

1 **Gestation and lactation exposure to nicotine induces transient**
2 **postnatal changes in lung alveolar development**

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13 Abbreviated title:

14 Alveolar development in mice: does nicotine interfere?

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16 Keywords: nicotine, lung development, alveolarization, developmental kinetics

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23 **ABSTRACT**

24 Harmful consequences of cigarette smoke (CS) exposure during lung development can already
25 manifest in infancy. In particular, early life exposure to nicotine, the main component of CS, was
26 shown to affect lung development in animal models. We aimed to characterize the effect of
27 nicotine on alveoli formation. We analyzed the kinetics of normal alveolar development during
28 the alveolarization phase and then looked at the effect of nicotine in a mouse model of
29 gestational and early life exposure. Immunohistochemical staining revealed that the wave of cell
30 proliferation (i.e. vascular endothelial cells, alveolar epithelial cells (AEC) type II and
31 mesenchymal cell) occurs at pnd8 in control and nicotine-exposed lungs. However, FACS
32 analysis of individual epithelial alveolar cells revealed nicotine-induced transient increase of
33 AEC type I proliferation and decrease of vascular endothelial cell proliferation at pnd8.
34 Furthermore, nicotine increased the percentage of endothelial cells at pnd2. Transcriptomic data
35 also showed significant changes in nicotine samples compared to the controls on cell cycle
36 associated genes at pnd2, but not anymore at pnd16. Accordingly, the expression of survivin,
37 involved in cell cycle regulation, also follows a different kinetics in nicotine lung extracts. These
38 changes resulted in an increased lung size detected by stereology at pnd16, but no longer in adult
39 age, suggesting that nicotine can act on the pace of lung maturation. Taken together, our results
40 indicate that early life nicotine exposure could be harmful to alveolar development independently
41 from other toxicants contained in CS.

42 **INTRODUCTION**

43 During fetal development, lungs undergo several orchestrated steps to ensure adequate
44 gas exchange. The successive embryonic, pseudoglandular, canalicular, saccular and alveolar
45 phases are required to achieve functionality of the various parts of the lung, such as the
46 conducting airways or respiratory units formed by alveoli (21, 53). Both genetic and
47 environmental factors can influence lung development (18). The consequences of harmful events
48 can manifest at birth, but also later in life. Exposure to cigarette smoke (CS) during early life has
49 been widely associated with wheezing in childhood, and later in life with the development of
50 chronic metabolic or cardiovascular diseases, such as diabetes or hypertension (5, 18), or chronic
51 obstructive pulmonary disease (COPD) (10, 46, 47). Nicotine, one of the main components of
52 CS, can freely cross the placenta and may harm the developing fetus (17). Nicotine exposure
53 during pregnancy and lactation has been studied in rats and monkeys where it reduces gas
54 exchange surface (39, 55). Furthermore, increased airway length and no change in the alveolar
55 intercepts in mice exposed to nicotine were detected (58, 60). Finally, accelerated development
56 of the bronchiolar tree was described in lambs (51). Most of the effects described worsened when
57 pups were exposed to nicotine during the prenatal and postnatal period (10). In humans, there is
58 no evidence that nicotine replacement therapy (NRT) induces major congenital abnormalities (8)
59 and very few information is available on e-cigarette use during pregnancy (59). Since the effect
60 of nicotine on alveolar development and in particular on the dynamics of cell death and
61 proliferation has been poorly studied, we aim to better characterize a) the dynamics of different
62 cell types during lung alveolarization, and b) the effect of *in utero* and lactation exposure to
63 nicotine on the development of alveoli. Mice are born at the end of the saccular phase of lung

64 development, and therefore the process of alveolarization occurs after birth and is easier to study
65 than in other animal species.

66 To address our questions we exposed mice to nicotine during gestation and lactation to
67 mimic pre- and postnatal exposure, and assessed lungs at post-natal day (pnd) 2 (end of saccular
68 phase), 8 (early alveolar phase) and 16 (middle alveolar phase). Our results indicate dynamic
69 processes of proliferation, with a peak detected at pnd8 and caused by three cell types: vascular
70 endothelial cells, mature AEC type II and mesenchymal cells. Nicotine had no effect on
71 proliferation dynamics when measured in the whole lung. Instead, we observed an effect on
72 individual cell proliferation levels of endothelial cells and AEC type I cells that was
73 accompanied by extensive genetic changes detected at the beginning of alveolar phase. Nicotine
74 also increased lung and parenchyma volume and septal surface area at pnd16. The study of the
75 cell cycle strongly suggest an acceleration in lung maturation. Majority of nicotine-induced
76 changes were lost by pnd16, indicating that these effects are transient, except for the bigger lung
77 volume detected at pnd16, that was no longer present in adult age.

78

79 **LIST OF ABBREVIATIONS**

80	AEC	alveolar epithelial cell
81	AQP5	Aquaporin 5
82	COPD	chronic obstructive pulmonary disease
83	CS	cigarette smoke
84	<i>Csf3</i>	Granulocyte colony stimulating factor 3
85	<i>Dlx5</i>	distal-less homebox 5
86	<i>Dmp1</i>	cyclin D binding myb-like protein
87	ECM	extracellular matrix
88	FACS	Fluorescence-activated cell sorting
89	<i>Fgf23</i>	Fibroblast growth factor 23
90	<i>Hox</i>	Homebox gene
91	HRP	Horseradish peroxidase
92	<i>Igf1</i>	Insulin like growth factor 1
93	<i>Il</i>	interleukin
94	<i>Irg1</i>	Immune-responsive gene 1
95	<i>Ltbp4</i>	Latent Transforming Growth Factor Beta Binding Protein 4
96	MHCII	major histocompatibility complex class II
97	MLI	mean linear intercept
98	<i>Nrp1</i>	Neuropilin 1
99	<i>Orm</i>	Orosomuroid
100	PBS	phosphate buffered saline
101	PCNA	proliferating cell nuclear antigen

102	<i>Pdgf-a</i>	Platelet Derived Growth Factor Subunit A
103	PDPN	Podoplanin
104	Pnd	post-natal day
105	RML	right middle lobe
106	RT	room temperature
107	<i>Serpina</i>	Serpin Peptidase Inhibitor Clade A gene
108	SPC	surfactant protein C
109	TUNEL	terminal deoxynucleotidyltransferase-mediated dUTP nick end
110		labeling

111 MATERIALS AND METHODS

112 *Animals*

113 The animal procedures were performed in accordance with the Institutional Ethics Committee on
114 Animal Care (Geneva, Switzerland) and the Cantonal Veterinary Office (authorization number
115 GE/32/15 and GE/62/14). C57BL/6J mice (*Mus Musculus*) obtained from the animal facility of
116 University of Geneva were kept under specific-pathogen-free conditions. Housing conditions
117 were 12 hours day and 12 hours night. Mice had access to food and drinking water ad libitum.
118 Nicotine (200 mg L⁻¹) was started immediately after mating and administered to the females in
119 drinking water supplemented with 2% saccharin for the whole period of gestation and lactation
120 (nicotine group). Exposure to nicotine was determined by measurements of cotinine
121 (measurements were done according to manufacturer protocol of ELISA kit, #501.301,
122 Immualysis, USA), the main metabolic product of nicotine, in the serum of the mothers and
123 pups upon sacrifice at pnd 2-16. The values obtained for the mothers were 130 ± 88 ng/ml (n=8)
124 and for the pups 70.6 ± 31 ng/ml (n=40), corresponding to values of medium to heavy smoker
125 (36). There was no statistical difference in cotinine values measured at different pnd. As a
126 control, we used drinking water with 2% saccharin (control group). Pups were sacrificed at pnd2,
127 8 and 16 with Esconarkon (150 mg/kg, Streuli Pharma SA, Uznach) and lungs subjected to
128 subsequent analysis. The litter size varied from 2-11 pups, and we did not normalize for it in our
129 experiments. The sex of the pups was determined by testing for the expression of sycp3-like y-
130 linked and X-linked lymphocyte-regulated complex genes via PCR (29). Furthermore, female
131 and male pups were analyzed together as one group as our preliminary experiments and literature
132 suggests no effect of sex on lung development (40). Pups were not weaned at different pnd
133 analyzed as they are normally weaned at pnd21.

134 Lung Instillation procedure was performed as described previously (34). Briefly, the
135 airspace of the lung was filled with solution of 4% paraformaldehyde in phosphate buffered
136 saline (10 mM sodium phosphate, containing 127 mM sodium chloride, pH 7.4) at a constant
137 pressure of 20 cm water column. At this pressure, the lung reaches roughly its total lung capacity
138 (49).

139

140 *Stereological analysis*

141 Lung volume was determined by applying the water displacement method (52). Five μm thick
142 paraffin sections of the embedded right middle lobe (RML) lobes were cut along the longitudinal
143 axis. Equally distributed sections obtained at 7 to 9 positions (depending on lung size) were dried
144 overnight at 37°C and stained with hematoxylin and eosin (26). Eighty to 100 images per RML
145 (i.e. per animal) were acquired using a Leica DM RB light microscope (Glattbrugg, Switzerland)
146 equipped with a motorized Maerzheuser XY stage (Wetzlar, Germany). N PLAN 20x/0.40 PH 1
147 (Leica P/N 506024) objective was used. Images were taken with the ColorView IIIu 5
148 MegaPixel CCD Color Camera (Olympus, Münster, Germany) provided by microscopy imaging
149 center core facility at the University of Bern. Finally, 40 images per animal were selected using a
150 systematic random sampling scheme (4).

