Sexual maturation protects against development of lung inflammation through estrogen.

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*Citation*

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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.
**Introduction**

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

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

This is of interest as estrogen can have direct effects on the immune system by binding to estrogen receptors expressed by several immune cells (10). Additionally, estrogen can influence airway responses indirectly by acting on structural cells, such as airway epithelial cells. In response to inhaled allergen, airway epithelial cells induce nuclear factor (NF)-κB-signaling and subsequently recruit and activate immune cells through production of pro-asthmatic mediators such as IL-33 and TSLP (17, 23). To protect the airways against escalating inflammation and subsequent damage, epithelial cells also secrete anti-inflammatory factors such as secretory leukoprotease inhibitor (SLPI)
SLPI exerts its anti-inflammatory effects via inhibition of NF-κB-signaling. Interestingly, SLPI expression has been found to be upregulated by estrogen treatment (9, 54, 55), but has not been linked to IL-33 signaling yet.

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

Animals

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

Ovariectomy and OVA model

Female BALB/c mice were ovariectomized (OVX) or sham-treated (SHAM) at 28 days of age before reaching sexual maturity (around day 35). OVX animals were either left untreated or subcutaneously received a 60-day slow release pellet of 0.1 mg estrogen (OVX+E2) or 15 mg progesterone (OVX+P) (IRA, Sarasota, FL, USA) at the time of ovariectomy. These E2 and P pellets were chosen because they have been shown to yield plasma levels about two times higher (E2 ±350 pg/ml, P ±55 ng/ml) than levels at estrus (for E2 ±150 pg/ml and for P ±25 ng/ml) (50). Uterine weight was assessed to check to quality of ovariectomy, estrogen and progesterone treatment (see figure S1 of the supplemental data). Ovariectomy strongly reduced uterine weight as compared to sham treatment. Estrogen treatment mostly prevented this low uterine weight, and progesterone treatment led to slightly, but significantly, higher uterine weight as compared to ovariectomy. Animals with normal (>40 mg) uterine weights after ovariectomy were excluded from the studies, as were animals with low (<15 mg) uterine weights after estrogen (indicating the pellet did not work) and or high (>50 mg) uterine weights after progesterone treatment (indicating that ovariectomy was not performed successfully).

Four weeks after ovariectomy (age 8 weeks), mice were sensitized intraperitoneally to 10 μg of ovalbumin (OVA, Grade V, Sigma-Aldrich Chemie BV, Zwijndrecht, The Netherlands) and 1.5 mg of
alum (Aluminject, Pierce Chemical, Etten-Leur, The Netherlands) on days 1 and 7, followed on day 11, by daily 20-min aerosol challenges with 1% w/v OVA in sterile PBS using a Pari LC Sprint Star nebulizer driven by a PARI Boy SX compressor (both kind gifts from Pari GmbH, Starnberg, Germany). Healthy controls (n=8) were not sensitized with alum and were exposed to PBS. An overview of the experimental design is depicted in figure 1.

On day 21 (age 11 weeks), serum was collected for OVA-specific IgE and animals were either sacrificed to collect bronchoalveolar lavage fluid and to isolate lung cells from digested lung for flow cytometry or sacrificed for histological analyses of lung tissue. The experimental groups consisted of 32 SHAM mice (15 for lavage/flow and 17 for histology), 18 OVX mice (10 for lavage/flow and 8 for histology), 11 OVX+E2 mice (6 for lavage/flow and 5 for histology), and 14 OVX+P (6 for lavage/flow and 8 for histology).

OVA-specific IgE

Serum levels of OVA-specific IgE were measured by ELISA as described previously (33). OVA-specific IgE titers are expressed as arbitrary ELISA units that were interpolated from a standard curve of a reference serum.

Bronchoalveolar lavage fluid

Bronchoalveolar lavage fluid was collected and cells were processed for cytospots to determine the percentage of eosinophils as described previously (33). Total and differential cell counts can be found in table S2 of the supplemental data. Lavage supernatants were used to assess the levels of secretory leucocyte protease inhibitor (SLPI) and IL-33.

