

Estrogen and progesterone decrease *let-7f* microRNA expression and increase IL-23/IL-23 receptor signaling and IL-17A production in patients with severe asthma

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Background: Women have an increased prevalence of severe asthma compared with men. IL-17A is associated with severe asthma and requires IL-23 receptor (IL-23R) signaling, which is negatively regulated by *let-7f* microRNA.

Objective: We sought to Determine the mechanism by which 17 β -estradiol (E2) and progesterone (P4) increase IL-17A production. **Methods:** IL-17A production was determined by using flow cytometry in T_H17 cells from women (n = 14) and men (n = 15) with severe asthma. Cytokine levels were measured by using ELISA, and IL-23R and *let-7f* expression was measured by using quantitative PCR in T_H17-differentiated cells from healthy women (n = 13) and men (n = 14). In sham-operated or ovariectomized female mice, 17 β -E2, P4, 17 β -E2+P4, or vehicle pellets were administered for 3 weeks before *ex vivo* T_H17 cell differentiation. Airway neutrophil infiltration and CXCL1 (KC) expression were also determined in ovalbumin (OVA)-challenged wild-type female recipient mice with an adoptive transfer of OVA-specific T_H17 cells from female and male mice. **Results:** In patients with severe asthma and healthy control subjects, IL-17A production was increased in T_H17 cells from women compared with men. IL-23R expression was increased and *let-7f* expression was decreased in T_H17-differentiated cells from women compared with men. In ovariectomized mice IL-17A and IL-23R expression was increased and *Let-7f* expression

was decreased in T_H17 cells from mice administered 17 β -E2+P4 compared with those administered vehicle.

Furthermore, transfer of female OVA-specific T_H17 cells increased acute neutrophil infiltration in the lungs of OVA-challenged recipient mice compared with transfer of male OVA-specific T_H17 cells.

Conclusions: 17 β -E2+P4 increased IL-17A production from T_H17 cells, providing a potential mechanism for the increased prevalence of severe asthma in women compared with men. (J Allergy Clin Immunol 2015;■■■■:■■■■-■■■■.)

Key words: Estrogen, IL-17A, IL-23 signaling, *Let-7f*, progesterone, severe asthma

Sexual dimorphism exists in the setting of asthma, including severe asthma.¹ In children, severe asthma is more prevalent in boys than in girls, but after puberty, there is a change in prevalence, with women being 2 times more likely than men to have severe asthma.^{1,2} This change in asthma prevalence suggests a role for sex hormones in asthma pathogenesis, and it is highly likely that sex differences in airway inflammation influence the sex predilection in asthmatic patients. However, the mechanisms that regulate age-related sex differences in asthma prevalence and severity remain unclear.

Asthma has long been associated with CD4⁺ T_H2-induced inflammation, with increased IL-4, IL-5, and IL-13 secretion and increased airway eosinophil counts.³⁻⁶ Patients with severe asthma might have T_H2 cytokine-mediated inflammation but might also have IL-17-mediated inflammation with increased neutrophil infiltration.^{3,7,8} IL-17A levels and T_H17 cell counts are increased in the bronchoalveolar lavage (BAL) fluid of patients with severe asthma compared with values in healthy control subjects or patients with mild asthma.^{9,10} In the lungs IL-17A is secreted by CD4⁺ T_H17 cells, as well as $\gamma\delta$ T cells and group 3 innate lymphoid cells (ILC3s).¹¹⁻¹³ Because sex has a role in the regulation of severe asthma prevalence and IL-17A is associated with severe asthma, the focus of our study was to determine the role of sex hormones in CD4⁺ T_H17 cell differentiation and IL-17A protein expression from CD4⁺ T_H17 cells by using PBMCs in human subjects and splenocytes in mice. We also wanted to determine the role of sex in IL-17A-mediated airway inflammation and neutrophil recruitment.

T_H17 cells, a distinct subset of CD4⁺ T cells, secrete IL-17A and IL-17F, as well as other cytokines. IL-23 signaling through the IL-23 receptor (IL-23R) is important in maintaining and stabilizing the T_H17 cell phenotype and increasing IL-17A protein expression.^{14,15} Recently, *let-7f*, a member of the *let-7* microRNA (miRNA) family, negatively regulated IL-17A protein expression

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Abbreviations used

AhR:	Aryl hydrocarbon receptor
BAL:	Bronchoalveolar lavage
E2:	Estradiol
FITC:	Fluorescein isothiocyanate
GAPDH:	Glyceraldehyde-3-phosphate dehydrogenase
ILC3:	Group 3 innate lymphoid cell
IL-23R:	IL-23 receptor
IRF-4:	Interferon regulatory factor 4
miRNA:	MicroRNA
OVA:	Ovalbumin
P4:	Progesterone
PE:	Phytoerythrin
qPCR:	Quantitative PCR
ROR:	Retinoic acid–related orphan receptor
SLE:	Systemic lupus erythematosus
STAT:	Signal transducer and activator of transcription
WT:	Wild-type

by decreasing IL-23R surface expression on T_H17 cells.¹⁶ CD4⁺ T cells, including T_H17 cells, express the estrogen nuclear receptors estrogen receptor α and β , progesterone receptors 1 to 4, and androgen receptors through which 17 β -E2 (estradiol), progesterone (P4), and testosterone signal, respectively.^{17,18} Estrogen signaling in the MCF-7 breast cancer cell line decreased *let-7f* expression, suggesting a role for estrogen in regulating *let-7f* expression.¹⁹ However, the role of sex hormones in regulating *let-7f* expression in CD4⁺ T cells has not been determined. Based on the increased prevalence of severe asthma in women compared with men and the increased airway neutrophil infiltration associated with IL-17A in patients with severe asthma, we hypothesized that T_H17 cells from women with severe asthma have increased IL-17A production compared with that seen in cells from men with severe asthma.

METHODS

A full description of the methods used in this study can be found in the **Methods** section in this article's Online Repository at www.jacionline.org. Data are presented as means $\pm$ SEMs, with a *P* value of less than .05 being significant.

Recruitment of participants with severe asthma

Patients with severe asthma (aged 18–45 years), as defined by the Severe Asthma Research Program,²⁰ were recruited from the Vanderbilt University. These patients with severe asthma were clinically stable and not undergoing an exacerbation and continued taking their asthma medications.

Isolation of memory or naive CD4⁺ T cells from participants

Memory (CD45RO⁺) or naive (CD45RA⁺) CD4⁺ T cells were isolated from PBMCs of patients with severe asthma or healthy subjects (catalog #130-094-125 and #130-094-131; Miltenyi Biotec, Bergisch Gladbach, Germany). Memory CD4⁺ T cells were then prepared for flow cytometry. Naive CD4⁺ T cells were activated and differentiated as described below and in the **Methods** section in this article's Online Repository.

Mice

Wild-type (WT) mice were purchased from Charles River Laboratories (Wilmington, Mass). Sham or ovariectomy surgeries were conducted at 3 to

4 weeks of age. D011.10 transgenic mice were purchased from Jackson Laboratories and bred at Vanderbilt University. Mouse experiments were performed in an Association for Assessment and Accreditation of Laboratory Animal Care–accredited facility with international animal care and use committee approval.

T_H17 cell differentiation from CD4⁺ naive T cells in human subjects and mice

Naive (CD45RA⁺) CD4⁺ T cells were isolated from the PBMCs of healthy subjects (aged 18–45 years) or the spleens of mice by using naive T-cell isolation kits (Miltenyi Biotec catalog nos. 130-094-131 and 130-093-227), activated with anti-CD3 and anti-CD28 antibodies, and differentiated into become T_H17 cells, as previously described and in the **Methods** section in this article's Online Repository.^{21,22}

RESULTS

Patients with severe asthma have an increased number of T_H17 cells in the peripheral blood compared with healthy control subjects.^{9,10,23} Therefore we hypothesized that IL-17A production is increased by T_H17 memory cells from women with severe asthma compared with T_H17 memory cells from men with severe asthma. To test our hypothesis, we recruited healthy subjects and patients with severe asthma who were clinically stable. All participants were 18 to 45 years old and did not have a viral or bacterial infection or a T_H17-associated disease (eg, multiple sclerosis, psoriasis, or systemic lupus erythematosus [SLE]). Women were excluded if they were pregnant, breast-feeding, or taking hormonal birth control medications. Patients with severe asthma were defined based on the guidelines used by the Severe Asthma Research Program.²⁰ There was no significant difference in age, race, body mass index, lung function, total IgE plasma levels, or severe asthma qualifying criteria between women and men with severe asthma (see **Tables E1** and **E2** in this article's Online Repository at www.jacionline.org). CD4⁺CD45RO⁺ memory T cells were isolated from PBMCs by using negative selection, and we first determined that IL-17A⁺CD4⁺ memory T_H17 cell counts, as identified based on CCR6 surface expression,^{24,25} were increased in patients with severe asthma (*n* = 29, both women and men) compared with those in healthy adult control subjects (*n* = 9; **Fig 1, A–C**). Next, we stratified patients with severe asthma based on sex, and women with severe asthma had increased percentages and total numbers of IL-17A⁺ memory T_H17 cells compared with men with severe asthma (**Fig 1, D and E**).

Patients with severe asthma can also have increased T_H2 cytokine production and increased IL-17A/IL-4–producing CD4⁺ T cells.^{8,26} Therefore total numbers of IL-17A/IL-4 dual-producing, CCR6⁺ memory T_H17 cells were also determined, and no difference was found (see **Fig E1, A–C**, in this article's Online Repository at www.jacionline.org). There was also no difference in IL-4 production in CD3⁺CD4⁺ memory T cells or total IgE plasma levels between women and men with severe asthma (see **Fig E1, D and E**, and **Table E2**). Furthermore, we found no association between total IgE plasma levels and IL-17A⁺ memory T_H17 cell numbers (see **Fig E1, F**). Collectively, these data showed that women with severe asthma had increased numbers of IL-17A–producing T_H17 cells compared with men with severe asthma, but the mechanism or mechanisms remained unclear.

