens) according to
153 manufacturer's instructions.

154

155 **Tissue homogenates**

156 Lungs were perfused with Isoton (Beckman Coulter) to flush the vascular content. Washed lungs
157 and ileum were homogenized by a rotor-stator (Ultra-turrax) in PBS with protease inhibitor
158 cocktail (Roche) for mediator measurement or in RIPA buffer with protease inhibitor cocktail

159 (ThermoFisher Scientific) for immunoblotting analysis. Extracts were centrifuged and
160 supernatants stored at -80°C.

161

162 **Lung histology**

163 Lung left lobe was fixed in 4% buffered formaldehyde (MMFrance), processed and paraffin
164 embedded under standard conditions. 3µm lung sections were stained with Direct Red 80 (Red
165 Sirius, Sigma-Aldrich). The slides were blindly examined at 10X magnification (Leica) and cell
166 infiltration assessed by a semi-quantitative score (with increasing severity 0-5) by two
167 independent investigators.

168

169 **Mediator measurements**

170 BALF supernatants and lung homogenates mediators were measured by ELISA assay kits for
171 murine MPO, IL-1β, CXCL1, CXCL5, CXCL15, BAFF, LCN2, MMP-9 and TIMP-1 (R&D
172 System) according to manufacturer's instructions.

173 **Air Liquide Interface (ALI) Trachea epithelial cells culture**

174 Mice trachea were collected from Wild type C57BL/6J (WT). Using pronase (1,5mg/mL)
175 (Roche®), tracheas were digested over night at 4°C. Digestion was stopped using MTEC/Basic
176 medium (Composition Table X) + 10% fetal bovine serum (FBS). DNAase (0,5mg/mL) (Sigma-
177 Aldrich®) step was realized in MTEC/Basic medium without FBS. Adherent cells were removed
178 by plating samples on specific petri dish (Primaria Corning). $33 \cdot 10^3$ of trachea epithelial cells
179 were plated in 150 µL of MTEC/Plus medium (Composition Table X) on rat-tail collagen
180 (0,05mg/mL diluted in acetic acid 20mM) (Thermofisher®) pre-coated transwells. Then,
181 MTEC/Plus medium was added in down transwell part. Trachea epithelial cell grown 4 days at
182 37°C in 5% of CO₂.

183 At day 4, medium was changed with MTEC/Plus medium until cell confluence. Then, trachea
 184 epithelial cells up medium was removed and cells were washed with Phosphate Buffered Saline
 185 (PBS). Down medium was changed every day during 1 week with MTEC/SF differentiation
 186 medium (Composition Table 1). Trachea epithelial cell in ALI grown at 37°C in 5% of CO₂ until
 187 cell collection or stimulation.

188

189

190

MTEC/Basic	Concentration
Hepes	15,000 mM
Pen/strep	1 X
MTEC/Plus	Concentration
Insulin Transferrin cocktail 1X (Thermofisher®)	10 µg/mL
Cholera toxin (Sigma Aldrich®)	0,1 µg/mL
Epidermal Growth Factor (EGF) (Sigma Aldrich®)	0,025 µg/mL
Bovin Pituitary Extract (Fisher Gibco®)	30 µg/mL
FBS	5%
Retinoic Acid B (RA-B) (Sgma Aldrich®)	0,05 µM
MTEC/NS	Concentration
NuSerum	2%
Retinoic Acid B (RA-B) (Sgma Aldrich®)	0,05µM
MTEC/SF	Concentration
Insulin Transferrin cocktail 1X (Thermofisher®)	5 µg/mL
Cholera toxin (Sigma Aldrich®)	0,025 µg/mL
Epidermal Growth Factor (EGF) (Sigma Aldrich®)	5ng/mL
Bovin Pituitary Extract (Fisher Gibco®)	30µg/mL
Commercial BSA solution (steril) (Sigma Aldrich®)	1mg/mL
Retinoic Acid B (RA-B) (Sgma Aldrich®)	0,05µM

191

192 **Table 1:** Trachea epithelial cells culture medium compositions

193

194 **Specific *Nlrp6* RT-PCR on trachea epithelial cells**

195 Total RNA was extracted from epithelial cells using isolation kit (RNeasy kit[®]) following the
 196 manufacturer's protocol. RNA concentration and integrity were determined using a Nanodrop
 197 (Nd-1000) spectrophotometer (Labtech[®]). Reverse transcription of RNA into cDNA was carried
 198 out with GoScript[™] Reverse Transcription System (Promega[®]). Using iScript RT supermix
 199 (BioRad[®]) 500ng of total RNA was subjected to Reverse Transcription. PCR was performed on
 200 8.75µg of synthesized cDNA. Specific primers used for *Nlrp6* mRNA amplification were as
 201 follows: *Nlrp6* forward 5'-GAC-CAG-TTT-AGC-CCA-GAA-AAG-G-3'; *Nlrp6* reverse 5'-CTC-
 202 CAG-TGT-AGC-CAT-AAG-CAG- 3'. PCR program is described in table 2.

203

Initialisation	94°C, 2min			
	5 Cycles	5 Cycles	5 Cycles	5 Cycles
Denaturation	94°C, 30s	94°C, 30s	94°C, 30s	94°C, 30s
Annealing	62°C, 30s	61°C, 30s	59°C, 30s	58°C, 30s
Elongation	72°C, 1min	72°C, 1min	72°C, 1min	72°C, 1min

204 **Table 2: PCR used program.**

208

209 **FLAG Immunohistochemistry**

210 Lungs were fixed with 4% paraformaldehyde (Sigma-Aldrich[®]) for 72h, embedded in paraffin
 211 and sectioned at 3 µm. Lung sections were dewaxed and rehydrated, then heated 20 min at 80°C
 212 in citrate buffer 10 mM pH=6 for antigen retrieval (unmasking step). Lung sections were
 213 permeabilized in PBS 0.5% triton X-100, blocked with 5% FCS for 1h at RT and then incubated
 214 overnight with primary mouse anti-FLAG (1:100, A9542 Sigma). After washing, sections were
 215 incubated with with the appropriate second antibody conjugated to horseradish peroxidase (1:200
 216 anti-mouse IgG, Sigma Aldrich[®]) in 1% FCS 1h at RT. Following washing, lung sections were
 217 incubated with HRP Substrate, DAB (Vector Laboratories[®]), following the manufacturer's
 218 protocol. After distilled water washing, Gill hematoxyline counterstaining on lung sections was

219 done. Then, lung sections were dehydrated, fixed and mounted onto microscope slides (Eukitt).
220 Slides were examined by using a scanner NDP view.