SLPI levels in lavage supernatants were determined by western blot analysis using a biotinylated goat polyclonal α-SLPI antibody (R&D Systems, Oxon, UK). Briefly, equal amounts of total protein (25 µg) were run on a 15% SDS-PAGE gel and subsequently transferred to polyvinylidene difluoride membranes (Roche, Mannheim, Germany). The membranes were blocked with 5% blotting grade...
non-fat milk (Biorad, Veenendaal, Netherlands) and incubated with an anti-SLPI antibody overnight (in 5% milk). Next, the blots were incubated with rabbit-anti-goat HRP-conjugated secondary antibody followed by a goat-anti-rabbit HRP-conjugated tertiary antibody (both DAKO, Glostrup, Denmark). Blots were subsequently developed using sensitive Western Lightning-ECL reagent (Perkin-Elmer, Boston, MA) in a G-Box (Syngene, Cambridge, U.K.). Molecular weights were estimated based on the migration of ten known molecular weight standards (Biorad, Veenendaal, The Netherlands). Digital evaluation of blots was performed by GeneTools analysis software (Syngene, Cambridge, U.K.), which assigns a peak height to each band. The relative SLPI expression is described as SLPI expression in lavage fluid of OVX and OVX+E2 mice in relation to the SLPI expression in lavage fluid of SHAM mice.

The levels of IL-33 in lavage supernatants were determined by ELISA kits according to the manufacturer’s instructions (R&D Systems).

**Lung digestion**

After bronchoalveolar lavage, lungs were minced and incubated in RPMI medium supplemented with 10% fetal calf serum (both Lonza, Verviers, Belgium), 10 μg/ml DNase I (grade II from bovine pancreas, Roche Applied Science, Almere, Netherlands), and 0.7 mg/ml collagenase A (Sigma-Aldrich) for 45 min at 37°C in a shaking water bath. Single cell suspensions of lungs were obtained by passing the digested lung tissue through a 70 μm nylon strainer (BD Biosciences, Breda, Netherlands). Contaminating erythrocytes were lysed using 10 times diluted Pharmlyse (BD Biosciences). Mononuclear cells were then enriched by performing discontinuous Percoll (Pharmacia, Uppsala, Sweden) gradient centrifugation (20 to 55%). Cells collected on the 55% and 45% layers were passed through a 35 μm nylon strainer (BD Biosciences) and counted using a Sysmex pocH-100i cell counter (Sysmex, Roche, Germany). Total cell counts can be found in table S3 of the supplemental data. Cells were subsequently used for flow cytometry.
Flow cytometric analysis

The single lung cell suspensions were stained for T-cell subsets and macrophages using two different mixes of antibodies for flow cytometry.

Frequencies of effector T cells (CD3+CD4+CD25+Foxp3-) and regulatory T cells (CD3+CD4+CD25+Foxp3+) were examined using anti-CD3-Pacific Blue (Biolegend, Fell, Germany), anti-CD4-PerCP (BD), anti-CD25-APC (BD), and anti-Foxp3-FITC (eBioscience, Vienna, Austria). An appropriate isotype control was used for the Foxp3 staining (rat IgG2a-FITC, eBioscience). In addition, frequencies of B cells (CD3-CD19+) were examined using anti-CD19-PE/Cy7 in this mix.

Frequencies of macrophages (autofluorescent+F4/80+CD11c+) and activated macrophages (autofluorescent+F4/80+CD11c+MHCIi+) were examined by using autofluorescence in the FITC channel and using biotin-labeled anti-MHC class II (BD Biosciences) followed by PerCP-labeled streptavidin (BD Biosciences), PE-labeled anti-CD11c (Biolegend) and pacific blue-labeled anti-F4/80 (Biolegend) (59).

Before staining with surface markers, 10⁶ cells per 25 μl were first incubated for 15 minutes on ice with cold 10% normal mouse serum in phosphate buffered saline solution (PBS) to block aspecific binding. Cells were subsequently incubated with the appropriate antibody mix for 30 minutes on ice, protected from light. After washing the cells with PBS supplemented with 2% FCS, the macrophage cell mix was incubated for 15 minutes with Streptavidin-PerCP, washed three times with PBS/2%FCS, resuspended in FACS lysing solution (BD Biosciences), and kept in the dark on ice until flow cytometry analysis. The cells of the T-cell mix were fixed and permeabilized for 30 minutes using a fixation and permeabilization buffer kit (eBioscience), and then washed with permeabilization buffer, blocked with 2% normal mouse serum and then incubated with anti-Foxp3 for 30 min. Thereafter the cells were washed with permeabilization buffer, resuspended in FACS lysing solution, and kept in the dark on ice until flow cytometric analysis. The fluorescent staining of the cells was measured on a LSR-II.
flow cytometer (BD Biosciences) and data were analyzed using FlowJo Software (Tree Star, Ashland, USA). Examples or our gating strategy can be found in figure S4 and S5 of the supplemental data.

