

The steroidogenic enzyme Cyp11a1 is essential for development of peanut-induced intestinal anaphylaxis

Meiqin Wang, MD, PhD,^a Julita Ramirez, DVM, PhD,^{b*} Junyan Han, PhD,^a Yi Jia, PhD,^a Joanne Domenico, BS,^a Max A. Seibold, PhD,^c James R. Hagman, PhD,^b and Erwin W. Gelfand, MD^{a,b} *Denver, Colo*

Background: Cytochrome P450, family 11, subfamily A, polypeptide 1 (Cyp11a1), a cytochrome P450 enzyme, is the first and rate-limiting enzyme in the steroidogenic pathway, converting cholesterol to pregnenolone. Cyp11a1 expression is increased in activated T cells.

Objectives: We sought to determine the role of Cyp11a1 activation in the development of peanut allergy and T_H cell functional differentiation.

Methods: A Cyp11a1 inhibitor, aminoglutethimide (AMG), was administered to peanut-sensitized and challenged mice. Clinical symptoms, intestinal inflammation, and Cyp11a1 levels were assessed. The effects of Cyp11a1 inhibition on T_H1, T_H2, and T_H17 differentiation were determined. Cyp11a1 gene silencing was performed with Cyp11a1-targeted short hairpin RNA.

Results: Peanut sensitization and challenge resulted in diarrhea, inflammation, and increased levels of *Cyp11a1*, *IL13*, and *IL17A* mRNA in the small intestine. Inhibition of Cyp11a1 with AMG prevented allergic diarrhea and inflammation. Levels of pregnenolone in serum were reduced in parallel. AMG treatment decreased *IL13* and *IL17A* mRNA expression in the small intestine without affecting Cyp11a1 mRNA or protein levels. *In vitro* the inhibitor decreased *IL13* and *IL17A* mRNA and protein levels in differentiated T_H2 and T_H17 CD4 T cells, respectively, without affecting *GATA3*, retinoic acid-related orphan receptor γ t (*ROR γ t*), or T_H1 cells and *IFNG* and *T-bet* expression. Short hairpin RNA-mediated silencing of Cyp11a1 in polarized T_H2 CD4 T cells significantly decreased pregnenolone and *IL13* mRNA and protein levels.

Conclusion: Cyp11a1 plays an important role in the development of peanut allergy, regulating peanut-induced

allergic responses through effects on steroidogenesis, an essential pathway in T_H2 differentiation. Cyp11a1 thus serves as a novel target in the regulation and treatment of peanut allergy. (*J Allergy Clin Immunol* 2013;■■■■:■■■-■■■.)

Key words: *Cyp11a1*, peanut allergy, T_H2, T_H17, CD4 T cells

Steroid hormones, including glucocorticoids (GCs), play an important role in the regulation of the immune system. The inhibitory role of GCs on immune cells is well characterized.^{1,2} GCs reduce inflammation through inhibition of nuclear factor κ B and by inducing the expression of anti-inflammatory proteins, including annexin 1 and the mitogen-activated protein kinase phosphatase 1.³ GCs and other synthetic derivatives have been used to treat a variety of diseases, including inflammatory diseases of the intestine and asthma.^{4,5} Although the anti-inflammatory activity of GCs is well described, accumulating evidence suggests that GCs can also enhance immune cell activation, inducing gene transcription and promoting the pathogenesis of allergic diseases.^{6,7} Steroid hormones are mainly produced in the adrenal glands, but other tissues also produce GCs through induction of steroidogenic enzymes.^{3,8} The intestinal mucosa contains steroidogenic enzymes, such as cytochrome P450, family 11, subfamily A, polypeptide 1 (Cyp11a1), and synthesizes potent GCs that exhibit both an inhibitory and a costimulatory role on intestinal T-cell activation.⁶

Cyp11a1 is a key regulator of steroid biogenesis as the first and rate-limiting enzyme in the steroidogenic pathway, converting cholesterol to pregnenolone.⁹ Cyp11a1 is expressed primarily in the cortex of the adrenal gland, but the testis, ovary, placenta, thymus, and intestine also express Cyp11a1.^{6,9} Depletion of Cyp11a1 in mice or rabbits results in steroid deficiency, female external genitalia, and death.¹⁰⁻¹² In human subjects mutations in the Cyp11a1 gene result in a steroid hormone deficiency, causing a rare and potentially fatal form of lipid congenital adrenal hyperplasia.^{13,14} Patients with a heterozygous or homozygous mutation of Cyp11a1 exhibit adrenal insufficiency and sex reversal.^{15,16}

We investigated the role of the Cyp11a1 enzyme in an experimental model of peanut-induced intestinal allergy and identified, for the first time, the essential role of this enzyme in the full development of intestinal allergic responses. Moreover, inhibition of the enzymatic activity of Cyp11a1 attenuated T_H2 and T_H17 differentiation and cytokine production.

METHODS

Mice

Five- to 6-week-old female BALB/c wild-type mice were purchased from the Harlan Laboratory (Indianapolis, Ind). All studies were conducted under a protocol approved by the Institutional Animal Care and Use Committee of National Jewish Health (Denver, Colo).

From ^athe Division of Cell Biology, Department of Pediatrics; ^bthe Integrated Department of Immunology; and ^cthe Center for Genes, Environment & Health, National Jewish Health.

*Julita Ramirez is currently at the Department of Comparative Medicine, University of Washington, Seattle, Wash.

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Corresponding author: Erwin W. Gelfand, MD, National Jewish Health, 1400 Jackson St, Denver, CO 80206. E-mail: gelfande@njhealth.org.

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Abbreviations used

AMG: Aminoglutethimide
 CFP: Cyan fluorescent protein
 Cyp11a1: Cytochrome P450, family 11, subfamily A, polypeptide 1
 GC: Glucocorticoid
 PE: Peanut extract
 ROR γ t: Retinoic acid-related orphan receptor γ t
 RT-PCR: Real-time PCR
 shRNA: Short hairpin RNA

Preparation of peanut protein

Crude peanut extract (PE) was prepared as described in the [Methods](#) section in this article's Online Repository at www.jacionline.org.

Sensitization and intragastric challenge

The experimental protocol for sensitization and challenge to peanut was previously described.¹⁷ Control animals were sham sensitized but challenged with peanut.

Cyp11a1 inhibitor treatment *in vivo* and *in vitro*

Aminoglutethimide (AMG) was obtained from Sigma (St Louis, Mo). PE-sensitized and challenged mice received different doses (0–20 mg/kg) of the inhibitor by means of gavage based on doses previously reported¹⁸ and described in the [Methods](#) section in this article's Online Repository.

Assessment of peanut intestinal sensitivity reactions

Clinical symptoms were evaluated, as previously reported,¹⁹ and are described in the [Methods](#) section in this article's Online Repository.

Histology

Jejunum was fixed in 10% formalin and processed into paraffin blocks. The number of mucus-containing cells from one side of the villus and the total number of villus cells on the same side were counted.

Mucosal mast cells were identified by means of chloroacetate esterase staining.¹⁷ Numbers of mucosal cells expressing Cyp11a1 were identified by using immunohistochemical staining with anti-human Cyp11a1 antibody (Abcam, Cambridge, Mass). At least 4 random fields per slide were examined and analyzed in a blinded manner. Quantification of stained mast cells or Cyp11a1-positive cells per square millimeter of lamina propria was performed with an Olympus microscope linked to the National Institutes of Health Image Analysis Program (NIH, Bethesda, Md).

Cytokines levels in cell culture

IL-4, IL-13, IL-17A, and IFN- γ levels in cell-culture supernatants were measured using ELISA (eBioscience, San Diego, Calif), as described by the manufacturer.

Measurement of peanut-specific antibody levels

Serum peanut-specific IgE, IgG₁, and IgG_{2a} levels were measured using an ELISA, as described previously.^{19,20}

Histamine levels in plasma

Levels of histamine in plasma were measured as described in the [Methods](#) section in this article's Online Repository.

Pregnenolone levels in serum and cell-culture supernatants

Pregnenolone levels in serum and cell-culture supernatants were measured by means of ELISA (ALPCO Diagnostics, Salem, NH), as described by the manufacturer.

T-cell differentiation *in vitro*

Differentiation of T_H1, T_H2, or T_H17 cells was performed, as previously described,^{21,22} and is described further in the [Methods](#) section in this article's Online Repository.

Western blot analysis

Cell lysates were prepared from cultured CD4 T cells, as previously described,⁷ and is described further in the [Methods](#) section in this article's Online Repository.

Quantitative real-time PCR

Real-time PCR (RT-PCR) was performed, as previously described,²³ and is described further in the [Methods](#) section in this article's Online Repository.

Expression constructs

The *Cyp11a1* short hairpin RNA (shRNA) sequences were generated by using the Dharmacon siDESIGN Center (Thermo Scientific, Uppsala, Sweden) Web site (<http://www.dharmacon.com/designcenter/designcenterpage.aspx>) and are described in the [Methods](#) section in this article's Online Repository.

Retrovirus production and transduction

Retrovirus production²⁴ and retroviral transduction of T_H2 cells²⁵ were performed as previously described and are detailed in the [Methods](#) section in this article's Online Repository.