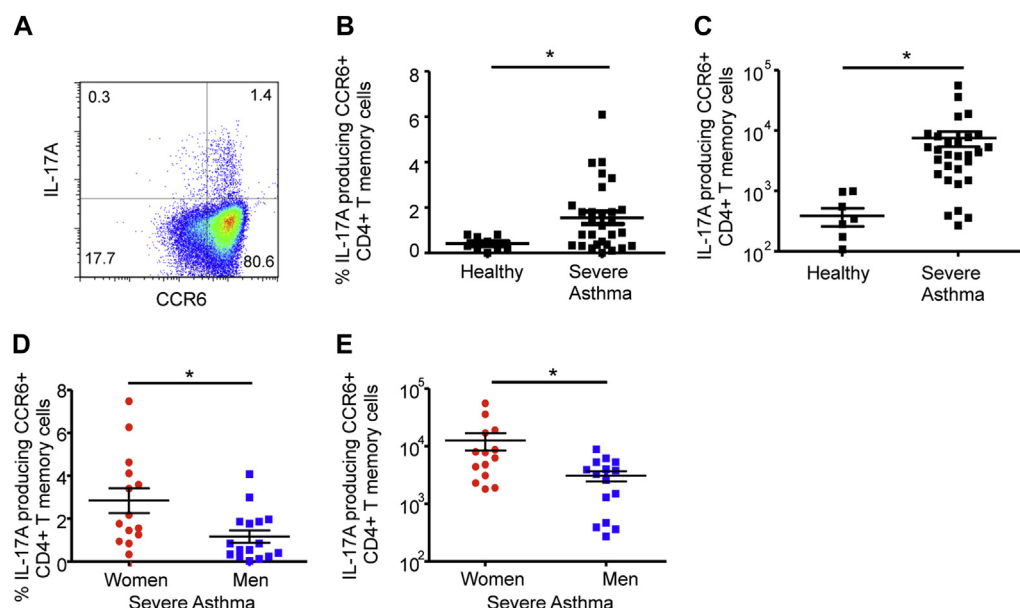


FIG 1. IL-17A⁺ memory T_H17 cell numbers are increased in women compared with men with severe asthma. **A–C**, Percentages and total numbers of IL-17A–producing CCR6⁺ memory T_H17 cells in healthy control subjects and patients with severe asthma. **P* < .05, Mann-Whitney *U* test; *n* = 9 healthy subjects and *n* = 29 patients with severe asthma patients (combined women and men). **D** and **E**, Percentages and total numbers of IL-17A⁺ memory T_H17 cells in women and men with severe asthma. **P* < .05, Mann-Whitney *U* test; *n* = 14 women and *n* = 15 men.

IL-17A protein expression is increased in T_H17-differentiated cells from women compared with expression in men

To determine the mechanisms by which sex and sex hormones regulate IL-17A protein expression and T_H17 cell differentiation, we next isolated naive T cells from healthy men and women and differentiated these naive T cells to T_H17 cells. We differentiated naive T cells to T_H17 cells from healthy patients for the following reasons: (1) to determine expression levels of transcription factors, cytokines, and cell-surface receptors required for T_H17 cell differentiation and (2) to generalize the effect of sex hormones on T_H17 development independent of disease state. T_H0 and T_H17 culture supernatants were analyzed for IL-17A and IL-17F protein expression, and as expected, IL-17A and IL-17F levels were significantly increased in T_H17 cells compared with those in T_H0 cells from both women and men (Fig 2, A and B). IL-17A, but not IL-17F, levels were significantly increased in T_H17 cells from women compared with levels in T_H17 cells from men. Protein expression of other cytokines produced by T_H17 cells (ie, IL-10, GM-CSF, and IL-21) was also determined. T_H17 cells from women had a significant increase in IL-10 protein expression compared with T_H17 cells from men; GM-CSF and IL-21 protein expression was similar in T_H17 cells from women and men (see Fig E2 in this article's Online Repository at www.jacionline.org).

We then determined whether increased IL-17A production in T_H17 cells from women compared with that in T_H17 cells from men was due to increased numbers of T_H17-differentiated cells or the amount of IL-17A produced in each T_H17 cell. Four days after T_H17 cell differentiation, cells were collected and restimulated with phorbol 12-myristate 13-acetates and ionomycin in the presence of GolgiStop (BD

Biosciences, San Jose, Calif), and IL-17A–producing T_H17 cell counts were determined. Although the number of CD3⁺CD4⁺ cells was similar between women and men (Fig 2, C), percentages, as shown in the representative dot plot, and total numbers of IL-17A–producing CD3⁺CD4⁺ T cells were significantly greater in the cells from women compared with those from men (Fig 2, D and E). Furthermore, the geometric mean fluorescence intensity for IL-17A⁺CD3⁺CD4⁺ cells was also increased in T_H17 cells from women compared with that in T_H17 cells from men (Fig 2, F). Combined, these data suggested that ovarian sex hormones increased T_H17 cell differentiation and IL-17A production from T_H17 cells.

We next determined whether 17β-E2 and P4 plasma levels at the time of the blood draw correlated with IL-17A protein expression in T_H17 cells from women. Neither 17β-E2 nor P4 plasma levels were significantly associated with IL-17A protein expression in T_H17 cells from women (see Fig E3, A and B, in this article's Online Repository at www.jacionline.org), suggesting circulating levels of 17β-E2 and P4 at the time of the blood draw were not responsible for the observed increase in IL-17A production from T_H17 cells. We next determined whether 17β-E2 (3–10 nmol/L) increased IL-17A protein expression from T_H17-differentiated cells by adding 17β-E2 to the T_H17 cell differentiation medium at the time of T-cell activation. Addition of 17β-E2 at the time of *in vitro* differentiation had no effect on IL-17A protein expression when compared with vehicle (0 nmol/L) in T_H17 cells from women or men (see Fig E3, C). These data suggest the increase in IL-17A production by T_H17 cells from women compared with men was not an acute effect of ovarian hormones but rather caused by the exposure of T cells to ovarian hormones during *in vivo* development before *ex vivo* T_H17 cell differentiation.

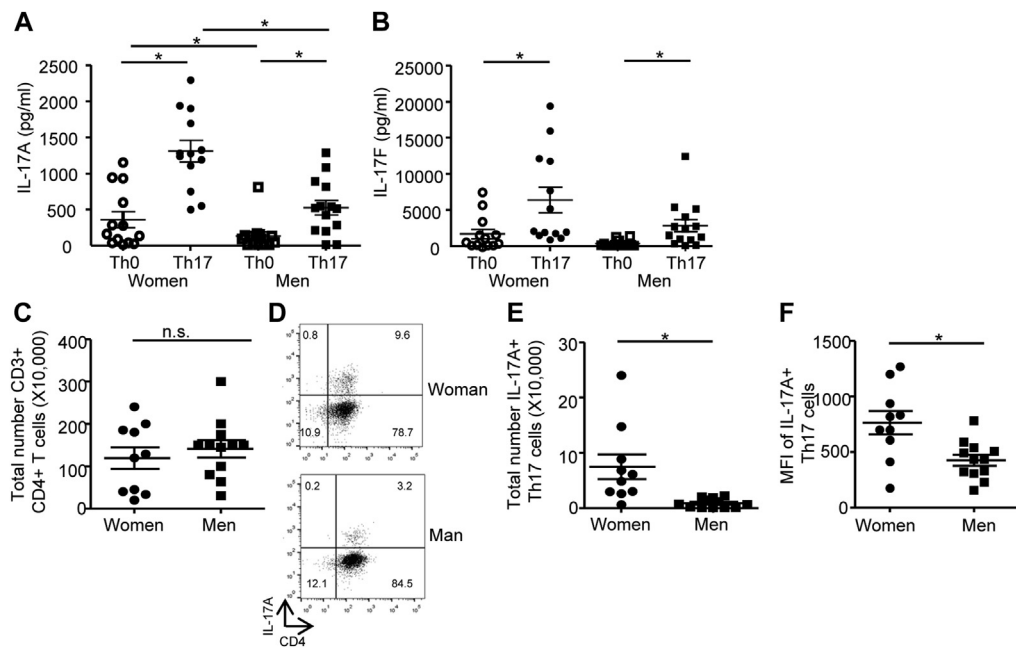


FIG 2. IL-17A protein expression is increased in Th0 and Th17 cells from women compared with those from men. **A** and **B**, Naive CD4⁺ T cells were differentiated into Th0 and Th17 cells. IL-17A and IL-17F protein expression was determined in Th0 and Th17 cells from healthy women (n = 13) or men (n = 14). **P* < .05, Kruskal-Wallis test with Dunn posttest analysis. **C**, Total number of CD3⁺CD4⁺ Th17 cells. **D-F**, Percentages, total numbers, and/or geometric mean fluorescence intensity for CD3-gated CD4⁺IL-17A⁺ Th17 cells. **P* < .05, Kruskal-Wallis test; n = 10 women and n = 12 men. n.s., Not significant.

IL-23R surface expression is increased and *let-7f* expression is decreased in Th17 cells from women compared with that seen in Th17 cells from men

To delineate the mechanism by which ovarian hormones increased IL-17A protein expression from Th17 cells, we next determined phosphorylation or expression levels of transcription factors involved with Th17 cell differentiation, including signal transducer and activator of transcription 3 (STAT3), retinoic acid–related orphan receptor C2 (*RORC2*), interferon regulatory factor 4 (*IRF4*), and aryl hydrocarbon receptor (*AHR*). No differences in STAT3 phosphorylation or *RORC2*, *IRF4*, and *AHR* mRNA expression were detected in Th17 cells from women compared with that in Th17 cells from men (data not shown). IL-23/IL-23R signaling is important for sustainability of the Th17 cell lineage and increased IL-17A protein expression.^{14,15} Therefore we also determined IL-23R surface expression in Th0 and Th17 cells from women and men using flow cytometry. Th17 cells from women had a significant increase in cell-surface expression of IL-23R compared with Th17 cells from men (Fig 3, A and B), and the total number of Th17 cells did not change between women and men (data not shown). Furthermore, the geometric mean fluorescence intensity for IL-23R surface expression on Th17 cells was also significantly increased in Th17 cells from women (1537.6 ± 297.3 cells) compared with that in Th17 cells from men (602.7 ± 181.5 cells, n = Th17 cells from 8 women and 7 men; *P* < .05, Mann-Whitney *U* test).

