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2 TITLE: **Neonatal endotoxin stimulation was associated with long- term innate**
3 **immune markers and an anti-allergic response in bronchiolar epithelium in spite**
4 **of allergen challenge**

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21 **ABSTRACT**

22 Asthma is a heterogeneous disease underlying different medical processes, being the
23 allergic asthma, with an early-onset in childhood, the most common type. In this
24 phenotype, the continuous exposure to allergens produces a Th2-driven airway
25 remodeling process that leads to symptoms and pathophysiological changes in asthma.
26 Strategies as the avoidance of aeroallergen exposure in early life have been tested to
27 prevent asthma, without a clear success. Alongside, several mouse models of
28 aeroallergen challenge have dissected potential homeostatic responses by which
29 environmental microbial stimulation reduces the subsequent allergic inflammation in
30 the offspring. This suggests the onset of underlying preventive mechanisms in the
31 beginning of asthma that have not been fully recognized. In this study, we aimed to
32 evaluate if neonatal LPS-induced stimulus in epithelial host defenses could contribute to
33 the prevent asthma in adult Balb/c mice. For this purpose, we studied the response of
34 bronchiolar club cells (CC) that are situated in the crossroads of the host defense and
35 allergic inflammation, and express specific pro and antiallergic proteins. LPS stimulus
36 in the neonatal life intensified the production of TLR-4, TNFα, and natural anti-allergic
37 products (CCSP and SPD), changes that contributed to prevent asthma triggering in
38 adulthood. At epithelial level, CC skipped the mucous metaplasia, declining the
39 overproduction of mucin via the EGFR pathway and the mice expressed normal
40 breathing patterns in front of OVA challenge. Furthermore, the overexpression of
41 TSLP, an epithelial pro-Th2 cytokine was blunted and normal TSLP and IL-4 levels
42 were found in bronchoalveolar lavage (BAL). Complementing this shift, we also
43 detected lower eosinophilia in BAL while an increase in phagocytes as well as in
44 regulatory cells (CD4+CD25+FOXP3+ and CD4+IL-10+) was seen, whit an elevation
45 in IL-12 and TNFα secretion. Summarizing, our study pointed to stable asthma-

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46 preventive effects promoted by neonatal LPS-stimulation; the main finding was the
47 increase of several anti-Th2 specific proteins at epithelial level, together with an
48 important diminution of pro-Th2 TSLP, conditions that promoted changes in the local
49 immune response with Treg. We thus evidenced several anti-allergic dynamic
50 mechanisms overlying in the epithelium that could be favored in an adequate
51 epidemiological environment

52 KEY WORDS:

53 Club cell, asthma, pulmonary host defense, antiallergic proteins

54 .

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55 **INTRODUCTION**

56 Asthma is a heterogeneous disease with diverse underlying processes and many
57 clinical expressions. The most common phenotype is the allergic asthma that has an
58 early-onset in childhood. This phenotype is associated with a family history of allergic
59 diseases, and is characterized by chronic airways inflammation, with activated mast
60 cells, increased numbers of eosinophils, T cells, natural killer T cells, and CD4+ T
61 helper (Th) 2 cells that release IL-4, IL-13, and IL-5. Additionally, IgE-secreting B cells
62 are induced during the asthma process (1, 2). In this phenotype, the continuous exposure
63 to allergens produces several consequences in the structure and function of the airways,
64 with the establishment of a remodeling process that includes mucus hypersecretion,
65 smooth muscle hyperplasia, subepithelial fibrosis, blood vessel proliferation and the
66 infiltration of inflammatory cells (3). All these effects provoke airways narrowing, a
67 common final pathway to symptoms and physiological changes in asthma (1). However,
68 the avoidance of airborne allergen exposure in the early life has been tested in
69 randomized clinical trials and has not been successful in preventing asthma
70 development, suggesting underlying mechanisms in the beginning of asthma that have
71 not been fully recognized so far (4, 5).

72 The progressive rise in allergic diseases in the last decades denotes the
73 involvement of environmental factors in their pathophysiology (6). Based on
74 epidemiological evidence, the hygiene hypothesis (HH) infers that the reduction of the
75 early life infections due to the modern lifestyle weakens their protective effects against
76 allergic disorders (7). In correlation, recent studies showed low childhood prevalence of
77 allergy/asthma in rural areas as compared with urban areas, related to the perinatal
78 microbial exposure, mainly to the high levels of endotoxins present in dust samples (8-
79 12). In addition, several mouse models have dissected potential immune mechanisms by
80 which environmental microbial stimulation, including the perinatal lipopolysaccharide
81 (LPS) stimulus, of the airways mucosa reduces the allergic inflammation to airborne
82 allergen challenge in the offspring, while favoring homeostatic responses (13-22).

83 In steady state, the homeostasis of the airways relays on the bronchioalveolar
84 cells (23, 24). For this purpose, airways epithelial cells (AECs) express inflammatory,
85 anti-inflammatory, chemoattractant, antimicrobial mediators, as well as pattern
86 recognition receptors (PRRs) to detect environmental molecules as endotoxin, and
87 initiate an innate immune response by activating dendritic cells (DC) (25). This link
88 between innate and adaptive immunity has evidenced a significant role of AECs in lung

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89 immunity and highlighted that an abnormal epithelial response may lead to a chronic
90 inflammatory response (26).

91 When AECs take contact with inhaled stimuli, which contain multiple
92 proteolytic allergens as well as microbial contaminant, they are induced to produce ROS
93 and pro-Th2 cytokines like TSLP, IL-25 and IL-33. These cytokines interact by cell–
94 cell communications with subepithelial DC, mast cells as well as innate lymphoid cells,
95 which in turn trigger the recruitment of Th 2 cells, leading to an amplified Th-2
96 cytokines production in the airways (27, 28) (29) (24, 30).

97 Additionally, there is accumulative evidence about AECs intrinsic alterations in
98 childhood asthma that render airways more vulnerable to airborne allergens and
99 predispose them to Th2- responses (31-34). These data indicate that AECs are essential
100 controller of the immune response to allergens and may be an early player in order to
101 bias a Th2 response in the immature immunity system. Therefore, AECs play a
102 particular role since they are situated at the crossroad of the innate host defense and
103 allergic inflammation.

104 Such contrasting activities are clearly exemplified by bronchiolar club cells
105 (CC). They perform a myriad of homeostatic mechanisms including detoxification of
106 xenobiotics and being a stem/progenitor cell to the airways AECs (35, 36).
107 Additionally, CC directly contribute to host defenses by secreting monocyte and
108 neutrophil chemoattractants, the antibacterial collectin surfactant protein (SP) D and the
109 anti-inflammatory club cell secretory protein (CCSP) (37-43). However, under allergic
110 genetic predisposition, CC can also activate elicited a Th2-inflammation via IL-4
111 receptor, driving eosinophil accumulation by producing eotaxin. Furthermore, they are
112 the principal cells to undergo epidermal growth factor receptor (EGFR)-mediated
113 mucous metaplasia as demonstrated in experimental models of asthma (29, 44-46).
114 Interestingly, both SP-D and CCSP, play a direct role suppressing allergic inflammation
115 in vivo and in vitro, inciting Th1 cytokines increment under LPS-stimulus. There is
116 quite evidence of the reduction of these mediators in allergic/asthmatic patients as well
117 as in mouse models of asthma (37, 40, 41, 47-54).

118 The potential of CC to respond to Th1 inflammatory stimulus, activating protective
119 mechanisms, has often been applied in studies to evidence if this protective role of
120 epithelium prevents the development of Th2 inflammation. In a previous study we

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121 reported that LPS pre-exposition to the allergen sensitization partially avoids mucous
122 metaplasia of CC. In consequence, the loss of anti-allergic products in CC and alveolar
123 adult mice macrophages were prevented. We observed a reduction of eosinophil influx,
124 Interleukin-4 levels and airway hyperreactivity, while the T-helper type 1 related
125 cytokines IL-12 and Interferon-g were enhanced (55). Considering early life as a better
126 window of opportunity for triggering an appropriate maturation of innate immunity. In
127 this study, we aimed to evaluate if LPS-stimulation during the neonatal lapse provides
128 better asthma-preventive effects to preserve adult AECs from the Th2-driven
129 inflammation. Mainly evaluating the role of bronchiolar CC and the preservation of
130 their pro and antiallergic proteins.
131
132

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133 ## MATERIALS AND METHODS

134 ### Animals

135 Balb/c mice were provided by Fun Vet (Universidad Nacional de La Plata,
136 Argentina) and housed under controlled temperature and lighting conditions, with free
137 access to tap water and commercial lab chow (GEPSA FEEDS, Buenos Aires,
138 Argentina). Animals were randomly assigned to four groups (n= 6 each) and
139 experiments were repeated at least three times.