221
222 **Treatments**
223 Mice were anesthetized by intra-muscular injection of ketamine (10mg/mL, Merial) and xylazine
224 (0.2%, Bayer). Recombinant murine CXCL5/LIX (9-78 amino acid, 1µg per mouse) was
225 administered intranasally at day 2 and 4 between the second and the third daily exposition. Anti-
226 CXCL5/LIX antibody was obtained from Jacques Van Snick. 150µg per mouse was injected
227 intraperitoneally at day 2 and 4 between the second and the third daily expositions. Antibiotic
228 cocktail containing Vancomycin 0.5g/L (Sandoz), Ampicillin 1g/L (Euromedex), Neomycin 1g/L
229 (Euromedex), Metronidazole 1g/L and sucrose 1% (Sigma-Aldrich) diluted in sterile water was
230 given in drinking water and replaced every 3 days.

231
232 **Generation of bone marrow chimeras**
233 CD45.1 WT and CD45.2 Nlrp6^{-/-} recipient mice were lethally irradiated [35] and 4x10⁶ NLRP6-
234 deficient or WT bone marrow cells were injected into the lateral tail vein 24h after. Four chimeric
235 mouse groups were obtained: WT>WT, NLRP6^{-/-}>WT, WT>NLRP6^{-/-}, NLRP6^{-/-}>NLRP6^{-/-}.
236 Bone marrow reconstitution was controlled by flow cytometry assessing the ratio of CD45.1
237 versus CD45.2 blood immune cells. Three months after bone marrow reconstitution, mice were
238 exposed to air or CS as described.

239
240 **Cohousing experiments**
241 Immediately after weaning, WT and Nlrp6^{-/-} gender-matched animals were co-housed at 1:1 ratio
242 for 12 weeks and mice were exposed to air or CS as described.

243

244 **Fecal transplantation**

245 Fecal transplantation was performed as previously described [35]. Briefly, 3-week old germ-free
246 C57BL6/J (WT) mice generated in-house in sterile isolators (TAAM-CNRS) were orally
247 transplanted with 200µl fecal homogenates from SPF-WT or SPF-Nlrp6^{-/-} mice. Mice were
248 maintained in isolator until 7 weeks of age, placed in conventional breeding facility for one week
249 for adaptation and exposed to CS for 4 days.

250

251 **Quantitative RT-PCR**

252 Total RNA was isolated from homogenized mouse lung using Tri Reagent (Sigma-Aldrich) and
253 quantified by NanoDrop (Nd-1000). Reverse transcription of RNA into cDNA was carried out
254 with GoScript™ Reverse Transcription System (Promega). RT-qPCR was performed with Fast
255 GoTaq qPCR Master Mix (Promega) on ARIA MX (Agilent Technologies). *Mmp12* primers
256 were purchased from Qiagen. RNA expression was normalized to *Gapdh* (Qiagen) expression
257 and analysed using the $\Delta\Delta$ Ct method.

258

259 **Immunoblotting**

260 Protein concentrations in tissue homogenates were determined using Pierce BCA protein assay
261 (ThermoFisher Scientific). 40 µg of proteins were denatured by boiling (95°C, 5 min) in reducing
262 SDS sample buffer, separated by SDS-PAGE and transferred to nitrocellulose membranes (GE
263 Healthcare). Membranes were blocked 2 hours in 5% Blotting-Grade Blocker (BioRad), washed
264 three times in Tris-Buffered saline (TBS)- 0,1% Tween 20 and incubated with primary rabbit
265 anti-murine LIX antibody (Peprotech) overnight at 4°C. Membranes were washed three times in
266 TBS- 0,1% Tween 20 and incubated with the appropriate secondary antibody conjugated to

267 horseradish peroxidase (HRP) 1 hour at room temperature (RT). Membranes were incubated with
268 mouse anti-actin HRP-conjugate (Sigma-Aldrich) in 5% Blotting-Grade Blocker in TBS-0,1%
269 Tween 20 for 1 hour at RT. Detection was performed with ECL Prime Western-blotting
270 Detection Reagent (GE Healthcare) and Multi-application gel imaging system PXi software
271 (Syngene).

272

273 **Immunostaining**

274 Lungs and ileum were fixed in 4% paraformaldehyde (PFA) (Sigma-Aldrich) and then
275 dehydrated in 30% sucrose (Sigma-Aldrich) solution for 2 weeks. Organs were embedded in
276 Tissue-Tek® OCT™ (Sakura) and store at -80°C prior to be sliced. Sections were incubated for
277 30 minutes in pre-heated antigen retrieval buffer (Citrate buffer 10mM pH=6). Sections were
278 incubated in TBS-Triton X-100 0.1% and then in blocking solution containing 1% bovine serum
279 albumin (BSA) -10% fetal bovine serum (FBS) -0.1% Triton X-100 in TBS. Primary antibody
280 directed against CXCL5 (Peprotech) was incubated in blocking solution over night at 4°C.
281 Sections were rinsed three times in TBS and incubated with appropriated secondary antibody.
282 Slides were counterstain using 4',6-diamidino-2-phenylindole (DAPI) for 10 minutes, rinsed and
283 coverslip were mount with Fluoromount-G medium (SouthernBiotech). Images were treated
284 using ImageJ software.

285

286 **Stool collection and DNA extraction**

287 Feces were collected and immediately frozen at -80°C for further analysis. DNA was extracted
288 from the fecal samples as described [36]. Following microbial lysis by both mechanical and
289 chemical methods, nucleic acids were precipitated in isopropanol for 10 minutes at room
290 temperature, incubated for 15 minutes on ice and centrifuged for 30 minutes at 20,000 g at 4°C.

