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# Sexual maturation protects against development of lung inflammation through estrogen

Running head: Puberty protects against development of lung inflammation

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## Abstract

Increasing levels of estrogen and progesterone are suggested to play a role in the gender switch in asthma prevalence during puberty. We investigated whether the process of sexual maturation in mice affects the development of lung inflammation in adulthood and the contributing roles of estrogen and progesterone during this process.

By inducing ovalbumin-induced lung inflammation in sexually mature and immature (ovariectomized before sexual maturation) adult mice, we showed that sexually immature adult mice developed more eosinophilic lung inflammation. This protective effect of 'puberty' appears to be dependent on estrogen, as estrogen substitution at the time of ovariectomy protected against development of lung inflammation in adulthood while progesterone substitution did not. Investigating the underlying mechanism of estrogen-mediated protection, we found that estrogen-treated mice had higher expression of the anti-inflammatory mediator secretory leukoprotease inhibitor (SLPI) and lower expression of the pro-asthmatic cytokine IL-33 in parenchymal lung tissue and that their expressions colocalized with type II alveolar epithelial. Cells (AECII) Treating AECII directly with SLPI significantly inhibited IL-33 production upon stimulation with ATP.

Our data suggest that estrogen during puberty has a protective effect on asthma development, which was accompanied by induction of anti-inflammatory SLPI production and inhibition of pro-inflammatory IL-33 production by AECII.

49 ## Introduction

50 Asthma prevalence is higher in boys than in girls during early childhood (1, 2, 3, 4). At the onset of
51 puberty, however, this sex difference reverses (11, 13, 20, 48, 60). Adult asthma is not only more
52 common in women, but asthma is also more severe in women than men (27, 48, 52, 62). Female sex
53 hormones, i.e. estrogen and progesterone, have been linked to the higher risk of girls to develop
54 asthma during puberty, when hormone levels increase (7). Girls with an earlier menarche have a
55 higher risk of developing asthma, which may be explained by exposure to greater cumulative
56 estrogen and progesterone levels than girls with late menarche (25, 28, 46, 58). These studies clearly
57 suggest a role of estrogen and progesterone in asthma development and severity, yet experimental
58 findings are anything but clear.

59 Some studies report worsening of asthma in women when estrogen and progesterone levels are
60 high, and others that low hormone levels coincide with worse asthma symptoms (6, 14, 16, 29, 30,
61 39, 40, 43). Contradictory results were also found in animal studies using mice and rats. Both pro-
62 inflammatory and anti-inflammatory effects of estrogen and progesterone have been reported in
63 mouse and rat models of asthma with a variety of explanations for these conflicting results (8, 18, 26,
64 35, 37, 38, 44). However, little is known about how puberty per se affects asthma development later
65 in life and how the changes in either estrogen or progesterone levels during puberty affect asthma
66 development.

67 This is of interest as estrogen can have direct effects on the immune system by binding to estrogen
68 receptors expressed by several immune cells (10). Additionally, estrogen can influence airway
69 responses indirectly by acting on structural cells, such as airway epithelial cells. In response to
70 inhaled allergen, airway epithelial cells induce nuclear factor (NF)-κB-signaling and subsequently
71 recruit and activate immune cells through production of pro-asthmatic mediators such as IL-33 and
72 TSLP (17, 23). To protect the airways against escalating inflammation and subsequent damage,
73 epithelial cells also secrete anti-inflammatory factors such as secretory leukoprotease inhibitor (SLPI)

74 (31). SLPI exerts its anti-inflammatory effects via inhibition of NF-κB-signaling. Interestingly, SLPI
75 expression has been found to be upregulated by estrogen treatment (9, 54, 55), but has not been
76 linked to IL-33 signaling yet.

77 We investigated the development of allergic lung inflammation subsequent to ovalbumin challenge
78 in adult female mice that did not go through puberty and assessed whether the increase in estrogen
79 or progesterone levels during puberty affect the severity of allergic lung inflammation in female adult
80 mice. Our studies showed that going through puberty actually leads to less severe lung inflammation
81 in adult female mice as compared to mice going through puberty. In addition, we also found that
82 allergic lung inflammation is inhibited by a sole increase in estrogen during puberty, but was not
83 affected by a sole increase in progesterone during puberty. This inhibition is associated with higher
84 SLPI expression that appears to inhibit the production of the pro-inflammatory cytokine IL-33.