Cell sorting and analysis of gene expression

Seventy-two hours after transduction, cells were collected and labeled with anti-mouse CD4 fluorescein isothiocyanate (eBioscience). Cyan fluorescent protein (CFP)-positive CD4⁺ cells were sorted with a Synergy cell sorter (iCyt). Sorted cells were stimulated with 2 μ g/mL anti-CD3/CD28 for quantitative RT-PCR and ELISA. Quantitative RT-PCR and ELISA were performed, as described above.

Estradiol levels in plasma

Estradiol levels in plasma were measured using ELISA (Oxford Biomedical Research, Oxford, Mich), as described by the manufacturer. The concentration of estradiol was calculated from a standard curve provided by the manufacturer.

Cell viability and growth

Cell viability was determined using the trypan blue dye exclusion assay. Cell growth was determined by counting the number of viable cells.

Statistical analysis

ANOVA was used to determine the levels of difference between all groups. Comparisons for all pairs used the Tukey-Kramer highest significance difference test. *P* values for significance were set at .05. All results were expressed as means $\pm$ SEMs.

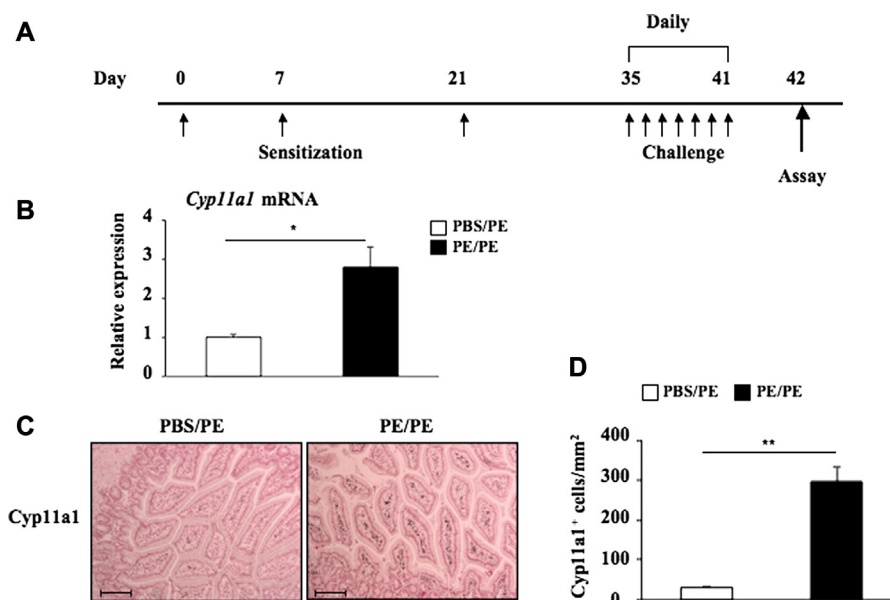


FIG 1. Cyp11a1 is expressed in mouse jejunum. **A**, Protocol for induction of peanut allergy. **B**, *Cyp11a1* mRNA expression detected using quantitative RT-PCR in peanut-sensitized and challenged versus sham-sensitized and peanut-challenged mice. **C**, Representative immunohistochemical staining for Cyp11a1 (magnification $\times 200$). Bar = 100 μ m. **D**, Quantitation of mucosal Cyp11a1-expressing cells. Results are from 3 independent experiments, and each experiment included 4 mice per group ($n = 12$). * $P < .05$ and ** $P < .01$. PBS/PE, Sham sensitized and peanut challenged; PE/PE, peanut sensitized and challenged.

RESULTS

Expression of Cyp11a1 in the small intestines of peanut-sensitized and challenged mice

We first examined the expression of *Cyp11a1* mRNA and protein in the jejunum of wild-type BALB/c mice. After PE sensitization and challenge (Fig 1, A), *Cyp11a1* mRNA expression was increased 3-fold in the jejunal homogenates (Fig 1, B). Immunohistochemical staining of jejunal tissue with an antibody specific for Cyp11a1 protein was mainly localized to the lamina propria of the small intestine (Fig 1, C). There were few Cyp11a1-positive cells in the mucosa of the small intestines of sham-sensitized mice, whereas numbers of Cyp11a1-positive cells were significantly increased in the PE-sensitized and challenged mice (Fig 1, D). Thus Cyp11a1 expression was induced after sensitization and challenge.

Inhibition of Cyp11a1 attenuates PE-induced allergic responses *in vivo*

To determine whether Cyp11a1 plays a role in the development of peanut allergy, we investigated the effects of inhibition of Cyp11a1 enzymatic activation on induction of peanut allergy using the inhibitor AMG. AMG is known to block the enzymatic activity of Cyp11a1, thus preventing conversion of cholesterol to pregnenolone.²⁶ To establish that AMG inhibitory activity was limited to the enzymatic activity, we measured pregnenolone levels in serum after PE sensitization and challenge. As shown in Fig 2, A, pregnenolone levels were significantly increased in the sera of peanut-sensitized and challenged mice (4.69 ± 0.92 ng/mL) compared with those seen in sham-sensitized but PE-challenged mice (1.99 ± 0.11 ng/mL). Pregnenolone levels were significantly decreased (2.98 ± 0.60 ng/mL) in peanut-sensitized and challenged mice after treatment with AMG (20 mg/kg). Although PE sensitization and

challenge increased *Cyp11a1* mRNA levels and numbers of Cyp11a1-positive cells in the small intestine, treatment with AMG (20 mg/kg) did not affect these results (Fig 2, B and C). These data confirmed that Cyp11a1 enzymatic activity, in parallel to mRNA and protein expression, was induced by peanut sensitization and challenge and that AMG specifically targeted the enzymatic activity but not protein expression *per se*.

Administration of the inhibitor to sensitized mice resulted in a dose-dependent inhibitory effect on intestinal allergy induction; 20 mg/kg of the inhibitor fully prevented development of diarrhea and significantly diminished clinical symptom scores in PE-sensitized and challenged mice (Fig 3, A and B). Lower doses of the inhibitor (10 mg/kg) partially inhibited diarrhea and symptom scores, whereas 5 mg/kg of the inhibitor had no observed inhibitory effects on diarrhea occurrence or clinical symptoms.

Mast cells are involved in allergic responses,^{17,27} and we demonstrated increased mast cell and mucus-producing goblet cell numbers in the small intestines of PE-sensitized and challenged mice (Fig 3, C and D, and see Figs E1 and E2 in this article's Online Repository at www.jacionline.org). Mice treated with the Cyp11a1 inhibitor at a dose of 20 mg/kg demonstrated markedly reduced numbers of mast cells, as well as mucus-producing goblet cells, in the mucosa of the small intestine. To detect mast cell degranulation, we measured plasma levels of histamine within 30 minutes of the last antigen challenge. Challenge of sensitized mice resulted in detection of increased levels of histamine in plasma; after treatment with AMG (20 mg/kg), significantly lower plasma histamine levels were detected (Fig 3, E).

Because the inhibitor was administered after sensitization, peanut-specific IgE, IgG₁, and IgG_{2a} levels were unaffected by AMG administration (see Fig E3 in this article's Online Repository at www.jacionline.org). To examine whether AMG treatment

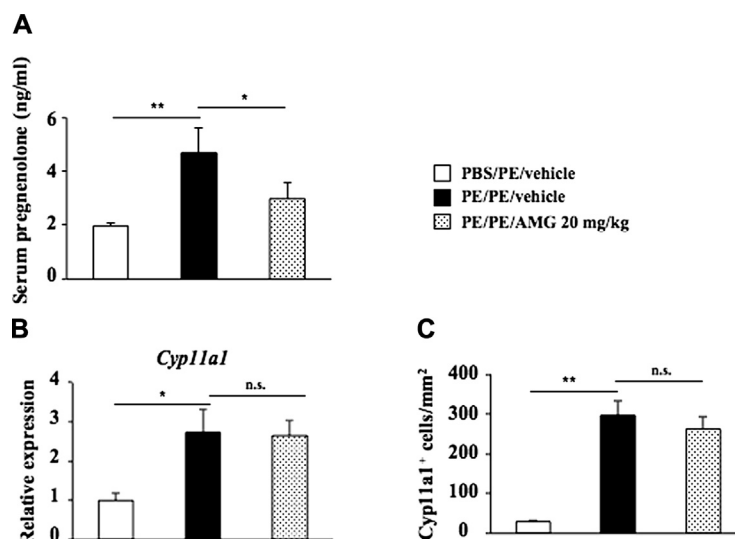


FIG 2. Inhibition of Cyp11a1 enzymatic activity does not affect Cyp11a1 protein and mRNA expression in the mouse jejunum. **A**, Pregnenolone levels were assessed in sera of mice. **B**, *Cyp11a1* mRNA expression in jejunum of mice treated with AMG or vehicle. **C**, Quantitation of mucosal Cyp11a1-expressing cells. Results are from 3 independent experiments, and each experiment included 4 mice per group ($n = 12$). * $P < .05$ and ** $P < .01$. n.s., Not significant; PBS/PE, sham sensitized and peanut challenged; PE/PE, peanut sensitized and challenged; PE/PE/AMG 20 mg/kg, peanut-sensitized and challenged and treated with AMG at 20 mg/kg.