291 Pellets were resuspended in 450 μ L of phosphate buffer and 50 μ L of potassium acetate. After
292 RNase treatment and DNA precipitation, nucleic acids were recovered via centrifugation at
293 20,000 g at 4°C for 30 minutes. The DNA pellet was resuspended in trypsin-EDTA buffer. DNA
294 samples were then subjected to 16S sequencing.

295

296 **16S rRNA sequencing**

297 Bacterial diversity in stools was determined by targeting a portion of the ribosomal genes in
298 extracted DNA. A 16S rRNA gene fragment comprising the V3 and V4 hypervariable regions
299 (16S sense 5'-TACGGRAGGCAGCAG-3' and antisense 5'-CTACCNGGGTATCTAAT-3') was
300 amplified using an optimized and standardized 16S-amplicon-library preparation protocol
301 (Metabio, GenoScreen, Lille, France). Briefly, 16S DNA PCR was performed using 5 ng of
302 genomic DNA according to the manufacturer's protocol (Metabio), 192 bar-coded primers
303 (Metabio MiSeq Primers) at final concentrations of 0.2 μ mol/L and an annealing temperature of
304 50°C for 30 cycles. The PCR products were purified using an Agencourt AMPure XP-PCR
305 purification system (Beckman Coulter, Brea, CA, USA), quantified according to the
306 manufacturer's protocol, and multiplexed at equal concentrations. Sequencing was performed
307 using a 300-bp paired-end sequencing protocol on an Illumina MiSeq platform (Illumina, San
308 Diego, CA, USA) at GenoScreen, Lille, France. Raw paired-end reads were subjected to the
309 following processes: (1) quality-filtering using the PRINSEQ-lite PERL script (Schmieder R,
310 Edwards R. Quality control and preprocessing of metagenomic datasets. *Bioinformatics*
311 2011;27:863-4), by truncating the bases from the 3' end, that did not exhibit a quality <30, based
312 on the Phred algorithm; (2) paired-end read assembly using fast length adjustment of short reads
313 to improve genome assemblies (FLASH) (Magoc T, Salzberg SL. FLASH: fast length adjustment

314 of short reads to improve genome assemblies. Bioinformatics 2011;27:2957-63.) with a minimum
315 overlap of 30 bases and a 97% overlap identity; and (3) searching for and removing both forward
316 and reverse primer sequences using CutAdapt, with no mismatches allowed in the primer
317 sequences. Assembled sequences, for which perfect forward and reverse primers were not found,
318 were eliminated.

319

320 **16S rRNA sequence analyses**

321 The sequences were demultiplexed and quality filtered using the QIIME version 1.9.1 software
322 package [37]. The sequences were then assigned to operational taxonomic units (OTUs) using the
323 UCLUST algorithm with a 97% pairwise identity threshold and classified taxonomically using
324 the Greengenes reference database (version 13.5). Rarefaction was performed (32,000 sequences
325 per sample) and used to compare the relative abundance of OTUs across samples. Beta diversity
326 was measured by a Bray Curtis distance matrix and was used to build principal coordinate
327 analysis (PCoA) plots. Raw sequence data are accessible in the European Nucleotide Archive.

328

329 **Statistical analysis.**

330 Statistical evaluation of differences between experimental groups was determined by one-way
331 ANOVA, analysis of variance, Bonferroni test for *in vivo* experiments and one-way ANOVA
332 using Prism software (La Jolla, CA, USA). *P* values <0.05 were considered statistically
333 significant. All tests were performed with Graphpad Prism, Version 8 for Windows (GraphPad
334 Software Inc.). Data are expressed as mean \pm SEM. Statistically significant differences were
335 defined as follow: **P*<0.05, ***P*<0.01, ****P*<0.001 and*****P*<0.0001).

336

337

338 **Results**

339 **Nlrp6 deficiency dampens lung inflammation and remodeling to CS exposure**

340 Since NLRP6 was shown to regulate intestinal epithelium homeostasis and immunity [24; 25;
341 28], we asked whether it could control pulmonary epithelium function and airway inflammation
342 following cigarette smoke (CS) exposure. Wild type (WT) and Nlrp6-deficient (Nlrp6^{-/-}) mice
343 were subchronically exposed to CS three times a day, five days a week for six weeks. As
344 compared to unexposed (Air) mice, subchronically-exposed WT mice (CS) display elevated
345 numbers of bronchoalveolar fluid (BALF) cells (Fig. 1A), mainly macrophages (Fig. 1B),
346 neutrophils (Fig. 1C) and lymphocytes (Fig. 1D), which were all strongly attenuated in CS-
347 exposed Nlrp6^{-/-} mice. Among immune cells, neutrophils play a major role in response to CS [38;
348 39]. As a marker of neutrophil recruitment, myeloperoxidase (MPO) was significantly reduced in
349 the BALF and the lungs (Fig. 1E and F) of CS-exposed Nlrp6^{-/-} mice. In comparison, neutrophilic
350 chemokine CXCL1 production was significantly decreased in the BALF of Nlrp6^{-/-} mice (Fig.
351 1G) but not in lung homogenates (Fig. 1H). Since NLRP6 was shown to regulate IL-1 β and IL-18
352 production through inflammasome activation, we analyzed IL-1 β lung expression and show that
353 its CS-mediated induction was significantly attenuated in Nlrp6^{-/-} mice (Fig. 1I). In addition, as a
354 correlate of B lymphocyte recruitment and disease severity [40], B cell activating factor (BAFF)
355 induction following CS-exposure was significantly reduced in the BALF of Nlrp6^{-/-} as compared
356 to WT mice (Fig.1J).