151 All the measurements were performed on lung RML. The volume of the RML, volume of the
152 lung parenchyma and septa, septal surface area density and absolute septal surface area were
153 estimated/calculated as described previously in (34).

154 *Gene expression*

155 Offspring lungs were collected at pnd2 and 16, snap frozen in liquid nitrogen and stored at -
156 80°C. RNA was isolated and purified using Macherey-Nagel purification kit (Germany). Six and
157 five offspring lungs were used for pnd2 and 16 in control group and five and three offspring
158 lungs were used for pnd2 and 16 in the nicotine group. cDNA libraries were constructed by the
159 Genomic platform of the University of Geneva using the Illumina TruSeq RNA Sample
160 Preparation Kit (CA, USA) according to the manufacturer's protocol. Libraries were sequenced
161 using single-end (50nt-long) on Illumina HiSeq2500. FastQ reads were mapped to the
162 ENSEMBL reference genome (GRCm38.80) using STAR version 2.4.0j (9) with standard
163 settings, except that any reads mapping to more than one location in the genome (ambiguous
164 reads) were discarded ($m = 1$). A unique gene model was used to quantify reads per gene.
165 Briefly, the model considers all annotated exons of all annotated protein coding isoforms of a
166 gene to create a unique gene where the genomic region of all exons are considered coming from
167 the same RNA molecule and merged together. All reads overlapping the exons of each unique
168 gene model were reported using feature Counts version 1.4.6-p1 (42). Gene expressions were
169 reported as raw counts and in parallel normalized in RPKM in order to filter out genes with low
170 expression value (1 RPKM) before calling for differentially expressed genes. Library size
171 normalizations and differential gene expression calculations were performed using the package
172 edgeR (48) designed for the R software (43). Only genes having a significant fold-change
173 (Benjamini-Hochberg corrected p-value < 0.05) were considered for the rest of the RNAseq
174 analysis. MDS plot was obtained with the package edgeR designed for R. Distances on the
175 Multidimensional scaling plot represent the expression differences between samples (based on
176 fold changes between samples). Data analysis was performed using MetaCore software
177 (<https://portal.genego.com/>). Complete results of RNAseq analysis are deposited in Gene

178 Expression Omnibus (GEO) repository under the following link
179 <http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE102239>.

180

181 *Immunohistochemistry staining*

182 Paraffin-embedded or cryosections of mouse lungs, collected at pnd2, 8 and 16, were stained
183 according to the cell signaling technology standard protocol (<https://www.cellsignal.com/>).
184 Briefly, paraffin-embedded samples were deparaffinized using xylene and 95-100% ethanol and
185 subsequently hydrated in H₂O. We applied the following antigen retrieval methods for single
186 epitope staining: heat-induced epitope retrieval for 10 min in 0.01 mol/L citrate buffer (pH 6.0)
187 for prosurfactant protein C (SPC) antigen and pressure-induced epitope retrieval (20 Bar) in 0.01
188 mol/L citrate buffer (pH 6.0) for Ki67. Endogenous peroxidases were blocked with DAKO
189 peroxidase block solution. Both primary and secondary antibodies were diluted with DAKO
190 antibody diluent. We applied the primary antibodies for 1 hour at room temperature (RT).
191 Finally, labelled polymer-HRP anti-rabbit or anti-mouse (Envision + system, DAKO, USA) was
192 used for 30 min at RT and the signal was visualized with diaminobenzidine (DAB, Envision +
193 system) or 3-amino-9-ethylcarbazole (AEC) (Dako SA, Geneva, Switzerland). Sections were
194 counterstained with Hematoxylin (BioGnost, Zagreb). Quantification of positive cells was
195 performed with Definiens software (Germany) provided by bioimaging core facility at the
196 University of Geneva or ImageJ software (U.S. National Institutes of Health). Images were taken
197 with Axio scan.Z1 (Carl Zeiss, Germany). Quantification of positive cells was performed with
198 Definiens software (Germany) provided by bioimaging core facility at the University of Geneva
199 or ImageJ software.

200 Cryosections were fixed for 15 minutes (min) at RT in 4% paraformaldehyde (PFA), and
201 subsequently washed 3 times for 5 min in PBS. The samples were further incubated in the
202 blocking solutions (5% normal goat serum + 0.3% triton X-100 in PBS) for 1 hour at RT, and
203 then exposed to primary antibodies (PDPN and CD31) diluted in the blocking solution for 1 hour
204 at RT. Sections were than washed with PBS (3x5 min) and exposed to fluorochrome coupled
205 secondary antibodies again for 1 hour at RT. The samples were then again washed with PBS and
206 the nuclei were stained with DAPI (5 min at RT) and slides were mounted with FluorSave™
207 reagent (Millipore). Images were acquired with Zeiss LSM800 confocal microscope with
208 Apochromat objective 63x/N.A. 1.4 oil immersion and further processed by Imaris
209 (<https://imaris.oxinst.com/>) and Huygens ([HuygensSoftware](https://www.scion.com/huygens)) software. For all the information
210 about the primary and secondary antibodies see the section “antibodies and reagents” in the
211 supplemental material.

212 *Measure of cell death*

213 For paraffin sections of fixed lung, terminal deoxynucleotidyltransferase–mediated dUTP nick
214 end labeling (TUNEL) was performed with an *in situ* apoptosis detection kit according to the
215 protocol of the manufacturer (ApopTag®, Chemicon, Temecula, CA). Briefly, 5 µm thick
216 paraffin sections were deparaffinized and then treated with proteinase K (ROCHE, 20 µg/ml
217 30min 37°C) to unmask DNA. The mix containing TdT enzyme and Dig-labelled dNTP in a
218 reaction buffer is added to the sections and allowed to react for 1h at 37°C with the free DNA
219 resulting from strand breaks or fragmentation. dNTP tail is revealed using a horse radish
220 peroxidase (HRP)-coupled anti-DIG antibody and AEC substrate. Sections were counterstained
221 with Hematoxylin (BioGnost, Zagreb). As reported in Immunohistochemistry staining section,

222 Images were taken with Axio scan.Z1 and quantification of positive cells was performed with
223 Definiens software.

224 *Western blot analysis*

225 Cells were lysed with the following buffer: TRIS 50 mM, NaCl 250 mM, Triton X-100 1%,
226 Sodium Deoxycholate 0.5%, SDS 0.1%, pH 8.0. Total protein extract was quantified by means
227 of a bicinchoninic acid protein assay reaction kit (Pierce, Rockford, IL, USA) and 40 µg were
228 separated on 10, 12 or 15% SDS-PAGE (depending of the targeted protein size) and
229 subsequently transferred to a nitrocellulose membrane. The membranes were blocked for 15-30
230 min at room temperature (RT) in a blocking buffer (phosphate buffered saline (PBS), 0.1%
231 Tween 20, 5% bovine serum albumin). Incubation with primary antibodies was performed
232 overnight, at 4⁰C and incubation with secondary antibodies was performed at RT for 1h. Proteins
233 were revealed with chemiluminescence reagents (ProtoGlow ECL, National Diagnostics, USA).
234 Images were quantified using ImageJ software.

235 *FACS analysis of cell cycle*

236 Tissue digestion and isolation of cells from control and nicotine-exposed mouse lungs for FACS
237 analysis was performed as described in Donati et al. (the full technical description is available in
238 the adjacent manuscript). The cells were always used fresh and analyzed by FACS at separate
239 times corresponding to harvest. We used CD31 to mark the endothelial cells and CD326 for the
240 epithelial cells. As described in the adjacent manuscript (method section), with the lung digestion
241 protocol we limited the contamination of bronchial cells, and further focused only on alveolar
242 cells by using markers that stain alveolar cells. To distinguish between type I and II epithelial

243 cells, we used podoplanin (PDPN) and major histocompatibility complex class II (MHCII) as
244 described in Donati et al. (see antibodies section in supplementary information). All the analysis
245 was performed in CD45 negative (CD45^{neg}) population, to exclude blood-derived cells. The cell
246 cycle analysis technique was adapted from Darzynkiewicz et al. (7). Briefly, 4×10^6 labelled cells
247 were washed with PBS and fixed in PBS-0.5% formaldehyde for 15min. the fixative solution
248 was removed by centrifugation, and the cell pellet was resuspended for 30min on ice in 1ml PBS,
249 1% BSA, and 0.1% saponin containing 2ug/ml of Hoechst33342. After washing, cells were kept
250 on ice until flow cytometer processing in 0.5ml PBS, 1% BSA containing 2ug/ml Hoechst33342.
251 Analysis events were recorded on a BD LSR Fortessa and data were analyzed with FlowJo
252 software (Version 10.5.0).

253 *Statistical analysis*

254 Results were expressed as mean \pm standard deviation (sd), except for supplementary Figure 1
255 (mean \pm s.e.m.), and were analyzed in PRISM by either a) 2-way ANOVA multiple t-test
256 analysis with recommended tukey or sidak correction for determining significance between
257 control and nicotine-treated samples at different days or b) nested 1-way ANOVA with tukey
258 correction for determining significance within the control samples at different days. The
259 threshold for the significance was set at $p \leq 0.05$. Any analysis that differs from these described
260 above is noted in the individual figure legend.