**Histology**

During sacrifice, the trachea was cannulated and both lungs were carefully inflated with 50% Tissue-Tek® O.C.T.™ compound (Sakura, Finetek Europe B.V., Zoeterwoude, The Netherlands) in PBS and the right lung was snap-frozen for histological analysis and the left lung fixed in formalin.

The number of IL-33-producing cells was determined in 4 µm cryosections of lung tissue with a goat polyclonal anti-IL-33 antibody and SLPI-producing cells were identified with a biotinylated goat polyclonal anti-SLPI antibody (both R&D Systems). The number of the IL-33-producing cells and intensity of SLPI-staining was measured separately in the infiltrates and parenchyma of whole tissue sections using ImageScope software (Aperio, Vista, CA, USA). Type II alveolar epithelial (AEC type II) cells were identified with a polyclonal rabbit anti-Pro-surfactant protein C antibody (anti-pro-SPC, Millipore, Amsterdam, Netherlands) and were double-stained with either anti-IL-33 or anti-SLPI using standard immunohistochemical procedures.

To determine the number of eosinophils in lavage fluid a Giemsa staining (Sigma-Aldrich) was performed on cytospots. The number of eosinophils was counted in a total of 300 cells and the percentage of eosinophils was used to calculate the absolute number of eosinophils as a fraction of total cells in lavage fluid.

**Epithelial cell line**

The murine type-II alveolar epithelial-like cell line C10 was a kind gift from Dr. H.I. Heijink (Department of Allergology, University Medical Center Groningen, The Netherlands). The cells were cultured in RPMI (Lonza) supplemented with 10% FCS and gentamycin under 5% CO₂ at 37° according to standard protocols.
C10 cells were plated into 12-wells plates at a density of $5 \times 10^5$ cells/well. At 90% confluence, the cells were starved for 24 hours. The cells were pre-incubated or not with human SLPI (10 µg/ml, R&D Systems) for 1 hour and subsequently stimulated with ATP (100 µg/ml, Sigma) or were not treated (control). After 24 hours, the cells were harvested and sonicated and levels of IL-33 were determined by ELISA (R&D Systems).

**Statistical Analysis**

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

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

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

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

Our previous studies showed that the severity of allergic lung inflammation correlated strongly with increased presence of eosinophils, (activated) macrophages, effector T lymphocytes, regulatory T lymphocytes and B lymphocytes in lung tissue (32–34). We therefore quantified these inflammatory cells in lavage fluid and lung tissue to assess severity of inflammation. Higher numbers of eosinophils, (activated) macrophages, effector T lymphocytes, regulatory T lymphocytes and B lymphocytes were induced in lungs of OVA-exposed SHAM-treated mice as compared to healthy controls (figure 3). We found higher numbers of eosinophils in lavage fluid and (activated) macrophages and B lymphocytes in lung tissue of ovariotomized mice compared to sham-treated mice. Significantly lower numbers of these cells were found in lung tissue of ovariotomized + estrogen-treated mice as compared to untreated ovariotomized mice (figure 3A-D). Higher levels of OVA-specific IgE levels were found in OVA-exposed SHAM-treated mice as compared to healthy controls. OVA-specific IgE levels in serum were not significantly affected by ovariotectomy, but treating ovariotomized mice with estrogen also led to significantly lower levels as compared to untreated ovariotomized mice. Ovariotomized + progesterone-treated mice and untreated ovariotomized mice had similar levels of OVA-specific IgE
While ovariectomy did not affect numbers of effector and regulatory T lymphocytes, treatment with estrogen led to significantly lower numbers of these cells in lung tissue as compared to untreated ovariectomized mice. Progesterone treatment of ovariectomized mice did not affect any of the aforementioned cells (figure 3F and G).