357 We next measured proteases involved in COPD establishment and in particular matrix
358 metalloproteinase (MMP) and their inhibitors (TIMP). MMP-9 protein levels in BALF and lungs
359 (Fig. 1K and L), *Mmp-12* mRNA expression in lungs (Fig. 1M) and tissue inhibitor of MMP
360 (TIMP)-1 protein levels in BALF and lungs (Fig. 1N and O) were significantly decreased in

361 subchronically CS-exposed *Nlrp6*^{-/-} mice as compared to WT mice. Lung microsections and
362 histology analysis showed reduced inflammation in *Nlrp6*^{-/-} mice in comparison to WT mice (Fig.
363 1P). In addition, acute CS exposure during 4 days confirmed reduced inflammation in *Nlrp6*^{-/-} in
364 comparison to WT mice. While total BAL cells numbers were not significantly changed (Fig.
365 S1A), we report a strong reduction of neutrophils (Fig. S1B), MPO levels (Fig. S1C) in the BAL,
366 IL-1 β levels (Fig. S1D) but not IL-18 (Fig. S1E) in the lungs. BALF levels of CXCL1/KC were
367 significantly reduced in *Nlrp6*^{-/-} mice (Fig. S1F) but not in lung homogenates (Fig. S1G) as
368 observed upon subchronical exposure. We also observed reduced MMP-9 levels in BALF and
369 lungs (Fig. S1, H and I), *Mmp-12* mRNA expression in lungs (Fig. S1J) and TIMP-1 protein
370 levels in BALF and lungs (Fig. S1, K and L) in *Nlrp6*^{-/-} mice as compared to WT mice. Lung
371 microsections and histology analysis showed reduced inflammation in *Nlrp6*^{-/-} mice in
372 comparison to WT mice (Fig. S1M).

373 In addition, to verify that mouse genetic background and/or housing were not responsible for the
374 difference in inflammatory response to CS observed, we performed breeding between *Nlrp6*^{+/-}
375 mice in order to obtain *Nlrp6*^{+/+} and *Nlrp6*^{-/-} littermates (Fig. S2A) and we acutely exposed them
376 to CS exposure. Importantly we confirmed that neutrophil number and percentage in BALF (Fig.
377 S2B-C), MPO levels (Fig. S2D-E), IL-1 β in lungs (Fig. S2F), CXCL1/KC and CXCL5/LIX were
378 decreased in BALF and lung homogenates (Fig. S2G-J) in *Nlrp6*^{-/-} mice as compared to WT mice.
379 Altogether, our results demonstrate that NLRP6 is central to pulmonary inflammatory responses
380 to CS exposure. Since NLRP6 plays comparable roles in pulmonary inflammation and
381 remodeling upon acute (1w) and subchronic (6w)-CS exposure in mice, we then preferentially
382 performed acute exposure to investigate the mechanism of NLRP6-mediated lung inflammation.

383 **NLRP6 expression in radioresistant cells is necessary for neutrophil influx and IL-1 β**
384 **production**

385 In order to investigate the cellular source of NLRP6 involved in acute CS-induced inflammation,
386 we addressed its respective contribution in bone marrow (BM)-derived versus resident cells using
387 bone marrow transplantation. WT (CD45.1) and Nlrp6^{-/-} (CD45.2) recipient mice were sub-
388 lethally irradiated (2 x 5.5 Gy, 3h apart) and reconstituted with either WT or Nlrp6^{-/-} BM cells.
389 CS-induced neutrophil recruitment (Fig. 2A) and MPO levels (Fig. 2B) in BALF were
390 significantly reduced in Nlrp6 deficient recipient mice (WT>Nlrp6^{-/-} and Nlrp6^{-/-}>Nlrp6^{-/-}) but
391 not in WT recipient mice (Nlrp6^{-/-}>WT and WT>WT). In addition, we observed a trend for
392 decreased lung IL-1 β levels in Nlrp6 deficient recipient mice (Fig. 2C). These data indicate that
393 NLRP6-dependent neutrophil influx, MPO and potentially IL-1 β productions in response to acute
394 CS-exposure, are dependent on Nlrp6 expression by non-immune/radioresistant cells rather than
395 BM-derived immune cells. In addition, while not significantly decreased in Nlrp6^{-/-}>Nlrp6^{-/-} and
396 Nlrp6^{-/-}>WT mice, lung CXCL1 expression seems to display a non-negligible BM-derived
397 contribution (Fig. 2D). In addition, remodeling factors MMP-9 (Fig. 2E) and TIMP-1 (Fig. 2F)
398 were decreased in Nlrp6^{-/-}>WT, WT>Nlrp6^{-/-} and Nlrp6^{-/-}>Nlrp6^{-/-} mice in comparison to WT>
399 WT mice suggesting an NLRP6-dependent production in both BM-derived and resident cells. As
400 a whole, these data indicate that BALF neutrophilic influx upon CS exposure depends on NLRP6
401 expression in resident cells.

402

403 **Acute CS-exposure induces NLRP6 expression in airway epithelial cells**

404 Since we observed that neutrophil influx upon CS-exposure depends on NLRP6 expression in
405 resident cells (Fig. 2, A and B), we investigated whether NLRP6 is expressed in epithelial cells.

406 We first isolated trachea epithelial cells from Wild type mice and performed air liquid interface
407 (ALI) in *in vitro* culture. We analyzed Nlrp6 mRNA basal expression by specific *Nlrp6* RT-PCR
408 and observed a 148 bp band corresponding to expected Nlrp6 cDNA size (Fig. 3A). Epithelial
409 cells markers (*Krt8* and *Ocln1*) were quantified by qPCR, confirming basal Nlrp6 expression in
410 trachea epithelial cells (Fig. 3B). Sequencing this amplified cDNA, we identified Nlrp6 sequence
411 (Fig. 3C). Then we exposed NLRP6-FLAG-IRES-GFP reported mice and WT C56BL/6 mice to
412 CS or air during 4 days. Performing FLAG-specific immunohistochemistry analysis of lung
413 sections, we observed increased FLAG expression in bronchial cells from CS-exposed mice in
414 comparison to air-exposed mice and no FLAG expression in WT C56BL/6 CS-exposed mice
415 (Fig. 3D). These results indicate that acute CS-exposure enhances NLRP6 expression in
416 bronchial airway epithelial cells in mice.