140 The animal care and experiments were conducted following the
141 recommendations of Helsinki convention, and in compliance with local laws on the
142 ethical use of experimental animals.

143 ### Experimental design

144 *Neonatal treatment:* Offspring Balb/c mice were exposed to intranasal applications on
145 every second day from days 3 to 13 of life. While one group of animals were sham
146 treated with PBS, the other received LPS (1µg/5µl; *Escherichia coli* O55:B5 Sigma-
147 Aldrich; St. Louis, MO, USA) according to protocols previous optimized for volume
148 (16) and treatment timing (19).

149 *Allergen sensitization:* At the age of 4 weeks, all female animals were selected and
150 sensitized by subsequent i.p injections of 0.1 ml of OVA grade VI (1000 µg, Sigma-
151 Aldrich) absorbed to 1 mg of imject Alum (Pierce Rockford, USA) on the 4 and 6
152 weeks of life.

153 *Airway challenge:* Ten days later, neonatally (n) LPS-treated mice as well as PBS-
154 exposed were divided into 2 groups. Whereas LPSn/OVA and PBSn/OVA mice were
155 challenged daily (on 10 consecutive days) by an intranasal application of 50µl of 1%
156 OVA, LPSn and PBSn mice were submitted to intranasal application of saline (Fig 1a).

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157 Then, after 24h, mice were sacrificed and processed according to the specific methods
158 outlined below.

159 **Fig 1. Experimental design and allergic inflammatory state.**
160 (a) Timeline diagram and protocols employed in this study. (b) Differential
161 quantification of cell populations in bronchioalveolar lavage (BAL). Bar graph
162 represent total number of macrophages, esosinophils, neutrophils and lymphocytes in
163 BAL. (c) and (d): Levels of IL-4 and TSLP by ELISA. (e) Score of respiratory
164 distress. The score represents increasing signs of respiratory distress obtained in the
165 first minute after intranasal challenge in all groups. Data represent mean ± SD
166 ***p<0.001 vs PBSn,** p<0.01 vs PBSn, *p<0.05 vs PBSn, ●● p <0.01 vs
167 PBSn/OVA

168

169 **Lung histopathology**

170 Morphological analysis was conducted in the right lungs of 3 mice per group as
171 previously described. Briefly, at least in 3 experiments, lungs were differentially fixed
172 for either ultrastructural or histopathology analysis by intratracheal perfusion and
173 processed in order to being examined under electron microscope (Zeiss LEO 906E) or
174 light microscope (Axiostar Plus, Zeiss, Germany).

175 **Mucous cell staining**

176 The Alcian blue-periodic acid Schiff (AB-PAS) staining technique as previously
177 described identified Mucous-secreting cells in the bronchiolar epithelium.
178 Photomicrographs at x400 were taken using a light microscope equipped with a digital
179 camera (Axiocam ERc5s). A total of 20-30 bronchioles (900-1700 μm diameter) per
180 mouse were analyzed, and the numbers of AB-PAS positive cells present in epithelia
181 lining per 100μm of basement membrane were quantified using Image J Software (NIH
182 version 1.43).

183 **Immunohistochemical analysis of lung tissue**

184 Immunohistochemical staining was performed as described elsewhere . Briefly, after
185 being blocked, the sections were incubated overnight at 4ºC with antibodies recognizing

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186 SP-D (1:1000-Chemicon, Temecula, CA, USA), TNFα (1:50 - Hycult, Plymouth
187 Meeting, USA), CCSP (CC10 antibody 1:1000 - Santa Cruz Biotechnology, Santa Cruz,
188 CA, USA), TLR4 (1:100- Santa Cruz Biotechnology), TSLP (1:200- Gene Tex, USA)
189 or pEGFR (1:50 - Santa Cruz Biotechnology), with bound antibodies being detected
190 using anti-rabitt (for SP-D, TNFα, TSLP and CCSP) or anti-goat (for TLR4 and
191 pEGFR) biotin-labeled antibodies (Vector Laboratories, Burlingame, CA, USA) in 1%
192 PBS-BSA. The sections were then incubated with ABC complex (VECTASTAIN
193 Vector Labs, Southfield, MI, USA). Diaminobenzidinde (DAB, Sigma-Aldrich), which
194 was used as a chromogen substrate, and the bronchioles (700-1400 μm diameter) were
195 analyzed and photomicrographs x 400 were taken.

196 **Bronchoalveolar lavage collection and cell counting**

197 Bronchoalveolar lavage (BAL) were obtained (n= 9 mice/group in three different
198 experiment) as described elsewhere (52). Briefly, after three serial intra-tracheal
199 instillations of 1 ml PBS, the cells obtained were centrifuged at 200g, resuspended and
200 counted meanwhile the supernatant was stored at -70ºC for ELISA.

201 For cytospin preparations, about $12.5x10^4$ cells from the pellets were cytocentrifuged
202 onto slide, whereas some slide were preserved at -70°C for immunofluorescence, others
203 were stained with May Grünwald-Giemsa (Biopur Diagnostic, Rosario, Argentina) and
204 counted. The cell populations were evaluated for two samples per mouse, and a total of
205 2400 cells per group were counted.

206 **Immunofluorescence**

207 Cytospin preparations (3 per mice) obtained from the BAL (3 mice per group)
208 were withdraw at room temperature at immediately fixed with 4% formaldehyde,
209 permeabilized with 0.25% Triton X-100 in PBS and incubated for 1 h in 5% PBS-BSA
210 to block non-specific binding. Slides were double immunostained by incubating

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211 overnight at 4ºC with a mix of anti-CD4 conjugated with PERCP (BioLegend, San
212 Diego, CA, USA) and anti IL-10 conjugated with PE (BD Biosciences Pharmingen, San
213 Diego, CA) and mounted using fluoromount containing DAPI. Afterwards, the cells
214 were viewed with Fluoview 1000 Confocal and laser scanning microscope, (Olympus,
215 Tokyo, Japan) and serial x 60 microphotographs (10 per coverslide) were collected,
216 with all double immunostained cells being evaluated in three different experiments and
217 the relative percentages were calculated.

218 **Flow cytometry**

219 Pellet cells obtained from BAL (n= 5 mice/group in three different experiment)
220 were incubated with a mix of conjugated antibodies (Biolegend) for the following T-cell
221 subset superficial markers: APC-Cy7 anti-mouse CD45 (1:600); FITC anti-mouse CD4
222 (1:200); PerCP anti- mouse CD25 (1:200) for 30 min at 4°C. Next, the cells were fixed
223 (CITOFIX; BD Biosciences Pharmingen, San Diego, CA) for 20 min at 4°C and
224 permeabilized with Perm/Wash (BD Biosciences Pharmigen), before being incubated
225 with a dilution 1:30 of the intracytoplasmic antibody: APC anti mouse FOXP3
226 (eBIoscience) for 30 min at 4°C. Finally, the cells were washed, suspended in filtered
227 PBS ($1x10^5$ events/experimental treatment), and analyzed by flow cytometry
228 (FACSCanto II Flow Cytometer, BD Biosciences, San Diego, CA, USA). Data analysis
229 was carried out using the FlowJo software (Tree Star, Ashland, OR).

230 **Immunobloting**

231 By Western Blot SP-D, TLR4 and TNFα levels were determined in total lung
232 homogenates from 3 animals per group in three different experiments as was described
233 (52). Briefly, after proteins were measured with a Bio-Rad kit (Bio-Rad Laboratories,
234 Hercules, CA, USA), the denatured protein samples were separated on 12% SDS-PAGE
235 and blotted onto a Hybond-C membrane (Amersham Pharmacia-GE, Piscataway, NJ,

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236 USA). Membranes were then blocked with 5% defatted dry milk in TBS/0.1% Tween
237 20, and incubated for 3h with one of the following antibodies: rabbit anti-SP-D (1:1000
238 - Chemicon, rabitt anti TNFα (1:50 – Hycult) or mouse anti-TLR4 (1:300 Abcam,
239 Maryland, USA). Blots were incubated with a peroxidase-conjugated (HRP) anti-rabbit
240 (Jackson Immunoresearch Labs Inc, West Grove, PA, USA), or anti-mouse (Jackson
241 Immunoresearch) secondary antibodies at a 1:2000 dilution. Finally, the membranes
242 were rinsed in TBS/0.1% Tween-20 and exposed to Pierce™ ECL Western Blotting
243 Substrate (Thermo Fischer Scientific) following the manufacturer's instructions.
244 Emitted light was captured on Hyperfilm (Amersham-Pharmacia) and a densitometry
245 analysis was performed by applying the Scion Image software (V. beta 4.0.2, Scion
246 Image Corp., Frederick, MD, USA). Additionally, the expression of ACTB (1: 4:000;
247 mouse anti-βactin; Sigma-Aldrich) was used as an internal control to confirm equivalent
248 total protein loading.