On the basis of these data, we determined IL-23R surface expression on a subset of memory Th17 cells from women and men with severe asthma. IL-23R surface expression was also increased on CD3⁺CD4⁺CCR6⁺ memory Th17 cells from

women with severe asthma compared with that in men with severe asthma (see Fig E4 in this article's Online Repository at www.jacionline.org). Combined, these data suggest that IL-23R surface expression is increased on Th17 cells from healthy women and women with severe asthma compared with that on Th17 cells from healthy men and men with severe asthma.

IL-23R expression was previously shown to be negatively regulated by the miRNA *let-7f* in CD4⁺ lymphocytes,¹⁶ and *let-7f* was negatively regulated by estrogen receptor signaling in the MCF-7 breast cancer cell line.¹⁹ Therefore we determined *let-7f* miRNA expression in Th17-differentiated cells from healthy women and men. Th17 cells from women had a significant decrease in *let-7f* expression compared with Th17 cells from men, but *let-7f* expression was similar in Th0 cells from women and men (Fig 3, C). These data suggest that decreased *let-7f* expression can be associated with increased IL-23R expression and IL-17A production in Th17 cells from women compared with that in Th17 cells from men.

Inhibition of *let-7f* miRNA increases IL-17A production in Th17 cells from both women and men

To determine whether *let-7f* miRNA regulated IL-17A protein expression differentially in women and men, we transfected a *let-7f* inhibitor (10 nmol/L) or negative control (10 nmol/L) into naive CD4⁺ T cells from healthy women and men and then differentiated the cells to Th17 cells. The *let-7f* inhibitor decreased *let-7f* expression by approximately 50% in Th17 cells from women and men (see Fig E5 in this article's Online Repository at www.jacionline.org). Inhibition of *let-7f* significantly increased IL-17A production in Th17 cells from both women and men

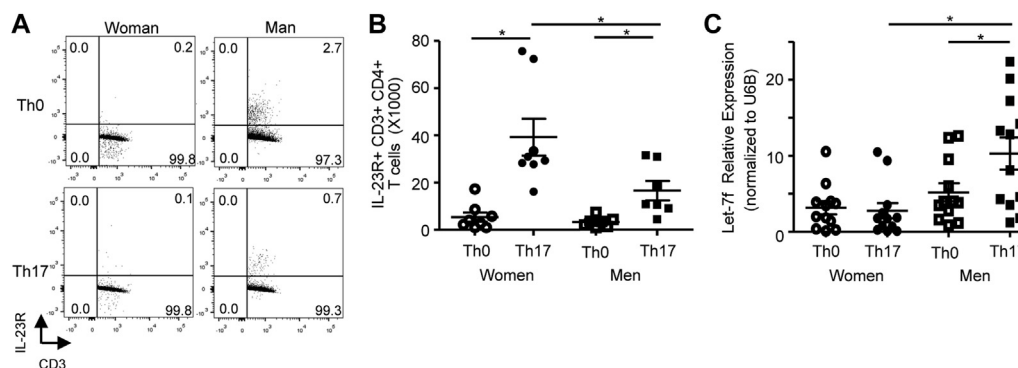


FIG 3. IL-23R surface expression is increased and *let-7f* expression is decreased in TH17 cells from women compared with values in men. **A** and **B**, Dot plot and total IL-23R⁺ CD3⁺ CD4⁺ T cells from women and men. **P* < .05, Kruskal-Wallis test with Dunn posttest analysis; *n* = 8 women and *n* = 7 men. **C**, *Let-7f* miRNA expression normalized to U6B expression in TH0 and TH17 cells from women and men. **P* < .05, Kruskal-Wallis test with Dunn posttest analysis; *n* = 12 women and men.

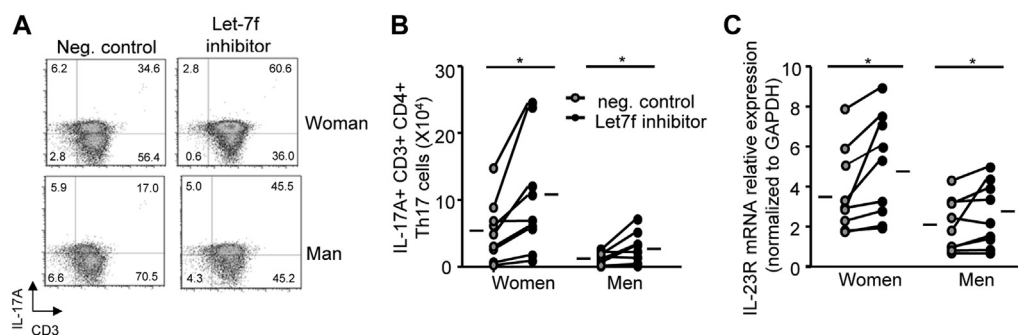


FIG 4. *Let-7f* decreased IL-23R surface expression and IL-17A production from TH17 cells. A 10 nmol/L *let-7f* inhibitor or negative control was transfected into naive T cells that were then differentiated into TH17 cells. **A** and **B**, Dot plots and total numbers of IL-17A⁺ TH17 cells. **C**, *IL23R* mRNA expression normalized to *GAPDH*. Dashes are means. **P* < .05, Wilcoxon matched pairs test; *n* = 9 women and *n* = 9 men.

compared with that in TH17 cells transfected with the negative control from both women and men, respectively (Fig 4, A and B). We also determined *IL23R* mRNA expression, and TH17 cells from women or men transfected with the *let-7f* inhibitor had increased *IL23R* mRNA expression compared with TH17 cells from women or men transfected with the negative control (Fig 4, C). These data show that *let-7f* negatively regulates *IL23R* expression and IL-17A production in TH17 cells and that the decreased *let-7f* expression in TH17 cells from women results in increased IL-17A production when compared with that in TH17 cells from men.

IL-17A protein expression is increased in TH17 cells from female mice compared with that in TH17 cells from male mice

Our data from human TH17 cells strongly suggest *in vivo* sex hormones regulated naive T-cell development before isolation and affected *in vitro* TH17 cell differentiation. Therefore we moved to a mouse model to test the *in vivo* effects of 17 β -E2 and P4 on TH17 cell differentiation and IL-17A protein expression. TH17 cells from female mice had increased IL-17A, but not IL-17F, production and increased *il23r* mRNA expression compared with that seen in TH17 cells from male mice (Fig 5, A-C). We also determined the requirement for IL-23R signaling

on increased IL-17A protein expression in TH17 cells from female and male mice by adding 0 to 30 ng/mL rmIL-23 to naive T cells during TH17 cell differentiation and keeping other TH17 differentiation conditions the same. TH17 cells from female and male mice differentiated without rmIL-23 (0 ng/mL) had similar IL-17A protein expression (Fig 5, D). However, IL-17A protein expression was increased in TH17 cells from female mice with the addition of 3 to 30 ng/mL rmIL-23 compared with that seen in TH17 cells from female mice with no rmIL-23 (0 ng/mL). Furthermore, the addition of rmIL-23 (10-30 ng/mL) increased IL-17A protein expression in TH17 cells from female mice to a significantly greater degree compared with that seen in TH17 cells from male mice.

In vivo exposure to 17 β -E2 and P4 increased IL-17A production from *in vitro* TH17-differentiated cells

To determine the mechanism or mechanisms by which ovarian hormones regulate IL-17A production in TH17 cells, we used female and male mice that were ovariectomized or sham operated before puberty. Slow-release pellets containing 17 β -E2 (0.1 mg), P4 (25 mg), 5 α -dihydrotestosterone (15 mg), the combination of 17 β -E2 (0.1 mg) and P4 (25 mg), or vehicle were subcutaneously implanted into adult ovariectomized female BALB/c mice. After 21 days, splenic naive T cells were differentiated into TH17 cells.