417

418 **Specific NLRP6 deficiency in lung cells dampens pulmonary inflammation to CS**

419 To confirm that NLRP6 expressed in lung cells may control airway and lung inflammation, we
420 generated mice deficient for Nlrp6 specifically in aquaporin-expressing lung epithelium ($Nlrp6^{fl/fl}$
421 $Acid^{+/CRE}$) as well as their control littermate mice ($Nlrp6^{fl/fl} Acid^{+/+}$) and exposed them to CS for 4
422 days. We show that $Nlrp6^{fl/fl} Acid^{+/Cre}$ mice displayed reduced BALF total cells influx and in
423 particular neutrophil counts and MPO levels in comparison to $Nlrp6^{fl/fl} Acid^{+/+}$ littermate mice
424 (Fig. 4, A and B). CXCL1 (Fig. 4, C and D) and CXCL5 (Fig. 4, E and F) levels were very
425 significantly decreased in the BALF whereas only a tendency was observed in the lungs of CS-
426 exposed $Nlrp6^{fl/fl} Acid^{+/Cre}$ as shown above in total $Nlrp6^{-/-}$ mice. Altogether, our findings indicate
427 that NLRP6 expression in lung cells controls pulmonary inflammation to CS exposure.

428

429

430

431 **NLRP6 inflammasome controls pulmonary inflammation and remodeling to CS exposure**

432 Since part of NLRP6 function relies on inflammasome activation, we investigated the role of
433 inflammasome-related molecules ASC, Casp-1 and Casp-11. After 4 days CS exposure, we
434 confirmed that BALF neutrophil numbers (Fig. 5A) and MPO levels (Fig. 5B), CXCL1 and
435 CXCL5 levels in BALF and lungs (Fig. 5C-F), were dramatically reduced in *Asc*^{-/-} or *Casp-1/11*^{-/-}
436 similarly than observed in *Nlrp6*^{-/-} mice suggesting an inflammasome-dependent function of
437 NLRP6 in neutrophilic inflammation.

438 Since we observed that resident pulmonary cells play an important role in NLRP6
439 inflammasome-mediated airway inflammation to CS (Fig. 2), we analyzed in more details the
440 role of CXCL5, a neutrophilic chemokine essentially produced by airway epithelial cells. CXCL5
441 and which is crucial for lung inflammation to CS exposure [11; 12; 38; 39]. We report that
442 CXCL5 levels, as well as CXCL1, were decreased in both BALF (Fig. 5, C and E) and lungs
443 (Fig. 5, D and F) of *Asc*^{-/-} or *Casp-1/11*^{-/-} mice indicating that Casp-1/11-dependent
444 inflammasome formation contributes to chemokine production.

445 To confirm that CXCL5 essentially produced by airway epithelial cells, is a crucial chemokine in
446 CS-induced pulmonary inflammation [11; 12; 38; 39], WT mice were intraperitoneally treated
447 with CXCL5 neutralizing antibodies. A very significant decrease in BALF CXCL5 levels after
448 anti-CXCL5 antibody treatment indicated efficient neutralization in the BALF (Fig. S3A). Of
449 note, we also observed decreased BALF CXCL1 levels suggesting that CXCL5 secretion
450 indirectly influences CXCL1 in CS-exposed mice (Fig. S3B), supposedly by decreasing
451 neutrophil-mediated macrophage activity. In line with decreased neutrophilic chemokines, BALF
452 total cells, neutrophil numbers (Fig. S3, C and D), and MPO levels (Fig. S3E) were reduced after
453 CXCL5 blockade. Conversely, airway instillation of recombinant CXCL5 (rCXCL5) in CS-

454 exposed *Nlrp6*^{-/-} mice was sufficient to restore most of CS-induced BALF neutrophil influx and
455 MPO levels (Fig. S3, F and G), demonstrating that CXCL5 is a major neutrophil chemokine in
456 CS-induced inflammation.

457

458 **Oral antibiotic treatment decreases pulmonary inflammation to CS**

459 Since NLRP6 was shown to control gut microbiota composition and inflammation with dysbiosis
460 in *Nlrp6*^{-/-} mice [25; 26; 27; 41; 42] we hypothesized that gut microbiota from *Nlrp6*^{-/-} mice could
461 influence pulmonary inflammation through a gut to lung axis. In that context, we analyzed
462 whether microbiota perturbation may disturb CS-induced lung inflammatory response and in
463 particular neutrophilic chemokine secretion and neutrophil influx. We treated WT mice with
464 broad-spectrum antibiotics ad libidum in drinking water for two weeks and exposed them to CS
465 during the last 4 days (Fig. 6A). Antibiotic treatment resulted in a drastic increased of caecum
466 size (Fig. 6B), as previously observed or in germ-free mice, as well as significant body weight
467 loss (Fig. 6C). Total cell counts (Fig. 6D), neutrophils percentage and numbers (Fig. 6, E and F)
468 in BALF, MPO (Fig. 6, G and H), CXCL1 (Fig. 6I and J), CXCL5 (Fig. 6K and L) levels were
469 decreased in BALF and lung homogenates. In addition, IL-1 β levels in the lungs (Fig. 6M),
470 MMP-9 (Fig. 6, N and O) and TIMP-1 (Fig. 6, P and Q) levels in BALF and lungs were
471 attenuated. These results show that whole body bacterial depletion in WT mice impairs
472 pulmonary inflammation upon CS exposure and recapitulates our results observed in *Nlrp6*^{-/-}
473 mice. The data suggest that gut microbiota is involved in CS-induced lung inflammation.

