

1 **SUPPLEMENTARY MATERIALS**

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3 **INFLAMMATORY CELLULAR RESPONSE TO MECHANICAL VENTILATION IN**
4 **ELASTASE-INDUCED EXPERIMENTAL EMPHYSEMA: ROLE OF PRE-EXISTING**
5 **ALVEOLAR MACROPHAGES INFILTRATION**

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10

11 **METHODS**

12

13 **Animal model**

14 All the experiments were performed in accordance with the official regulations of the French Ministry
15 of Agriculture and the US National Institute of Health guidelines for the experimental use of animals,
16 were approved by the local institutional animal care and use committee, and were conducted in a
17 specific “little animal” platform (Plateforme Exploration Fonctionnelle du Petit Animal, INSERM
18 U955, Créteil, France). Six-weeks-old male C57BL/6 mice (Janvier Labs, Le Genest Saint-Isle,
19 France) were used. Under anesthesia, mice received the instillation of either 5 IU of porcine
20 pancreatic elastase (Elastin Products, Owensville, MO, USA) diluted in 50 µl of saline (Elastase
21 mice), or 50 µl of saline (Saline mice) into the surgically exposed trachea. Mice were then subjected
22 to subsequent ventilation at two time points, 14 and 21 days after instillation.

23

24 **Mechanical ventilation**

25 Mice were anesthetized with intraperitoneal thiopental (30 $\mu\text{g/g}$ of body weight; Hospira, Meudon-La-
26 Forêt, France) followed by continuous inhaled 5% isoflurane (Abbott, Rungis, France). The larynx
27 was surgically exposed and the trachea was intubated orally under direct vision with a metal cannula
28 (internal diameter of 1 mm, Harvard Apparatus, Les Ulis, France). Mice in mechanical ventilation
29 (MV) group were ventilated in the supine position using humidified gas (20mgH₂O/L absolute
30 humidity, MR410 humidifier, Fischer & Paykel Healthcare, Courtaboeuf, France), with a Vt of 8 $\mu\text{L/g}$
31 of body weight, a respiratory rate of 180 /min, an inspiratory to expiratory time ratio of 67%, an end-
32 expiratory pressure of 1.5 cmH₂O, and a fraction of inspired oxygen of 0.4 - 0.6, by means of a
33 computer-driven small-animal ventilator (flexiVent, Scireq, Montreal, Canada), as previously
34 reported. The tracheal cannula was properly secured with surgical thread (Ethicon 3-0, Ethicon,
35 Auneau, France) before connection to mechanical ventilator in order to avoid leaks. The cervicotomy
36 was closed with surgical thread (Ethicon 6.0, Ethicon, Auneau, France). Mechanical ventilation lasted
37 two hours with continuous anesthesia maintained by 1.5% inhaled isoflurane and muscle paralysis
38 using intraperitoneal pancuronium at the onset of the experiment (0.8 $\mu\text{g/g}$ of body weight; Organon,
39 Puteaux, France) to ensure passive mechanical conditions. The body temperature was monitored using
40 a rectal probe and was maintained at 36.5°C with a blanket connected to a homeothermic regulator
41 (Homeo-blanket system 50-7221F, Harvard Apparatus, Les Ulis, France). An intraperitoneal warm
42 fluid bolus of 20 $\mu\text{L/g}$ of saline was performed immediately after the induction of anesthesia. A
43 control group (SV) consisted of anesthetized, intubated mice, maintained on spontaneous ventilation
44 for two hours, under conditions identical to MV mice, except for muscle paralysis. At the end of the
45 timed ventilator protocol, mice were exsanguinated via sectioning the left carotid artery. Arterial
46 blood was obtained, using a heparinized needle and syringe, then transported in ice rapidly after
47 collection, for determination of blood gases, using a GEM Premier 3000 analyser (Instrumentation
48 Laboratory, Lexington, MA, USA) in D21 mice. Blood gases data were similar between the 4 groups
49 at D21 (n=10-13/group), allowing us to validate fluid replacement strategy, and both ventilator

50 settings in MV mice, and anesthesia depth in SV mice (table S1). To note, blood gases analysis were
 51 not performed in D14 mice, and the validity of the experimental protocol has been extrapolated to this
 52 group.

TABLE S1. Arterial blood gases in SV and MV mice, 21 days after saline or elastase tracheal instillation.