249 **Dot Blot Analysis**

250 The CCSP protein expression was evaluated in lung homogenates after protein
251 measurement was performed using a Bio-Rad kit. Samples were then adjusted to 5μg/μl
252 in PBS, pH 7.4, and 10μl of each sample were spotted onto a Hybond C membrane
253 (Amersham Pharmacia). Then, the membrane was blocked with 5% fat-free milk in
254 PBS buffer for 1h and then incubated for 3h with a rabbit primary antibody anti-CC10
255 1:500 (Santa Cruz Biotechnology) in blocking buffer at room temperature. After
256 washing with TBS–Tween-20 buffer, the membrane was treated with a HRP-conjugated
257 anti-rabbit antibody (Jackson Immunoresearch) and the next handle was as described
258 above for Western blot.

259 **Cytokine detection by ELISA**

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260 Cytokines production was measured, in BAL supernatant, following the manufacturer´s
261 instructions. It was applying commercially available sandwich ELISA kits for IL-4 (BD
262 Biosciences), IL-12 and TSLP (Biolegend, San Diego, CA, USA), as well as TNFα and
263 IFNγ (eBioscience, San Diego, CA, USA).

264 **RNA isolation and gene expression analysis**

265 Total RNA was extracted from right lung tissue samples (~0,01mg) with Trizol reagent.
266 RNA was subsequently purified using Direct-zol RNA min prep kit (Zymo Research)
267 following the manufacturer's instructions and quantified with a ND-1,000, NanoDrop
268 spectrophotometer (Thermo Scientific) at 260 nm. Measurements of A260/280 were
269 used to determine the purity of the RNA. After that, 1μg of RNA was used as template
270 for reverse transcription following the manufacturer's instructions (EpiScript^{TM} Reverse
271 Transcriptase System kit, Epicentre, USA), using random hexamer primers (Fermentas,
272 Thermo Fisher Scientific, MA, USA) and utilizing a My Cicle rTM BIO-RAD (Thermal
273 Cycler System, CA, USA).

274 Real-Time PCR analysis was performed on an ABI Prism 7500 detection system
275 (Applied Biosystem, CA, USA) using Power SYBR Green PCR Master Mix (Applied
276 Biosystems, Thermo Fisher Scientific). Relative changes in gene expression were
277 calculated using the 2-ΔΔCt method normalized against the housekeeping gene 18s. For
278 each pair of primers, a dissociation plot resulted in a single peak, indicating that only
279 one cDNA species was amplified. Amplification efficiency for each pair of primers was
280 calculated using standard curves generated by serial dilutions of cDNA. All primers
281 were from Invitrogen (Buenos Aires, Argentina). The specific primers pairs used were:
282 TSLPfp: 5'-AGAGAAATGACGGTACTCAGG-3', TSLPrp: 5'-
283 TTCTGGAGATTGCATGAAGGA-3'; 18sfp 5'-ATGCGGCGGCGTTATTCC-3',
284 18srp: 5'-GCTATCAATCTGTCAATCCTGTCC-3'; CCSPfp: 5'-

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285 GATCGCCATCACAATCACTG-3', CCSPrp: 5'-CTCTTGTGGGAGGGTATCCA-3';
286 SP-Dfp: 5'-TGGACCCAAAGGAGAGAATG-3', SP-Drp: 5'-
287 CATGCCAGGAGCACCTACTT-3'.

288 **Clinical score assessment of the degree of respiratory distress**

289 In three different protocols, at days 7-10 of the allergen challenge, the breathing patterns
290 of mice (n=6/group) were video recording during the first minute after OVA instillation.
291 The values assigned to increasing signs of respiratory distress resulted from the
292 adaptation of the respiratory failure clinical score system developed by Wood (56). The
293 scoring was performed, via a double-blind procedure, by three different physician
294 operators and analyzed by a nonparametric statistical test (see below).

295 **Statistical analysis**

296 In general, data obtained were analyzed by one-way ANOVA, followed by post-hoc
297 comparison with the Tukey-Kramer test. In particular, for the analysis of clinical score
298 data, we applied the Kruskal Wallis test. For all test a $p<0.05$ significance level was
299 used.

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304 **RESULTS**

305 **Neonatal LPS stimulation inhibited OVA-induced allergic airways inflammation**
306 **triggered in the adulthood**

307 Firs, we evaluated the effect of an early exposition with LPS (represented in the
308 LPSn/OVA group) over an ulterior OVA allergic response in the airway's analyzing
309 BAL cytokines and cellular inflammatory content as well as the breathing pattern
310 recorded in the different mice groups.

311 We detected that neonatal LPS treatment affected the development of
312 experimental asthma triggered in adulthood. As shown in Fig.1, the establishment of
313 experimental asthma in adult mice was largely prevented as indicated by a significantly
314 lower influx of both total inflammatory cells and eosinophils into the airways lumen of
315 LPSn/OVA mice compared to the PBSn/OVA group (Fig.1b). In addition, the number
316 of macrophages remained unchanged while neutrophils increased significantly (Fig 1a).
317 Surprisingly, IL-4 and TSLP, both associated to Th2 inflammation, exhibited normal
318 levels in BAL of LPS pre-treated mice in spite of the allergen-challenge, while they
319 were significant higher in PBSn/OVA group (Fig. 1c and 1d respectively). As expected,
320 in the LPSn group neither BAL cell count nor IL-4 content were different from controls
321 (Fig 1b and c); TSLP was remarkable reduced to non-detectable levels (Fig. 1d).

322 To test whether the inflammatory parameters were accompanied by changes in
323 the degree of respiratory distress, a clinical scoring system was carried out (See
324 Supplementary material). While most of the neonatal PBS-exposed mice displayed
325 higher signs of respiratory distress after OVA challenge, the breathing pattern of
326 LPSn/OVA mice was not different from control mice (Fig 1e).

327

328 **Neonatal LPS application promoted innate immunity mediators, antimicrobial**
329 **cytokines and Treg cells in the airway's microenvironment**

330 After demonstrating the abrogation of a Th2 inflammatory response, we
331 investigated whether neonatal LPS exposure influences other components of the
332 immune response. In the airway's milieu LPS stimulus increased inflammatory
333 cytokines as TNFα and IL-12 in both LPSn and LPSn/OVA mice groups (Fig. 2c and
334 2b, respectively). It was noteworthy that LPS induced high levels of IFNγ, a
335 prototypical Th1 cytokine, in the LPSn group but not in LPSn/OVA animals (Fig. 2a).

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336 By contrast, the latter exhibited the higher influx of CD25+/FOXP3+ Treg cells (48,16
337 % ± 10,2 LPSn/OVA vs 19.65 % ± 4,22 PBSn/OVA) (Fig.2d). Furthermore,
338 immunofluorescence performed in cytospins displayed an increased level of IL-10
339 positive cells in LPSn/OVA group compared with PBSn animals, although the analysis
340 of the IL-10+/CD4+ cells ratio revealed a significant change in both group (LPSn and
341 LPSn/OVA) compared to control. (Fig. 2e).
342

343 **Fig 2. Modulatory response of the airway's environment in BAL.**
344 a), b) and c) IFNγ, IL-12 and TNFα levels by ELISA, respectively. d) Percentage of
345 CD4+CD25+FOXP3+ cells obtained in BAL by flow cytometry. The plots correspond
346 to a representative experiment analysis in all groups, the % of CD25+FOXP3+ cells
347 are shown in the Q2 quadrant. e) Immunofluorescence count of CD4+, IL 10+ and
348 ratio of IL10+/CD4+cells performed in cytospin. Data are represented as mean ±SEM,
349 *p< 0.05 vs PBSn, **p<0.01 vs PBSn, ***p<0.001vs PBSn.
350

351 We next characterized the epithelial response in this long-lasting anti-allergic
352 modulation in order to evidence if the early LPS stimulus can trigger local epithelial
353 mechanisms involved in prevention of asthma development in adulthood.
354

355 **Neonatal LPS exposure abrogated the development of mucous metaplasia and pro-**
356 **allergic mediators in the bronchiolar epithelium**

357 We first analyzed changes in the expression of specific pro-allergic mediators
358 that are known to increase in bronchiolar epithelium during asthma,

359 As was shown before (55), the OVA-allergic inflammation incited mucous cell
360 metaplasia in the bronchiolar Club cell via EGFR signaling. In this way, Figure 3 shows
361 an increased number of mucous secreting cells (AB-PAS panel in Fig 3a and Fig. 3b) as
362 well as the overexpression of phosphorylated-EGFR in the apical cytoplasm of CC in
363 PBSn/OVA mice (pEGFR panel in Fig. 3a). Whereas in LPSn/OVA mice, both, pEGFR
364 overexpression (pEGFR panel in Fig. 3a) and mucous metaplasia (Fig. 3b), were largely
365 reduced by the neonatal endotoxin- treatment.