*Estrogen treatment increases SLPI expression and decreases IL-33 production in alveolar epithelial cells in mice ovariectomized before puberty*

Based on our recent studies and in order to explain the mechanism behind this estrogen modulation of lung inflammation we focused on secretory leukoprotease inhibitor (SLPI), an anti-inflammatory factor that is known to be upregulated by estrogen in airway epithelial cells 9). We therefore studied the expression of both SLPI in lung tissue in combination with the expression of the pro-asthmatic cytokine IL-33. SLPI is an inhibitor of NFkB, which in turn regulates expression of IL-33. Double stainings of lung tissue for these two mediators showed that both IL-33 and SLPI colocalize with pro-SPC, a marker for type II alveolar epithelial cells (figure 4), indicating that IL-33 and SLPI have a similar source, namely the type II alveolar epithelial cells.

The SLPI levels we determined in lavage fluid of control mice were too low to be detected but these levels increased significantly in mice subjected to OVA-induced lung inflammation (figure 5A and B). Ablating estrogen before sexual maturity led to lower SLPI levels in lavage fluid of ovariectomized mice as compared to sham-treated mice. Subsequently treating ovariectomized mice with estrogen restored higher SLPI levels as compared to untreated ovariectomized mice. We also tried to assess IL-33 in lavage fluid, but found no detectable levels (data not shown).

We then quantified the number of SLPI- and IL-33 producing cells in parenchymal lung tissue to assess if the number of cells producing these mediators had changed. The expression of SLPI in parenchymal lung tissue was similarly low in healthy mice, sham-treated mice and ovariectomized mice, but ovariectomized + estrogen-treated mice had significantly more cells expressing SLPI than untreated ovariectomized mice (figure 5C).
Higher numbers of IL-33-producing cells were found in lung tissue of OVA-exposed SHAM-treated mice as compared to healthy controls. The number of IL-33-producing cells was comparable in ovariectomized mice and sham-treated mice, but was lower in parenchymal tissue of ovariectomized + estrogen-treated mice as compared to untreated ovariectomized mice (Figure 5D). A double staining of SLPI and IL-33 showed that there are almost no cells that expressed both SLPI and IL-33 at the same time. Figure 5E shows an example of the double staining on lungs of ovariectomized + estrogen-treated mice (figure 5E).

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

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

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

Similar as seen in humans, female mice develop more severe allergic lung inflammation than male mice and therefore this model was used to study effects of sexual maturation and sex hormones in asthma development (34). Contrary to our findings, previous studies on rat and mouse ovalbumin-induced allergic airway inflammation have shown that depletion of sex hormones before sensitization inhibits lung inflammation and that treatment with estrogen before and during ovalbumin immunization re-established lung inflammation in these ovariectomized animals (26, 37, 44). In addition, both pro-and anti-inflammatory effects of progesterone on asthma development have been described (18, 35, 37). These previous studies have in common that ovariectomy or hormone treatments were performed in adult animals with different timings of ovariectomy/treatment with respect to induction of lung inflammation, which may be an important determinant in the outcome of those studies. Since we focused on the relationship between
increasing estrogen and progesterone levels during puberty and the development of lung inflammation, we performed ovariectomy and started estrogen or progesterone treatment before sexual maturation and induced lung inflammation in adulthood. Contrary to our expectations, our data clearly showed that the hormone-driven process of sexual maturation ameliorates some aspects of the development of lung inflammation in adulthood. The disparity with the clinical situation may be explained by the observational nature of the clinical studies and the fact that sexual maturation in humans is a multi-factorial process that involves more than just an elevation of and the start of cycling of sex hormones.