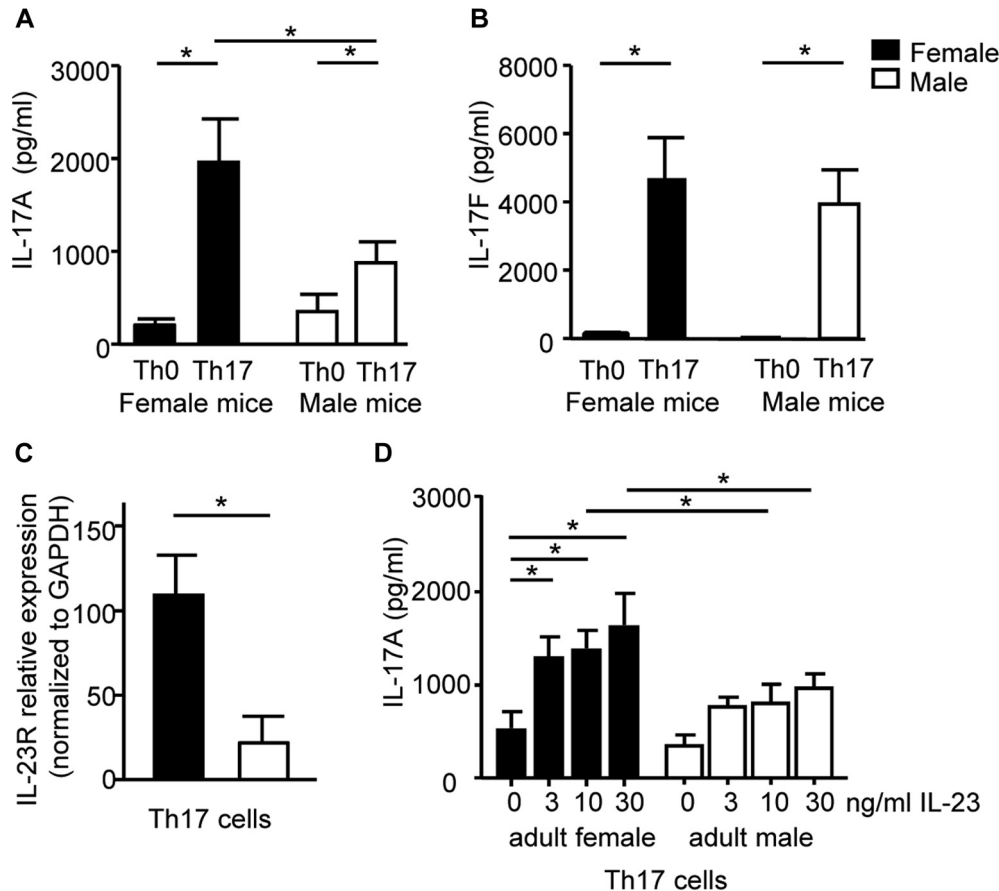


FIG 5. IL-17A protein expression is increased in Th0 and Th17 cells from female mice compared with that in Th0 and Th17 cells from male mice. **A-C**, IL-17A and IL-17F protein expression or *il23r* mRNA expression normalized to *gapdh* in Th0 and Th17 cells from female and male adult or prepubescent mice. **D**, IL-17A protein expression from adult Th17 cells differentiated with rmlL-23 (0-30 ng/mL). * $P < 0.05$, ANOVA with Tukey posttest analysis; $n = 6$, 2 experiments.

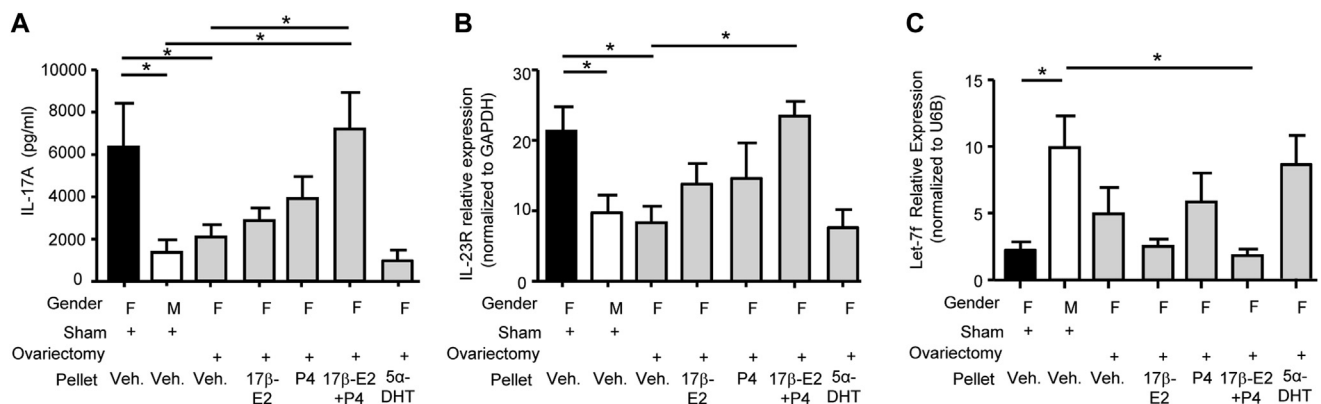


FIG 6. *In vivo* administration of 17β-E2 and P4 increased IL-17A protein expression and *il23r* mRNA expression from *in vitro* Th17 cells. Hormones or vehicle pellets (Veh.) were implanted into mice for 21 days, followed by naive T-cell isolation and Th17 cell differentiation. **A**, IL-17A protein expression. **B**, *il23r* mRNA expression normalized to *gapdh*. **C**, *Let-7f* miRNA expression normalized to *u6b* expression. * $P < .05$, Kruskal-Wallis test with Dunn posttest analysis; $n = \text{Th17 cells from 5 to 10 mice for each group}$, 2 experiments.

IL-17A protein expression was increased in Th17 cells from sham-operated female mice administered vehicle pellets compared with that in Th17 cells from both sham-operated male mice administered vehicle pellets and ovariectomized

female mice administered vehicle pellets (Fig 6, A). Furthermore, IL-17A protein expression was similar in Th17 cells from sham-operated male mice administered vehicle pellets and Th17 cells from ovariectomized female mice administered vehicle pellets.

A significant increase in IL-17A protein expression was found in T_H17 cells from ovariectomized female mice administered the combination of 17 β -E2 and P4 compared with that in T_H17 cells from ovariectomized female mice administered vehicle pellets (Fig 6, A). This significant increase in IL-17A protein expression was not detected in T_H17 cells from ovariectomized female mice administered 17 β -E2, P4, or 5 α -dihydrotestosterone pellets. Furthermore, IL-17A protein expression was similar in T_H17 cells from sham-operated female mice administered vehicle pellets and T_H17 cells from ovariectomized female mice administered the combination of 17 β -E2 and P4. IFN- γ and IL-13 production was also determined in T_H17 cells from all groups, and no differences were detected (see Fig E6 in this article's Online Repository at www.jacionline.org).

We also determined there were no statistical differences in ROR γ T, AhR, or IRF-4 mRNA expression in T_H17 cells from ovariectomized female mice administered hormone pellets and T_H17 cells from ovariectomized female mice administered vehicle pellets (data not shown). However, T_H17 cells from ovariectomized female mice administered the combination of 17 β -E2 and P4 had a significant increase in *il23r* mRNA expression compared with that in T_H17 cells from sham-operated male mice administered vehicle pellets and ovariectomized female mice administered vehicle pellets (Fig 6, B). Furthermore, *il23r* mRNA expression was similar in T_H17 cells from ovariectomized female mice administered the combination of 17 β -E2 and P4 and T_H17 cells from sham-operated female mice administered vehicle pellets.

Let-7f expression was significantly decreased in T_H17 cells from sham-operated female mice administered vehicle pellets compared with that in T_H17 cells from sham-operated male mice administered vehicle pellets (Fig 6, C). *Let-7f* expression was also decreased in T_H17 cells from ovariectomized female mice administered the combination of 17 β -E2 and P4 compared with that in T_H17 cells from sham-operated male mice administered vehicle pellets, but *let-7f* expression was not significantly different in T_H17 cells from ovariectomized female mice administered the combination of 17 β -E2 and P4 compared with that in T_H17 cells from ovariectomized female mice administered vehicle pellets. Combined, these data suggest that *in vivo* exposure to the combination of 17 β -E2 and P4 decreased *let-7f* expression in T_H17 cells and therefore increased IL-23R and IL-17A protein expression.

Ovarian hormones increase IL-17A-mediated neutrophilic airway inflammation in mice

Next, we wanted to determine whether female mice had increased IL-17A-mediated acute airway inflammation from T_H17 cells compared with male mice. As shown in Fig 7, A, CD4⁺ T cells from D011.10 intact female and male donor mice, which have an ovalbumin (OVA)-specific T-cell receptor, were differentiated into T_H17 cells and adoptively transferred into WT female recipient mice. IL-17A protein expression was measured from pooled D011.10 T_H17 culture supernatants (4484 $\pm$ 74.2 pg/mL IL-17A in T_H17 cells from female D011.10 mice and 2377 $\pm$ 44.1 pg/mL IL-17A in T_H17 cells from male D011.10 T_H17 mice; $P < .05$, *t* test), which is similar to findings in T_H17 cells from female and male WT mice. WT female recipient mice were challenged with OVA aerosol 1 day before adoptive transfer and for 3 days after. WT female recipient

mice were killed on days 2 and 4, and end points were determined. We first determined OVA-specific (KJ126⁺) T_H17 cell migration to the lung, and similar numbers of female or male OVA-specific CD3⁺CD4⁺ T cells migrated to the lung at 4 days after adoptive transfer (Fig 7, B). Next, IL-17A production from the OVA-specific T_H17 cells was determined, and adoptive transfer of OVA-specific T_H17 cells from female D011.10 mice increased the percentages and total numbers of IL-17-producing CD3⁺CD4⁺ T cells in WT female recipient mice compared with those in OVA-specific CD3⁺CD4⁺ T cells from male D011.10 mice (Fig 7, C and D). These data support our earlier *in vitro* findings in T_H17-differentiated cells from women and men (Fig 2, A) and showed that T_H17 cell differentiation and IL-17A production were increased in CD4⁺ T cells from female compared with male mice.

We next determined that protein expression of CXCL1 (KC), a neutrophil-attracting cytokine known to be upregulated in airway epithelial cells in response to IL-17A signaling, was significantly increased on days 2 and 4 in whole-lung homogenates from WT female recipient mice adoptively transferred with D011.10 T_H17 cells from female mice compared with adoptive transfer of D011.10 T_H17 cells from male mice or lack of adoptively transferred cells (Fig 7, E). Neutrophil counts were also increased on day 4 in the BAL fluid of WT female recipient mice administered D011.10 T_H17 cells from female mice compared with those in WT female recipient mice administered D011.10 T_H17 cells from male mice or no cells (Fig 7, F). These data show that the adoptive transfer of OVA-specific T_H17 cells from female mice increased neutrophilic airway inflammation in recipient mice compared with adoptive transfer of OVA-specific T_H17 cells from male mice.