261 RESULTS

262 *In utero* exposure to nicotine leads to increased lung size at pnd16

263 To address whether nicotine exposure *in utero* can affect lung morphology, we performed
264 stereological analysis of lungs collected at three different postnatal days, namely pnd2, pnd8 and
265 pnd16. All the analyses were performed on RML. We first examined the lung volume,
266 parenchyma and septal volume, as well as septal surface area in control mice. All examined
267 parameters increased significantly during normal alveolar development (Table 1). Mean linear
268 intercept (MLI), describing the mean free distance in the air spaces, decreased as expected during
269 alveolar development and significantly between pnd8 and 16 (Table 1). We then analyzed
270 nicotine effect on these parameters. Nicotine increased lung and parenchyma volumes as well as
271 septal surface area, slightly but significantly, at pnd16, but not at pnd2 and 8 (Fig. 1A, B, C and
272 E). Septa volume and MLI values were not affected by nicotine (Fig. 1A, D and F).

273

274 Nicotine affects gene profile at pnd2 but not pnd16

275 *In utero* exposure to nicotine has been reported to affect gene expression in the brain (24, 56).
276 We examined the whole lungs by RNAseq analysis at the end of saccular phase (pnd2) and then
277 in the middle of alveolar phase (pnd16). Principal component analysis showed a nice segregation
278 between nicotine and the control samples at pnd2, but not anymore at pnd16 (Fig. 2A). At pnd2,
279 we obtained 3957 genes whose mRNA expression was significantly modified by nicotine.
280 Amongst them, 2098 were upregulated and 1859 downregulated in the nicotine group compared
281 to the control. The top 10 most upregulated and downregulated genes upon nicotine exposure are
282 presented in Table 2. The majority of upregulated genes are bone-secreted proteins that do not
283 normally have a lung function and are involved in bone and muscle energy metabolism.

284 However, some of the genes have been reported to promote lung cancer, such as distal-less
285 homeobox 5 (*Dlx5*), or act as tumor suppressors such as cyclin D binding myb-like protein
286 (*Dmp1*) (20, 27). Among the downregulated top 10 genes, the majority are immune system
287 related and some of them are associated with lung-related diseases such as asthma (*Orosomucoid*
288 *family*, several single nucleotide polymorphisms in ORMDL3 were enriched in asthmatic
289 population)(32), emphysema (*Fgf23*, increased in emphysema and COPD)(23) and COPD (*Csf3*,
290 1719T single nucleotide polymorphism associated with protection against low lung function in
291 COPD patients)(16). Furthermore, 31% of top 100 downregulated proteins are relevant for lung
292 injury repair (for example, *Il22*, *Il17f*, *Irg1*, *Il1b*) (25, 45, 61). Amongst other nicotine
293 downregulated genes is Serpin Peptidase Inhibitor Clade A (*Serpina*) genes whose deficiency has
294 been associated to severe emphysema (31).

295 Pathway analysis for data from pnd2 was performed with MetaCore using threshold 1.5 and
296 significance ≤ 0.05 . Results for the top 10 upregulated and downregulated pathways is shown in
297 Fig. 2B and C. Top 10 pathways upregulated by nicotine can be sorted into several categories:
298 cell cycle, epithelial to mesenchymal transition, stem cell related pathway, WNT signaling
299 pathway, stromal-epithelial interaction, cell adhesion and DNA damage (Fig. 2B). On the other
300 hand, the top 10 of nicotine downregulated pathways are associated with general immune
301 response or asthma associated immune response (Fig. 2C).

302
303 **Kinetics of lung cell proliferation and apoptosis during development are not modified by**
304 **nicotine**

305 First, we addressed the expression of Ki67, normally found only in proliferating cells (Fig. 3A),
306 throughout lung development. We detected a proliferation peak at pnd8. On the other hand,

307 apoptosis analyzed by TUNEL staining showed no significant difference between analyzed days
308 (Fig. 3B). Neither cell proliferation nor apoptosis was modified by nicotine. Similar results were
309 obtained for the other two proliferation markers: cyclins, in particular cyclin A and D, and
310 proliferating cell nuclear antigen (PCNA) (Fig. S1A-F). As we previously showed that nicotine
311 affects the intrinsic apoptotic pathway in mouse epithelial cells (63), we addressed the expression
312 of BCL and BAX molecules, key players in this pathway, at pnd2, 8 and 16 but again confirmed
313 an absence of effect with nicotine (Fig. S2A and B). To understand the increase in lung volume
314 observed with nicotine at pnd16, we further investigated RNAseq results that were linked to cell
315 proliferation/apoptosis. We focused on cell cycle related genes and genes involved in the WNT
316 pathway upregulated at pnd2 and reported to be crucial in the development of distal lung
317 involved in gas exchange (13, 33). We found a stable expression of β -catenin, the central
318 component of canonical WNT signaling pathway, throughout the three analyzed post-natal days,
319 but detected an upregulation of survivin at pnd8, a downstream target of the β -catenin pathway
320 involved in cell cycle and apoptosis regulation (Fig. 3C and D). The expression of both proteins
321 was affected by nicotine, showing a significant increase at pnd2. This effect was again transient
322 and no longer detected at pnd16 (Fig. 3C and D). The survivin peak detected at pnd8 in the
323 control was not present at any of the analyzed days in nicotine samples, suggesting that survivin
324 expression could follow different kinetics or that the peak, observed at pnd8 in the control,
325 occurred earlier (Fig. 3D).

326

327 **Nicotine affects proliferation rates of AEC type I and endothelial cells at pnd8 and 16**

328 As some of the effect caused by nicotine might be cell specific and thereby lost in the whole lung
329 population, we analyzed the cell cycle of individual lung cell types by FACS (see explanation of

330 the analysis in Fig. S3). Characterization of lung alveolar populations is described in detailed in
331 Donati and colleagues (see adjacent manuscript). The major proliferating populations were
332 vascular endothelial cells (CD31^{pos}), mature AEC type II (MHCII^{pos}) and CD31^{neg}CD326^{neg}
333 population containing mesenchymal cells, with the highest values of 10.8, 5.4 and 4.8%,
334 respectively, detected at pnd8 (Fig. 4A, B and D), which follow the same trend as the total lung
335 cell populations analyzed by Ki67 (see Fig. 3A). The population of AEC type I
336 (CD326^{pos}PDPN^{high}) showed low proliferation rates of 0.6% and 0.7% at pnd2 and 8,
337 respectively, which increased to 1.9% at pnd16 (Fig.4C). Nicotine significantly increased the
338 proliferation rate of AEC type I at pnd8 from 0.7 to 1.6% and also decreased significantly the
339 proliferation rate in endothelial cells at pnd8 and 16 from 10.8 to 9% and 3.65 to 1.4%,
340 respectively (Fig. 4A and C). The proliferative value of AEC type II and CD31^{neg}CD326^{neg}
341 population was not affected by nicotine (Fig. 4B and D).

342

343 **Nicotine accelerates early alveolar maturation of AEC type I and II cells**

344 We wished to verify our results on nicotine affected cycling of endothelial and AEC type I cells
345 and analyzed the remaining alveolar population. We therefore stained by IHC mouse lungs at
346 pnd2, 8 and 16 with established alveolar cell type markers, namely PDPN for AEC type I (37),
347 SPC for AEC type II (19) and CD31 for endothelial cells of lung capillaries and vessels (41).
348 Because of their very thin and circle shaped cytoplasm, it is often hard to see the nucleus of
349 endothelial cells of capillaries (Fig. 5B). Furthermore, it was also not easy to adequately match
350 individual nuclei with membranous PDPN staining and therefore quantify the AEC type I cells
351 by IHC (Fig. 5B). We were only able to quantify the SPC staining (Fig. 5A).

352 The density of AEC type II (SPC^{pos}) remained stable throughout all the pnds between 4 and
353 $6 \times 10^4/\text{cm}^2$. Nicotine had no effect on the amount of SPC stained cells at any of the days analyzed
354 (Fig. 5A).

355 Determination of percentages of endothelial and AEC type I cells was based on FACS method
356 published by Donati and colleagues (see Materials & Methods section in adjacent manuscript).
357 Furthermore, we used the same approach to address the percentages of other major alveolar cell
358 types, such as Immune ad mesenchymal cells.

359 We found that during development, nicotine caused a significant increase in the number of
360 CD45^{pos} cells, reflecting marginated immune cells (2), and therefore causing a relative decrease
361 in CD45^{neg} cells at pnd2 and 16 (Fig. 6A). The CD31^{neg}CD326^{neg} population, that should contain
362 the mesenchymal cells decreased towards pnd16 and was not affected by nicotine (Fig. 6B and
363 Table 3). We further found a linear increase of vascular endothelial cells during alveolarization,
364 from 7.7% at pnd2 to 15.5% at pnd16 (Fig. 6C and Table 3). At pnd2 nicotine caused an increase
365 in the percentage of endothelial cells compared to control (Fig. 6C). As suggested by Donati and
366 colleagues (see adjacent manuscript), the smaller percentage of AEC type I isolated at pnd8 and
367 pnd16 compared to pnd2 probably reflects lung maturity. Indeed, in a more mature lung AEC
368 type I form cell junctions with the extracellular matrix (ECM) and neighboring cells and
369 isolation of these cells becomes more difficult by FACS. In fact, the highest yield of 8.1% for
370 AEC type I at pnd2 had drastically dropped by pnd16 (Fig. 6D and Table 3). Nicotine induced a
371 significant decrease of the number of extractable AEC type I to 6.2% at pnd2 and 1% at pnd8,
372 suggesting that nicotine accelerates lung maturation (Fig. 6D).