85

86 **Materials and methods**

87 *Animals*

88 Female BALB/c mice (aged 3 weeks) were obtained from Harlan (Horst, The Netherlands). The mice
89 were fed *ad libitum* with standard food and water and were kept in a temperature and light-
90 controlled room (lights on from 7.30 am until 7.30 pm). Animal procedures were approved by the
91 Institutional Animal Care and Use Committee of the University of Groningen (application number
92 4609) and were performed under strict governmental and international guidelines. All surgeries were
93 performed under isoflurane/oxygen anesthesia and all efforts were made to minimize suffering
94 including the use of buprenorphine for pain management after surgery.

95

96 *Ovariectomy and OVA model*

97 Female BALB/c mice were ovariectomized (OVX) or sham-treated (SHAM) at 28 days of age before
98 reaching sexual maturity (around day 35). OVX animals were either left untreated or subcutaneously
99 received a 60-day slow release pellet of 0.1 mg estrogen (OVX+E2) or 15 mg progesterone (OVX+P)
100 (IRA, Sarasota, FL, USA) at the time of ovariectomy. These E2 and P pellets were chosen because they
101 have been shown to yield plasma levels about two times higher (E2 ±350 pg/ml, P ±55 ng/ml) than
102 levels at estrus (for E2 ±150 pg/ml and for P ±25 ng/ml) (50). Uterine weight was assessed to check to
103 quality of ovariectomy, estrogen and progesterone treatment (see figure S1 of the supplemental
104 data). Ovariectomy strongly reduced uterine weight as compared to sham treatment. Estrogen
105 treatment mostly prevented this low uterine weight, and progesterone treatment led to slightly, but
106 significantly, higher uterine weight as compared to ovariectomy. Animals with normal (>40 mg)
107 uterine weights after ovariectomy were excluded from the studies, as were animals with low (<15
108 mg) uterine weights after estrogen (indicating the pellet did not work) and or high (>50 mg) uterine
109 weights after progesterone treatment (indicating that ovariectomy was not performed successfully).
110 Four weeks after ovariectomy (age 8 weeks), mice were sensitized intraperitoneally to 10 μg of
111 ovalbumin (OVA, Grade V, Sigma-Aldrich Chemie BV, Zwijndrecht, The Netherlands) and 1.5 mg of

112 alum (Aluminject, Pierce Chemical, Etten-Leur, The Netherlands) on days 1 and 7, followed on day
113 14-20 by daily 20-min aerosol challenges with 1% w/v OVA in sterile PBS using a Pari LC Sprint Star
114 nebulizer driven by a PARI Boy SX compressor (both kind gifts from Pari GmbH, Starnberg, Germany).
115 Healthy controls (n=8) were not sensitized with alum and were exposed to PBS. An overview of the
116 experimental design is depicted in figure 1.
117 On day 21 (age 11 weeks), serum was collected for OVA-specific IgE and animals were either
118 sacrificed to collect bronchoalveolar lavage fluid and to isolate lung cells from digested lung for flow
119 cytometry or sacrificed for histological analyses of lung tissue. The experimental groups consisted of
120 32 SHAM mice (15 for lavage/flow and 17 for histology), 18 OVX mice (10 for lavage/flow and 8 for
121 histology), 11 OVX+E2 mice (6 for lavage/flow and 5 for histology), and 14 OVX+P (6 for lavage/flow
122 and 8 for histology).
123
124 *OVA-specific IgE*
125 Serum levels of OVA-specific IgE were measured by ELISA as described previously (33). OVA-specific
126 IgE titers are expressed as arbitrary ELISA units that were interpolated from a standard curve of a
127 reference serum.
128
129 *Bronchoalveolar lavage fluid*
130 Bronchoalveolar lavage fluid was collected and cells were processed for cytospots to determine the
131 percentage of eosinophils as described previously (33). Total and differential cell counts can be found
132 in table S2 of the supplemental data. Lavage supernatants were used to assess the levels of secretory
133 leucocyte protease inhibitor (SLPI) and IL-33.
134 SLPI levels in lavage supernatants were determined by western blot analysis using a biotinylated goat
135 polyclonal α-SLPI antibody (R&D Systems, Oxon, UK). Briefly, equal amounts of total protein (25 µg)
136 were run on a 15% SDS-PAGE gel and subsequently transferred to polyvinylidene difluoride
137 membranes (Roche, Mannheim, Germany). The membranes were blocked with 5% blotting grade