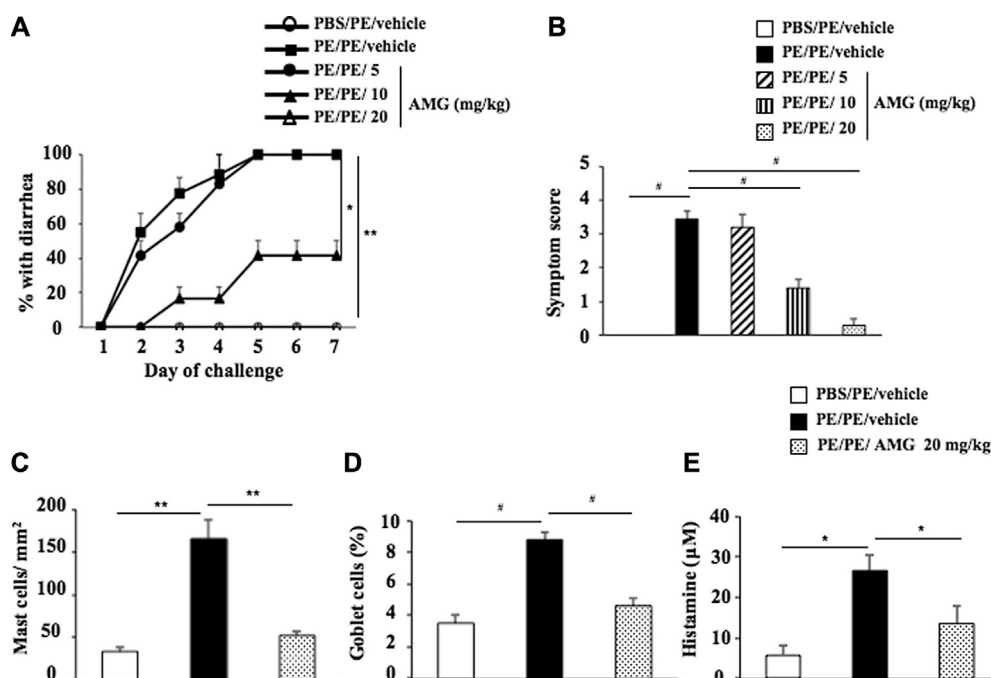


FIG 3. Inhibition of Cyp11a1 enzymatic activity *in vivo* reduces intestinal responses. **A**, Kinetics of the development of diarrhea after treatment with AMG (Cyp11a1 inhibitor) versus vehicle. **B**, Scores based on the severity of clinical signs were assessed 30 minutes after oral challenge. **C** and **D**, Quantitation of mucosal mast cell and goblet cell numbers in jejunum. **E**, Plasma histamine levels were assessed within 30 minutes of the last oral challenge. Results are from 3 independent experiments, and each experiment included 4 mice per group. * $P < .05$, ** $P < .01$, and # $P < .001$.

has an effect on estrogen secretion, we measured estradiol levels in plasma after PE sensitization and challenge. Estradiol levels did not significantly increase in the plasma of peanut-sensitized and challenged mice compared with those seen in sham-

sensitized but PE-challenged mice (see Fig E4 in this article's Online Repository at www.jacionline.org). Estradiol levels were unaffected by AMG (20 mg/kg) administration (see Fig E4). Together, these results demonstrated that AMG was a potent

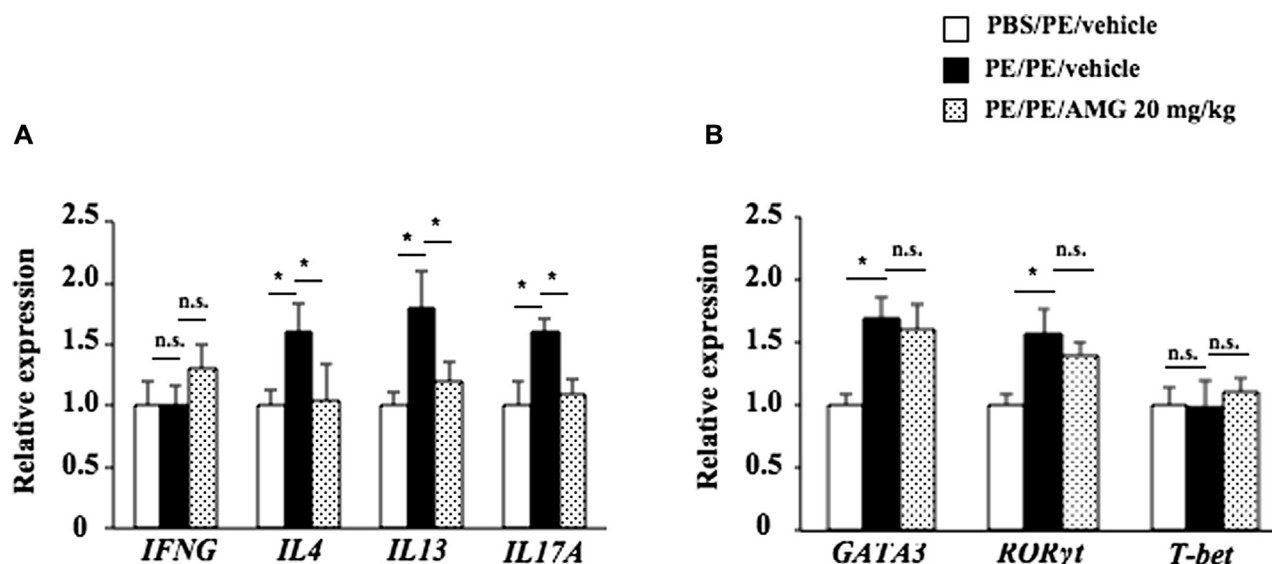


FIG 4. Effects of Cyp11a1 inhibition on cytokine and lineage-specific transcription factor expression in the mouse jejunum. **A**, *IFNG*, *IL4*, *IL13*, and *IL17A* mRNA expression in jejunum of mice treated with AMG or vehicle. **B**, T_H1 , T_H2 , and T_H17 transcription factors *T-bet*, *GATA3*, and *RORγt* expression in jejunum of mice treated with AMG or vehicle. Results are from 3 independent experiments ($n = 12$). * $P < .05$. n.s., Not significant.

inhibitor of the enzymatic activity of Cyp11a1 *in vivo* without affecting mRNA expression or protein levels of the enzyme. These data identified Cyp11a1 as playing an essential role in the triggering of allergic diarrhea and symptoms, intestinal inflammation, and goblet cell metaplasia.

Inhibition of Cyp11a1 enzymatic activity suppresses T_H2 and T_H17 cytokine production without affecting expression of lineage-specific transcription factors *in vivo*

T_H2 and T_H17 cells have been implicated in the development of allergic disorders, including asthma and food allergy.^{24,28-31} PE sensitization and challenge increased *IL4*, *IL13*, and *IL17A* but not *IFNG* mRNA expression in the small intestine (Fig 4, A). In parallel, mRNA expression of the lineage-specific transcription factors *GATA3* and retinoic acid-related orphan receptor γt (*RORγt*) were significantly increased in sensitized and challenged mice, whereas levels of *T-bet* mRNA were not altered (Fig 4, B). After treatment with AMG (20 mg/kg), *IL4*, *IL13*, and *IL17A* mRNA expression was reduced to baseline levels, but *T-bet*, *GATA3*, or *RORγt* mRNA expression was not affected (Fig 4, B), indicating that the effects on cytokine transcription were mediated downstream of these transcription factors. Given that transcription factor expression was still increased in AMG-treated animals, drug toxicity as an explanation of the effects on cytokine expression was eliminated.

Inhibition of Cyp11a1 enzymatic activity suppresses T_H2 and T_H17 cell differentiation *in vitro* without affecting lineage-specific transcription factor or Cyp11a1 expression

Naive T_H cells differentiate into T_H1 , T_H2 , and T_H17 cells under the control of specific polarizing cytokines and master

transcription factors.³² We investigated the effect of Cyp11a1 inhibition on T_H cell differentiation *in vitro*. Isolated $CD4^+CD45RB^+$ T cells from spleens of naive T-cell receptor transgenic mice (OT II mice) were cultured under T_H1 -, T_H2 -, and T_H17 -polarizing conditions in the presence or absence of the inhibitor AMG for 6 days and then stimulated with anti-CD3/anti-CD28.