373 As expected the mature type II population, detected by the presence of MCHII, increased
374 significantly during alveolarization from 0.4% at pnd2 to 34.8% at pnd16 (Fig. 6E and Table 3).

375 Here again, we found a significantly higher percentage of mature AEC type II in nicotine-treated
376 lungs at pnd8 (from 18.55 to 25%, Fig. 6E), a finding that goes along with nicotine-induced
377 increase in lung developmental kinetics.

378 **DISCUSSION**

379 Nicotine is one of the main components of CS that affects early lung development (44).
380 Animal studies associated nicotine exposure to decreased airways diameter, enlarged airspace or
381 faster lung development, depending on the time frame and concentration of exposure, and also to
382 the species exposed (51, 60). However, studies looking at alveolarization are rare and
383 controversial (28, 54, 62).

384 The purpose of this study was a) to analyze the normal alveolar lung development and b)
385 to assess the effect of nicotine exposure during gestation and lactation on the alveolar phase of
386 lung development.

387 In control mice we observed a cell proliferation peak at pnd8 during lung alveolarization,
388 confirmed by the analysis of three different proliferation markers: Ki67, PCNA, and cyclins
389 (cyclin A and D). Furthermore, levels of survivin, a downstream molecule in the WNT pathway
390 and involved in proliferation, also peaked at pnd8 (57). Levels of apoptosis were constant and
391 low at all analyzed days. The three most abundant proliferating populations, vascular endothelial
392 cells, mature AEC type II and CD31^{neg}CD326^{neg} containing mesenchymal cells, determined by
393 FACS, also showed a cell cycle peak at pnd8. AEC type I showed the lowest level of
394 proliferation, reaching 1.9% at pnd16. These data are in agreement with those published by Yang
395 and colleagues suggesting that AEC type I retain some degree of proliferative potential (62).
396 Because detection of AEC type I by IHC is not accurate, as due to their elongated shape is
397 sometimes hard to match the stained cell with its nuclei, our FACS results are all the more
398 interesting as they enabled specific identification of AEC type I (see adjacent manuscript).
399 Concurrently with lung maturation and extension of the capillary bed during the alveolar phase,
400 vascular endothelial cells and mature AEC type II increased in percentage from pnd2 to pnd16.

401 The percentages of AEC type II were between 5 and $7 \times 10^4/\text{cm}^2$ of the lung (Fig. 5A), which is
402 significantly lower than reported for adult rat and human lung (12-16%) (38). As it has been
403 suggested that the alveolar phase lasts until young adulthood, it is probable that the percentage of
404 AEC type II reported at pnd16 was not final and might increase further (54). Finally, we were
405 not able to determine the total amount/percentage of AEC type I due to the limitation of available
406 techniques. The percentage of AEC type I isolated at pnd2 (8.1%) and the corresponding low
407 levels of cell cycling at all measured days suggest that the percentage detected at pnd2 is stable
408 throughout alveolarization. The reported percentages of AEC type I in adult rat or human lung
409 are 8.1% and 8.3% respectively, and are within the range of data described in this manuscript
410 (38). Finally, stereological analysis, reported for RML, nicely reflected lung maturation during
411 alveolar development and showed an increase of both, lung and parenchyma volume, as well as
412 septal surface area from pnd2 to pnd16. On the other hand, mean linear intercept decreased
413 during alveolarization, as expected, due to the process of lung septation. Values for lung RML
414 volume range from 10 μl at pnd2 to 20 and 40 μl at pnd8 and 16, respectively. This is in line
415 with values reported by Yang and colleagues, ranging from 90 to 100 μl for the entire left lung at
416 pnd14 (62). Furthermore, the values obtained for MLI in our study are similar to those published
417 by Pozarska and colleagues (40).

418 We then assessed the effects of nicotine exposure on offspring's lung development.
419 Nicotine is very unstable and is indirectly measured by levels of cotinine, the main metabolic
420 product of nicotine. There is no clear consensus on the levels of cotinine reported for passive
421 smokers; the values vary from study to study and tissue analyzed (saliva, blood or urine). The
422 values range between 5 (measured in saliva) and 30 ng/ml (measured in urine) (12, 14). In our
423 study, the values for cotinine were 130 ± 88 ng/ml, measured in gestant mice and 70.6 ± 31

424 ng/ml measured in the pups after birth. Based on significantly higher level of cotinine detected in
425 gestant mice, one might expect the highest effect of nicotine at pnd2. However, this trend was
426 confirmed only for the results of RNAseq analysis, where we observed extensive genetic changes
427 induced by nicotine at pnd2 affecting the cell cycle, the canonical WNT pathway, cell adhesion
428 and immune system and for the percentage of endothelial cells. Other parameters, such as
429 percentages of other lung populations and the analysis of their cell cycle obtained by FACS were
430 also affected at later analyzed days. Nevertheless in almost all analyses, the effect of nicotine
431 was no longer present by pnd16, except for the “bigger lung” that was no longer observed in
432 adult mice (Fig. S4). Taken together, these data suggest, that while nicotine induces a variety of
433 changes, these changes are not permanent. Such a transient effect was reported previously, in
434 rats, in the study by Petre et al, where nicotine induced initial decrease in the airspace size
435 immediately after the rats are born, that was followed by a catch-up increase detected in 3 weeks
436 old rats and complete loss of the effect by week 12 after birth (39).

437 Several results from our FACS analysis hint towards nicotine-induced shift in phase and
438 acceleration of lung maturation, a hypothesis already brought forward by Sandberg and
439 colleagues in 2004 (51), although metabolic pathways have not been addressed in our study.

440 First, we detected a nicotine-induced increase in percentages of vascular endothelial cells at pnd2
441 and mature AEC type II at pnd8 (Fig. 6C and E). In both cases, the control lung eventually
442 reached the same values by pnd8 and 16 respectively. Second, in spite of nicotine-induced
443 increase in cell cycle detected for AEC type I cells at pnd8, we could isolate lower amount of
444 this cell type at both pnd2 and 8. As explained in Donati et al (see adjacent manuscript), the
445 isolation of AEC type I cells becomes more difficult with aging because a) they undergo
446 extensive structural changes during development, including cell flattening, elongation and

447 folding (62) and b) they develop an increasing number of cell-cell and cell-ECM interactions.
448 We have confirmed this phenomenon in control mice, where after pnd4-5 the percentage of AEC
449 type I isolation drastically drops (data not shown). Therefore, we believe that the lower isolation
450 efficiency of AEC type I at pnd2 and 8 from nicotine-exposed lungs reflects the increased
451 maturation of this lung structure. Third, nicotine-induced increase in lung volume, observed at
452 pnd16, did not persist in adult age. Such transient effect of nicotine on lung volume is consistent
453 with the shift in phase hypothesis. Forth, results from RNAseq analysis at pnd2 showed nicotine-
454 induced upregulation of genes involved in alveolarization, such as genes related to elastin
455 deposition (insulin like growth factor 1 (*Igf1*) and its receptor, *Ltbp4*, fibrilin) and septa
456 formation (*Hox*, *Pdgf-a*, *Nrp1*) (3, 11, 15, 22, 30). Finally, two genes involved in different stages
457 of lung development were upregulated in our transcriptomic analysis from nicotine samples at
458 pnd2. IGF1 receptor, whose absence is associated with delayed saccular development (11) and
459 WNT5a related to alveolar development in mice. All these results could support the hypothesis
460 of nicotine-induced lung maturation. Furthermore, ECM proteins such as collagens, known to be
461 involved in cell shape regulation, were also upregulated in nicotine samples. Despite the fact that
462 gene up- or downregulation disappeared at pnd16, changes in lung cell dynamics is of
463 importance in the context of *in utero* and early life programming, leading to increased
464 susceptibility of adults to lung and non-lung diseases, a concept suggested as “fetal origin
465 hypothesis” by Barker and Osmond (1). In line with this, WNT signaling pathway, already
466 shown to be affected by nicotine in various lung cell lines (50, 64, 65) as well as *in vivo* in mice
467 (6) was also reported to be involved in maintaining AEC type II stemness. In particular, Nabhan
468 and colleagues, found that high levels of WNT pathway maintained the progenitors pool while
469 the decrease in WNT lead to transdifferentiation of this pool into a mature AEC type I (35).

470 Therefore, nicotine effect on alveolar stem cell niche via WNT as well as nicotine-induced
471 transcriptomic changes in genes such as Serpina involved in emphysema and genes related to
472 lung injury might contribute to a higher susceptibility to diseases in adulthood. This hypothesis
473 remains an open question and requires further research. Finally, understanding the impact of
474 nicotine vs CS is required in the context of the increasing interest in CS replacement therapies.