DISCUSSION

The increased prevalence of severe asthma in female subjects begins around the time of puberty,^{1,2} suggesting a role for sex hormones. IL-17A is associated with severe asthma, but the role of sex and sex hormones on T_H17 cell differentiation remains unclear. Determining whether sex hormones regulate T_H17 cell differentiation is vital for understanding the mechanisms responsible for severe asthma. Prior research showed that estrogen signaling negatively regulated *let-7f* in the MCF-7 breast cancer cell line.¹⁹ *Let-7f* also decreased IL-23R surface expression and IL-17A protein expression in T_H17 cells.¹⁶ Our study is the first to show that (1) CD4⁺ T cells from women have increased T_H17 cell differentiation and IL-17A protein expression and (2) *let-7f* is differentially expressed in T_H17 cells from women and men and alters T_H17 cell expression of IL-23R and production of IL-17A. We extended our findings in human T_H17 cells and showed that T_H17 cells from ovariectomized female mice exposed to the combination of 17 β -E2 and P4 had decreased *let-7f* expression, increased *il23r* mRNA expression, and increased IL-17A protein expression compared with values in T_H17 cells from sham-operated male mice, T_H17 cells from female ovariectomized mice administered vehicle pellets, or both (see Fig E7 in this article's Online Repository at www.jacionline.org). Furthermore, we showed that adoptive transfer of OVA-specific T_H17 cells from female mice into WT female recipient mice followed by OVA aerosol challenge resulted in increased neutrophilic inflammation compared with adoptive transfer of OVA-specific T_H17 cells from male mice. Combined, these data strongly suggest

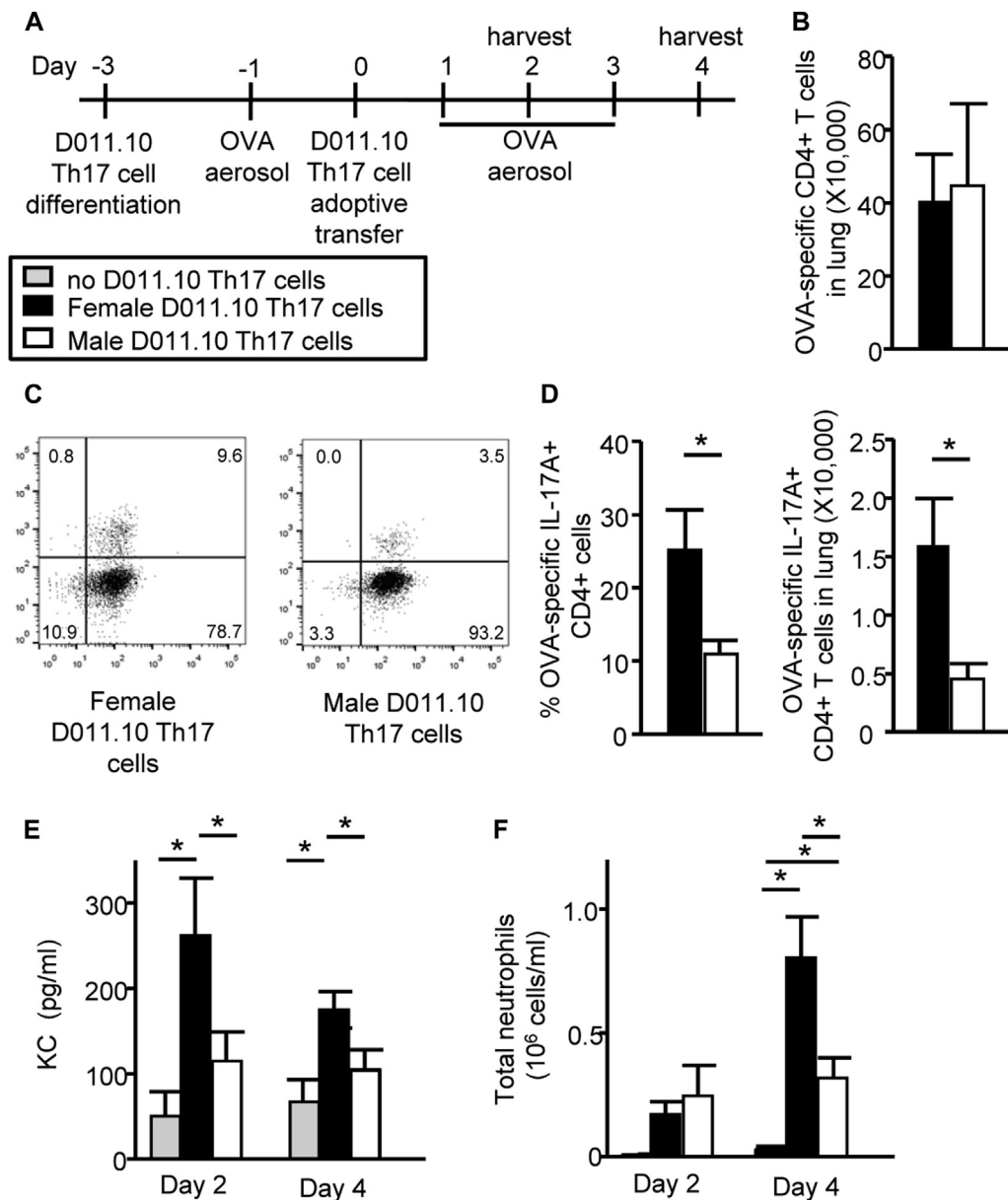


FIG 7. Adoptive transfer of T_H17 cells from female D011.10 mice increases neutrophilic infiltration in the airways of WT female recipient mice. **A**, Schematic of experimental protocol. **B-D**, Total number of OVA-specific $CD3^+CD4^+$ T cells (Fig 7, B) or percentage and total number of IL-17A $^+$ OVA-specific $CD3^+CD4^+$ T_H17 cells (Fig 7, C and D) in lungs of recipient mice on day 4. **E**, CXCL1 (KC) protein expression in whole-lung homogenates. **F**, Total neutrophils in BAL fluid. * $P < .05$, ANOVA with Tukey posttest analysis; $n = 10$ to 12 recipient mice per group administered D011.10 T_H17 cells from female or male mice combined from 2 experiments.

that *in vivo* exposure to both 17 β -E2 and P4 might differentially regulate IL-17A protein expression in T_H17 cells through a Let-7f/IL-23R pathway.

T_H17 cells are also associated with autoimmune conditions, including multiple sclerosis, SLE, and rheumatoid arthritis. A sex disparity also exists in these autoimmune conditions, with women being 2 to 3 times more likely to have multiple sclerosis and 9 times more likely to have SLE compared with men.²⁷ However, the role of sex hormones in patients with T_H17 -associated diseases remains unclear, with variable findings in both *in vitro* and *in vivo* studies. Similar to our *in vitro* findings,

IL-17A protein expression was increased in T_H17 cells from mice administered 17 β -E2 compared with that seen in T_H17 cells from mice administered vehicle.²⁸ However, other *in vitro* studies reported 17 β -E2 signaling decreased IL-17A production in $CD4^+$ T cells. These variable findings might be due to different T-cell activation and differentiation protocols.²⁸⁻³³ *In vivo* mouse models of multiple sclerosis and rheumatoid arthritis showed 17 β -E2 signaling decreased T_H17 -mediated inflammation.²⁸⁻³⁴ $CCR6^+CXCR3^+IL-17A^+IFN-\gamma^+$ cells are vital for the pathogenesis of multiple sclerosis and rheumatoid arthritis³⁵ but not severe asthma. Therefore the divergence of our *in vivo* data and data

from mouse models of IL-17A-mediated autoimmune conditions suggests that severe asthma and IL-17A-mediated autoimmune diseases are mediated by different subsets of IL-17A-producing T_H17 cells.

The *Il17a* and *Il17f* genes are found on chromosome 1 in a tail-to-tail configuration.³⁶ IL-17A and IL-17F have approximately 50% homology, the most closely related members of the IL-17 family of proteins.³⁶ Many studies have shown that IL-17A and IL-17F levels are increased by similar mechanisms, but our findings and others show differential production of IL-17A and IL-17F. In our study we found that IL-17A, but not IL-17F, protein expression was increased in T_H17 cells from women and female mice compared with that in T_H17 cells from men and male mice, respectively. Furthermore, in mouse models of multiple sclerosis and rheumatoid arthritis, *Il17a*^{-/-}, but not *Il17f*^{-/-}, mice have decreased disease severity compared with WT mice.^{37,38} These results suggest that 17β-E2 and P4 can differentially regulate IL-17A and IL-17F protein expression, potentially through an epigenetic mechanism or different enhancer/promoter states of the genes.

Let-7f expression was significantly decreased in T_H17 cells from women and female mice compared with that in T_H17 cells from men and male mice, respectively. *In vivo* administration of pellets containing the combination of 17β-E2 and P4 decreased *let-7f* expression in T_H17 cells from ovariectomized female mice compared with T_H17 cells from sham-operated male mice administered vehicle pellets. However, there was not a significant difference in *let-7f* expression between T_H17 cells from ovariectomized female mice administered the vehicle pellet and T_H17 cells from ovariectomized female mice administered the combination of 17β-E2 and P4. These data suggest that other factors in conjunction with or independent of *let-7f* also regulate IL-23R expression. *Sgk1* and *Foxo1* were recently described to increase and decrease, respectively, IL-23R expression and IL-17A protein expression in T_H17 cells.³⁹ We determined *SGK1* and *FOXO1* mRNA expression in T_H17 cells from women and men, as well as T_H17 cells from sham-operated and ovariectomized female mice administered hormone or vehicle pellets. No differences in *SGK1* and *FOXO1* mRNA expression were detected in T_H17 cells from human subjects or mice (data not shown). Furthermore, we also examined expression of *let-7e*, another *let-7* family member, which increased allergic airway inflammation in mice,⁴⁰ and found no differences in *let-7e* expression in T_H17 cells from healthy women compared with that seen in men (data not shown). Combined, these results suggest that 17β-E2 and P4 regulate IL-23R expression partially through *let-7f* but that other unidentified factors are likely involved in regulating IL-17A protein expression.