Addition of AMG to cultured CD4 T cells under T_H1 -, T_H2 -, and T_H17 -polarizing conditions had significant and distinct effects. In polarized cells *Cyp11a1* mRNA expression was approximately 300-fold higher in T_H2 cells compared with T_H1 cells and 10-fold higher in T_H17 cells compared with T_H1 cells (Fig 5, A). The addition of AMG (400 $\mu\text{mol/L}$) to the cell cultures did not suppress expression levels of *Cyp11a1* mRNA or protein in the polarized T_H1 , T_H2 , or T_H17 cells (Fig 5, B and C). As shown in Fig 5, D, pregnenolone levels were highest in the culture supernatants from polarized T_H2 cells, with lower levels released from T_H17 cells, followed by release from T_H1 cells. Addition of AMG (400 $\mu\text{mol/L}$) during polarization of T_H cells *in vitro* significantly decreased levels of pregnenolone in culture supernatants from T_H2 cells but not in T_H1 cells. Levels in cultures of polarized T_H17 cells were also reduced by AMG, but the decreases did not reach statistical significance. These results confirmed the findings that the inhibitory activity of AMG appeared restricted to the enzymatic activity of Cyp11a1 without affecting gene transcription or translation. Furthermore, the data demonstrated the highest levels of Cyp11a1 expression and enzymatic activity in T_H2 cells, with little to no expression or activity in T_H1 cells.

In the culture supernatants of polarized T_H2 cell cultures, dose-dependent inhibitory effects of AMG on IL-13 secretion were observed. IL-13 levels were significantly decreased in the culture supernatants of polarized T_H2 cell cultures in the presence of AMG at 400 and 600 $\mu\text{mol/L}$ (Fig 5, E, and see Fig E5 in this article's Online Repository at www.jacionline.org). IL-13 levels were decreased in the presence of AMG at 200 $\mu\text{mol/L}$, but the

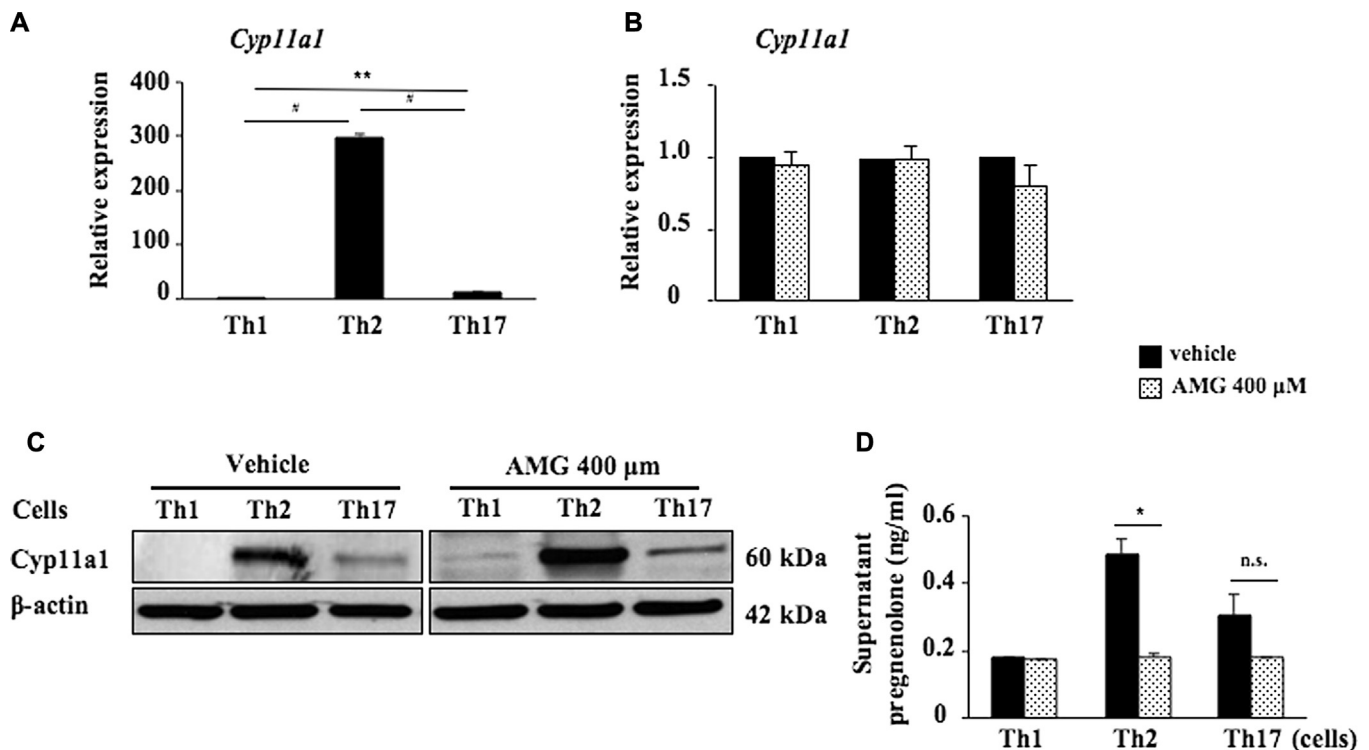


FIG 5. Inhibition of *Cyp11a1* enzymatic activity suppresses differentiation of naive CD4 T cells into T_H2 and T_H17 cells without affecting lineage-specific transcription factor and *Cyp11a1* expression. **A**, Relative *Cyp11a1* expression in naive CD4 T cells differentiated *in vitro* into T_H1 , T_H2 , and T_H17 cells from spleens of naive T-cell receptor transgenic mice (OT II mice) determined using RT-PCR. **B**, *Cyp11a1* mRNA expression in polarized CD4 T cells in the presence of AMG or vehicle. **C**, Western blot analysis of *Cyp11a1* protein in polarized T_H1 , T_H2 , or T_H17 cells treated with AMG or vehicle. **D**, Pregnenolone levels were assessed in supernatants of cultured CD4 T cells under T_H1 -, T_H2 -, and T_H17 -polarizing conditions. **E**, Cytokine levels in supernatants of cultured CD4 T cells treated with inhibitor or vehicle under T_H1 -, T_H2 -, and T_H17 -polarizing conditions. **F**, T_H1 , T_H2 , and T_H17 cytokine and lineage-specific transcription factor mRNA expression in polarized T_H1 , T_H2 , or T_H17 cells treated with inhibitor or vehicle. Data are from 3 independent experiments ($n = 12$). * $P < .05$, ** $P < .01$, and # $P < .001$. n.s., Not significant.

decreases did not reach statistical significance. IL-17A levels were significantly decreased by AMG at 400 and 600 μ mol/L in polarized T_H17 cell cultures. However, IFN- γ levels were not affected by AMG at any concentration tested in polarized T_H1 cell cultures. In parallel, the inhibitor decreased *IL13* and *IL17A* mRNA levels in polarized T_H2 and T_H17 cells, respectively (Fig 5, F), but no significant effects of the inhibitor were detected on *IFNG* mRNA expression in T_H1 cells. Consistent with results from the *in vivo* studies, the inhibitor (400 μ mol/L) did not have any effect on lineage-specific transcription factor mRNA expression, *T-bet*, *GATA3*, or *ROR γ t* mRNA in polarized T_H1 , T_H2 , and T_H17 cells, respectively (Fig 5, F). Further, addition of the inhibitor at 400 μ mol/L or less during the T_H cell polarization *in vitro* had no effect on T_H cell viability; in the presence of 600 μ mol/L of the inhibitor, cell viability was marginally affected (see Fig E6 in this article's Online Repository at www.jacionline.org).

shRNA-mediated silencing of *Cyp11a1* reduces T_H2 cytokine expression

To confirm the importance of *Cyp11a1* in T_H cell differentiation, we used shRNA-mediated silencing of *Cyp11a1* in polarized T_H2 cells. Polarized T_H2 cells were transduced with retroviruses coexpressing CFP with control (*luc*) or *Cyp11a1* shRNA.

Seventy-two hours after transduction, CFP⁺CD4⁺ cells were sorted and stimulated with 2 μ g/mL anti-CD3/anti-CD28 for 6 and 24 hours. To confirm the effectiveness of *Cyp11a1* gene silencing, we demonstrated reduced *Cyp11a1* mRNA levels in T_H2 cells compared with silencing with the control shRNA (Fig 6, A). As a result, levels of pregnenolone in supernatants of cultured T_H2 cells transfected with *Cyp11a1* shRNA were significantly reduced compared with those transfected with control shRNA (Fig 6, B).

IL4 and *IL13* mRNA levels were decreased in T_H2 cells transfected with *Cyp11a1* shRNA compared with those transfected with control shRNA, without affecting levels of *GATA3* mRNA (Fig 6, C). In parallel, IL-4 and IL-13 levels were reduced in supernatants of T_H2 cell cultures transfected with *Cyp11a1* shRNA (Fig 6, D). These results demonstrated that silencing of *Cyp11a1* in polarized T_H2 cells resulted in decreased *IL4* and *IL13* mRNA and protein levels without affecting *GATA3* transcription. These results indicated that *Cyp11a1* upregulation and activation are downstream of *GATA3*.