475 **CONCLUSIONS**

476 Our study describes the dynamics of cell cycle and lung alveoli composition during the
477 developmental alveolar phase and identifies three main types of cells responsible for the
478 proliferation peak observed at pnd8, namely vascular endothelial cells, mature AEC type II, and
479 CD31^{neg}CD326^{neg} population containing, amongst others, mesenchymal cells. We found that
480 nicotine induces a variety of strong effects immediately after birth that transiently increase the
481 kinetics of lung development, and are then lost by pnd16. Finally, we also found that nicotine
482 decreases the level of cell cycle of vascular endothelial cells at pnd8 and 16, thereby affecting
483 one of the cell types involved in gas exchange. Even so the morphological changes do not seem
484 to persist beyond pnd16, prenatal exposure to nicotine as well as to other compounds such as
485 alcohol, stress, famine or toxicants contained in CS might result in a poor health outcome in
486 adulthood. Indeed, epigenetic changes in different genes have been reported for nicotine. It will
487 be important to understand whether this effect persists during the whole alveolar development
488 phase and also later in life, and to address whether these subtle and transient changes detected at
489 the beginning of alveolarization can leave a more permanent mark and affect lung health later in
490 life.
491

492 **ACKNOWLEDGEMENTS**

493 We thank Eveline Yao for expert technical assistance and Stephan Tschanz for writing the
494 software STEPanizer (www.steapanizer.com), the tool used for the stereological counting, and for
495 helpful discussions. We also thank the Immunohistochemistry laboratory of the Pathology
496 department, HUG, for their advices and help. We thank Genomic and Bioimaging core facilities
497 of the Faculty of Medicine, University of Geneva, for their assistance and technical expertise.
498 Finally, we thank Aliko Buhayer, Prism Scientific Sàrl (www.prismscientific.ch), for scientific
499 and English writing support.

500 This work was supported by grants to Constance Barazzone-Argiroffo (Swiss National Science
501 Foundation grant 310030-159500/1) and to Johannes Schittny (Swiss National Science
502 Foundation grant 310030_175953), the Swiss Lung Liga and the Ligue pulmonaire Genevoise
503 and the OPO-stiftung.

504

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- 693

694 **FIGURE LEGENDS**

695 Figure 1. Morphology and stereological analysis of pup lungs. Stereological analysis
696 was performed on H&E lung sections of post-natal day (pnd) 2, 8 and 16 from control
697 and nicotine group (A). lung volume (B), parenchyma volume (C), septal volume (D),
698 septal surface area (E) and mean linear intercept (MLI, F) were measured at pnd2, 8
699 and 16. Control/Nicotine group; For pnd2 and 16 n=5 animals was used for each
700 group (for pnd2, 2 females (F), 2 males (M) and one not determined in the control
701 group and 3M and 2F in the nicotine group; for pnd16, 5M in the control group and
702 3F and 2M in the nicotine group), for pnd8 n=3 animals for each group (2F and 1M in
703 the control group and 1F and 2M in the nicotine group). Results are presented as
704 individual plots and mean values \pm SD. * $p < 0.05$; *n.s.* not significant. The comparison
705 was done between control and nicotine group for each time point with two way
706 ANOVA and Bonferroni correction.

707

708 Figure 2. Comparison of RNAseq analysis of lung extracts of control and nicotine
709 exposed mice. Analysis of RNAseq data, was done at pnd2 (n=6 animals for control
710 group (4F and 2M) and n=5 animals for nicotine group (3F and 2M)) and pnd16 (n=5
711 animals for control group (2F and 3M) and n=3 animals for nicotine group (2F and
712 1M)) and is represented as a two-dimensional MDS plot with samples in red for the
713 control group and in purple for the nicotine group (A). Pathway analysis performed at
714 pnd2 with MetaCore software (with threshold of two and significance $p < 0.05$) is
715 shown in (B) for nicotine-induced pathways and in (C) for nicotine-downregulated
716 pathways.

717 Figure 3. Nicotine upregulates canonical WNT/ β -catenin signaling pathway. Lung
 718 tissues were processed at pnd2, 8 and 16 from control and nicotine group and
 719 analyzed by immunohistochemistry (A and B) and western blot (C and D). To address
 720 cell proliferation we measured Ki67 expression (A, n=4-11 animals/pnd/condition)
 721 and for apoptosis we used TUNEL staining (B, n=4-8 animals/pnd/condition). WNT
 722 pathway was accessed by measuring the expression of β -catenin (C, n=3
 723 animals/pnd/condition) and survivin (D, n=3 animals/pnd/condition). Actin was used
 724 as a loading control in western blot analysis (C and D).* significance for nicotine
 725 treatment vs. control between different pnds. #significance pnd16/8 vs pnd2; †
 726 significance pnd16 vs pnd8; #####/†††† p<0.0001; *n.s.*, not significant (A and B). The
 727 average of separate western blot experiments is shown. p<0.05; *n.s.*, not significant. T-
 728 test analysis was performed between control and nicotine group for each time point (C
 729 and D).

730

731 Figure 4. Nicotine affects cell cycling of vascular endothelial cells and AEC type I.
 732 Cells were isolated from mouse lung at pnd2, 8 and 16 and percent of cycling cells
 733 was determined by Hoechst staining via FACS in different cell populations: vascular
 734 endothelial cells (CD31^{pos}) (A), mature AEC type II cells (CD326^{pos}MHCII^{pos}) (B),
 735 AEC type I cells (CD326^{pos}PDPN^{high}) (C) and mesenchymal cells (CD31^{neg}CD326^{neg})
 736 (D). n = 5-10 animals/condition and pnd were used for the analysis. Graphical
 737 representation of the data are shown; * significance for nicotine treatment vs. control
 738 between different pnds. #significance pnd16/8 vs pnd2; † significance pnd16 vs pnd8;
 739 */# p<0.05; ###/†† p<0.01; #####/†††† p<0.0001; *n.s.* not significant.

740

741 Figure 5. IHC of alveolar cells in nicotine-exposed and non-exposed pups. Lung
 742 paraffin sections of the control and nicotine-exposed pups from pnd2, 8 and 16 were
 743 stained with IgG antibody control (upper panel) and AEC type II marker (SPC)
 744 (middle and lower panel)(A). Graphical quantification for this marker is also shown in
 745 (A). SPC positive cells at pnd2 and 16 are indicated with red arrows. Analysis was
 746 performed on 10 different fields/lung (dimensions 210 μ m x 110 μ m) and 3-5
 747 animals/group. Results are presented as total number of positive cells per cm² of lung
 748 tissue. * significance for nicotine treatment vs. control between different pnds.
 749 #significance pnd16/8 vs pnd2; † significance pnd16 vs pnd8; */# p<0.05; ###†† p<0.01;
 750 ### p<0.001; *n.s.* not significant. Lung cryosections of the control pups from pnd2 and
 751 16, stained for AEC type I marker PDPN and the marker of vascular endothelial cells,
 752 CD31, are shown in (B). The first column represents staining with IgG control, and
 753 the middle and last column show staining with the corresponding antibodies.

754

755 Figure 6. FACS isolation of alveolar cells during lung development in nicotine-
 756 exposed and non-exposed pups. Cells were isolated from mouse lung at pnd2, 8 and
 757 16 and sorted by FACS based on expression of CD45 marker. CD45^{neg} population (A)
 758 was further analyzed for the expression of following markers: mesenchymal cells
 759 (CD31^{neg}CD326^{neg}) (B), CD31^{pos}PDPN^{neg} (vascular endothelial cells) (C),
 760 CD326^{pos}PDPN^{high} (AEC type I cells) (D) and CD326^{pos}MHCII^{pos} (mature AEC type
 761 II cells) (E). n = 5-10 animals/condition and pnd were used for the analysis. Graphical
 762 representation of the data are shown; * significance for nicotine treatment vs. control
 763 between different pnds. #significance pnd16/8 vs pnd2; † significance pnd16 vs pnd8;
 764 */# p<0.05; ### p<0.001; ****/#####††††† p<0.0001; *n.s.* not significant.