366 **Fig 3. Mucous metaplasia analysis in Club cells and epithelial TSLP expression.**
367 a) Representative photomicrographs of Alcian blue-periodic acid Schiff (AB-PAS),
368 TSLP and pEGFR staining of bronchiolar sections. Scale bars: 20µm. In AB-PAS
369 panel arrows indicate AB-PAS positive cells in PBSn/OVA and LPSn/OVA groups,
370 while arrowheads indicate infiltrating inflammatory cells. In TSLP panel, arrows
371 indicate positive cells in PBSn/OVA although some cells (arrowhead) from te

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372 inflammatory response also expressed TSLP, inset selection demonstrated the lack of
373 staining in club cells LPSn/OVA groups.. In pEGFR panel arrows indicate positive
374 cells in PBSn/OVA, while the inset demonstrated the apical expression of the
375 activated receptor in club cells. b) Graph represents AB-PAS cells count per 100µm.
376 c) TSLP mRNA expression by Real-Time PCR analysis. Graph represents fold
377 increase expression in lung tissue homogenate. Data are represented as mean ±SEM,
378 *p<0.05 vs PBSn, ***p<0.001 vs PBSn, ### p<0.001 vs PBSn/OVA.

379 We also studied the effect of neonatal-LPS on the expression of TSLP, an
380 epithelial cell cytokine that promotes Th2 differentiation after allergen contact. In
381 accordance with the pEGFR and mucous metaplasia induction, bronchiolar epithelial
382 cells of PBSn/OVA group, showed strong TSLP immunoreactivity in the apical
383 cytoplasm; meanwhile CC of LPSn/OVA animals skipped of the overexpression of
384 TSLP (TSLP panel in Fig 3a). These results were corroborated by the quantitative PCR
385 analysis (Fig. 3c), showing that lung TSLP mRNA almost duplicated its expression in
386 PBSn/OVA animals (1.85 ± 0.09 PBSn/OVA vs 1 ± 0.1 PBSn) while remained
387 unchanged in LPSn/OVA mice (1.12 ± 0.19).

388 In previous studies, we have demonstrated that the ultrastructure of CC is a
389 sensitive parameter of the airways allergic inflammatory affectation (Roth 2007, 2013,
390 Garcia 2014). For this reason, we studied CC morphological profile in all groups by
391 electron microscopy (Fig. 4). At this level, we corroborated the preservation of the
392 typical cellular profile in PBSn mice, characterized by the presence of a dome-shape
393 cupola, numerous polymorphic mitochondria in the cytoplasm, along with scarce
394 spherical electron-dense secretory granules under the plasma membrane (Fig. 4a). These
395 parameters could also be seen in LPSn/OVA mice, which differed only by an increase
396 on the number of normal electron-dense granules as well as a mayor development of
397 RER (Fig. 4d). Meanwhile, PBSn/OVA animals displayed characteristic mucous cell
398 metaplasia featured as a hypertrophied cytoplasm filled up with numerous large
399 electron-lucent secretory granules, slim mitochondria and abundant RER (Fig. 4b). In
400 control mice, only exposed to LPS, CC also developed an increased number of electro-
401 dense granules, as was shown by LPSn/OVA animals (Fig. 4c). In this group, the
402 evident diminution of their CC cupola is probably due to the repeated LPS instillation
403 they received in neonatal life.

404 **Fig 4. Club cell ultrastructural features.**

405 Representative electron micrograph images of club Cell morphology of PBSn (a),
406 PBSn/OVA (b), LPSn/OVA(c) and LPSn/OVA (d) groups are shown. Scale bar
407 represents 5µm. Nu: nucleus, Mi: mitochondria, Ci: ciliated cells, Golgi: Golgi

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408 apparatus, RER: rough endoplasmic reticulum. Arrowheads: normal electron dense
409 granules, arrows: electron lucid granules.

410

411 **Neonatal LPS stimulus promoted a long-lasting increase of mediators of innate**
412 **response and Th2-immunomodulatory proteins on bronchiolar epithelium**

413 Next, we analyzed whether the mucous metaplasia prevention by neonatal LPS
414 treatment correlated with changes in the expression of epithelial host defense mediators,
415 mainly CCSP and SP-D. As it was described (55), OVA-allergic inflammation induced
416 a diminution in the imunoreactivity of CCSP and SP-D in CC of PBSn/OVA group
417 when compared to its control group (CCSP and SP-D panels in Fig 5a). Meanwhile for
418 both LPSn and LPSn/OVA groups, a strong CCSP and SP-D immunolabelling was
419 observed (Fig 5a). These changes in protein expression of CCSP and SP-D were also
420 verified by immunoblottting (Fig. 5b and 5c). However, the neonatal LPS-instillation
421 did not increase mRNA expression of CCSP or SP-D in LPSn and LPSn/OVA (Fig. 5f
422 and 5g, respectively). This may be due to the stimulus for protein secretion provided by
423 the allergen challenge in LPS/OVA group, and to the contribution of SP-D of the Type
424 II alveolar cells; which could explain the highest SP-D content by western blot analysis
425 in these group.

426 Regarding the microbial recognition and cytokine response, both the toll like
427 receptor 4 (TLR4) and TNFα, increased their expression in CC as well as in lung tissue
428 of both LPS neonatal stimulated groups (Fig 5d and 5e, respectively). These suggest a
429 specific LPS-response in bronchiolar epithelium that induced a persistent elevation of
430 these defense molecules and seemed to be preserved in spite of allergen stimulus.

431 **Fig 5. Club cell's expression of host defense proteins and antimicrobial cytokines.**
432 a) Immunostaining of CCSP, SP-D, TLR4 and TNFα performed on lung section of all
433 groups. Positive cells appear in brown against the blue counter stain of haematoxylin.
434 Scale bars: 20μm. b) Dot blot of CCSP in lung homogenates. Graph represents fold
435 increase of the relative CCSP/β-actin expression in lung homogenate by densitometric
436 analysis. c), d) and e) Western Blot of SP-D, TLR4 and TNFα lung content,
437 respectively. Graph represents fold increase of the relative expression in lung
438 homogenate by densitometric analysis.f) and g) CCSP and SP-D mRNA respectively
439 expression by Real-Time PCR analysis. Graph represents fold increase expression in
440 lung tissue homogenate. Data are represented as mean ±SEM, *$p < 0.05$ vs PBSn,
441 **$p<0.01$ vs PBSn, ● $p<0.05$ vs PBSn.

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445 **DISCUSSION**

446 In the present work, we reported that neonatal LPS-treatment triggers anti-
447 allergic secretory products of the local airway epithelium that persist in adulthood.
448 Among these products, we demonstrated, the increase of CCSP and SP-D content,
449 together with the upregulation of TLR-4 and TNFα, both related to innate host defenses,
450 in epithelial CC and lung tissue. In correlation, CC skipped the mucous metaplasia
451 pathway in front of airborne allergen challenge, preserving their typical phenotype and
452 declining EGFR, mucins, and TSLP, a pro-Th2 cytokine, overexpression. Furthermore,
453 under allergic stimulus, animals with neonatal LPS treatment exhibited normal
454 breathing patterns, normal IL-4 and TSLP levels and a lower eosinophilia. By contrast,
455 in those mice, an increase in phagocytes and in regulatory cells (CD4+CD25+FOXP3+
456 and CD4+IL-10+), as well as in IL-12 and TNFα levels was observed. These evidences
457 are remarkable considering that they reveal a possible new preventative and therapeutic
458 approach to asthma focused on increasing the airways resistance to environmental
459 insults rather than suppressing Th2 downstream inflammation once it is established. The
460 finding of anti-allergic effects associated to CCSP and SP-D is consistent with previous
461 results (19) (41, 49-51) (40, 48). For instance, there is evidence that these proteins down
462 regulate the type 2 differentiation of Th cells, inhibits the allergen-activation of innate
463 immune cells (eosinophils, basophils, and mast cells) and are reduced in both BAL and
464 serum of asthmatic individuals (40, 41, 50, 51, 57) (47, 48, 58). Moreover, previous
465 reports from our laboratory showed the interplay between the establishment of an
466 experimental asthma model in mice and the diminution of CCSP and SP-D levels,
467 which were restored by Budesonide or Montelukast treatment (52).