474

475

476

477
478 **Impaired CS-induced airway inflammation in *Nlrp6*^{-/-} mice is transferable to wild type mice**
479 **by cohousing**

480 To decipher whether gut microbiota from *Nlrp6*^{-/-} influences pulmonary inflammation, WT mice
481 were co-housed (WT CH) for 3 months with *Nlrp6*^{-/-} mice (*Nlrp6*^{-/-} CH). Single-housed WT (WT
482 SH) and *Nlrp6*^{-/-} (*Nlrp6*^{-/-} SH) mice were used as controls. Then, mice were exposed to air or CS
483 for 4 days (Fig. 7A) and their gut microbiota composition analyzed using unbiased 16S ribosomal
484 RNA (rRNA) gene high throughput sequencing. As expected, the dominant phyla were
485 represented by Firmicutes, Bacteroidetes, Proteobacteria and Actinobacteria and differences
486 appeared comparing WT versus *Nlrp6*^{-/-} mice (Fig. S4 and S5). No significant change in alpha
487 diversity was observed in intestinal microbiota among the different mouse groups (Fig. S6).
488 However, principal component analysis (PCA) of beta diversity showed sharp differences
489 comparing WT SH and *Nlrp6*^{-/-} SH mice, which were abrogated upon cohousing. This confirms
490 differences in gut microbiota composition between WT and *Nlrp6*^{-/-} mice as previously reported
491 [25; 41; 43; 44]. Indeed, WT SH and *Nlrp6*^{-/-} SH mice exposed to air (Fig. 7B) or to CS (Fig. 7C)
492 cluster separately on the PC1 axis while the cohoused clusters became indistinguishable. Using
493 LDA Effect Size (LEfSe) [45], specific differences stand out in gut microbiota composition
494 between WT and *Nlrp6*^{-/-} mice exposed to air or CS (Fig. S4, S5 and S7). These differences
495 mostly disappear upon co-housing (Fig. S8) demonstrating the efficacy of the microbiota transfer
496 by this method.

497 In addition, analysis of gut microbiota from single-housed WT or *Nlrp6*^{-/-} mice exposed to air or
498 to CS revealed that CS exposure affects intestinal microbiota composition in both WT (Fig. 7D)
499 and *Nlrp6*^{-/-} (Fig. 7E) mice indicating that CS airway exposure may influence gut homeostasis
500 and/or inflammation. However, CS-driven gut microbiota alterations were distinct in WT versus

501 Nlrp6^{-/-} (Fig. S7) suggesting possible connections linking CS, gut microbiota and NLRP6-
502 mediated airway inflammation. In line with this hypothesis, Nlrp6^{-/-} phenotype characterized by
503 decreased cell recruitment (Fig. 7F) and in particular neutrophil influx (Fig. 7G) as well as BALF
504 CXCL1 (Fig. 7H) and CXCL5 (Fig. 7I) levels was transferred to WT mice upon cohousing.
505 These results indicate that intestinal microbiota influences CS-induced airway inflammation.
506 Noteworthy, we observed that total cells (Fig. 7F), especially neutrophils influx (Fig. 7G),
507 CXCL1 (Fig. 7H) and CXCL5 (Fig. 7I) secretions into the BALF remained low in Nlrp6^{-/-}
508 cohoused with WT mice (Nlrp6 CH), indicating that microbiota from Nlrp6^{-/-} mice displays a
509 dominant effect. These results highlight differences in gut microbiota composition between WT
510 and Nlrp6^{-/-} mice and show that gut microbiota from Nlrp6^{-/-} mice limits airway inflammation to
511 CS exposure.

512
513 **Oral transplantation of WT germ-free mice with fecal microbiota from Nlrp6^{-/-} mice**
514 **attenuated CS exposure-induced lung inflammation**

515 To confirm that gut microbiota regulates airway inflammation to CS exposure in an NLRP6-
516 dependent manner, we colonized germ-free (GF) WT mice (WT^{GF}) with fecal microbiota
517 obtained from WT or Nlrp6^{-/-} mice bred in the same EOPS mouse facility. Transplanted mice
518 were maintained in isolators for 4 weeks to allow microbiota reconstitution and immune system
519 maturation. Following one week of acclimation in a conventional breeding facility, mice were
520 exposed to CS for 4 days (Fig.8A). While GF mice are unable to mount proper inflammatory
521 response [43], we observed that reconstitution of WT^{GF} mice with WT microbiota (WT>WT^{GF})
522 allows a normal lung inflammatory response to CS with CXCL5 secretion in the bronchoalveolar
523 space (Fig. 8, B-F). In contrast, WT^{GF} mice colonized with microbiota from Nlrp6^{-/-} mice (Nlrp6^{-/-}
524 ^{-/-}>WT^{GF}) displayed decreased cellular influx in BALF (Fig. 8B), in particular of neutrophil (Fig.

525 8C) associated with decreased MPO (Fig. 8D), CXCL1 (Fig. 8E) and CXCL5 (Fig. 8F) levels in
526 BALF as compared to WT>WT^{GF} mice. These results confirm that gut microbiota shapes lung
527 inflammation and that gut microbiota from Nlrp6^{-/-} mice negatively regulates lung inflammation
528 upon acute CS exposure.

535

536 **Discussion**

537 NOD-like receptor family pyrin domain containing (NLRP)6 inflammasome was shown to
538 regulate microbiota homeostasis as well as antibacterial and antiviral immunity in the intestine
539 [24; 25; 26; 27; 28; 41]. However, the role of this multifaceted immune sensor in the lungs
540 remains poorly understood. Studies showed that NLRP6 controls neutrophil recruitment and
541 function during pulmonary bacterial infection [29; 30; 46; 47; 48] but its involvement in the
542 context of sterile lung injury remains elusive. We chose to understand early inflammation
543 mechanisms because acute inflammation repetition often lead to chronicity and lung pathology.
544 In addition, episodes of acute inflammatory exacerbations due to bacterial infections, frequently
545 observed in COPD patients, are associated with dramatic increased pathology severity. Therefore,
546 a better characterization of the mechanisms of acute phases of inflammation appears crucial.