IL-17A can also be produced by other cells, including γδ T cells and ILC3s.^{11,12} IL-23/IL-23R signaling is important for γδ T-cell and ILC3 production of IL-17A,^{11,12} but it is unknown whether *let-7f* regulates IL-23R expression in γδ T cells and ILC3s. Further studies are needed to elucidate these mechanisms. Therapeutics targeting IL-17A and IL-17RA are currently undergoing clinical trials for patients with multiple sclerosis, psoriasis, or severe asthma. Brodalumab, a humanized IL-17RA mAb, decreased disease severity in patients with moderate-to-severe plaque psoriasis,⁴¹ but brodalumab had little effect in decreasing asthma symptoms in patients with moderate-to-severe asthma.⁴² However, the brodalumab asthma clinical trial did not specifically recruit patients with high IL-17A

production,⁴² and brodalumab can have a more pronounced effect on asthma symptoms in patients with IL-17A-mediated airway inflammation and asthma. Our findings suggest that women with severe asthma might respond better to medications that target IL-17A and IL-17RA signaling.

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Key messages

- 17β-E2 plus P4 increase IL-17A production in T_H17 cells by decreasing *let-7f* miRNA expression and increasing IL-23R expression, providing a potential mechanism for the increased prevalence of severe asthma in women compared with men.
- Transfer of OVA-specific T_H17 cells from female mice increased neutrophil infiltration in the lungs of OVA-challenged recipient mice compared with transfer of male OVA-specific T_H17 cells.

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METHODS

Recruitment of participants

Patients with severe asthma (aged 18–45 years), as defined by the Severe Asthma Research Program,^{E1} were recruited from Vanderbilt University. These patients with severe asthma were clinically stable, not undergoing an exacerbation, and continued taking their asthma medications. Table E1 shows the percentage of women and men in our study meeting the severe asthma criteria, including use of oral and inhaled corticosteroids, daily controller medications, and short-acting β -agonists. Participants were excluded for viral or bacterial symptoms in the previous week or any T_H17 -associated disease, including multiple sclerosis, psoriasis, SLE, or rheumatoid arthritis. Women were excluded if they were pregnant; breast-feeding; taking hormonal birth control medications; receiving estrogen replacement therapy; or menopausal or if they had undergone an oophorectomy or hysterectomy. At the time of the blood draw, the first day of the last menstrual period was recorded for each woman. Healthy participants (aged 18–45 years) with no diagnosis of asthma were also recruited with the same exclusion criteria described for patients with severe asthma. All participants were consented in accordance with Vanderbilt University's Institutional Review Board policies.

Flow cytometry of memory T cells from participants with severe asthma

In Figs 1 and E1, one million restimulated memory $CD4^+$ T cells from healthy subjects or patients with severe asthma were harvested, and cells were surface stained in the dark for 45 minutes at 4°C with fluorescein isothiocyanate (FITC)-conjugated anti- $CD4$ (5 μ L per 1×10^6 cells, catalog #555346, BD Biosciences), Pacific Blue-conjugated anti- $CD3$ (0.5 μ L per 1×10^6 cells, catalog #558117, BD Biosciences), and biotinylated anti- $CCR6$ (2 μ L per 1×10^6 cells, catalog #559561, BD Biosciences). For Fig E4, cells were also surface stained with phycoerythrin (PE)-conjugated anti- $IL-23R$ (2 μ L per 1×10^6 cells, catalog #FAB14001P; R&D Systems, Minneapolis, Minn). Cells were then stained with PE-Cy7 conjugated to streptavidin (1:500 diluted, catalog #557598, BD Biosciences), permeabilized with Cytotfix/Cytoperm in the dark at 4°C for 20 minutes to overnight (catalog #00-5523-00; eBioscience, San Diego, Calif), washed thoroughly, and intracellularly stained in the dark with peridinin-chlorophyll-protein complex Cy5.5 anti- $IL-17A$ (5 μ L per 1×10^6 cells, catalog #45-7179-41, eBioscience), and PE-conjugated anti- $IL-4$ (2 μ L per 1×10^6 cells, catalog #554435, BD Biosciences). In Fig 3, A, T_H17 -differentiated cells from healthy women and men were surface stained with FITC-conjugated anti- $CD4$, Pacific Blue-conjugated anti- $CD3$, and PE-conjugated $IL-23R$. For experiments in Fig 4, T_H17 -differentiated cells from healthy women and men were surface stained with FITC-conjugated anti- $CD4$ and Pacific Blue-conjugated anti- $CD3$. Cells were then fixed and permeabilized with Cytotfix/Cytoperm solution and intracellularly stained in the dark with peridinin-chlorophyll-protein complex Cy5.5 anti- $IL-17A$. For all experiments, cells were analyzed with an LSR II flow cytometer (BD Biosciences), and data were analyzed with Flow Jo 7.2 software (TreeStar, Ashland, Ore).

Hormone analysis

17β -E2 and testosterone levels were determined by using the radioimmunoassay double-antibody procedure at the Vanderbilt University Hormone Assay Core. Final analysis was accomplished by quantifying the bound radioactive counts with a Packard Gamma counter connected to a computerized data reduction station. Progesterone levels were determined by means of ELISA (catalog #11-PROHU-E01; ALPCO, Salem, NH).

T_H17 cell differentiation from human $CD4^+$ naive T cells

Human PBMCs were isolated from the buffy coats of participants by using Ficoll-Paque Plus (catalog #17-1440-02; GE Healthcare, Fairfield, Conn). Naive $CD4^+$ T cells were isolated from the PBMCs by using a naive T cell isolation kit (catalog #130-094-131; Miltenyi Biotec, Bergisch Gladbach, Germany) per the manufacturer's instruction. $CD4^+$ naive T cells were activated by using an activation/expansion kit (catalog #130-091-441, Miltenyi

Biotec) with anti- $CD3$ and anti- $CD28$ bound to bead particles at the ratio of 1 bead particle per 2 cells. $CD4^+$ T cells were cultured and differentiated into become T_H17 cells with hIL-2 (10 ng/mL), rhIL-1 β (10 ng/mL), rhTGF- β (1 ng/mL), rhIL-6 (10 ng/mL), rhIL-23 (10 ng/mL), anti-IFN- γ (10 μ g/mL), and/or anti-IL-4 (10 μ g/mL) in T-cell culture medium. T-cell culture medium was RPMI containing 10% FBS, 1% penicillin/streptomycin, 2 mmol/L L-glutamine, 10 mmol/L HEPES, 0.1 mmol/L nonessential amino acids, and 1 mmol/L sodium pyruvate (Gibco, Carlsbad, Calif). All antibodies and rhIL-1 β , rhIL-6, and rhIL-23 were purchased from R&D Systems. rhIL-2 and rhTGF- β were purchased from PeproTech (Rocky Hill, NJ).

Cytokine measurements

Cytokine levels were measured from T_H0 and T_H17 cell-culture supernatants, BAL fluid, or whole-lung homogenates with available Duoset ELISA kits (R&D Systems), according to the manufacturer's instructions, or by using a cytokine bead array (BD Technologies) from the Vanderbilt Immunology Core. Cell-culture supernatants were collected from 400,000 to 500,000 T_H0 or T_H17 cells, and no differences were detected in T_H0 and T_H17 cell numbers from men and women at the time of supernatant collection. Any value less than the lower limit of detection was assigned half the value of the lowest detectable standard.

IgE measurements

Total and allergen-specific plasma IgE concentrations were determined by using a commercially available Phadiatop assay (ImmunoCAP; Phadia, Kalamazoo, Mich) per the manufacturer's directions.

RNA/miRNA isolation and quantitative PCR

Total RNA was isolated with an RNeasy Mini Kit (catalog #74106; Qiagen, Hilden, Germany). For studies on human T cells, cDNA was generated by using 100 to 500 ng of total RNA, with the same concentration of total RNA used within each independent experiment. TaqMan quantitative PCR (qPCR) analysis of *IL23R* and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) mRNA expression was conducted by using commercially available primers and FAM/MGB probes (catalog #4331182 Hs00332759_m1 for *IL23R* and catalog #4352934E for *GAPDH*; Applied Biosystems, Foster City, Calif). For studies on mouse T cells, cDNA was generated by using 100 to 200 ng of total RNA, with the same concentration of total RNA used within each independent experiment. qPCR was conducted by using SYBR green mix (catalog #170-8882; Bio-Rad Laboratories, Hercules, Calif) and *il23r* and *gapdh* primers from Integrated DNA Technologies (Coralville, Iowa) with the following sequences: *il23r* forward, 5'-GGTCCAAGCTGTCAATTCCTAGGC-3'; *il23r* reverse, 5'-AGCCCTGGAAATGATGGACGCA-3'; *gapdh* forward, 5'-GGCCCCCTCTGGAAAGCTGTGG-3'; *gapdh* reverse, 5'-CCCGGCATCGAAGGTGGAAGA-3'. Data were reported as relative expression normalized to the housekeeping gene *GAPDH* for humans and *gapdh* for mice.

For the detection and quantification of miRNA, we used the NCode miRNA first-strand cDNA synthesis and qRT-PCR kit (catalog #MIRQ-100; Life Technologies, Grand Island, NY). Per the manufacturer's instructions, 500 ng of total RNA underwent a polyadenylation reaction to add a poly(A) 3' tail to miRNAs. cDNA synthesis was then performed with 4 μ L of polyadenylated RNA, Superscript III reverse transcriptase, and a universal reverse transcriptase primer. Finally, qPCR was conducted with SYBR green mix and a provided universal qPCR reverse primer (200 nmol/L) and forward primers for miRNA (200 nmol/L). Forward primer sequences were as follows: *let-7f* forward primer, TGAGGTAGTAGATTGTATAGTT; *u6b* forward primer, TGACACGCAAATTCGTGAAG (Integrated DNA Technologies). Data were reported as relative expression normalized to the housekeeping gene, U6B.

Transfection of the *let-7f* miRNA inhibitor

Two million isolated human $CD4^+$ naive T cells were transfected by using nucleoporation with the commercially available mirVana *let-7f* inhibitor (10 nmol/L, catalog #4464084 hsa-let-7f-, Life Technologies) or mirVana Negative Control #1 (10 nmol/L, catalog #4464076, Life Technologies), according to the manufacturer's protocol (Lonza, Basel, Switzerland) and as

previously described.^{E2} Cells rested for 4 hours after transfection at 37°C/5% CO₂, and transfected cells were activated and differentiated into T_H17 cells, as described above, for 3 days.