DISCUSSION

CD4 T_H cells play a pivotal role in the induction and control of allergic inflammation, including food allergy.³³ In a mouse model

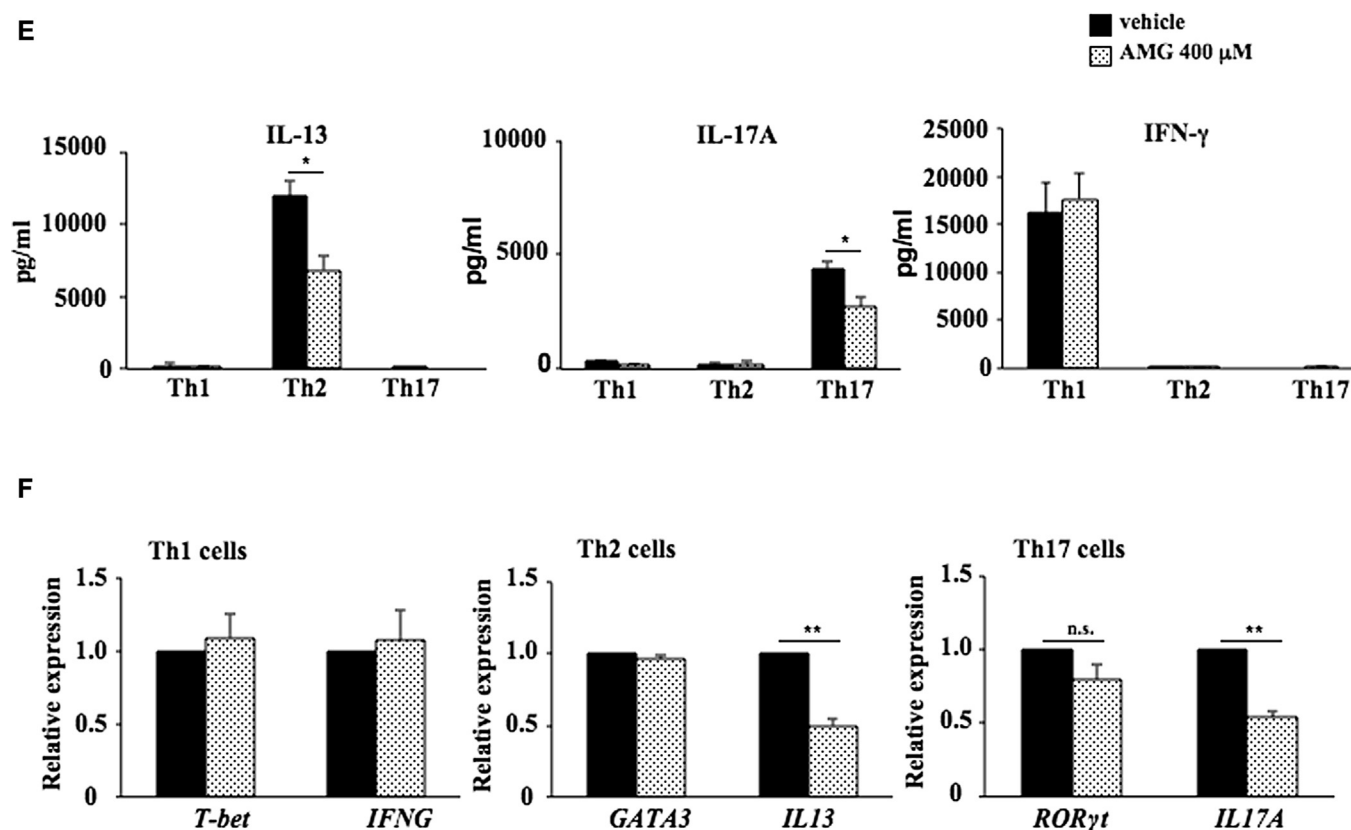


FIG 5. (Continued)

of food allergy, allergen-specific CD4 T cells were activated in the mesenteric lymph nodes and recruited to the small intestine, resulting in increased T_H2 cytokine levels in the inflamed small intestine.³⁴ We showed previously that increased numbers of CD4 T cells accumulated in the small intestine accompanied by increases in T_H2 cytokine (*IL4* and *IL13*) mRNA expression in a mouse model of peanut allergy.²³ Here we demonstrated not only that peanut sensitization and challenge resulted in inflammatory and cytokine changes in the small intestine but also that mRNA, protein, and enzymatic activity levels of the steroidogenic enzyme *Cyp11a1* were also markedly increased. Administration of an inhibitor of *Cyp11a1* enzymatic activity, AMG, prevented development of allergic diarrhea and accumulation of inflammatory cells in the small intestine in a dose-dependent manner. Serum pregnenolone levels were reduced in parallel. AMG treatment decreased *IL13* and *IL17* mRNA expression in the small intestine without affecting *Cyp11a1* mRNA or protein levels. *In vitro* the inhibitor decreased *IL13* and *IL17* mRNA levels in polarized T_H2 and T_H17 cells, respectively, without affecting levels of *GATA3* or *ROR γ t* or the polarization of T_H1 cells, *IFNG*, and *T-bet* expression. The importance of *Cyp11a1* was further demonstrated using shRNA-mediated silencing of *Cyp11a1* in polarized T_H2 cells, which resulted in significantly decreased *IL4* and *IL13* mRNA and protein levels. These data indicated that *Cyp11a1* played an important role in the development of peanut allergy through its effects on steroidogenesis, a critical pathway in T_H2 differentiation.

In human subjects allergen-specific T_H2 cells were essential in the development and maintenance of both type I IgE-mediated and non-IgE-mediated food-induced allergic responses. In

patients with anaphylactic peanut allergy, increased numbers of peanut-specific IL-5- and IL-4-producing T_H2 cells were found in peripheral blood.³⁵ In addition, peanut-specific T-cell lines from subjects with peanut-induced anaphylaxis primarily produced T_H2 cytokines (IL-4 and IL-13).³⁶ Other food allergies were also characterized by increased T_H2 cytokine levels; in patients with milk-induced gastrointestinal diseases, milk-specific CD4 T cells derived from the duodenal mucosa produced high levels of T_H2 cytokines, especially IL-13.³⁷

GCs are steroid hormones with important functions in regulating immune responses and inflammation.³ Endogenous GC synthesis is controlled by the hypothalamic-pituitary-adrenal axis^{3,38} and regulated by the transcriptional control of steroidogenic enzymes of the cytochrome P450 gene family.³⁹ Corticosteroids have been used in the treatment of allergic diseases because of their anti-inflammatory activity,⁴ but somewhat paradoxically, increasing evidence indicates that corticosteroids might also enhance disease pathogenesis by activating and enhancing growth of CD4 T cells and inhibiting T_H1 cytokine production.⁴⁰ GCs amplified immune responses in steroid-insensitive $CD8^+$ T cells.⁷ In addition, the corticosteroids themselves might induce T_H2 cytokine production while simultaneously suppressing T_H1 cytokine production.⁷

Cyp11a1 is the first and rate-limiting enzyme in the steroid biosynthetic pathway, catalyzing the conversion of cholesterol to pregnenolone. Transcription factors, such as steroidogenic factor 1, activator protein 2, and several tissue-specific GATA family proteins, enhance the transcription of *Cyp11a1* through interactions with activator protein 1, specificity protein 1, and activator protein 2.¹⁰ In particular, the GATA protein family plays an

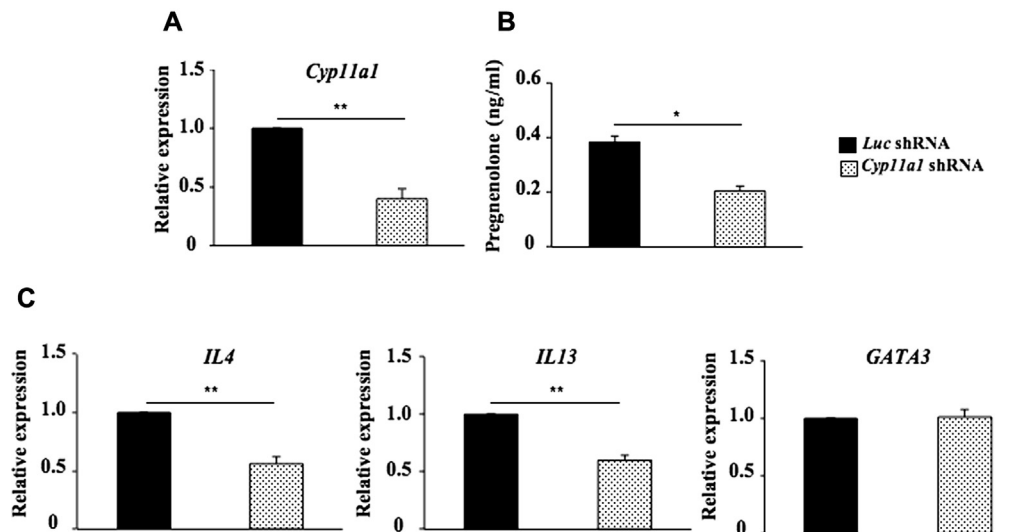


FIG 6. shRNA-mediated silencing of *Cyp11a1* regulates levels of IL-13 without affecting levels of GATA3 transcripts in T_H2 cells. **A**, *Cyp11a1* mRNA expression in shRNA-transduced T_H2 cells. **B**, Pregnenolone levels were assessed in supernatants of cultured T_H2 cells transduced with *Cyp11a1* or *luc* shRNA. **C**, Levels of *IL4*, *IL13*, and *GATA3* mRNA expression in cultured T_H2 cells transduced with *Cyp11a1* or *luc* shRNA. **D**, Levels of IL-4 and IL-13 in supernatants of cultured T_H2 cells transduced with *Cyp11a1* or *luc* shRNA. Results are from 3 independent experiments ($n = 12$). * $P < .05$ and ** $P < .01$.

important role in the regulation of *Cyp11a1* expression.⁴¹ GATA-binding elements have been identified in the *Cyp11a1* promoter, and *Cyp11a1* expression was decreased in GATA3-deficient mice.⁴² GATA4 significantly upregulated *Cyp11a1* expression in granulosa cells.⁴³ These results identify important events in the transcriptional regulation of *Cyp11a1* that directly affect steroid synthesis and release.