138 non-fat milk (Biorad, Veenendaal, Netherlands) and incubated with an anti-SLPI antibody overnight
139 (in 5% milk). Next, the blots were incubated with rabbit-anti-goat HRP-conjugated secondary
140 antibody followed by a goat-anti-rabbit HRP-conjugated tertiary antibody (both DAKO, Glostrup,
141 Denmark). Blots were subsequently developed using sensitive Western Lightning-ECL reagent
142 (Perkin-Elmer, Boston, MA) in a G-Box (Syngene, Cambridge, U.K.). Molecular weights were
143 estimated based on the migration of ten known molecular weight standards (Biorad, Veenendaal,
144 The Netherlands). Digital evaluation of blots was performed by GeneTools analysis software
145 (Syngene, Cambridge, U.K.), which assigns a peak height to each band. The relative SLPI expression is
146 described as SLPI expression in lavage fluid of OVX and OVX+E2 mice in relation to the SLPI
147 expression in lavage fluid of SHAM mice.
148 The levels of IL-33 in lavage supernatants were determined by ELISA kits according to the
149 manufacturer's instructions (R&D Systems).
150
151 *Lung digestion*
152 After bronchoalveolar lavage, lungs were minced and incubated in RPMI medium supplemented with
153 10% fetal calf serum (both Lonza, Verviers, Belgium), 10 µg/ml DNAse I (grade II from bovine
154 pancreas, Roche Applied Science, Almere, Netherlands), and 0.7 mg/ml collagenase A (Sigma-Aldrich)
155 for 45 min at 37°C in a shaking water bath. Single cell suspensions of lungs were obtained by passing
156 the digested lung tissue through a 70 µm nylon strainer (BD Biosciences, Breda, Netherlands).
157 Contaminating erythrocytes were lyzed using 10 times diluted Pharmlyse (BD Biosciences).
158 Mononuclear cells were then enriched by performing discontinuous Percoll (Pharmacia, Uppsala,
159 Sweden) gradient centrifugation (20 to 55%). Cells collected on the 55% and 45% layers were passed
160 through a 35 µm nylon strainer (BD Biosciences) and counted using a Sysmex pocH-100i cell counter
161 (Sysmex, Roche, Germany). Total cell counts can be found in table S3 of the supplemental data. Cells
162 were subsequently used for flow cytometry.
163

164

165

166 *Flow cytometric analysis*

167 The single lung cell suspensions were stained for T-cell subsets and macrophages using two different
168 mixes of antibodies for flow cytometry.
169 Frequencies of effector T cells (CD3+CD4+CD25+Foxp3-) and regulatory T cells
170 (CD3+CD4+CD25+Foxp3+) were examined using anti-CD3-Pacific Blue (Biolegend, Fell, Germany),
171 anti-CD4-PerCP (BD), anti-CD25-APC (BD), and anti-Foxp3-FITC (eBioscience, Vienna, Austria). An
172 appropriate isotype control was used for the Foxp3 staining (rat IgG2a-FITC, eBioscience). In addition,
173 frequencies of B cells (CD3-CD19+) were examined using anti-CD19-PE/Cy7 in this mix.
174 Frequencies of macrophages (autofluorescent+F4/80+CD11c+) and activated macrophages
175 (autofluorescent+F4/80+CD11c+MHCII+) were examined by using autofluorescence in the FITC
176 channel and using biotin-labeled anti-MHC class II (BD Biosciences) followed by PerCP-labeled
177 streptavidin (BD Biosciences), PE-labeled anti-CD11c (Biolegend) and pacific blue-labeled anti-F4/80
178 (Biolegend) (59).
179 Before staining with surface markers, $10^6$ cells per 25 µl were first incubated for 15 minutes on ice
180 with cold 10% normal mouse serum in phosphate buffered saline solution (PBS) to block aspecific
181 binding. Cells were subsequently incubated with the appropriate antibody mix for 30 minutes on ice,
182 protected from light. After washing the cells with PBS supplemented with 2% FCS, the macrophage
183 cell mix was incubated for 15 minutes with Streptavidin-PerCP, washed three times with PBS/2%FCS,
184 resuspended in FACS lysing solution (BD Biosciences), and kept in the dark on ice until flow cytometry
185 analysis. The cells of the T-cell mix were fixed and permeabilized for 30 minutes using a fixation and
186 permeabilization buffer kit (eBioscience), and then washed with permeabilization buffer, blocked
187 with 2% normal mouse serum and then incubated with anti-Foxp3 for 30 min. Thereafter the cells
188 were washed with permeabilization buffer, resuspended in FACS lysing solution, and kept in the dark
189 on ice until flow cytometric analysis. The fluorescent staining of the cells was measured on a LSR-II