	Saline SV	Elastase SV	Saline MV	Elastase MV
PaO₂/FiO₂	450 ± 100	371 ± 124	417 ± 99	460 ± 84
PaCO₂, mmHg	27 ± 7	34 ± 21	34 ± 10	30 ± 6
pH	7.31 ± 0.08	7.30 ± 0.16	7.23 ± 0.09	7.28 ± 0.12
HCO₃⁻, mmol/L	14.6 ± 3.7	15.1 ± 5.1	16.4 ± 3.9	15.3 ± 7.0
Lactates, mmol/L	3.2 ± 2.4	3.2 ± 3.0	2.8 ± 0.7	2.5 ± 1.0

Definition of abbreviations: PaO₂, partial pressure of oxygen in arterial blood; PaCO₂, partial pressure of carbone dioxide in arterial blood; SV, spontaneous ventilation; MV, mechanical ventilation.

Values are expressed as median ± interquartile range; n = 10-13 animals /group.

No significant difference was found between groups. Ventilator settings (MV mice) and anesthesia depth (SV mice) allowed suitable minute ventilation.

53

54 **Respiratory mechanics**

55 Special features of the *flexiVent* ventilator include a continuous monitoring of airway pressures and a
 56 precision computer-controlled piston that is capable of accurately measuring the delivered volume
 57 (with appropriate corrections for gas compression) and to produce any desired waveform, allowing
 58 respiratory mechanics assessment with the forced oscillation technique and pressure-volume curves.
 59 Before each mouse was connected to the ventilator, pressure and piston displacement calibration data
 60 were collected to correct the respiratory mechanics data. Mice were allowed to stabilize on the
 61 ventilator for 5 minutes and were then inflated three times to a transrespiratory pressure of 30 cmH₂O
 62 to establish a standard volume history. Forced oscillation technique were assessed at initiation of
 63 mechanical ventilation, before (H0) and after (H0') volume history standardization (recruitment
 64 maneuver), and then repeated hourly (H1 and H2), to capture the time course and the detailed
 65 response to mechanical ventilation. Respiratory system dynamic resistance, elastance, and compliance
 66 were calculated in the *flexiVent* software by fitting the single frequency forced oscillation technique
 67 data (a 1.2 second, 2.5 Hz single-frequency signal called SnapShot-150 perturbation) to the single
 68 compartment model. In addition, a continuous pressure-volume curve (pressure-driven from 3 to 30

69 cmH₂O) was performed at start and end of mechanical ventilation. The loops were processed by the
70 *flexiVent* software to fit the Salazar-Knowles equation and to calculate parametric results, such as the
71 quasi-static compliance of the respiratory system.

72

73 **Specimen collection**

74 After sacrifice by exsanguination via sectioning the left carotid artery, two sets of samples collection
75 were performed. In a first set of experiments, a thoracotomy was immediately performed, exposing
76 heart and lungs. Pulmonary circulation was flushed with 2 mL of saline directly injected into the right
77 ventricle after ablation of the left atrium. Lungs were then harvested, fixed in 4% paraformaldehyde by
78 tracheal injection at a constant transpleural pressure of 20cmH₂O, held in 4% paraformaldehyde and
79 finally embedded in paraffin. In a second set of experiments, bronchoalveolar lavage (BAL) was first
80 performed with two separate 1 mL aliquots of saline at 20°C injected through tracheal tube. Then
81 immediately following thoracotomy, pulmonary circulation was identically flushed (to remove
82 residual blood within pulmonary vasculature and reduce nonadherent leukocytes from subsequent
83 flow cytometric analysis), and both lungs were collected for flow cytometry analysis.

84

85 **Morphometric analysis**

86 Great-axis sagittal sections of 5 μm thickness of the left lung were cut in a systematic fashion and
87 were stained with hematoxylin and eosin. Five digital photomicrographs were acquired from the
88 cranial, medial, and caudal regions of each slide at x200 magnification, excluding areas where large
89 bronchi or vessels predominated, resulting in a total of 15 images per lung (Axioplan 2 microscope
90 *equipped with an MRc digital color camera (Zeiss, Oberkochen, Germany)*). Emphysema was
91 quantified by measurement of alveolar diameters with an image analysis software (ImageJ, NIH,
92 Bethesda, USA). This automated analysis was made vertically and horizontally on each
93 photomicrograph. The mean chord length of alveoli was obtained by averaging those measurements.

94

95 **Bronchoalveolar lavage**

96 The total cell count of BAL was determined for a fresh fluid specimen using a Malassez
97 hemocytometer (Hycor biomedical, Indianapolis, IN, USA). BAL fluid was centrifuged (1500 rpm, 15
98 min at 4°C). The cell pellet was diluted in saline, and differential cell counts were done on
99 cytocentrifuge preparations (Cytospin3; Shandon Scientific, Cheshire, UK) stained with Diff-Quick
100 stain (Baxter Diagnostics, McGaw Park, IL, USA).