In this study we investigated the role of *Cyp11a1* in peanut-induced allergic intestinal responses. We demonstrated that *Cyp11a1* protein and mRNA levels were increased in the jejunum of sensitized and challenged mice. In parallel, enzymatic activity was increased, as demonstrated by increased levels of pregnenolone in the sera of sensitized and challenged mice. Next, we determined whether *Cyp11a1* enzymatic activity was essential for induction of peanut allergy using the inhibitor AMG. AMG is a recognized inhibitor of *Cyp11a1* enzymatic activity and has been used for the suppression of adrenal function in patients with Cushing syndrome⁴⁴ and as second-line therapy for patients with breast cancer^{45,46} and prostate cancer.⁴⁷

Administration of this inhibitor during the oral challenge phase and after sensitization resulted in significantly lower serum pregnenolone levels and reduced the incidence and severity of diarrhea and intestinal inflammation (mast cell accumulation and goblet cell metaplasia) accompanied by decreases in *IL13* and *IL17A* mRNA levels in the intestine. The inhibitor did not alter the development of specific antibodies, including peanut-specific IgE, likely because sensitization was completed before treatment in the challenge phase. The inhibitor did not affect estradiol secretion after peanut sensitization and challenge nor were serum levels affected by AMG administration. In part, this might reflect the finding that AMG, *per se*, does not suppress plasma estrogen levels by inhibiting the adrenal secretion of precursor androgen.⁴⁸ Although administration of the inhibitor *in vivo* could not identify specific target cells, these data demonstrated, for the first time, that *Cyp11a1* functioned as a key regulator of the development of peanut-induced allergic responses.

The data showed that inhibition of *Cyp11a1* significantly reduced T_H2 and T_H17 cytokine production *in vivo*. Interestingly, the inhibitor did not affect expression of the T_H1 , T_H2 , and T_H17 lineage-specific transcription factors *T-bet*, *GATA3*, or *ROR γ t*. The results suggested that suppression of T_H2 and T_H17 cytokine production was not mediated through effects on lineage-specific transcription factor expression but on cytokine transcription. The primary action of *Cyp11a1* enzymatic activity in this model thus appeared to manifest downstream of these lineage-specific transcription factors.

To investigate the function of *Cyp11a1* in CD4 T cells, we monitored T_H1 , T_H2 , and T_H17 polarization *in vitro* in the presence of AMG. The highest levels of *Cyp11a1* protein and enzymatic activity were detected in polarized T_H2 cells, with significantly lower levels in T_H1 cells and virtually no activity in T_H1 cells. On the basis of AMG dose-dependent inhibitory effects on T_H2 and T_H17 cytokine secretion, as well as cell viability, we chose 400 μ mol/L AMG to carry out the *in vitro* T_H cell polarization experiments. The inhibitor decreased IL-13 cytokine production in polarized T_H2 cells; however, IFN- γ production was not affected by the inhibitor in polarized T_H1 cells. Similar to our *in vivo* data, the inhibitor did not affect *GATA3* mRNA expression in polarized T_H2 cells or levels of *T-bet* or *ROR γ t* in polarized T_H1 and T_H17 cells, respectively. Thus inhibition of *Cyp11a1* enzymatic activity impaired CD4 T_H2 and T_H17 cell differentiation, which in turn decreased production of the T_H2 cytokine IL-13 and the T_H17 cytokine IL-17A, and these effects were mediated downstream of their respective and essential lineage-specific transcription factors.

To confirm that the results with AMG were specific to inhibition of *Cyp11a1*, we silenced *Cyp11a1* mRNA in cultured T_H2 cells using an shRNA. During T_H2 polarization, cells were transduced with retrovirus expressing *Cyp11a1*-targeted shRNA or control (*luc*) shRNA and activated under T_H2 conditions. *Cyp11a1* shRNA decreased the expression of *Cyp11a1* mRNA levels by $58\% \pm 5.2\%$, and enzymatic activity of *Cyp11a1*,

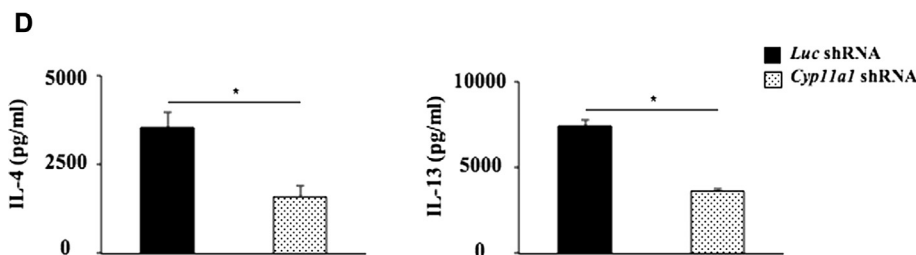


FIG 6. (Continued)

monitoring pregnenolone levels, was reduced by $47\% \pm 4.5\%$. T_H2 cytokine (IL-4 and IL-13) mRNA and protein levels were decreased on transduction of *Cyp11a1* shRNA. As we observed with *Cyp11a1* inhibition *in vivo* and *in vitro* with AMG, *GATA3* mRNA levels remained unaffected after silencing of *Cyp11a1*. These data confirmed *in vivo* and *in vitro* AMG inhibition data, indicating that *Cyp11a1* critically regulates T_H2 cell differentiation and cytokine production.

For the first time, these studies demonstrate that activation of the steroidogenic enzyme *Cyp11a1* plays a critical role in the development of intestinal allergic responses through its effects on T_H2 polarization and IL-13 production. Although studies in experimental animal models are difficult to extrapolate to human conditions, identification of the functional significance of *Cyp11a1* represents a novel and potential target for the regulation and treatment of peanut-induced allergy.

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Clinical implications: At present, the only recognized therapy for peanut-sensitive subjects is peanut avoidance. The data identify the important role of the steroidogenic pathway in the triggering of peanut-induced intestinal anaphylaxis and identify potential targets for intervention.

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METHODS

Preparation of peanut protein

Crude PE was prepared from defatted raw flours (Golden Peanut Company, Alpharetta, Ga), as previously described.^{E1} Briefly, the flour (1:10 wt/vol) was extracted in 10× PBS overnight at 4°C. After centrifugation at 30,000g for 60 minutes, the supernatant was filter sterilized, measured for protein concentrations by using the BCA method (Pierce, Rockford, Ill), and stored as aliquots at −20°C. Endotoxin levels in PE solutions were less than 0.1 EU/mL, as assessed by using a Chromogenic LAL endotoxin assay kit (GeneScript, Piscataway, NJ).

Sensitization and intragastric challenge

The experimental protocol for sensitization and challenge to peanut was previously described.^{E1} Briefly, mice were sensitized 3 times with 500 µg of PE together with 2.0 mg of alum (Pierce) by means of intraperitoneal injection in a total volume of 100 µL on days 1, 7, and 21. Two weeks later, mice received 20 mg of PE (in 250 µL of PBS) by means of gavage with a 22-gauge feeding needle (Fisher Scientific, Pittsburgh, Pa) every day for 1 week. Thirty minutes after the last challenge, plasma was collected and stored at −80°C. Twenty-four hours after the last challenge, mice were killed. Serum and jejunal tissue were collected for further analyses. Control animals were sham sensitized but challenged with peanut.

Cyp11a1 inhibitor and treatment

AMG was dissolved in 1 mol/L hydrogen chloride and diluted with saline for *in vivo* study or diluted with RPMI medium for *in vitro* study. The final concentration of 1 mol/L hydrogen chloride was less than 1% and 0.05% for *in vivo* and *in vitro* study, respectively. PE-sensitized and challenged mice received different doses (5, 10, and 20 mg/kg) of the Cyp11a1 enzyme inhibitor (PE/PE/AMG) by means of gavage with a 22-gauge feeding needle (Fisher Scientific) twice a day during the peanut challenge phase. Control animals included PE-sensitized and challenged but vehicle (saline)-treated (PE/PE/vehicle) or sham-sensitized but PE-challenged and vehicle-treated (PBS/PE/vehicle) mice.

Histology

Jejunum was fixed in 10% formalin and processed into paraffin blocks. Five-micrometer-thick tissue sections were cut and stained with periodic acid-Schiff for detection of mucus-containing cells.^{E2} The number of mucus-containing cells from one side of the villus and the total number of villus cells on the same side were counted. The number of mucus-containing cells was expressed as the percentage of mucus-containing cells divided by the total number of cells counted. At least 10 randomly selected villi were counted on each slide.