190 flow cytometer (BD Biosciences) and data were analyzed using FlowJo Software (Tree Star, Ashland,
191 USA). Examples or our gating strategy can be found in figure S4 and S5 of the supplemental data.
192
193 *Histology*
194 During sacrifice, the trachea was cannulated and both lungs were carefully inflated with 50% Tissue-
195 Tek® O.C.T.™ compound (Sakura, Finetek Europe B.V., Zoeterwoude, The Netherlands) in PBS and
196 the right lung was snap-frozen for histological analysis and the left lung fixed in formalin.
197 The number of IL-33-producing cells was determined in 4 µm cryosections of lung tissue with a goat
198 polyclonal anti-IL-33 antibody and SLPI-producing cells were identified with a biotinylated goat
199 polyclonal anti-SLPI antibody (both R&D Systems). The number of the IL-33-producing cells and
200 intensity of SLPI-staining was measured separately in the infiltrates and parenchyma of whole tissue
201 sections using ImageScope software (Aperio, Vista, CA, USA). Type II alveolar epithelial (AEC type II)
202 cells were identified with a polyclonal rabbit anti-Pro-surfactant protein C antibody (anti-pro-SPC,
203 Millipore, Amsterdam, Netherlands) and were double-stained with either anti-IL-33 or anti-SLPI using
204 standard immunohistochemical procedures.
205 To determine the number of eosinophils in lavage fluid a Giemsa staining (Sigma-Aldrich) was
206 performed on cytospots. The number of eosinophils was counted in a total of 300 cells and the
207 percentage of eosinophils was used to calculate the absolute number of eosinophils as a fraction of
208 total cells in lavage fluid.
209
210 *Epithelial cell line*
211 The murine type-II alveolar epithelial-like cell line C10 was a kind gift from Dr. H.I. Heijink
212 (Department of Allergology, University Medical Center Groningen, The Netherlands). The cells were
213 cultured in RPMI (Lonza) supplemented with 10% FCS and gentamycin under 5% $CO_2$ at 37° according
214 to standard protocols.

215 C10 cells were plated into 12-wells plates at a density of 5 x $10^5$ cells/well. At 90% confluence, the
216 cells were starved for 24 hours. The cells were pre-incubated or not with human SLPI (10 µg/ml, R&D
217 Systems) for 1 hour and subsequently stimulated with ATP (100 µg/ml, Sigma) or were not treated
218 (control). After 24 hours, the cells were harvested and sonicated and levels of IL-33 were determined
219 by ELISA (R&D Systems).

220

221 *Statistical Analysis*

222 Data are represented as mean ± standard error of the mean. To determine the normality of the data
223 the Kolmogorov-Smirnov test was used. Data were log-transformed to fit a normal distribution when
224 not normally distributed. Differences between groups were tested using a one-way ANOVA followed
225 by Sidak's multiple comparisons test to compare Healthy vs SHAM, SHAM vs. OVX, OVX vs. OVX+E2,
226 and OVX vs. OVX+P. P-values $<0.05$ were considered to be statistically significant.

227

228 **Results**

229 *The process of sexual maturation dampens the severity of OVA-induced lung inflammation in adult*
230 *female mice*

231 To test whether preventing puberty would affect development of allergic lung inflammation in adult
232 mice we ovariectomized mice (OVX) or sham-treated them (SHAM) before they reached sexual
233 maturity. After inducing lung inflammation in these animals in adulthood we quantified a number of
234 parameters of allergic lung inflammation.

235 General histology showed that sham-treated, ovariectomized and ovariectomized + progesterone-
236 treated mice responded to OVA challenges with lung inflammation as compared to healthy controls.
237 Especially lungs of sham-treated and ovariectomized mice, and to a lesser extent ovariectomized +
238 progesterone-treated, showed thickening of the alveolar walls. Ovariectomized + estrogen-treated
239 mice demonstrated less lung inflammation and no thickening of the alveolar walls (figure 2).