An explanation for the protective effect of hormone-driven sexual maturation may be found in the changes in expression of immune genes that was shown to occur during sexual maturation in mice (22). Lamason et al. showed a sexual dimorphism in innate and adaptive immune functions in post-pubertal mice, which was dependent on puberty. Female mice had higher expression of adaptive immune genes while males had higher expression of innate immune genes after sexual maturation. The ovariectomy that we performed before this “puberty”-induced difference in immune function could occur, may have prevented the upregulation of genes of the adaptive immune system and thereby make female mice more susceptible to allergic lung inflammation. Here we did not study the expression of immune genes, but it would be interesting to know what happens after substitution of estrogen and progesterone, even though this would be without the natural cycling changes in sex hormone levels. Both hormones were given at levels exceeding estrus levels (E2: ±150 pg/ml, P:±25 ng/ml) around a factor of 2 (E2: ±350 pg/ml, P:±55 ng/ml) (50). In sexually mature animals these levels have been shown to promote lung inflammation and T helper 2 responses when exposed to allergens, and also to induce regulatory T cells in healthy animals (18, 35, 36, 56, 57). However, in our case estrogen substitution after ovariectomy strongly inhibited lung inflammation and this was accompanied with lower numbers of regulatory T cells, while progesterone substitution had no effect at all as compared to untreated ovariectomy. It therefore seems likely that estrogen substitution induces changes in immune gene expression or immune function that is still possible in sexually
immature animals but can no longer occur in animals that have gone through normal sexual maturation. The fact that normal sexual maturation only moderately protects against lung inflammation as compared to the strong protection of estrogen substitution, may be explained by the lack of corresponding progesterone increases in our model. The increasing levels of progesterone may counteract the effects of estrogen during normal development. The importance of the ratio of circulating estrogen-to-progesterone levels in the response to allergens was recently also suggested by others (37, 57).

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

Currently, little is known about the molecular mechanisms involved in production of IL-33 (21), but the NF-κB signaling pathway appears to be involved. In human corneal epithelial cells it was demonstrated that IL-33 was produced in response to microbial pathogens through Toll like receptor (TLR)/NF-κB signaling pathways (61). The same mechanism was confirmed in a newly identified source of IL-33, i.e. dendritic cells (53). We therefore further investigated mediators known to inhibit the NF-κB signaling pathway to explain how estrogen may inhibit IL-33 expression.
Previously, Chotirmall et al. found that estrogen inhibits the expression of the NF-κB-dependent chemokine IL-8 in bronchial epithelial cells by upregulating SLPI. There is a partial estrogen response element in the SLPI promoter and they showed that E2 upregulates expression of SLPI in bronchial epithelial cells via estrogen receptor-β (9). SLPI is known to inhibit NF-κB activation by binding directly to NF-κB binding sites in a site-specific manner (54) and we therefore investigated the expression of SLPI in our model and indeed found higher levels in lavage fluid and lung parenchyma of estrogen-treated ovarioctomized mice than in lungs of ovarioctomized mice. The highest SLPI expression was found in the lung parenchyma and double stainings revealed that SLPI was also produced by AECII. Since these are the same cells that expressed IL-33, we performed double stainings for SLPI and IL-33. Interestingly, these mediators did not colocalize, meaning that the same cell did not express SLPI and IL-33 at the same time. High expression of SLPI in lung tissue was associated with low expression of IL-33, and vice versa. This suggested to us a direct inhibitory effect of SLPI on IL-33 expression, a finding confirmed in our *in vitro* model of ATP-induced IL-33 production by AECII. Of relevance to asthma, OVA-sensitized and challenged mice have increased levels of ATP in the lungs and extracellular ATP modifies the recruitment/function of inflammatory cells, suggesting that it is a key mediator in asthma (19). In response to extracellular ATP, IL-33 expression was higher in AECII cells, but the production was significantly lower when SLPI was added. These findings confirm the anti-inflammatory effects of SLPI as described previously in monocytes (12). Exogenously applied SLPI to monocytes is taken up into the cells and is distributed in the cytoplasm and nucleus. There it blocks NF-κB activation by inhibiting the degradation of IκBa and IκBb and by competing with p65 for binding to NF-κB sites in the promoters of NF-κB-responsive genes and inhibits the production of pro-inflammatory cytokines (15, 54, 55).

Taken together, our data show that the sexual maturation (puberty) ameliorates the development of lung inflammation in mice. This protective effect of ‘puberty’ appears to be dependent on estrogen and may be induced by production of the anti-inflammatory mediator SLPI and thereby inhibition of
production of pro-inflammatory IL-33 in parenchymal lung tissue. These data provide a new perspective regarding the role of the female sex hormones estrogen and progesterone in asthma pathogenesis.


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

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

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