468 Recently, we determined that the pre-treatment of adult mice with LPS before an
469 allergic inflammation partially prevented CCSP and SP-D reduction in CC, and the
470 increase of IL-4 levels and airways hyperresponsiveness (55). However, in the present
471 work we demonstrate that when endotoxin treatment is performed in neonatal life it
472 achieved a more extensive asthma prevention in adulthood. The neonatal treatment not
473 only avoided the metaplastic changes in these cells, but also preserved the mRNA levels
474 of CCSP and SP-D; moreover, the characteristic increment of IL-4 and respiratory
475 distress in front of OVA challenge were damped. These results are similar to the
476 blunting of a Th-2 allergic response and airways hiperresponsiveness (AHR) reported

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477 by other authors using either, infant or pregnant mice and microbial stimulus (16, 17,
478 19) (13)

479 As expected, an increase in TNFα and IL-12 was observed in both groups exposed
480 to LPS; nevertheless, a robust Th1 response was only seen in LPS exposure animals as
481 indicated by the high IFNγ levels meanwhile in LPSn/OVA the most important
482 immunological change was the increased number of Treg found. In addition, whereas
483 LPSn/OVA group was the only one that reached a significant number of CD4+IL10+
484 cells, both groups (LPSn and LPSn/OVA) demonstrated a significant ratio of IL10+
485 /CD4+ cells ex vivo versus PBSn animals. Although the experimental design of our
486 study cannot explain the IL-12 elevation coexisting with a Treg response in LPSn/OVA
487 group, other authors have related the persistent increment of IL-12 cytokine as a
488 stimulus of phagocyte activity (59, 60).

489 Meanwhile studies conducted by Gerhold in adults Balb/c with systemic
490 administration of an anti- IL-12 before LPS stimulus, demonstrated that the reduction of
491 an ulterior allergic inflammation occurs in an IL-12 dependent way (17). Regarding the
492 Treg response, Nguyen et al previously described that TSLP directly impairs the
493 function of pulmonary Treg cells obtained of allergic asthma patients (61). This was
494 indicated by a significant decrease in suppressive activity and IL-10 production
495 compared to healthy control and non-allergic asthmatic counterparts, which were
496 associated with the TSLP expressions levels in BAL. Therefore, it is probable that the
497 diminution of TSLP induced by LPS-pretreatment in this study, had the additional effect
498 of restoring Treg.

499 In accordance with our results, experimental studies conducted by other authors
500 in neonatal Balb/c mice exposed to LPS and different models of sensitization and
501 exposition to OVA,also evidenced the occurrence of a response involving the
502 expression of IL-10 and IFN-γ in the re-exposition to allergen (16) (19). Furthermore,
503 Gerhold demonstrated that LPS, either in prenatal or postnatal stimulus, induces a
504 persistent elevation in soluble factors such as CD14 and Lipopolysaccharide binding
505 protein-LBP, as well as TLR4 mRNA expression in young mice (16). More recently,
506 the gene expression levels of innate and adaptive immunity essential markers in white
507 blood cells in farmers' children were assessed in the multinational and prospective
508 epidemiological study PARSIFAL (62). This study compared farmers to non-farmers'
509 essential markers expressions and the prevalence of asthma; the authors determinate an

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510 enhanced expression of genes of the innate immunity such as IRAK-4 and RIPK1 as
511 well as regulatory molecules such as IL-10, TGF-beta, SOCS4, and IRAK-2. (62).
512 Although the correlation of Treg and host defense molecules described is similar to our
513 results, our finding pointed to the epithelium involvement in this persistence immune
514 response.

515 As was descripted before, several experimental and clinical studies established the
516 correlation between LPS pre-exposure and asthma phenotype abrogation; our study
517 attempted to dissect the changes of a pro-allergic cytokine secreted by the epithelium
518 such as TSLP in this context. In this sense, our results demonstrated that LPS neonatal
519 exposition correlated with the abrogation of TSLP expression in epithelium and in BAL.
520 Therefore, meanwhile allergen-induced TLSP recruits dendritic cells that amplify the
521 Th2 response and reduces Treg cells expansion (27). In the last decades, several
522 neonatal and pregnancy animal models suggested that the transition from the quiescent
523 Th2-polarized fetal immune phenotype towards the more active Th1-pattern of mature
524 adaptive immunity was intrinsically slower in the atopic population, thus increasing the
525 risk of an allergen priming response against environmental antigens. (21, 22, 63-68).
526 Thus, it would be important to future evaluate whether this early proinflammatory
527 stimulus by LPS could cooperate with the progression of this transition.

528 In our study, LPS diminished the TSLP mRNA basal expression consistent with its
529 intrinsic capacity to counterbalance different pro- allergic action; thus blunted the
530 subsequent overproduction of TSLP in front of OVA exposition. In a model of human
531 bronchial epithelial cell line, Lin et al also demonstrated that LPS pre-treatment could
532 reduce the induction of TSLP mRNA levels by a virus that causes neonatal respiratory
533 disease (69). Interestingly, in this study the basal mRNA levels of different signaling
534 proteins involved in the TSLP overproduction were downregulated only when a
535 repeated LPS- preventive treatment were applied. In a process that, similar to ours
536 results, the authors attributed to the modulation in the expression of innate immunity
537 signaling molecules of the airway epithelial cells to mitigate the allergic response.

538 The main contribution of our study is highlighting the involvement of
539 bronchioalveolar epithelium in this early microbial protection from allergic disorder, a
540 topic that remains unaddressed. This was demonstrated by the stable changes in the
541 expression of antiallergic proteins as well as host defense factors by CC and the
542 reduction to basal levels of potent epithelial-Th2 mediators, all of which was promoted

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543 by neonatal LPS-stimulation that further polarized Treg response in front of allergen
544 exposition. Altogether, our results point to several anti-allergic dynamic mechanisms
545 overlying in the epithelium that could be favored in an adequate epidemiological
546 environment.

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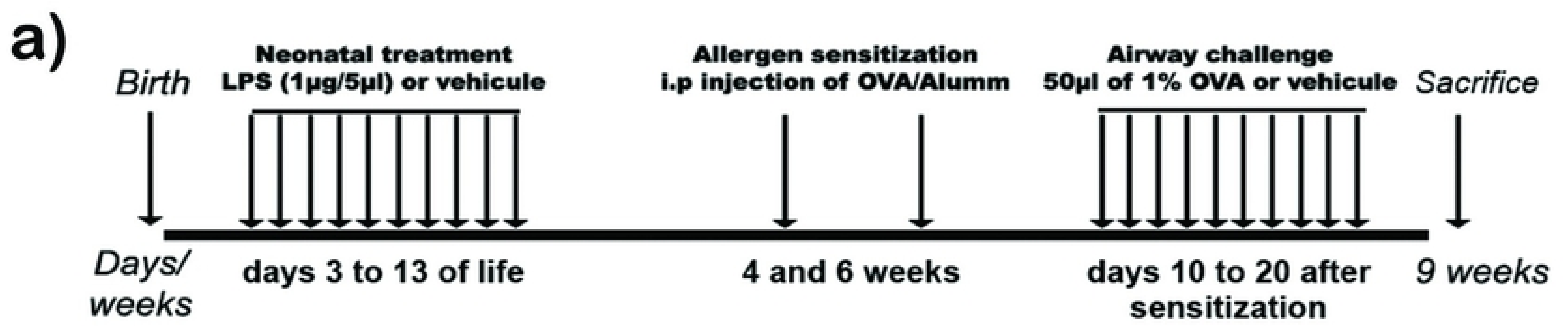
736

a)



Group	Neonatal treatment	Allergen sensitization	Airway challenge
PBSn	*PBS*	*OVA*	*Saline*
LPSn	*LPS*	*OVA*	*Saline*
PBSn/OVA	*PBS*	*OVA*	*OVA*
LPSn/OVA	*LPS*	*OVA*	*OVA*