240 Our previous studies showed that the severity of allergic lung inflammation correlated strongly with
241 increased presence of eosinophils, (activated) macrophages, effector T lymphocytes, regulatory T
242 lymphocytes and B lymphocytes in lung tissue (32–34). We therefore quantified these inflammatory
243 cells in lavage fluid and lung tissue to assess severity of inflammation. Higher numbers of eosinophils,
244 (activated) macrophages, effector T lymphocytes, regulatory T lymphocytes and B lymphocytes were
245 induced in lungs of OVA-exposed SHAM-treated mice as compared to healthy controls (figure 3). We
246 found higher numbers of eosinophils in lavage fluid and (activated) macrophages and B lymphocytes
247 in lung tissue of ovariectomized mice compared to sham-treated mice. Significantly lower numbers of
248 these cells were found in lung tissue of ovariectomized + estrogen-treated mice as compared to
249 untreated ovariectomized mice (figure 3A-D). Higher levels of OVA-specific IgE levels were found in
250 OVA-exposed SHAM-treated mice as compared to healthy controls. OVA-specific IgE levels in serum
251 were not significantly affected by ovariectomy, but treating ovariectomized mice with estrogen also
252 led to significantly lower levels as compared to untreated ovariectomized mice. Ovariectomized +
253 progesterone-treated mice and untreated ovariectomized mice had similar levels of OVA-specific IgE

254 (figure 3E). While ovariectomy did not affect numbers of effector and regulatory T lymphocytes,
255 treatment with estrogen led to significantly lower numbers of these cells in lung tissue as compared
256 to untreated ovariectomized mice. Progesterone treatment of ovariectomized mice did not affect
257 any of the aforementioned cells (figure 3F and G).
258
259 *Estrogen treatment increases SLPI expression and decreases IL-33 production in alveolar epithelial*
260 *cells in mice ovariectomized before puberty*
261 Based on our recent studies and in order to explain the mechanism behind this estrogen modulation
262 of lung inflammation we focused on secretory leukoprotease inhibitor (SLPI), an anti-inflammatory
263 factor that is known to be upregulated by estrogen in airway epithelial cells 9). We therefore studied
264 the expression of both SLPI in lung tissue in combination with the expression of the pro-asthmatic
265 cytokine IL-33. SLPI is an inhibitor of NFkB, which in turn regulates expression of IL-33. Double
266 stainings of lung tissue for these two mediators showed that both IL-33 and SLPI colocalize with pro-
267 SPC, a marker for type II alveolar epithelial cells (figure 4), indicating that IL-33 and SLPI have a similar
268 source, namely the type II alveolar epithelial cells.
269 The SLPI levels we determined in lavage fluid of control mice were too low to be detected but these
270 levels increased significantly in mice subjected to OVA-induced lung inflammation (figure 5A and B).
271 Ablating estrogen before sexual maturity led to lower SLPI levels in lavage fluid of ovariectomized
272 mice as compared to sham-treated mice. Subsequently treating ovariectomized mice with estrogen
273 restored higher SLPI levels as compared to untreated ovariectomized mice. We also tried to assess IL-
274 33 in lavage fluid, but found no detectable levels (data not shown).
275 We then quantified the number of SLPI-and IL-33 producing cells in parenchymal lung tissue to assess
276 if the number of cells producing these mediators had changed. The expression of SLPI in parenchymal
277 lung tissue was similarly low in healthy mice, sham-treated mice and ovariectomized mice, but
278 ovariectomized + estrogen-treated mice had significantly more cells expressing SLPI than untreated
279 ovariectomized mice (figure 5C).

280 Higher numbers of IL-33-producing cells were found in lung tissue of OVA-exposed SHAM-treated
281 mice as compared to healthy controls. The number of IL-33-producing cells was comparable in
282 ovariectomized mice and sham-treated mice, but was lower in parenchymal tissue of ovariectomized
283 + estrogen-treated mice as compared to untreated ovariectomized mice (Figure 5D). A double
284 staining of SLPI and IL-33 showed that there are almost no cells that expressed both SLPI and IL-33 at
285 the same time. Figure 5E shows an example of the double staining on lungs of ovariectomized +
286 estrogen-treated mice (figure 5E).