101

102 **Flow cytometric analysis**

103 *Preparation of lung cell suspensions*

104 Freshly collected lungs were immediately placed in a storage medium consisting of RPMI 16/40
105 supplemented with 0.1% L-glutamine and 0.1% nonessential amino acids and kept on ice. Mechanical
106 disruption followed by enzymatic digestion of murine lungs were then performed, as previously
107 described. Lung tissue, placed in previously described medium, supplemented with 5% fetal bovine
108 serum (FBS), 0.35 mg/mL collagenase XI (Sigma-Aldrich, Saint Louis, MO, USA) and 0.1 mg/mL
109 type IV bovine pancreatic DNase (Sigma-Aldrich), was cut into small pieces by rapid chopping using
110 a scalpel. The dissected tissue was then incubated at 37°C with 5% CO₂ during 35 minutes.
111 Enzymatic action was stopped by adding 9 mL of previous medium storage containing 10% FBS, and
112 digested lungs were further disrupted by gently pushing the tissue through a 70 µm nylon cell strainer.
113 Cell suspensions were then centrifuged at 900 rpm. To lyse contaminating red blood cells, the cell
114 pellet was incubated during 5 minutes at room temperature with 5 ml of ACK Lysing Buffer (NH₄Cl
115 and KHCO₃, ThermoFisher Scientific Waltham, MA, USA). Cells were then washed with saline,
116 recentrifuged, and the pellet resuspended in 4 ml of saline. Cell counts were performed using a
117 Malassez hemocytometer after Trypan blue staining. Cell suspensions were lastly centrifuged and
118 resuspended in saline in order to obtain approximately 10 millions cells per mL. In cytometry

119 dedicated tubes, 500,000 cells (50 μ L) were incubated with 50 μ L of anti-mouse CD16/CD32 (Fc
 120 Block, 10 μ g/mL, clone 2.4G2, BD Biosciences, San Jose, CA, USA) at 4°C during 15 minutes, to
 121 prevent nonspecific binding of antibodies to the Fc receptors, and then stained with fluorochrome-
 122 conjugated anti-mouse antibodies for CD11b, CD11c, Gr1 (Ly6C/G), F4/80 and CD62L (L-selectin)
 123 or appropriate isotype-matched controls. All antibodies used are listed in table S2. Finally, cytometry
 124 tubes were centrifuged, cell pellets resuspended in 500 μ L saline with 1% formaldehyde, and tubes
 125 were stored away from light at 4°C.

TABLE S2. Fluorochrome conjugated anti-mouse antibodies.

Surface molecule	Fluorochrome	Clone	Isotype control	Manufacturer
Ly6G/Ly6C (Gr1)	FITC	RB6-8C5	Rat IgG2a, κ	BD Biosciences
Ly6G/Ly6C (Gr1)	PE/Cy7	RB6-8C5	Rat IgG2b, κ	Biolegend
CD11c	PE	HL3	Hamster IgG1, λ 2	BD Biosciences
F4/80	PE/Cy7	BM8	Rat IgG2a, κ	Biolegend
CD62L	APC	MEL-14	Rat IgG2a, κ	Biolegend
CD11b	APC/Cy7	M1/70	Rat IgG2b, κ	BD Biosciences

BD Biosciences, San Jose, CA, USA; Biolegend, San Diego, CA, USA

126

127 *Identification of inflammatory cell populations*

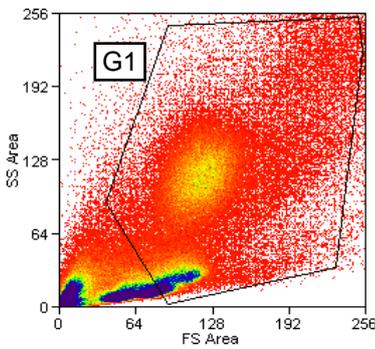
128 Cells were analysed the following day by a 7channel cytometer (CyAn ADP Analyser, Beckman
 129 Coulter, Brea, USA). One channel was dedicated to measurement of cell autofluorescence. Complete
 130 data analysis was performed with Summit software (Summit v4.3, Dako, Cambridge, UK). Three
 131 inflammatory cell populations in murine lung were identified (figure S1). CD11b⁻, CD11c⁺
 132 phenotype, with high autofluorescence defined alveolar macrophages (figure S1c). Monocytes and
 133 neutrophils were identified as CD11b⁺, CD11c⁻ cells but differ in their granularity (side-scatter, SS),
 134 and F4/80 and Gr1 expression. High SS and F4/80⁻, Gr1⁺ phenotype defined neutrophils, whereas low
 135 SS, and F4/80⁺, Gr1^{mid} phenotype characterized monocytes (figures S1d, S1e, S1f). Cells activation
 136 state were assessed using expression of CD62L and CD11b adhesion molecule, as well as Gr1. All
 137 flow cytometric results were presented as relative values, called percentage of gated cells. Indeed, our
 138 cytometer did not allow automated simultaneous counting of collected lung cells, resulting in
 139 inaccurate absolute values.