547 We demonstrate that NLRP6 positively controls both pulmonary inflammation and remodeling to
548 cigarette smoke exposure. Nlrp6 deficient mice (Nlrp6^{-/-}) displayed drastic reduction of airway
549 cell recruitment particularly neutrophils, macrophages and lymphocytes and attenuated lung
550 tissue inflammation in comparison to wild type (WT) mice. Interestingly, NLRP6 contribution is
551 likely context-dependent as it was shown to negatively regulate pulmonary host defense after
552 gram-positive bacterial infection through neutrophil influx modulation [29]. COPD patients often
553 suffer from disease exacerbations due to bacterial infections and despite strong neutrophil-driven

554 inflammation, these cells are largely ineffective leading to impaired bacterial clearance [49; 50;
555 51]. A possibility is that NLRP6 favors chronic detrimental neutrophilic inflammation but
556 suppresses anti-bacterial immunity. If true, targeting NLRP6 in COPD patients might be
557 beneficial by preventing chronic inflammation and bacterial infection-driven disease
558 exacerbation. This hypothesis could be tested in COPD exacerbation models by infecting
559 chronically CS exposed mice to bacteria such as *Streptococcus pneumoniae* or *Haemophilus*
560 *influenzae*.

561 Our data demonstrate for the first time that NLRP6 is a sensor of lung injury here induced by CS
562 but possibly by other chemicals and toxics, opening new investigation fields. We verified that
563 mouse genetic background and/or housing were not responsible for the difference in
564 inflammatory response to CS observed using *Nlrp6*^{+/+} and *Nlrp6*^{-/-} littermate mice and observed
565 similar decreased pulmonary inflammation in *Nlrp6*^{-/-} mice in comparison to *Nlrp6*^{+/+} littermate.

566 We provide evidence that NLRP6 expression in radioresistant cells positively regulates CS-
567 mediated airway neutrophil inflammation. Our results show that NLRP6 is expressed in tracheal
568 and lung epithelial cells and indicate that acute CS-exposure enhances NLRP6 expression in
569 airway epithelial cells in mice. Although much less than in the intestine, NLRP6 was shown
570 expressed in the lungs in epithelial cells and tissue infiltrating neutrophils and macrophages after
571 bacterial infection of human or mice [29; 30; 46; 47; 48]).

572 Then, using mice deficient for *Nlrp6* specifically in aquaporin-expressing lung cells (*Nlrp6*^{fl/fl}
573 *Acid*^{+/^{CRE}}) and their control littermate (*Nlrp6*^{fl/fl} *Acid*^{+/+}) we demonstrate that NLRP6 expressed
574 in lung controls neutrophilic airway and lung inflammation in response to acute CS exposure.

575 We confirmed that CXCL5, a neutrophilic chemokine essentially produced by airway epithelial
576 cells, is a crucial chemokine in CS-induced pulmonary inflammation [11; 13; 39; 52; 53]. In
577 addition, exogenous recombinant CXCL5 was sufficient to restore neutrophilic inflammation in

578 *Nlrp6*^{-/-} mice suggesting that NLRP6 expressed in airway epithelial cells plays a key role in
579 CXCL5 production and secretion and leads to neutrophil recruitment and airways inflammation.
580 We report a new role for the NLRP6 inflammasome in pulmonary inflammation to CS exposure
581 through production of the neutrophilic chemokine CXCL1/KC and CXCL5/LIX leading to
582 neutrophils influx and airway inflammation. NLRP6 expression and function in airway epithelial
583 cells highlights active contribution of tissue constitutive cells in pulmonary inflammation as
584 supported recently [16; 17]. In addition, the NLRP6, ASC, Casp-1 and/or Casp-11
585 inflammasome-related proteins contribute to CS-induced pulmonary inflammation. CS-exposure
586 induced NLRP6 expression in AEC suggesting the formation of the NLRP6 inflammasome in
587 these cells. We recently reported that CS-exposure induces NLRP3-dependent caspase-1
588 activation in bronchoalveolar space macrophages and NLRP3-dependent gasdermin D activation
589 in both bronchoalveolar space macrophages and bronchial epithelial cells [54]. This indicate that
590 both NLRP3 and NLRP6 inflammasomes are involved in CS-induced pulmonary inflammation.
591 However, inflammasome-independent role of NLRP6 was reported in intestinal epithelial cells
592 [28]. Indeed NLRP6 was showed to control enteric virus infection through its binding to viral
593 RNA via the RNA helicase Dhx15 and interacted with mitochondrial antiviral signaling protein
594 to induce type I/III interferons and IFN-stimulated genes [28]. Even if we observed a similar
595 reduction in inflammation in the absence of NLRP6, ASC or Casp1/11, we cannot exclude that
596 NLRP6 could play a role independently of inflammasome via unknown partners.
597 Here we hypothesized that gut microbiota from *Nlrp6*^{-/-} mice could influence pulmonary
598 inflammation through a gut to lung axis. We showed that whole body bacterial depletion in WT
599 mice impairs pulmonary inflammation upon CS exposure and recapitulates our results observed
600 in *Nlrp6*^{-/-} mice. Interestingly, *Nlrp6*^{-/-} mice presented defective mucus secretion and intestinal
601 barrier, reduced control of colonic host-microbial interface and microbiota dysbiosis [25; 26].

602 These results were challenged when $Nlrp6^{+/+}$ and $Nlrp6^{-/-}$ littermate were used, both displaying
603 intact mucus layer [55]. However, *ex vivo* LPS-induced mucus secretion was shown to be
604 defective in intestinal goblet cells of colonic tissue from $Nlrp6^{-/-}$ mice in comparison to $Nlrp6^{+/+}$
605 littermate [55].

606 Importantly we report for the first time the existence of an NLRP6-dependent gut to lung axis
607 controlling pulmonary inflammation to CS-induced injury. Cohousing and fecal transplantation
608 experiments both revealed that $Nlrp6^{-/-}$ gut microbiota transfer to WT mice impedes pulmonary
609 inflammation in response to CS. During cohousing or fecal transplantation, metabolites generated
610 by $Nlrp6^{-/-}$ gut microbiota upon CS-exposure may inhibit NLRP6 in airway epithelial cells of WT
611 mice. In addition, antibiotic-mediated microbiota depletion indicates that microbiome is required
612 for efficient lung immunity. While antibiotic treatment probably affects both gut and lung
613 microbiota, these data suggest that the predominant gut microbiota is necessary for CS-induced
614 lung inflammation through NLRP6 activation and subsequent lung inflammation. Lung
615 microbiota composition analysis is difficult to perform because of the weak amount of bacteria.
616 However, we believe that as shown, NLRP6-dependent perturbation of the gut microbiome may
617 generate specific metabolites or compounds [43; 56] that might modulate NLRP6 inflammasome
618 activation in lung.