CD4⁺ T_H17 cell differentiation from mice

Naive CD4⁺ T cells isolated from the spleens of WT mice were activated by using anti-CD3 (5 µg/mL, catalog #553057, BD Biosciences) and anti-CD28 (1 µg/mL, catalog #553295, BD Biosciences) in 96-well plates. Naive CD4⁺ T cells from D011.10 mice were activated by using OVA peptide 323-339 (5 µg/mL; catalog #O164; Sigma Chemical, St Louis, Mo) and anti-CD28 (1 µg/mL). CD4⁺ T cells from both WT and D011.10 mice were differentiated into T_H17 cells by adding rmIL-23 (10 ng/mL), rhTGF-β (1 ng/mL), rmIL-6 (20 ng/mL), anti-IFN-γ (10 µg/mL), and anti-IL-4 (10 µg/mL). In select experiments T_H17 cells were differentiated with varying concentrations of rmIL-23 ranging from 0 to 30 ng/mL. All antibodies and rmIL-23 were purchased from R&D Systems. rmIL-6 and rhTGF-β were purchased from PeproTech.

Administration of hormone pellets *in vivo* to mice

At 7 weeks of age, 60-day slow-release pellets (Innovative Research of America, Sarasota, Fla) containing 17β-E2 (0.1 mg), P4 (25 mg), 5α-dihydrotestosterone (15 mg), or a combination of 17β-E2 (0.1 mg) and P4 (25 mg) were surgically placed subcutaneously into ovariectomized BALB/c mice, as previously described.^{E3} As a control, vehicle pellets (Innovative Research of America) were surgically placed into intact male, intact female, or ovariectomized female mice. Three weeks (21 days) after pellets were implanted, naive CD4⁺ T cells were harvested from the spleens of mice and differentiated into T_H17 cells. Serum was also collected and analyzed for hormone levels by the Vanderbilt Hormone Core Facility, as described in the section above. Four days after T_H17 cell differentiation, cell-culture supernatants and cells were collected for various end points.

Adoptive transfer of D011.10 cells into WT female recipient mice

As depicted in Fig 7, A, naive CD4⁺ T cells were isolated from the spleens of female and male D011.10 donor mice by using a commercially available CD4 negative selection isolation kit from Miltenyi Biotec (catalog #130-093-227) per the manufacturer's directions. Naive CD4⁺ T cells from donor mice were stimulated with 5 µg/mL OVA peptide 323-339 and 1 µg/mL anti-CD28 and differentiated into T_H17 cells for 4 days, as described in the section above. One day before adoptive transfer (day -1), recipient WT female

BALB/c mice were aerosol challenged with 1% OVA for 40 minutes to facilitate migration of adoptively transferred CD4⁺ D011.10 T_H17 cells to the lung. On day 0, 2 × 10⁶ T_H17-differentiated cells from male or female donor D011.10 mice were adoptively transferred by means of tail vein injection into WT female recipient BALB/c mice. As a control, a group of recipient WT female BALB/c mice received no adoptive transfer of cells (depicted by the gray bars in Fig 7). On days 1, 2, and 3, after the adoptive transfer of D011.10 OVA-specific T_H17 cells, all recipient mice were challenged with 1% OVA protein aerosol for 40 minutes each day to induce T_H17-mediated airway inflammation. BAL fluid and lungs were collected on days 2 and 4 for analysis of KJ126⁺, OVA-specific T_H17 cells in the lung (see methods below), inflammatory cell infiltrate (see methods below), and cytokine or chemokine expression in whole-lung homogenates measured by means of ELISA.

Analysis of inflammatory cell infiltration into the airway

BAL was performed by instilling 800 µL of saline through a tracheostomy tube and then withdrawing the fluid with gentle suction through a syringe, as previously described.^{E4} The total cell count in BAL fluid was determined by using a hemocytometer and Trypan Blue Exclusion dye (catalog #T8154, Sigma). Cells from the BAL fluid were then fixed to a slide and stained by using a Three-Step Stain (catalog #22-050-272; Richard-Allan Scientific, Thermo Scientific, Waltham, Mass). Two hundred leukocytes were classified as neutrophils, eosinophils, lymphocytes, or monocytes by using standard morphologic criteria, and neutrophil percentage was determined. Total neutrophil numbers were determined by multiplying percentages of neutrophils times total numbers of viable cells in BAL fluid.

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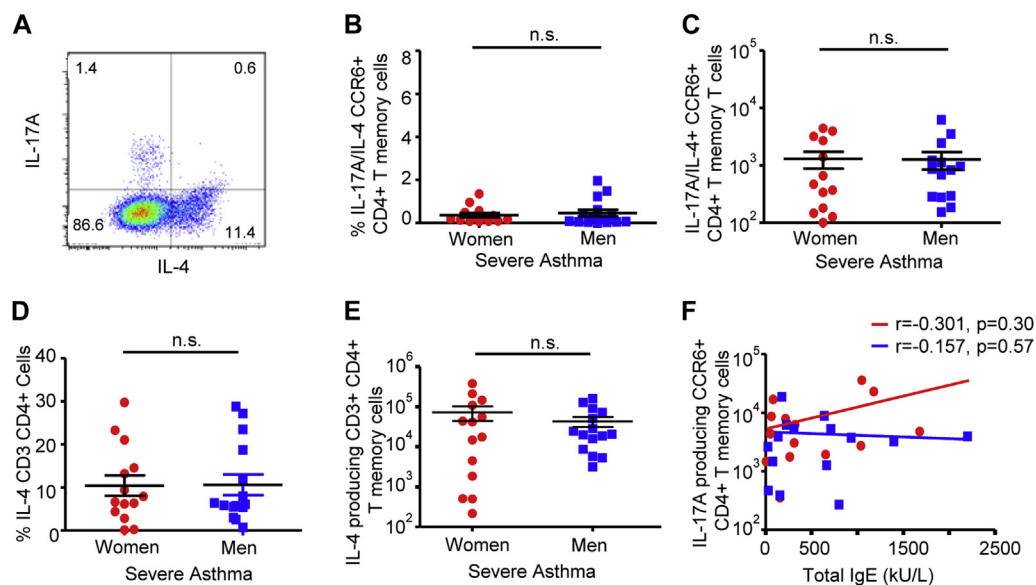


FIG E1. IL-4⁺ memory T-cell counts are similar in women and men with severe asthma. **A–C**, Percentages and total numbers of IL-17A/IL-4⁺ memory T_H17 cells in women and men with severe asthma. **D** and **E**, Percentages and total numbers for IL-4⁺CD3⁺CD4⁺ memory T cells in women and men with severe asthma. $n = 14$ women and $n = 15$ men. **F**, Spearman correlation of total IgE plasma levels and IL-17A–producing CCR6⁺ memory T_H17 cells. *n.s.*, Not significant.

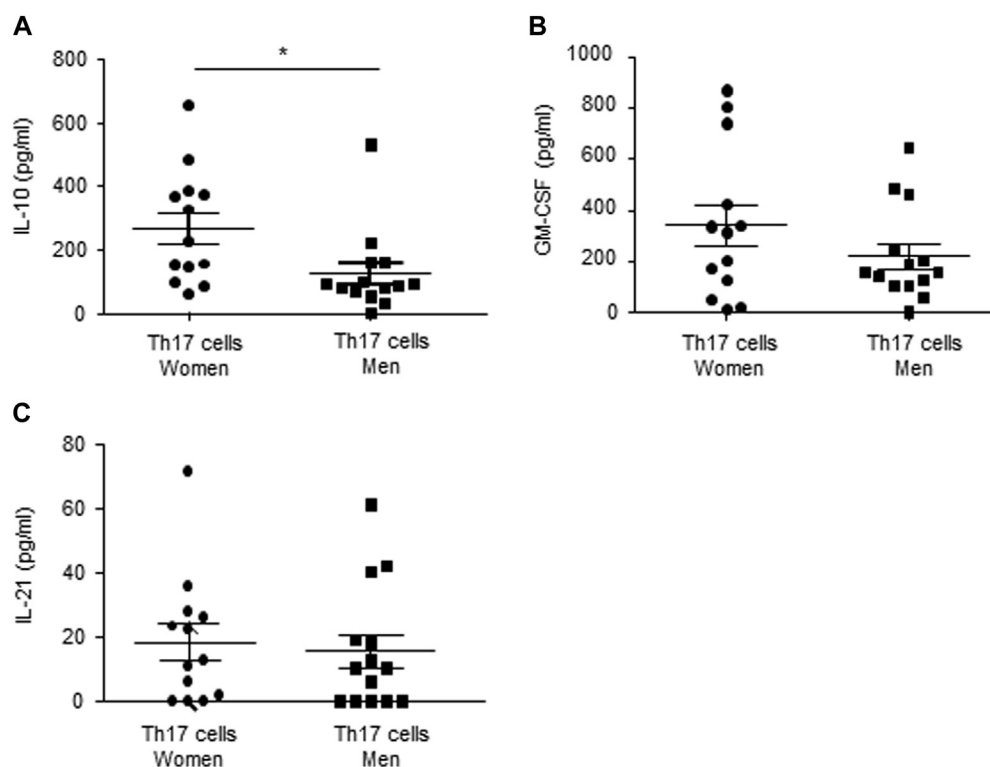


FIG E2. Cytokine expression in human T_H17 -differentiated cells from women and men. **A-C**, Naive T cells isolated from healthy women (circles) and men (squares) were differentiated into T_H17 cells. Cell-culture supernatants were harvested and analyzed for protein expression. * $P < .05$, Mann-Whitney U test; $n = 13$ T_H17 cells from women and $n = 14$ T_H17 cells from men.