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b)

Total cells (mean± SEM)	4.33x10⁵ ± 0.8	75x10⁵ ± 9.4***	6.54x10⁵ ± 0.8	37.42x105 ± 3.3**/●●

c)

d)

e)

Figure 1

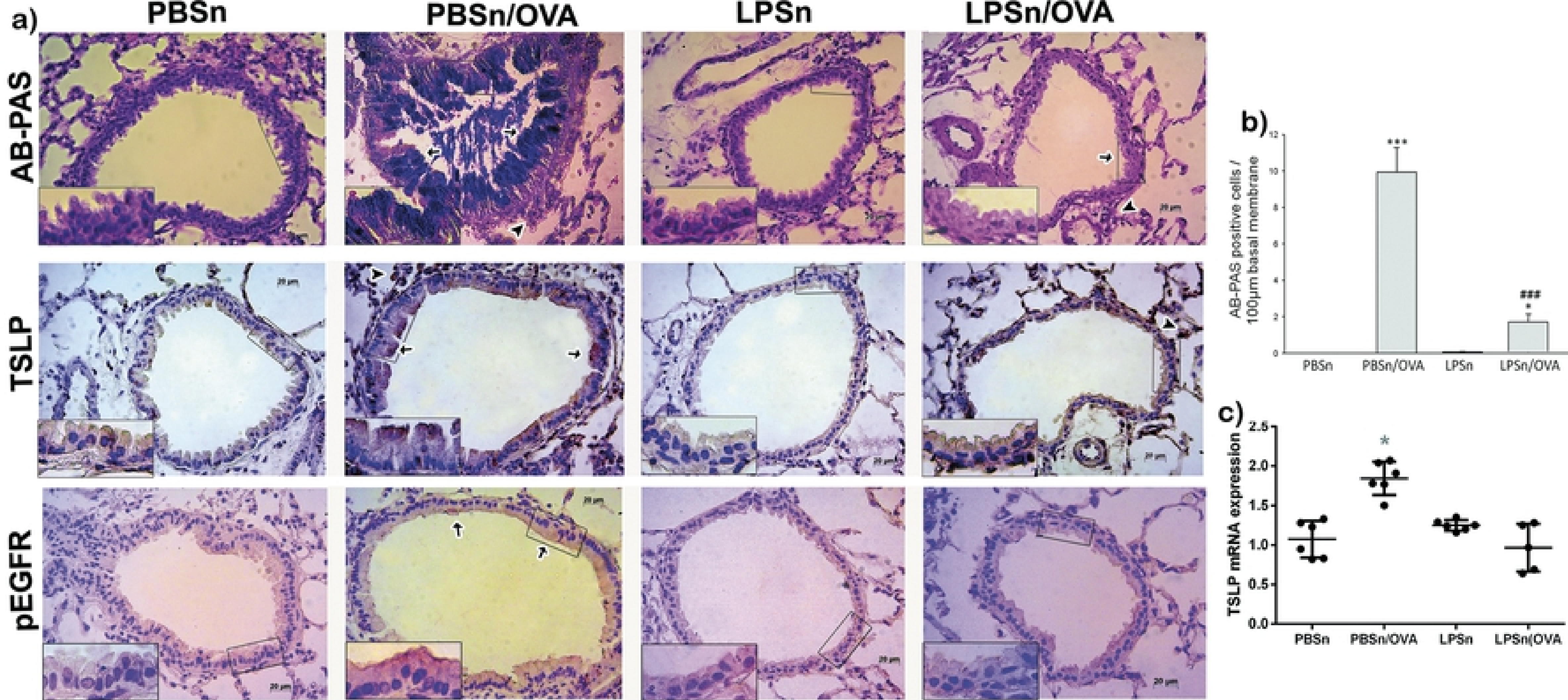
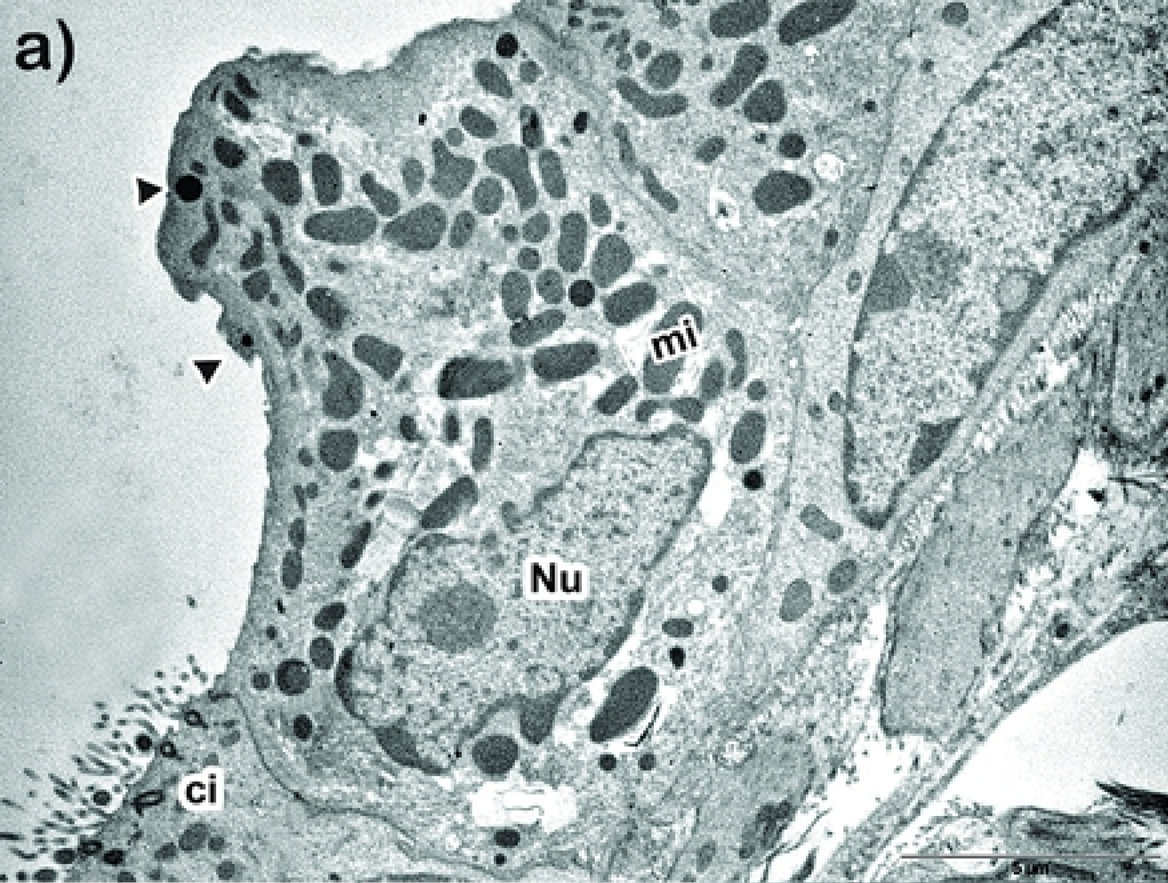
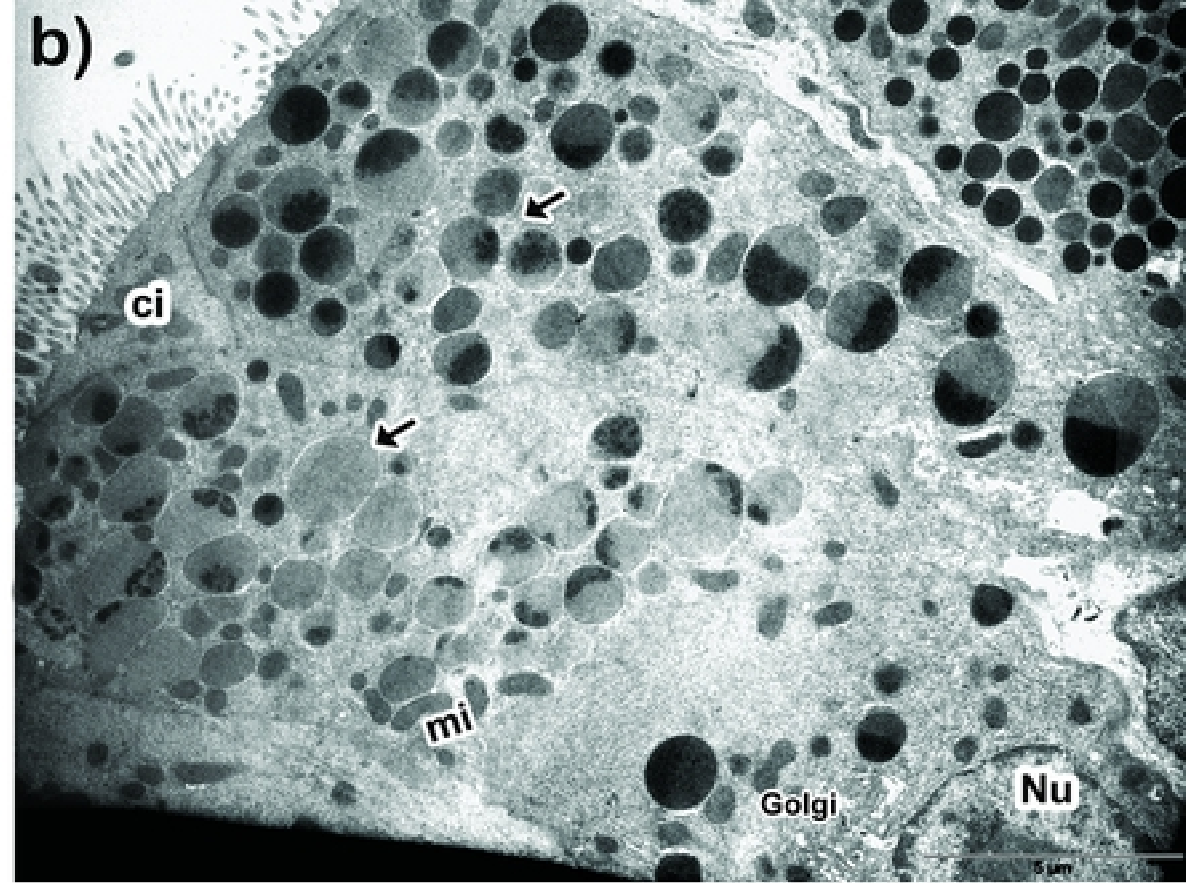
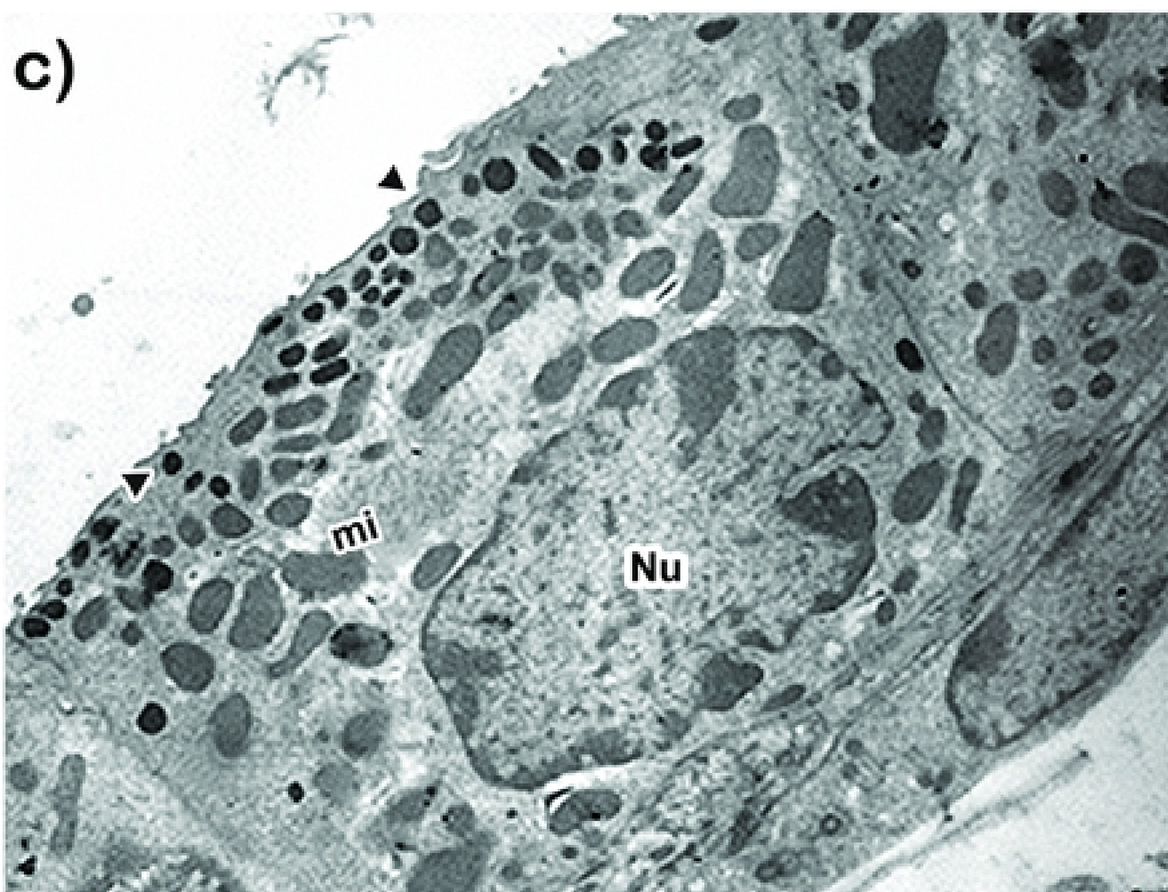