619 Our study supports evidence of an altered gut microbiota between WT and $Nlrp6^{-/-}$ mice as
620 previously reported [25; 41; 43; 44]. Two studies suggested that NLRP6 inflammasome does not
621 shape commensal gut microbiota composition by analyzing gut microbiota phylogenetic after
622 DSS-induced colitis of ASC-deficient mice [57; 58]. However, two other laboratories reported
623 that littermate breeding led to the development of distinct microbiome compositions in NLRP6
624 inflammasome-deficient mice housed in two different facilities [27; 43].

625 In addition, two independent groups showed that NLRP6 deficiency lead to impaired intestinal
626 mucus secretion, disrupted mucus barrier and mucosal surface invasion by enteric pathogens and
627 finally to dysbiosis [25; 26] but this was discussed by another group [55]. These seemingly
628 conflicting results might be explained by differences in microbiome compositions between
629 laboratories. Indeed, familial transmission was shown to significantly influence microbiota
630 composition in conventionally housed *Nlrp6*^{-/-} mice. In addition, introduction of potential
631 pathobionts revealed effects of *Nlrp6* deficiency on gut microbiome and increased abundance of
632 pathobionts was observed in *Nlrp6*^{-/-} mice indicating that *Nlrp6*^{-/-} microbiota depends on
633 community structure [42]. Moreover, littermate approach has been coupled with generation of
634 germ-free mice. De novo dysbiosis was observed in spontaneous recolonization of germ-free
635 NLRP6^{-/-} mice [43] as well as after fecal transfer of diverse microbiomes in germ-free NLRP6^{-/-}
636 mice, as compared to germ-free WT mice [27; 42; 43].

637 Our results obtained after cohousing or fecal transplantation experiments, or antibiotics
638 treatments demonstrate that airway exposure to CS significantly alters gut microbiota
639 composition and hence may influence gut homeostasis and/or inflammation. These observations
640 are in line with data showing increased risk of smokers and COPD patients to develop intestinal
641 diseases and support the notion of a complex interplay between environmental factors, gut
642 microbiota and organ disease [59; 60]. Epidemiological evidence linked gut dysbiosis and COPD
643 [61; 62]. Interestingly, some specific changes we observed in gut microbiota of CS-exposed
644 *Nlrp6*^{-/-} mice are inversely correlated to faecal microbiome taxonomic indicators described in
645 COPD patients. For example, upon CS-exposure Clostridia are increased in gut microbiota of
646 *Nlrp6*^{-/-} mice in comparison to WT mice (Fig. S5) but depleted in faecal microbiome of COPD
647 patients in comparison to healthy subject. Inversely Bifidobacteriaceae are decreased in gut
648 microbiota of *Nlrp6*^{-/-} mice whereas increased in faecal microbiome of COPD patients [63].

649 In addition, severe COPD was associated with reduced sputum microbiota diversity [64] and lung
650 microbiome dynamics during stability and exacerbation, influences COPD pathogenicity [65;
651 66]. It is likely that the lung microbiota may be influenced by CS exposure in our mouse model
652 as shown in COPD patients [66]. In particular, during infection-triggered acute COPD
653 exacerbations, pathogens promote lung microbiota dysbiosis that may favor intestinal diseases,
654 pointing to the existence of a bidirectional connection between the lungs and the gut in COPD
655 [67]. Altered lung microbiota in *Nlrp6*^{-/-} mice could interfere with host defense against lung
656 infection in particular triggered by *Streptococcus pneumoniae* (*S.p.*) and frequently associated with
657 COPD exacerbation [68]. However, NLRP6 was shown to negatively regulate inflammation to
658 *S.p.* [31; 48] or to *Staphylococcus aureus* infections [29] suggesting a beneficial role of *Nlrp6*
659 depletion or inhibition in these secondary infections. However, NLRP6 might exhibit detrimental
660 functions in other infection settings. We show that both NLRP6 expression in lung and gut
661 controls CS-induced pulmonary inflammation. Gut microbiota shaped by NLRP6 expressed in
662 gut, regulates pulmonary inflammation to CS probably via circulating microbiota-derived
663 metabolites [69]. In addition to gut metabolites, gut-microbiota-derived components can also
664 modulate lung inflammation, such as *lipoteichoic acid* (*LTA*), a major constituent of the cell wall
665 of gram-positive bacteria that was shown to induce inflammation through NLRP6 sensing in
666 wild-type mice but not in *Nlrp6*^{-/-} mice [70]. As a whole, we report new data supporting
667 important NLRP6 functions in basal and pathological lung settings that could be translated to the
668 clinic following preclinical validations. Gut microbiota-derived metabolites or components might
669 represent biomarkers present in the blood of COPD patients [43; 71]. In addition, this study
670 uncovers NLRP6 as a new target for the development of potential therapeutic strategies against
671 COPD by specific inhibition of NLRP6 or gut microbiota transfer approaches might be useful in
672 the treatment of COPD.

673

674 **Author contributions**

675 MN, MF, SHM, MS, FS, NR and AG performed the experiments.

676 MN, NR, AG, PG, HS and IC conceived the experiments and analyzed the data.

677 MLB supervised mouse breeding.

678 LA, MC provided mouse strains.

679 JVS provided antibodies.

680 MN, NR, MLB, BR, VFJQ, FT, PG, HS and IC discussed the results.

681 MN, NR, BR, AG, HA and IC wrote the manuscript.

682 IC, FT, HS, PG and VJFQ provided funding and IC overall supervision of this study.

683

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690

691 **Declaration of Interests**

692 All authors declare no competing interests.

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700 **References**

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