Assessment of hypersensitivity reactions

Anaphylactic symptoms were evaluated 30 minutes after the oral challenge, as previously reported^{E3} and as follows: 0, no symptoms; 1, scratching and rubbing around the nose and head; 2, puffiness around the eyes and mouth, diarrhea, pilar erecti, reduced activity, and/or decreased activity with increased respiratory rate; 3, wheezing, labored respiration, and cyanosis around the mouth and tail; 4, no activity after prodding or tremor and convulsion; and 5, death. Scoring of symptoms was performed in a blinded manner by an independent observer.

Measurement of peanut-specific antibody levels

Serum peanut-specific IgE, IgG₁, and IgG_{2a} levels were measured by means of ELISA, as described previously.^{E3,E4} Briefly, Immulon II 96-well plates (Dynatech Laboratories, Chantilly, Va) were coated with 2 µg/mL PE in coating buffer (eBioscience). Detection was performed with horseradish peroxidase-conjugated goat anti-mouse IgG₁ and IgG_{2a} (SouthernBiotech, Birmingham, Ala) and biotin rat anti-mouse IgE (BD PharMingen, Franklin Lakes, NJ), respectively. The peroxidase activity was measured with tetramethylbenzidine (eBioscience) at 450 nm.

Histamine levels in plasma

Levels of histamine in plasma were measured with an enzyme immunoassay histamine kit (Beckman Coulter, Fullerton, Calif), as described by the manufacturer. The concentration of histamine was calculated from a standard curve provided by the manufacturer.

T-cell differentiation and treatment with the Cyp11a1 inhibitor *in vitro*

Differentiation of T_H1, T_H2, or T_H17 cells was performed, as previously described, with minor changes.^{E5,E6} CD4⁺CD45RB⁺ T cells were isolated from naive T-cell receptor transgenic mouse (OT II mice) spleens by using a cell sorter (MoFlo XDP, Beckman Coulter). In the presence of mitomycin C-treated spleen cells, 5 µg/mL OVA₃₂₃₋₃₃₉ peptide, and the inhibitor AMG (400 µM), isolated naive CD4 T cells were cultured with rmIL-2 (10 ng/mL; R&D Systems, Minneapolis, Minn), rmIL-12 (10 ng/mL; PeproTech, Rocky Hills, NJ), rmIFN-γ (5 ng/mL, PeproTech), and anti-IL-4 mAbs (10 µg/mL, eBioscience) to induce T_H1 cell differentiation; with rmIL-2 (10 ng/mL, R&D Systems), rmIL-4 (5 ng/mL, PeproTech), and anti-IFN-γ mAb (10 µg/mL, eBioscience) for differentiation of T_H2 cells; and with rhIL-6 (50 ng/mL, PeproTech), rhTGF-β (2 ng/mL, PeproTech), rmIL-23 (10 ng/mL, PeproTech), anti-IL-4 mAb (10 µg/mL, eBioscience), and anti-IFN-γ mAb (10 µg/mL, eBioscience) for differentiation of T_H17 cells. After 6 days of culture, the cells were washed with fresh medium and restimulated with anti-CD3/anti-CD28 for 24 hours for assay of cytokine production. The cells were collected for quantitative RT-PCR and Western blotting. For transduction experiments, the cells were cultured under T_H1-, T_H2-, and T_H17-polarizing conditions for 5 days, as described in the Methods section.

Western blot analysis

Cultured cells were lysed, as previously described.^{E7} Lysates were resolved by means of SDS-PAGE and transferred to nitrocellulose membranes. Proteins were detected by using antibodies specific for Cyp11a1 (LifeSpan Biosciences, Seattle, Wash), followed by chemiluminescence detection (GE Healthcare, Little Chalfont, United Kingdom).

Quantitative RT-PCR

RNA was extracted from jejunal tissue homogenates or from CD4 T cells cultured *in vitro* by using Trizol (Invitrogen, Carlsbad, Calif). cDNA was generated with the iScript cDNA synthesis kit (Bio-Rad Laboratories, Hercules, Calif). Quantitative RT-PCR was performed on the ABI Prism 7300 sequence detection system (Applied Biosystems, Foster City, Calif). All primers and probes used were purchased as TagMan Gene Expression Assays from Applied Biosystems. Fold change was calculated by using the ΔΔ cycle threshold (ΔΔC_T) method.

Expression constructs

The *Cyp11a1* shRNA sequence was generated by using the Dharmacon siDESIGN Center (Thermo Scientific) Web site (<http://www.dharmacon.com/designcenter/designcenterpage.aspx>), as follows: *Cyp11a1* sense, 5'-TTCAATAAAGCTGATGAGTATTCAAGAGATACTCATCAGCTTTATTGATTTTTC-3'; *Cyp11a1* anti-sense, 5'-TCGAGAAAAAATCAATAAAGCTGATGAGTATCTCTGAATACATCATCAGCTTTATTGAA-3'; and control firefly luciferase (*luc*) shRNA was described previously.^{E8} PAGE-purified and phosphorylated oligonucleotides (Integrated DNA Technologies, Coralville, Iowa) encoding *Cyp11a1* shRNA were annealed and ligated into a modified pQCXIP vector (Clontech, Mountain View, Calif) expressing CFP to construct the shRNA expression vectors.^{E8} Plasmid DNA encoding mouse *Cyp11a1* and control firefly *luc* were purified by using EndoFree Plasmid Maxi Kit (Qiagen, Valencia, Calif) and sequenced (Eton Bioscience, San Diego, Calif).

Retrovirus production and transduction

Retrovirus production was performed, as previously described.^{E9} Phoenix packaging cells were plated on poly-d-lysine-coated 100-mm dishes and cultured overnight to reach 60% to 80% confluency. Cells were cotransfected

with the pCL-Eco viral packaging plasmid and plasmid DNA encoding *Cyp11a1* or control *luc* shRNA by using Lipofectamine 2000 (Invitrogen), according to the manufacturer's instructions. Two days after transfection, the virus-containing supernatant was collected and used to transfect cells.

Retroviral transduction of T_H2 cells was performed, as previously described.^{E10} Sorted CD4⁺ T cells were cultured under T_H2 cell differentiation conditions, as previously reported.^{E11} Cells were transduced with retroviruses (control *luc* shRNA or *Cyp11a1* shRNA) by means of centrifugation in the presence of 8 µg/mL Polybrene (Sigma). Cells were expanded and analyzed on day 5.

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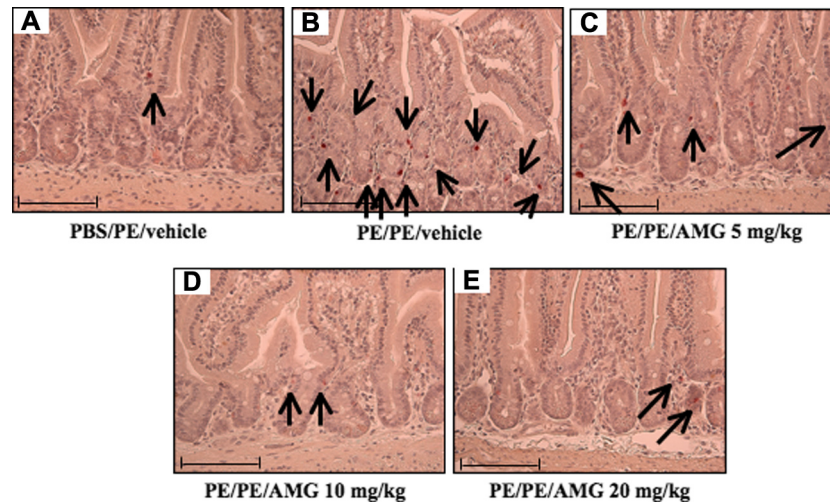


FIG E1. Decreased mast cell infiltration in the intestinal wall of PE/PE mice treated with AMG. Intestinal mucosal mast cells were quantified in jejunum by using chloroacetate esterase staining. Representative sections of PBS/PE/vehicle mice (**A**), PE/PE/vehicle mice (**B**), PE/PE/AMG (5 mg/kg) mice (**C**), PE/PE/AMG (10 mg/kg) mice (**D**), and PE/PE/AMG (20 mg/kg) mice (**E**) are shown (magnification $\times 400$). Bar = 100 μ M.