287

288 *SLPI decreases IL-33 production in alveolar epithelial type II cells*

289 To determine the direct effect of SLPI on IL-33 production, the murine C10 cell line (alveolar type II
290 cells) was treated with ATP, a danger signal that is an important effector molecule in the OVA model
291 of allergic lung inflammation, to induce the production of IL-33 (21). Untreated cells and cells treated
292 with SLPI alone were used as controls. We were unable to find any secreted IL-33 (data not shown)
293 and found all IL-33 present within cells. The amount of IL-33 in C10 type II epithelial cells was
294 significantly higher after treatment with ATP. After SLPI treatment, the ATP-induced IL-33 production
295 by C10 cells was significantly lower (Figure 6).

296

297 **Discussion**

298 Before puberty more boys suffer from asthma than girls, while this distribution changes during
299 puberty and more females develop asthma after puberty and suffer from more severe disease as
300 compared to males (1, 2, 13, 20, 27, 48, 60). Although many studies have focused on the female sex
301 hormones estrogen and progesterone to explain this dichotomy, their roles remain inconclusive. We
302 found that preventing sexual maturation by ablating sex hormones before "puberty" in mice
303 significantly increased the number of eosinophils and B lymphocytes as compared to sham-treated
304 mice, suggesting that the process of sexual maturation actually ameliorates some aspects of
305 subsequent lung inflammation in adult mice. Interestingly, this beneficial effect of sexual maturation
306 appears to solely depend on estrogen, as estrogen but not progesterone substitution after
307 ovariectomy inhibited lung inflammation. This effect of estrogen is associated with higher production
308 of SLPI and lower production of IL-33 by cells in lung parenchyma. Our further findings showed that
309 SLPI could directly inhibit IL-33 production in alveolar epithelial cells explaining how estrogen may
310 play a protective role in the development of lung inflammation.

311 Similar as seen in humans, female mice develop more severe allergic lung inflammation than male
312 mice and therefore this model was used to study effects of sexual maturation and sex hormones in
313 asthma development (34). Contrary to our findings, previous studies on rat and mouse ovalbumin-
314 induced allergic airway inflammation have shown that depletion of sex hormones before
315 sensitization inhibits lung inflammation and that treatment with estrogen before and during
316 ovalbumin immunization re-established lung inflammation in these ovariectomized animals (26, 37,
317 44). In addition, both pro-and anti-inflammatory effects of progesterone on asthma development
318 have been described (18, 35, 37). These previous studies have in common that ovariectomy or
319 hormone treatments were performed in adult animals with different timings of
320 ovariectomy/treatment with respect to induction of lung inflammation, which may be an important
321 determinant in the outcome of those studies. Since we focused on the relationship between

322 increasing estrogen and progesterone levels during puberty and the development of lung
323 inflammation, we performed ovariectomy and started estrogen or progesterone treatment before
324 sexual maturation and induced lung inflammation in adulthood. Contrary to our expectations, our
325 data clearly showed that the hormone-driven process of sexual maturation ameliorates some aspects
326 of the development of lung inflammation in adulthood. The disparity with the clinical situation may
327 be explained by the observational nature of the clinical studies and the fact that sexual maturation in
328 humans is a multi-factorial process that involves more than just an elevation of and the start of
329 cycling of sex hormones.

330 An explanation for the protective effect of hormone-driven sexual maturation may be found in the
331 changes in expression of immune genes that was shown to occur during sexual maturation in mice
332 (22). Lamason *et al.* showed a sexual dimorphism in innate and adaptive immune functions in post-
333 pubertal mice, which was dependent on puberty. Female mice had higher expression of adaptive
334 immune genes while males had higher expression of innate immune genes after sexual maturation.
335 The ovariectomy that we performed before this "puberty"-induced difference in immune function
336 could occur, may have prevented the upregulation of genes of the adaptive immune system and
337 thereby make female mice more susceptible to allergic lung inflammation. Here we did not study the
338 expression of immune genes, but it would be interesting to know what happens after substitution of
339 estrogen and progesterone, even though this would be without the natural cycling changes in sex
340 hormone levels. Both hormones were given at levels exceeding estrus levels (E2: ±150 pg/ml, P:±25
341 ng/ml) around a factor of 2 (E2: ±350 pg/ml, P:±55 ng/ml) (50). In sexually mature animals these
342 levels have been shown to promote lung inflammation and T helper 2 responses when exposed to
343 allergens, and also to induce regulatory T cells in healthy animals (18, 35, 36, 56, 57). However, in our
344 case estrogen substitution after ovariectomy strongly inhibited lung inflammation and this was
345 accompanied with lower numbers of regulatory T cells, while progesterone substitution had no effect
346 at all as compared to untreated ovariectomy. It therefore seems likely that estrogen substitution
347 induces changes in immune gene expression or immune function that is still possible in sexually

348 immature animals but can no longer occur in animals that have gone through normal sexual
349 maturation. The fact that normal sexual maturation only moderately protects against lung
350 inflammation as compared to the strong protection of estrogen substitution, may be explained by
351 the lack of corresponding progesterone increases in our model. The increasing levels of progesterone
352 may counteract the effects of estrogen during normal development. The importance of the ratio of
353 circulating estrogen-to-progesterone levels in the response to allergens was recently also suggested
354 by others (37, 57).