765

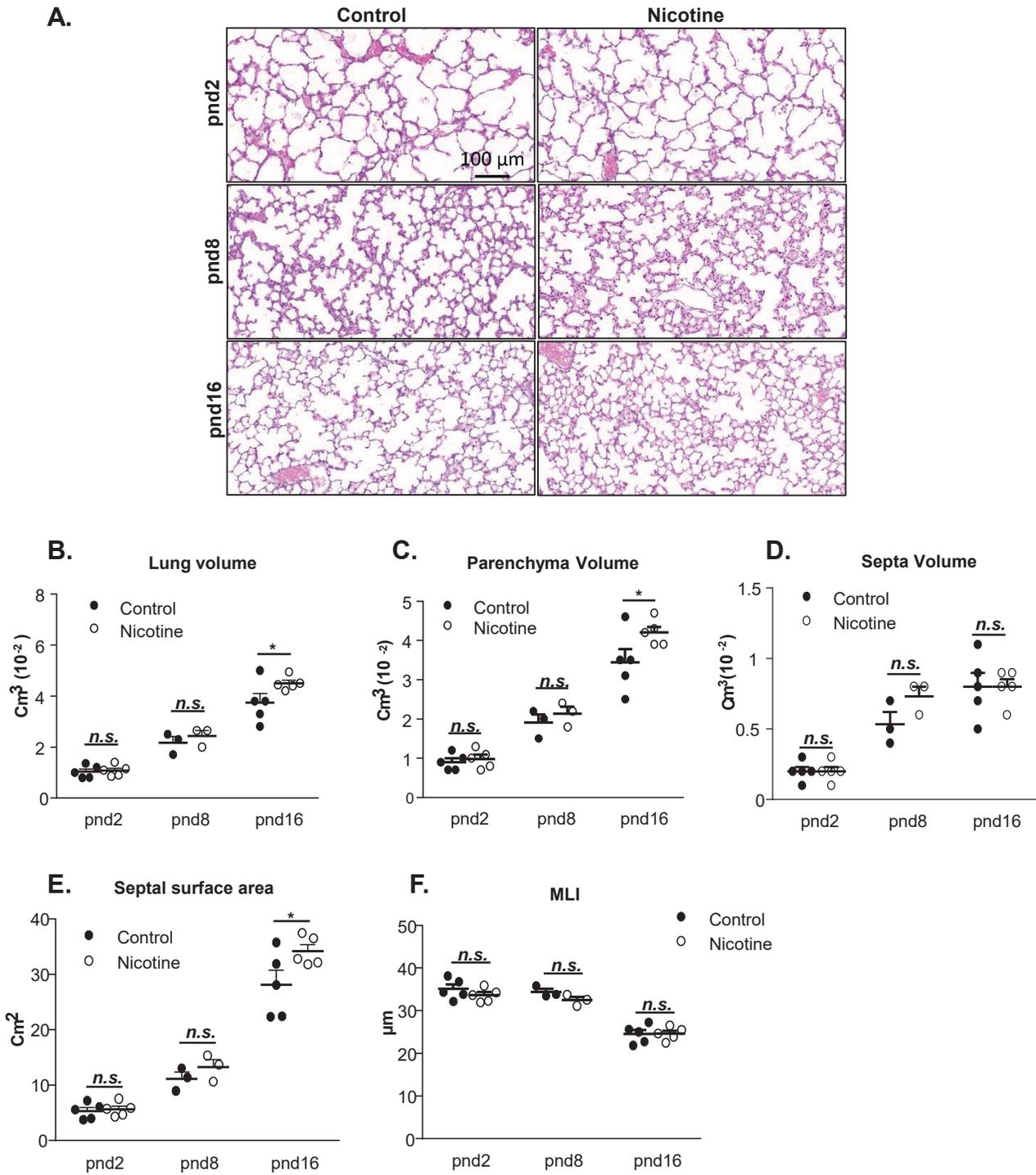
766 **SUPPLEMENTAL MATERIAL**

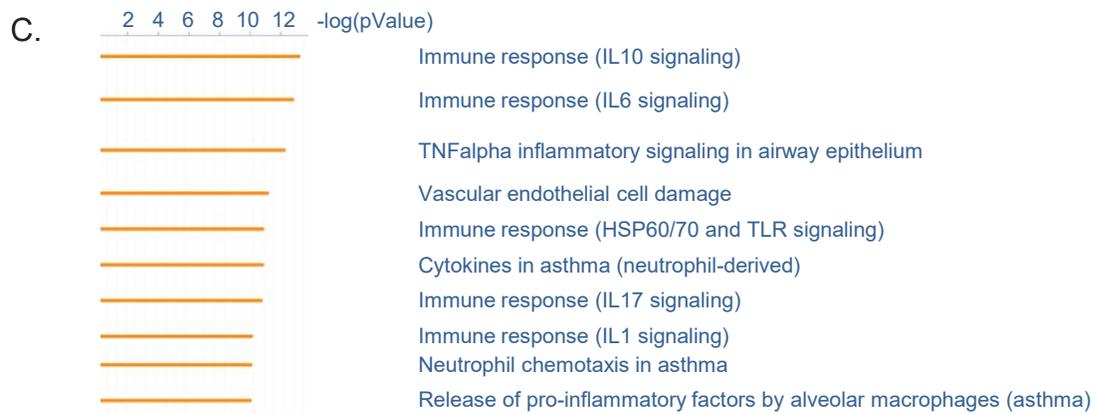
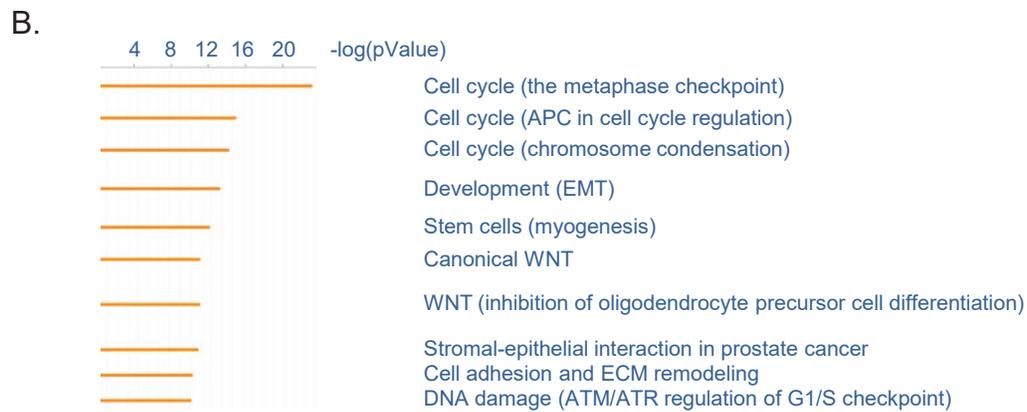
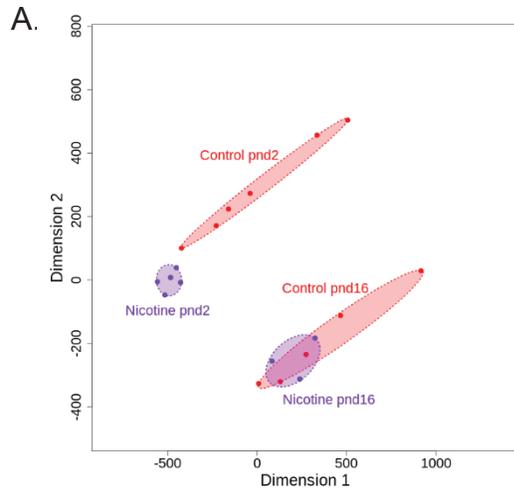
767 The supplemental material (Figures S1 to S4 and the list of antibodies and reagents) is

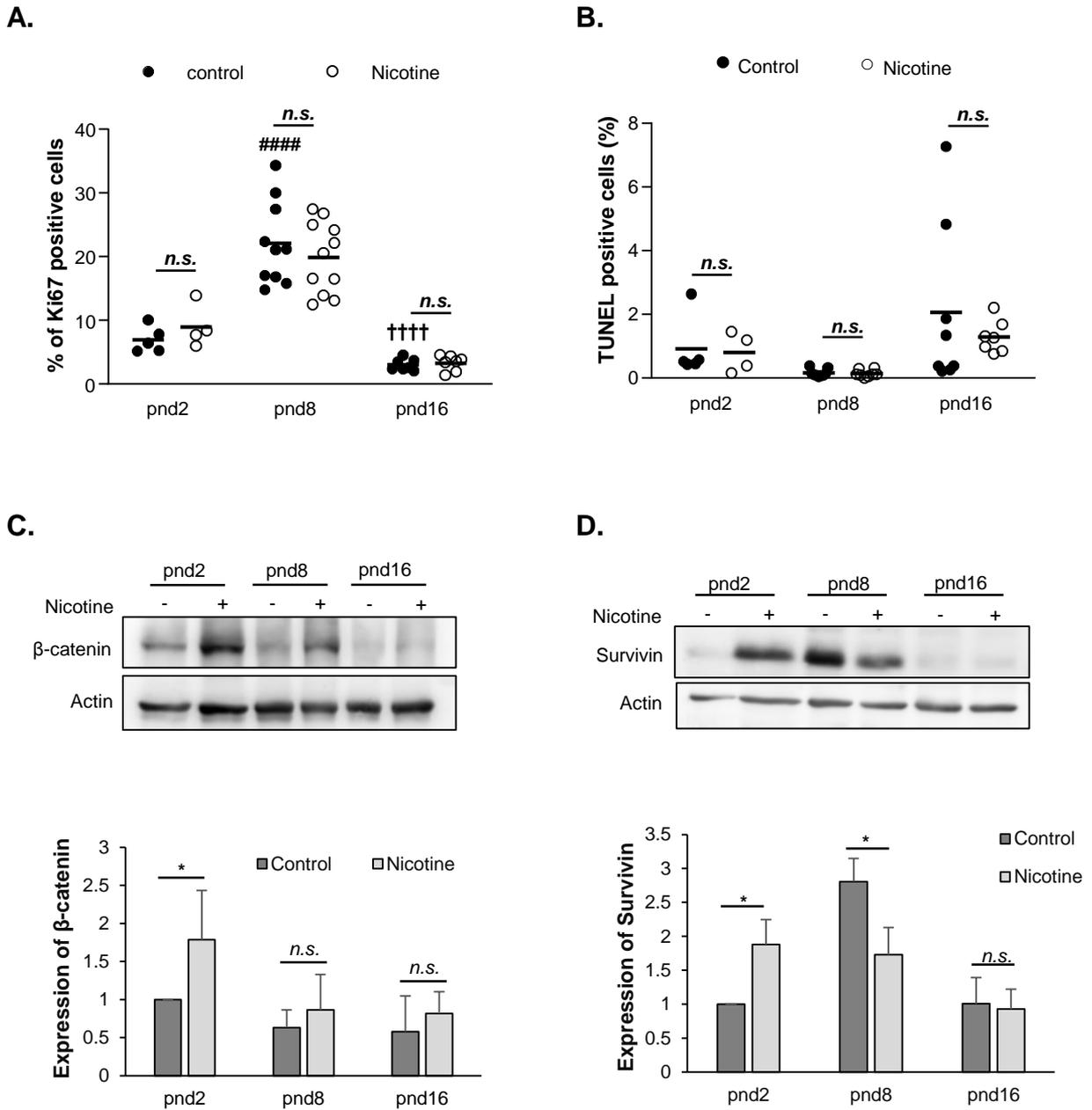
768 deposited in a generalist public access repository figshare