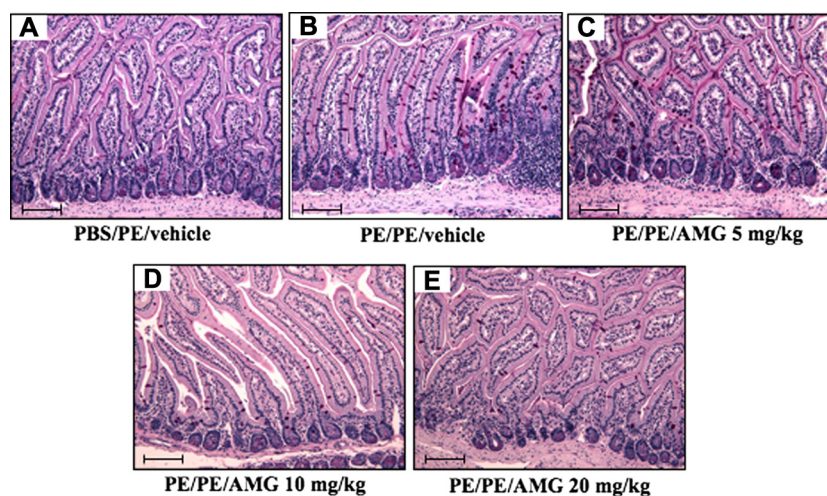


FIG E2. Decreased numbers of goblet cells in the intestinal epithelium of sensitized and challenged mice treated with AMG. Goblet cells were identified by means of periodic acid-Schiff staining 24 hours after the last challenge. Representative sections of PBS/PE/vehicle mice (**A**), PE/PE/vehicle mice (**B**), PE/PE/AMG (5 mg/kg) mice (**C**), PE/PE/AMG (10 mg/kg) mice (**D**), and PE/PE/AMG (20 mg/kg) mice (**E**) are shown (magnification $\times 200$). *Bar* = 100 μ M.

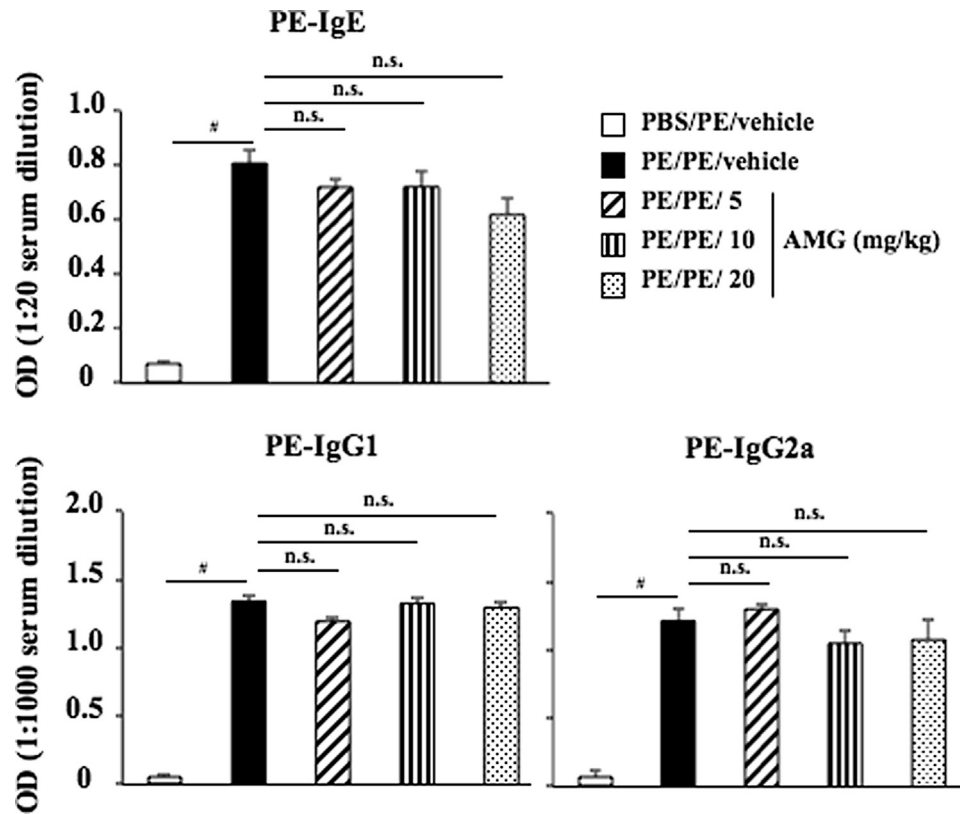


FIG E3. Treatment with AMG had no effect on serum immunoglobulin production in peanut-sensitized and challenged mice. Serum peanut-specific IgE, IgG₁, and IgG_{2a} levels were assessed by means of ELISA 24 hours after the last challenge and expressed as the OD of diluted serum, as described in the Methods section. Results were obtained from 3 individual experiments with 4 mice per group. #*P* < .001. n.s., Not significant.

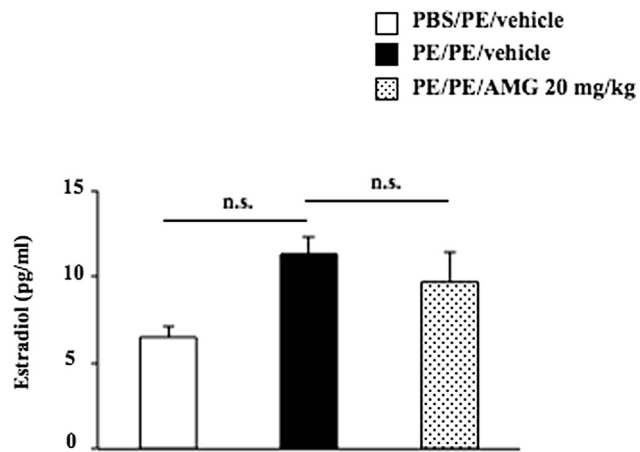


FIG E4. Treatment with AMG had no effect on plasma estradiol production in peanut-sensitized and challenged mice. Plasma levels of estradiol were assessed by means of ELISA 30 minutes after the last challenge. Results were obtained from 3 individual experiments with 4 mice per group. *n.s.*, Not significant.

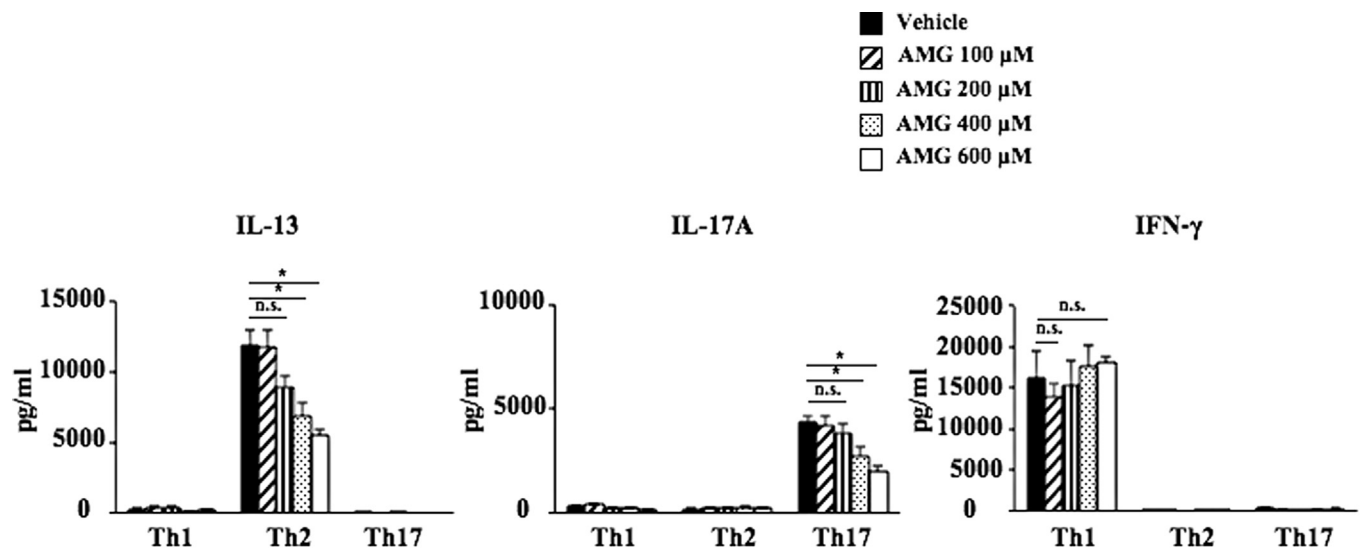


FIG E5. Dose-dependent inhibitory effects of AMG on cytokine production in *in vitro* polarized T_H cells. Cytokine levels in supernatants of cultured CD4 T cells treated with different doses of the inhibitor or vehicle under T_H1 -, T_H2 -, and T_H17 -polarizing conditions. * $P < .05$. n.s., Not significant.

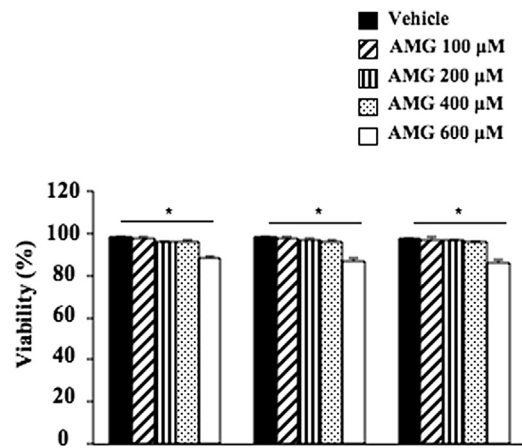


FIG E6. Cell viability in the presence of different concentrations of AMG in *in vitro* cultures of polarized T_H cells. Results were obtained from 3 individual experiments. * $P < .05$.