140

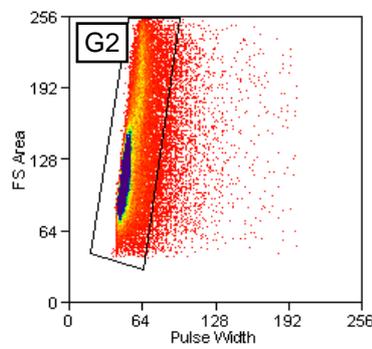
141 **Figure S1.** Flow cytometric analysis of different inflammatory cell populations in mouse lungs. **a.**
142 Dot-plot showing side-scatter (SS) versus forward-scatter (FS) within total cell population. Cellular
143 debris and lymphocyte were excluded from the analysis. Myeloid cells were gated (G1) on compatible
144 size (FS) and granularity (SS). **b.** Dot-plot showing FS versus pulse width within G1 gated events.
145 Doublets and triplets were subtracted from cell analysis through a second gate (G2) defined on pulse
146 width, which distinguished cell aggregates from unique events (called singlets). From there, only
147 combined G1 and G2 gated events were analysed. **c.** Dot-plot showing CD11c versus CD11b
148 expression within combined G1 and G2 gated events. Alveolar macrophages were identified by
149 CD11c+ CD11b- phenotype. Autofluorescence level was high in this cell population. **d.** Dot-plot SS
150 versus CD11b expression within combined G1 and G2 gated events. G3 gated CD11b+ cells with
151 neutrophils compatible granularity, G4 gated CD11b+ cells with monocytes compatible granularity. **e.**
152 Dot plot CD11c versus Gr1 expression within combined G1, G2 and G3 gated events. Neutrophils
153 were defined as CD11c- Gr1+ cells. **f.** Dot plot CD11c versus Gr1 expression within combined G1,
154 G2 and G4 gated events. Monocytes were defined as CD11c- Gr1mid cells.

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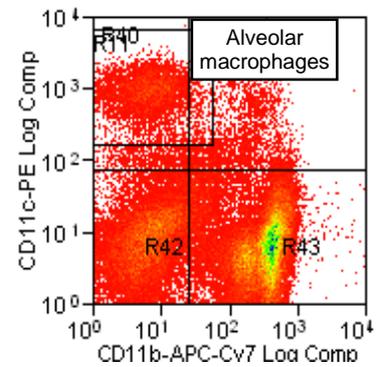
a.



b.



c.

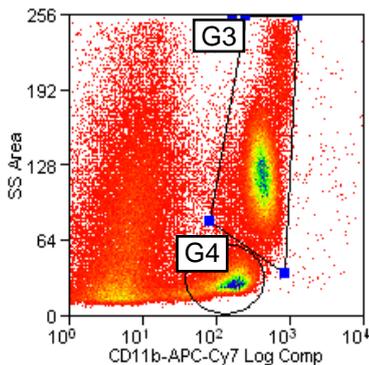


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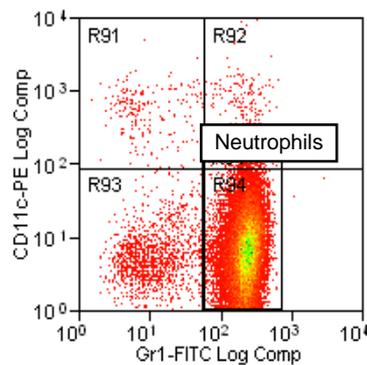
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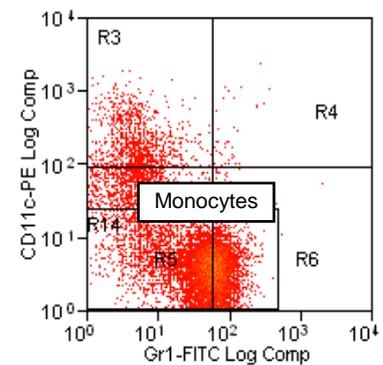
d.



e.



f.



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