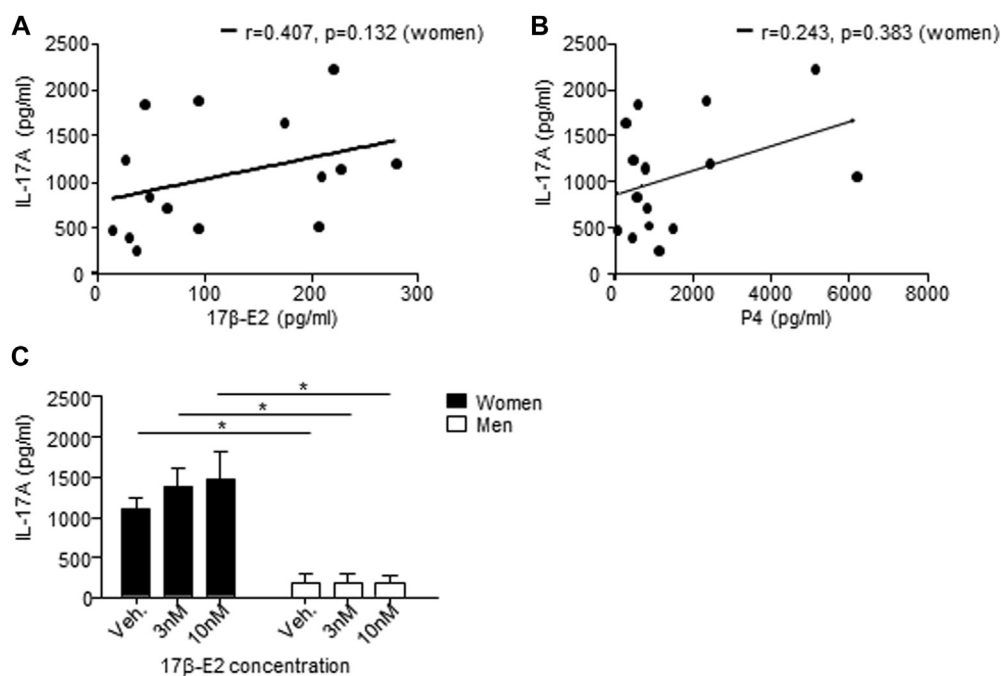


FIG E3. IL-17A protein expression in T_H17 cells from women does not correlate with 17β -E2 and P4 plasma levels. **A** and **B**, Spearman nonparametric correlations of 17β -E2 or P4 plasma concentrations and IL-17A protein expression from T_H17 cell-culture supernatants; r and P values are listed on the graphs. **C**, Naive T cells isolated from healthy women and men were differentiated into T_H17 cells in the presence of 17β -E2 or vehicle (ethanol). Four days after differentiation, IL-17A protein expression was determined from cell-culture supernatants. Data are representative of 8 T_H17 cells from women and 5 T_H17 cells from men. * $P < .05$, ANOVA with Tukey posttest analysis.

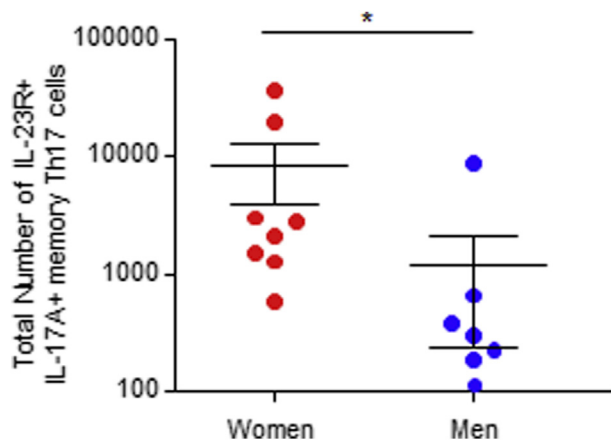


FIG E4. IL-23R surface expression is increased in memory T_H17 cells from women with severe asthma compared with that in men with severe asthma. IL-23R surface expression was determined on $CD3^+CD4^+CCR6^+$ memory T_H17 cells from women and men with severe asthma. $*P < .05$, Mann-Whitney U test; $n = 8$ women and $n = 7$ men.

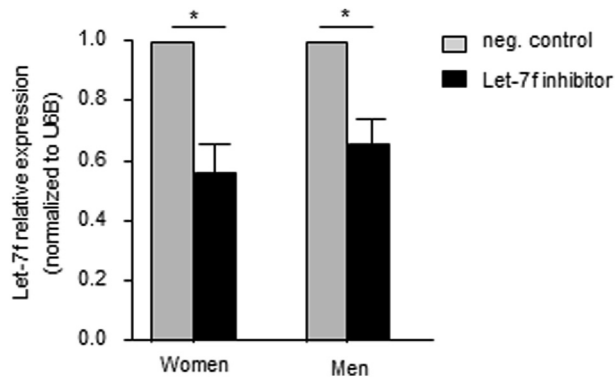


FIG E5. *Let-7f* miRNA expression is decreased in T_H17 cells transfected with *let-7f* inhibitor. *Let-7f* inhibitor (10 nmol/L) or negative control (10 nmol/L) were transfected into naive T cells isolated from healthy women and men. Cells were differentiated into T_H17 cells, and *Let-7f* miRNA expression was determined by using qPCR and normalized to U6B expression. * $P < .05$, Wilcoxon matched paired test; $n = 9$ T_H17 cells from women and $n = 9$ T_H17 cells from men.

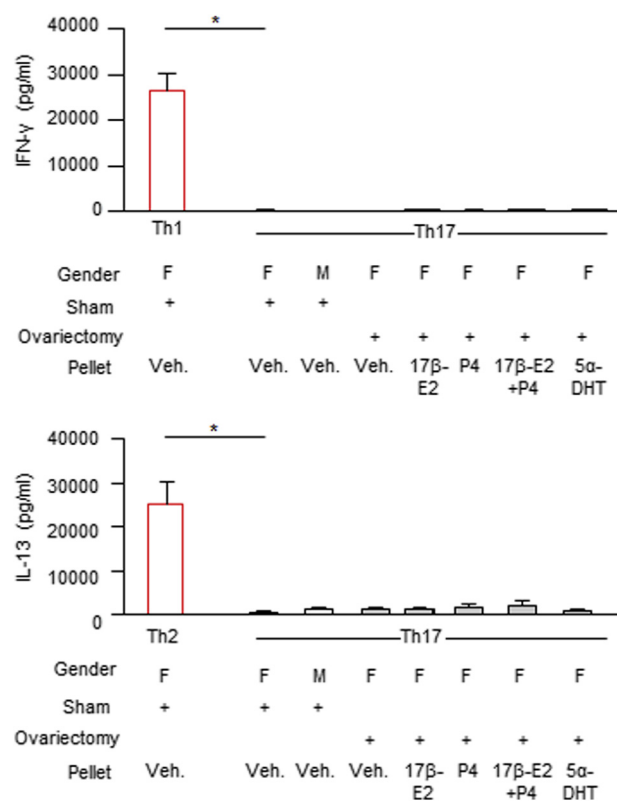


FIG E6. IL-13 and IFN- γ protein expression in T_H17 cells. Hormones or vehicle pellets (*Veh.*) were implanted into mice for 21 days. Naive T cells were isolated from the spleens of mice, and T_H17 cells were differentiated. As positive controls, T_H1 and T_H2 cells were differentiated from sham-operated female mice administered vehicle pellets. IFN- γ and IL-13 protein expression was determined by using ELISA. * $P < .05$ compared with T_H17 cells from sham-operated female mice administered vehicle pellets, Kruskal-Wallis test with Dunn posttest analysis; $n = 5$ to 10 mice per group combined from 2 experiments.

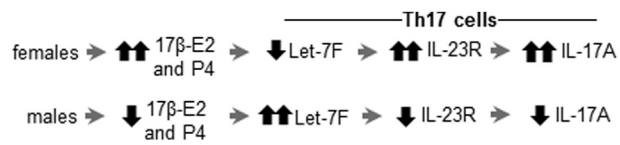


FIG E7. Role of 17β-E2 and P4 in IL-17A protein expression from T_H17 cells. The schematic proposes how increased 17β-E2 and P4 concentrations in women affect IL-17A protein expression from T_H17 cells.

TABLE E1. Percentage of women and men meeting each of the severe asthma criteria, as defined by the Severe Asthma Research Program^{E1*}

	Women (%)	Men (%)	<i>P</i> value [†]
Major criteria (must have 1)			
Oral corticosteroids >50% of year	35.7	20	.362
High-dose inhaled corticosteroids	78.6	82.4	.928
Minor criteria (must have 2)			
Daily treatment with controller medication	100	86.7	.082
Daily or near-daily use of short-acting β_2 -agonist	35.7	46.7	.566
FEV ₁ <80% of predicted value	42.9	73.3	.103
≥1 Urgent care visits for asthma per year	28.6	6.7	.127
≥3 Oral corticosteroid “bursts” per year	7.1	6.7	.961
Prompt deterioration in asthma control when corticosteroids were reduced <25%	0	0	—
Near-fatal asthma event in the past	0	0	—

*Some patients might have met more than the minimum requirements.

[†]*P* values were determined by using the Mann-Whitney *U* test; a *P* value of less than .05 was considered significant.

TABLE E2. Characteristics of patients with severe asthma

	Women	Men	<i>P</i> value*
No.	14	15	
Age (y)	35.3 (1.8)	35.7 (1.8)	.983
Race (%)			
African American	14.3	5.9	
Asian	14.3	0	
White	64.3	88.2	
Hispanic	0	0	
Other	7.1	5.9	
BMI (kg/m ²)	34.8 (9.7)	31.1 (4.8)	.418
Baseline lung function			
FEV ₁ (% predicted)	78.0 (6.4)	72.6 (4.5)	.458
FVC (%)	93.2 (5.0)	85.2 (3.6)	.199
FEV ₁ /FVC ratio	0.86 (0.04)	0.85 (0.03)	.927
Total IgE levels	465.2 (131.2)	592.2 (162.0)	.76

Data are listed as means (SDs).

BMI, Body mass index.

**P* values were determined by using the Mann-Whitney *U* test; a *P* value of less than .05 was considered significant.