355 To elucidate the mechanism behind the estrogen-mediated protective effects on lung inflammation,
356 we first focused on quantifying known pro-asthmatic mediators like TSLP and IL-33. Our previous
357 studies in males and females did not show differences in TSLP (32), so we decided to study IL-33 first.
358 We found its expression to be significantly lower in lung parenchyma of ovariectomized mice treated
359 with estrogen as compared to untreated ovariectomized mice. IL-33 is a member of the IL-1 family
360 and it activates group 2 innate lymphoid cells (ILC2 cells), Th2 lymphocytes and mast cells to secrete
361 Th2 cytokines and chemokines leading to allergic inflammation (24, 49, 51). IL-33 expression in the
362 lung is higher in asthmatics than healthy controls (5, 42, 45) and polymorphisms in the IL-33 gene
363 have been associated with the development of asthma (47). In humans IL-33 expression has mainly
364 been shown in bronchial epithelial cells, but we found that IL-33 in mice was solely expressed in type
365 II alveolar epithelial cells, confirming that there are species-specific differences between human and
366 mice in IL-33 expression (41).

367 Currently, little is known about the molecular mechanisms involved in production of IL-33 (21), but
368 the NF-κB signaling pathway appears to be involved. In human corneal epithelial cells it was
369 demonstrated that IL-33 was produced in response to microbial pathogens through Toll like receptor
370 (TLR)/NF-κB signaling pathways (61). The same mechanism was confirmed in a newly identified
371 source of IL-33, i.e. dendritic cells (53). We therefore further investigated mediators known to inhibit
372 the NF-κB signaling pathway to explain how estrogen may inhibit IL-33 expression.

373 Previously, Chotirmall *et al.* found that estrogen inhibits the expression of the NF-κB-dependent
374 chemokine IL-8 in bronchial epithelial cells by upregulating SLPI. There is a partial estrogen response
375 element in the SLPI promoter and they showed that E2 upregulates expression of SLPI in bronchial
376 epithelial cells via estrogen receptor-β (9). SLPI is known to inhibit NF-κB activation by binding
377 directly to NF-κB binding sites in a site-specific manner (54) and we therefore investigated the
378 expression of SLPI in our model and indeed found higher levels in lavage fluid and lung parenchyma
379 of estrogen-treated ovariectomized mice than in lungs of ovariectomized mice. The highest SLPI
380 expression was found in the lung parenchyma and double stainings revealed that SLPI was also
381 produced by AECII. Since these are the same cells that expressed IL-33, we performed double
382 stainings for SLPI and IL-33. Interestingly, these mediators did not colocalize, meaning that the same
383 cell did not express SLPI and IL-33 at the same time. High expression of SLPI in lung tissue was
384 associated with low expression of IL-33, and vice versa. This suggested to us a direct inhibitory effect
385 of SLPI on IL-33 expression, a finding confirmed in our *in vitro* model of ATP-induced IL-33 production
386 by AECII. Of relevance to asthma, OVA-sensitized and challenged mice have increased levels of ATP in
387 the lungs and extracellular ATP modifies the recruitment/function of inflammatory cells, suggesting
388 that it is a key mediator in asthma (19). In response to extracellular ATP, IL-33 expression was higher
389 in AECII cells, but the production was significantly lower when SLPI was added. These findings
390 confirm the anti-inflammatory effects of SLPI as described previously in monocytes (12). Exogenously
391 applied SLPI to monocytes is taken up into the cells and is distributed in the cytoplasm and nucleus.
392 There it blocks NF-κB activation by inhibiting the degradation of IκBa and IκBb and by competing
393 with p65 for binding to NF-κB sites in the promoters of NF-κB-responsive genes and inhibits the
394 production of pro-inflammatory cytokines (15, 54, 55).