Figure 3







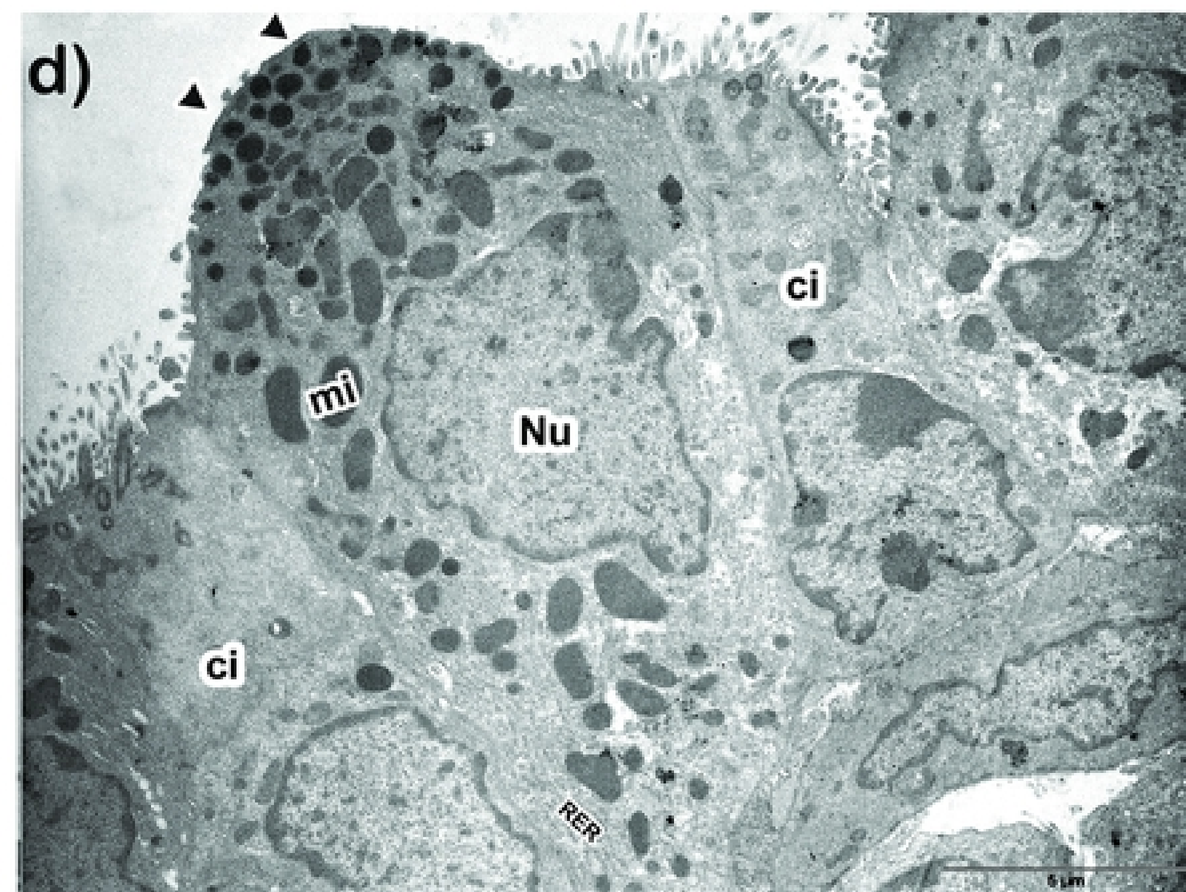


Figure 4

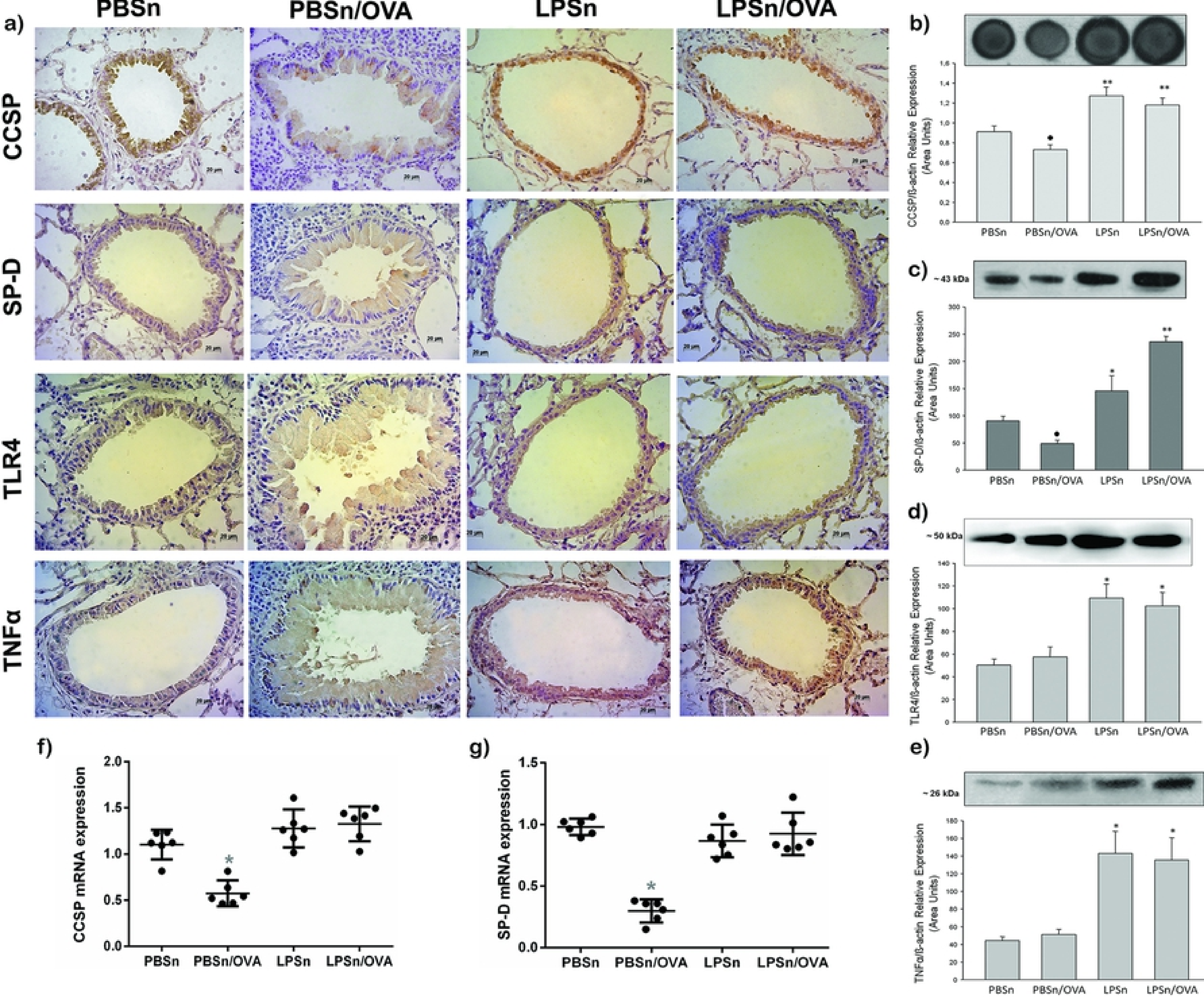
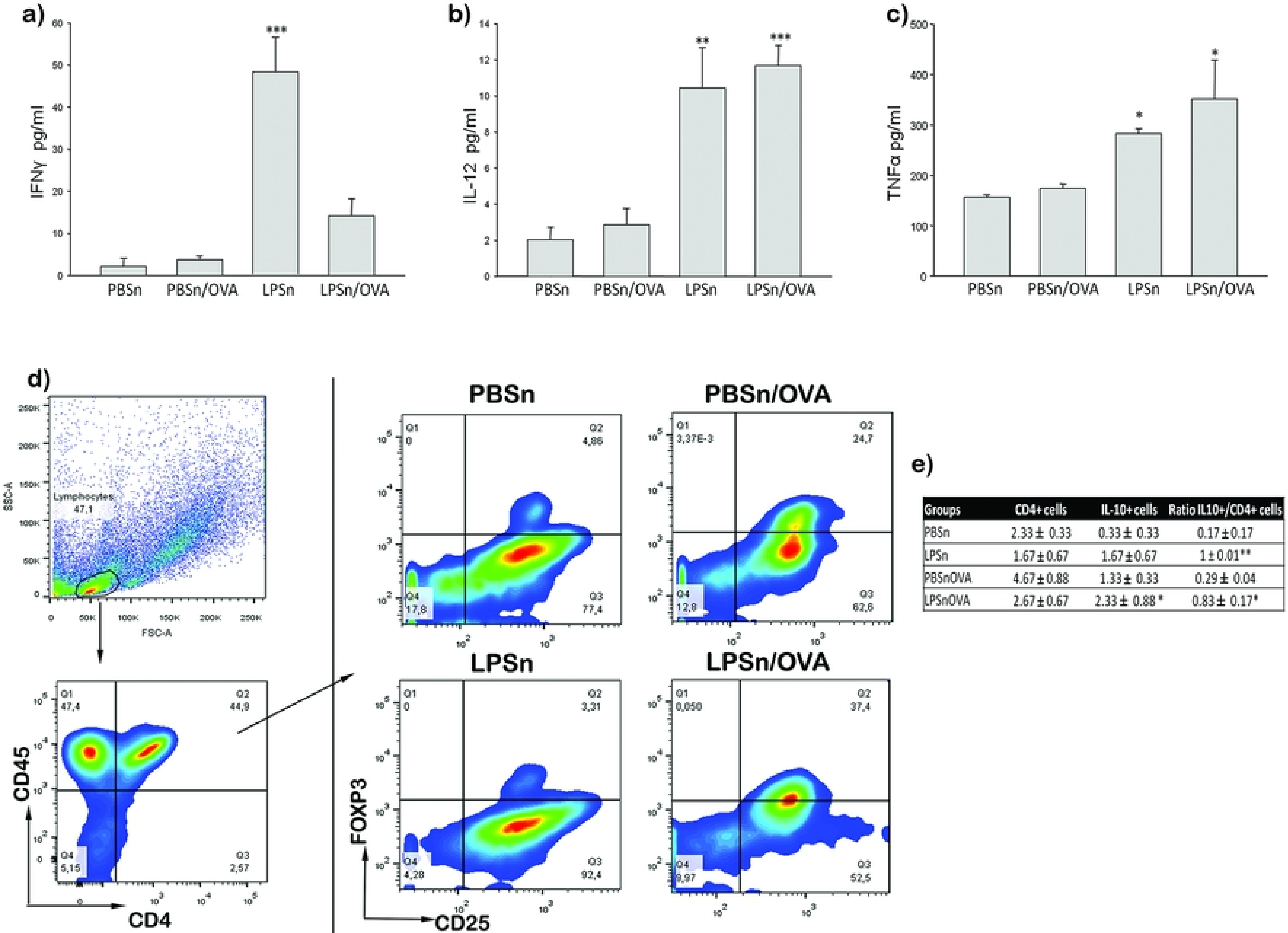


Figure 5



Groups	CD4+ cells	IL-10+ cells	Ratio IL10+/CD4+ cells
PBSn	2.33 ± 0.33	0.33 ± 0.33	0.17 ± 0.17
LPSn	1.67 ± 0.67	1.67 ± 0.67	1 ± 0.01**
PBSnOVA	4.67 ± 0.88	1.33 ± 0.33	0.29 ± 0.04
LPSnOVA	2.67 ± 0.67	2.33 ± 0.88 *	0.83 ± 0.17*

Figure 2