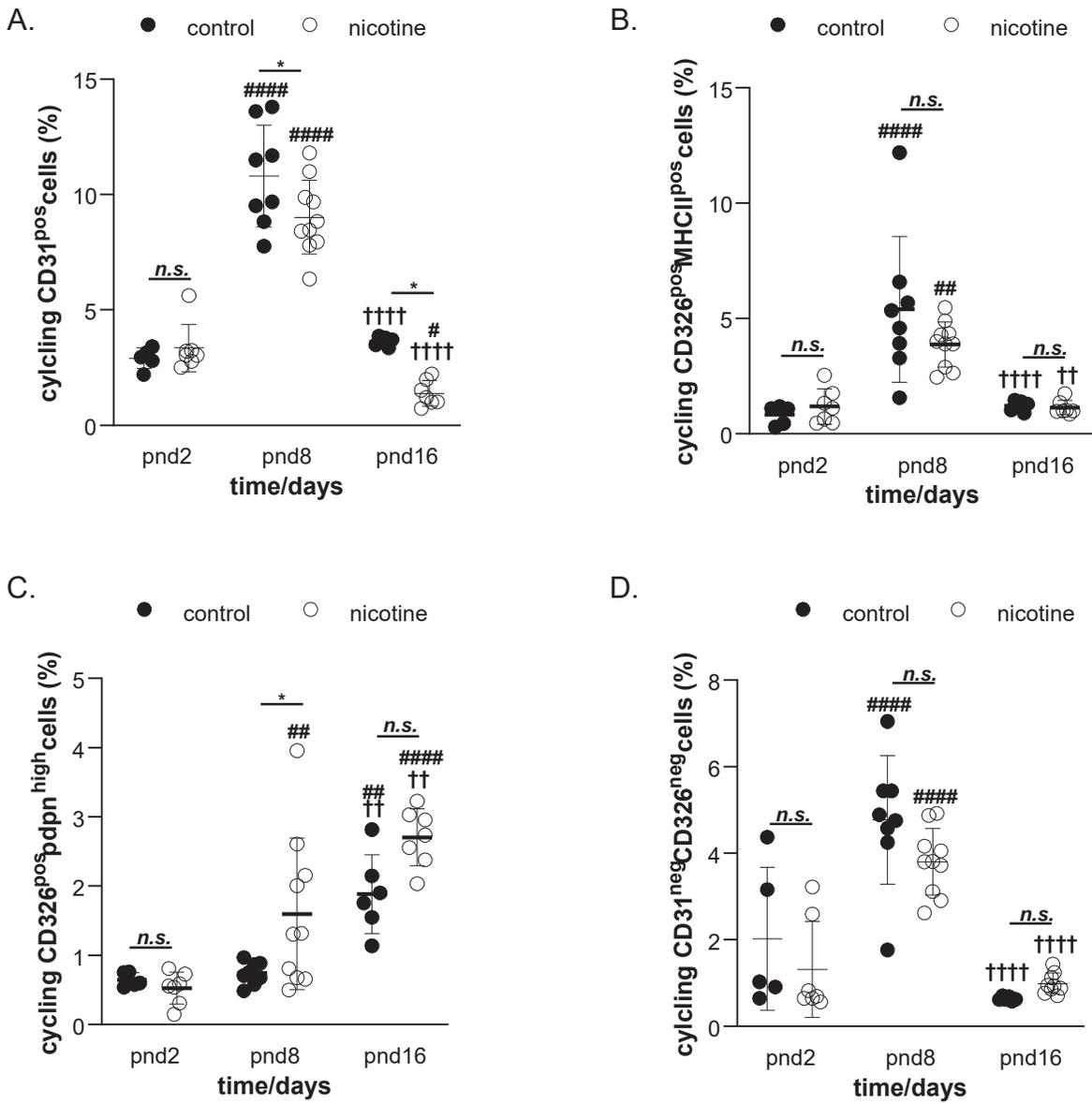
769 (<https://figshare.com/s/56282a06102729a7ee90>) (DOI:[10.6084/m9.figshare.8201696](https://doi.org/10.6084/m9.figshare.8201696)).