395 Taken together, our data show that the sexual maturation (puberty) ameliorates the development of
396 lung inflammation in mice. This protective effect of ‘puberty’ appears to be dependent on estrogen
397 and may be induced by production of the anti-inflammatory mediator SLPI and thereby inhibition of

398 production of pro-inflammatory IL-33 in parenchymal lung tissue. These data provide a new
399 perspective regarding the role of the female sex hormones estrogen and progesterone in asthma
400 pathogenesis.

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## Figure legends

***Figure 1.*** *Experimental design of the study. OVX, ovariectomy; SHAM, SHAM-operated mice; OVA, ovalbumin; i.p., intra-peritoneal.*

***Figure 2.*** *Hematoxylin and eosin staining of healthy, SHAM-treated, OVX, OVX+P and OVX+E2 mice. Sham-treated, OVX and OVX+P mice responded to OVA challenges with lung inflammation and thickening of the alveolar walls. OVX mice treated with estrogen demonstrated less lung inflammation and no thickening of the alveolar walls (magnification 100x).*

***Figure 3.*** *Inflammatory parameters in bronchoalveloar lavage fluid, lung tissue and serum of healthy, SHAM-treated, OVX, OVX+E2 and OVX+P mice. Higher numbers of eosinophils, (activated) macrophages and B lymphocytes were induced in lungs of OVA-exposed SHAM-treated mice as compared to healthy controls. OVX mice had higher numbers of eosinophils in lavage fluid and B lymphocytes in lung tissue as compared to SHAM-treated mice and significantly lower numbers of eosinophils, activated/macrophages and B lymphocytes were found in OVX mice treated with estrogen as compared to untreated OVX mice (A, B, C and D). Higher levels of OVA-specific IgE were detected in serum of SHAM-treated mice as compared to healthy mice. OVA-specific IgE levels in serum were not significantly affected by OVX, but OVX mice treated with estrogen had lower levels as compared to untreated OVX mice (E). Higher numbers of effector and regulatory T cells were found in lung tissue of SHAM-treated mice as compared to healthy mice. OVX did not affect numbers of effector and regulatory T lymphocytes in lung tissue, but E2 treatment led to significantly lower numbers of these cells in lung tissue as compared to untreated OVX (F and G). Progesterone treatment of OVX mice did not affect any of the indicated inflammatory parameters. P<0.01 statistical trend, *P<0.05 and **P<0.01 and ***P<0.001.*

***Figure 4*** *Immunohistochemical double stainings for alveolar epithelial cells (pro-SPC, red) and IL-33 (blue) showed colocalization of IL-33 expression in type II epithelial cells (A). Double stainings for*

598 alveolar epithelial cells (pro-SPC, blue) and SLPI (red) showed colocalization of SLPI expression in type
599 II epithelial cells (B) (magnification 200x).

600 **Figure 5.** SLPI and IL-33 expression in bronchoalveloar lavage fluid and lungs of healthy, SHAM-
601 treated, OVX and OVX+E2 mice. SLPI expression could not be detected in in BALF of healthy mice (ND,
602 not detected). Lower levels of SLPI were detected in lavage fluid of OVX mice as compared to sham-
603 treated and estrogen-treated OVX mice (A). Representative Western blot quantification of SLPI levels
604 in lavage fluid of 2 OVX, 1 SHAM and 1 OVX+E2 mice. Blots were rearranged, but all samples were
605 derived at the same time and processed in parallel (B). OVX+E2 mice had significantly more cells
606 expressing SLPI than SHAM mice and untreated OVX mice (C). Higher numbers of IL-33 producing cells
607 were induced in lungs of SHAM-treated mice than in lungs of healthy mice. Lower numbers of IL-33-
608 producing cells were found in OVX+E2 mice as compared to OVX (D). An example of a double staining
609 of lung tissue of ovariectomized + estrogen-treated mice for SLPI (red) and IL-33 (blue). The double
610 staining showed no colocalization of IL-33 and SLPI (magnification 200x) (E). *P<0.05, **P<0.01,
611 ***P<0.001 and ****P<0.0001.

612 **Figure 6.** Relative IL-33 concentration in C10 type II alveolar epithelial cells. ATP treatment induced
613 higher levels of IL-33 within cells as compared to control (**P<0.01). After SLPI treatment, the ATP-
614 induced IL-33 production was lower (**P<0.01). SLPI treatment alone did not affect IL-33 levels in
615 cells.

616