770

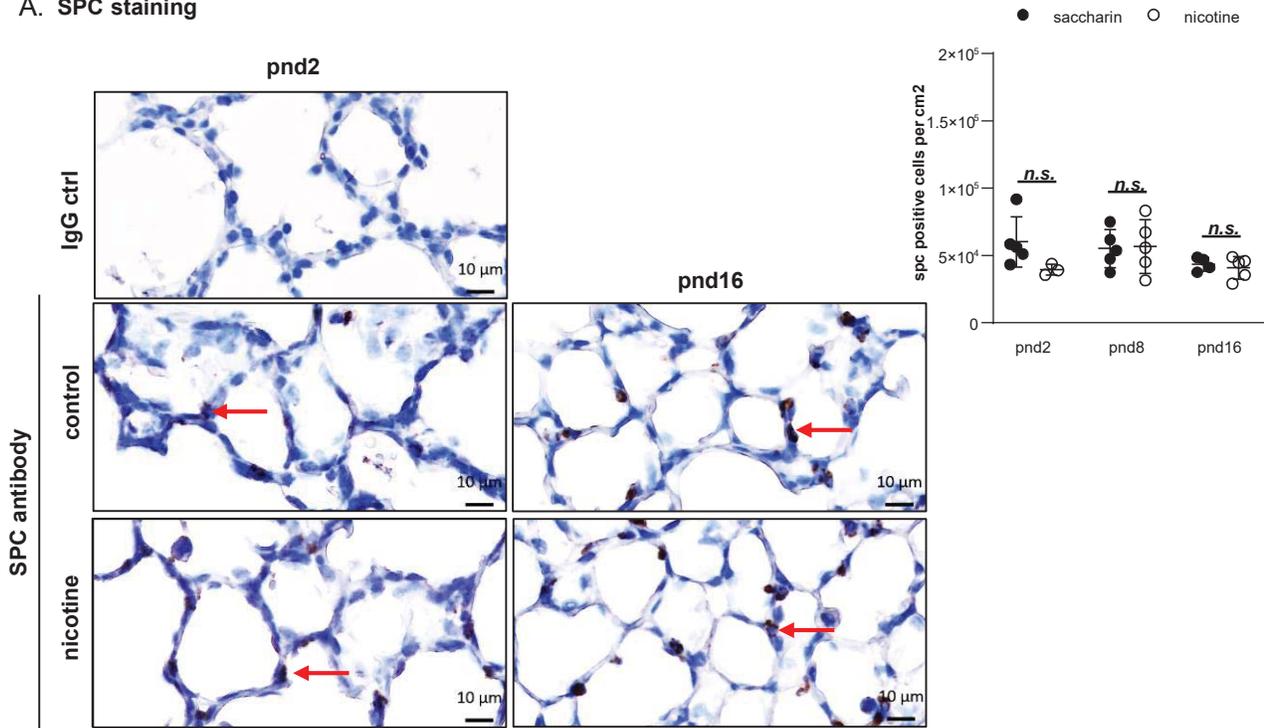




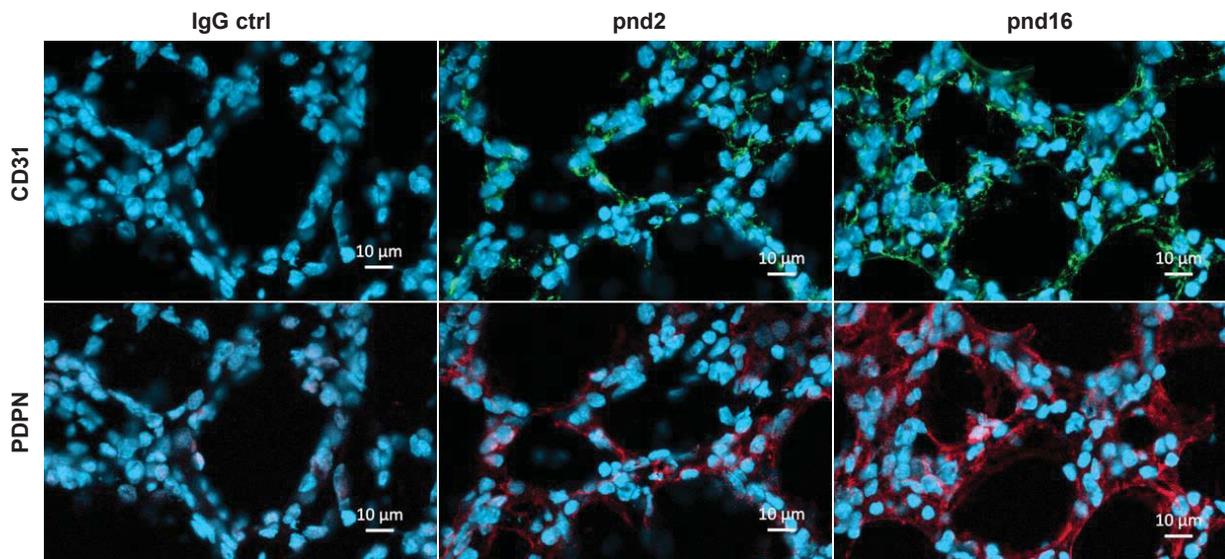


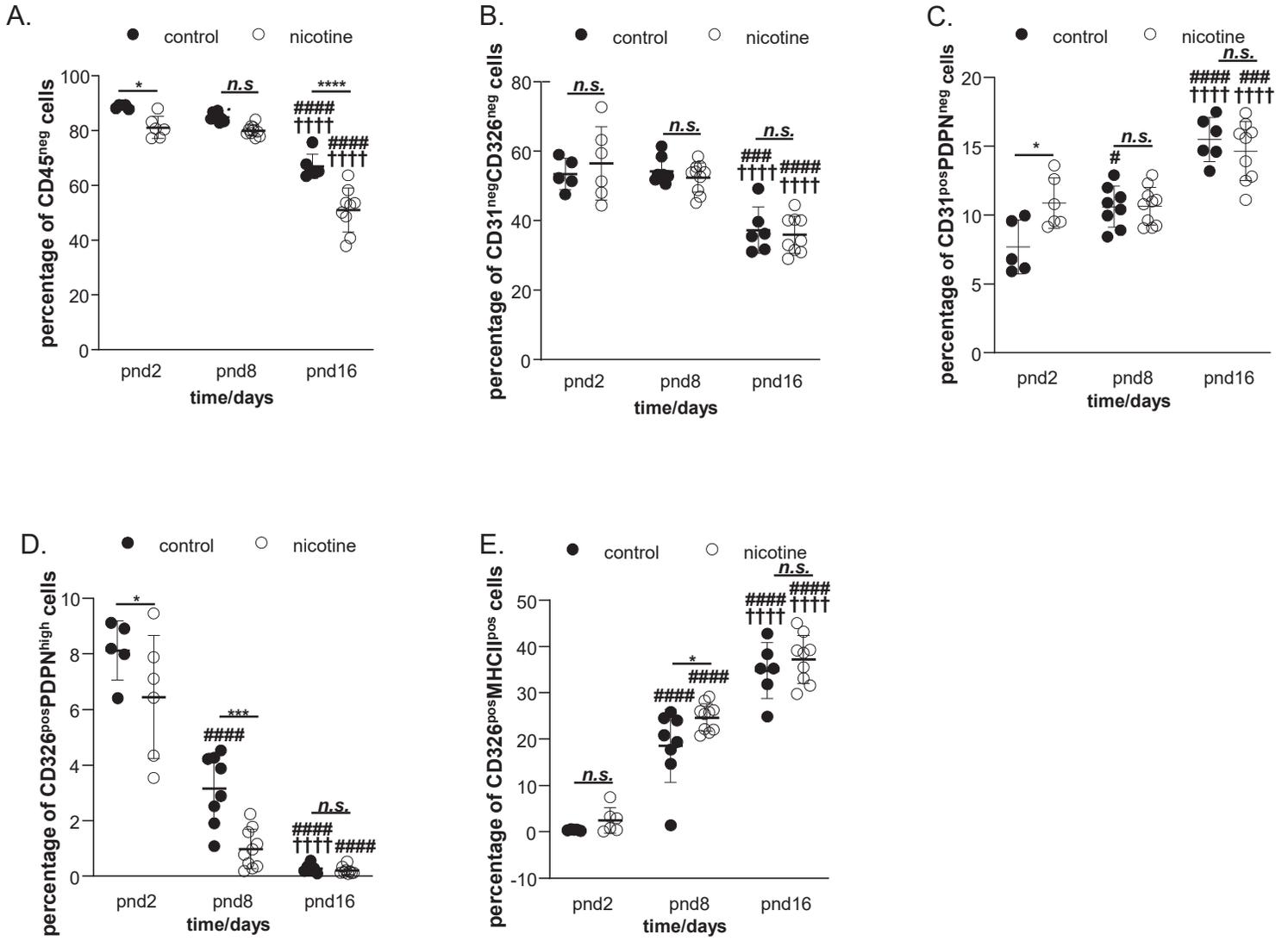


A. SPC staining



B. PDPN and CD31 staining





TABLES

Table 1. Lung morphometry of mouse lungs analyzed from pnd2 to pnd16

Morphometry	control			P^a	P^b	P^c
	pnd2 (n=5)	pnd8 (n=3)	pnd16 (n=5)			
Lung volume (cm ³)	0.01 ± 0.002	0.022 ± 0.004	0.04 ± 0.008	0.0513	<0.0001	0.0092
Parenchyma volume (cm ³)	0.009 ± 0.002	0.019 ± 0.004	0.03 ± 0.008	0.0638	<0.0001	0.0066
Septa volume (cm ³)	0.002 ± 0.001	0.005 ± 0.001	0.008 ± 0.002	0.0458	0.0005	0.1128
Septal surface area (cm ²)	5.3 ± 1.4	11.2 ± 2.1	28.15 ± 5.9	0.1566	<0.0001	0.0004
Mean linear intercept (μm)	35.1 ± 2.4	34.4 ± 1.2	24.5 ± 2.2	0.8916	<0.0001	0.0002

pnd = post-natal day;

Mean values ± SD are reported.

P Pairwise statistical comparison one-way ANOVA with tukey correction was performed between day 2 and 8 (P^a), day 2 and 16 (P^b), and day 8 and 16 (P^c)

Values reported are for lung right middle lobe.

Table 2. Top 10 of UP- and DOWN-regulated genes upon nicotine *in utero* exposure at pnd2

Symbol	Gene name	Function	Lung-related function	FC
Bglap	Osteocalcin	Bone remodeling/energy metabolism	N/A	316
Mepe	Matrix Extracellular Phosphoglycoprotein	Bone ECM	N/A	274
Bglap2	Bone Gamma-Carboxyglutamate Protein 2	Bone remodeling/energy metabolism	N/A	180
Dlx5	Distal-Less Homeobox 5	Osteoblast differentiation	Oncogene in lung cancer	175
Dmp1	Cyclin D binding myb-like protein 1	P53 pathway regulator	Lung cancer	124
Myoz3	Myozenin 3	Regulator of calcineurin	N/A	119
Atp1b4	ATPase Na ⁺ /K ⁺ Transporting Family Member Beta 4	Regulation of active transport	N/A	81
Ostn	Osteocrin	Glucose metabolism in skeletal muscle/osteoblasts differentiation	N/A	80.5
Myh2	Myosine heavy chain	Muscle contraction	pneumonia	64
Ctrnd	Cholinergic receptor Nicotinic Delta Subunit	Acetylcholine/nicotine receptor	Lung cancer	63
Fgf23	Fibroblast growth factor 23	Phosphate and vitamin D metabolism	Fgf-23 ^{-/-} mouse exhibit premature aging and lung emphysema	-606.5
Csf3	Granulocyte colony stimulating factor 3	production, differentiation, and function of granulocytes	Lung metastasis ;	-219
8030474K03Rik		N/A	N/A	-196
Il22	Interleukin 22	Innate and adaptive immune response	Lung epithelial repair after injury	-178.5
Cxcl2	C-X-C Motif Chemokine Ligand 2	Angiogenic chemokine	Regulation of airway smooth muscle cell migration	-148.4
Irg1	Aconitate Decarboxylase 1	Inhibitor of toll-like receptor-mediated inflammatory response	Suppression improves immune lung injury after RSV infection by reducing ROS production	-132
Il17f	Interleukin17f	Innate and adaptive immune response	Protection from bacteri/fungi and involved in repair	-129
Orm2	Orosomuroid 2	modulating immunity, maintaining the barrier function of capillary, sphingolipid metabolism	asthma	-96
Orm3	Orosomuroid 3	modulating immunity, maintaining the barrier function of capillary, sphingolipid metabolism	asthma	-92
Orm1	Orosomuroid 1	modulating immunity, maintaining the barrier function of capillary, sphingolipid metabolism	asthma	-90

FC = fold change

Table 3. Relative abundance of alveolar cells analyzed by FACS during lung development from pnd2 to pnd16

Cell type %	control			P^a	P^b	P^c	F(DFn, Dfd)
	pnd2 (n=5)	pnd8 (n=8)	pnd16 (n=6)				
CD45 ^{neg}	88.8 ± 0.8	84.9 ± 1.5	67 ± 4.5	0.1362	0.0004	0.0006	104(2,4)
CD45 ^{neg} CD31 ^{neg} CD326 ^{neg}	53.4 ± 4.5	54.25 ± 3.7	37.3 ± 6.6	0.9898	0.0300	0.0194	13.2(2,4)
EC (CD31 ^{pos} PDPN ^{neg})	7.7 ± 1.9	10.6 ± 1.5	15.5 ± 1.6	0.0788	0.0034	0.0125	31(2,4)
AEC type I(CD326 ^{pos} PDPN ^{high})	8.1 ± 1.1	3.2 ± 1.3	0.3 ± 0.2	0.0049	0.0011	0.0271	54.3(2,4)
AEC type II(CD326 ^{pos} MHCII ^{pos})	0.4 ± 0.1	18.55 ± 7.9	34.8 ± 6.1	0.0659	0.0082	0.0584	18.4(2,4)

Percentages of endothelial and epithelial populations are expressed as mean ± SD after exclusions of CD45^{pos} population.

Nested one-way ANOVA was performed to determine statistical significance.

P Pairwise statistical comparison one-way ANOVA with Turkey correction was performed between day 2 and 8 (P^a), day 2 and 16 (P^b), and day 8 and 16 (P^c); degrees of freedom are reported with degrees of freedom numerator (DFn) and denominator (DFd) values.

pnd = post-natal day; EC = endothelial cells; PDPN = podoplanin; MHCII = major histocompatibility complex class II