The Steroidogenic Enzyme Cyp11a1 is Essential for Development of Peanut-Induced Intestinal Anaphylaxis

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Supplementary Materials and Methods

Preparation of peanut protein

Crude peanut extract (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 10x PBS overnight at 4°C. After centrifugation at 30,000*g* for 60 minutes, the supernatant was filter-sterilized, measured for protein concentration using the BCA method (Pierce, Rockford, IL), and stored as aliquots at -20°C. Endotoxin levels in PE solutions were less than 0.1 EU/ml as assessed by 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 (17). Briefly, mice were sensitized 3 times with 500 μg of PE together with 2.0 mg of alum (Pierce, Rockford, IL) by means of intraperitoneal injection in a total volume of 100 mL on days 1, 7, and 21. Two weeks later, mice received 20 mg of PE (in 250 mL 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 M hydrogen chloride and diluted with saline for in vivo studies or diluted with RPMI medium for in vitro study. The final concentration of 1 M hydrogen chloride was less than 1% and 0.05% for in vivo and in vitro, respectively. PE sensitized and challenged mice received different doses (5, 10, 20 mg/kg) of the Cyp11a1 enzyme inhibitor (PE/PE/AMG) by means of gavage using a 22-gauge feeding needle (Fisher Scientific) twice a day during the peanut challenge phase. Controls 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 (20). 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 (E2). Briefly, 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 the 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

Serum peanut-specific IgE, IgG1, and IgG2a levels were measured by ELISA, as described previously (19, 21). 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 horse-radish peroxidase-conjugated goat anti-mouse IgG1 and IgG2a (SouthernBiotech, Birmingham, AL) 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 using an enzyme immunoassay histamine kit (Beckman Coulter, Fullerton, CA), 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 Th1, Th2, or Th17 cells was performed as previously described with minor changes (E3, E4). $CD4^+CD45RB^+$ T cells were isolated from naive TCR-transgenic mice (OT II mice) spleen using a cell sorter (MoFlo XDP, Beckman Coulter). In the presence of mitomycin-C-treated spleen cells, 5 μg/ml $OVA_{323-339}$ peptide, and the inhibitor AMG (400 μm), isolated naive CD4 T cells were cultured with rmIL-2 (10 ng/ml, R/D Systems), rmIL-12 (10

ng/ml, Peprotech), rmIFN-γ (5 ng/ml, Peprotech), and anti-IL-4 mAbs (10 μg/ml, eBioscience) to induce Th1 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 Th2 cells; and with rhIL-6 (50 ng/ml, Perotech), 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 Th17 cells. After 6 days of culture, the cells were washed with fresh medium and restimulated with anti-CD3/anti-CD28 for 24 hrs for assay of cytokine production. The cells were collected for quantitative RT-PCR and Western blot. For transduction experiments, the cells were cultured under Th1, Th2, and Th17 polarizing conditions for 5 days as described in Methods.

Western blot analysis

Cultured cells were lysed as previously described (E5). Lysates were resolved by means of SDS-PAGE and transferred to nitrocellulose membranes. Proteins were detected using antibodies specific for Cyp11a1 (LifeSpan Biosciences. Seattle, WA) followed by chemiluminescence detection (GE Healthcare, Little Chalfont, UK).

Quantitative real-time PCR

RNA was extracted from jejunal tissue homogenates or from CD4 T cells cultured in vitro using Trizol (Invitrogen). cDNA was generated using the iScript cDNA synthesis kit (Bio-Rad Laboratories, Hercules, CA). Quantitative RT-PCR was performed on the ABI Prism 7300 sequence detection system (Applied Biosystems, Foster City, CA). All primers and probes used

were purchased as Tagman Gene Expression Assays from Applied Biosystems. Fold change was calculated using the Delta Delta cycle threshold ($\Delta\Delta C_T$) method.

Expression constructs

The *Cyp11a1* short hairpin RNA (shRNA) sequence was generated using the Dharmacon siDESIGN center (Thermo Scientific) web site (http://www.dharmacon.com/designcenter/designcenterpage.aspx) and are described in the Online Supplement. *Cyp11a1* sense 5′-TTCAATAAAGCTGATGAGTATTCAAGAGATACTCATCAGCTTTATTGATTTTTTC-3', anti-sense 5′-TCGAGAAAAAATCAATAAAGCTGATGAGTATCTCTTGAATACTCATCAGCTTTATTGAA-3'. Control firefly *luciferase (luc)* shRNA was described previously (25). To construct the shRNA expression vectors, PAGE-purified and phosphorylated oligonucleotides (Integrated DNA Technologies, Coralville, IA) encoding *Cyp11a1* shRNA were annealed and ligated into a modified pQCXIP vector (Clontech, Mountain View, CA) expressing cyan fluorescent protein (CFP) (25). Plasmid DNA encoding mouse Cyp11a1 and control firefly *luc* were purified using endofree plasmid maxi kit (Qiagen, Valencia, CA) and sequenced (Eton Bioscience, San Diego, CA).

Retrovirus production and transduction

Retrovirus production was performed as previously described (26). Phoenix (ΦNX) packaging cells were plated on poly-d-lysine-coated 100-mm dishes and cultured overnight to reach 60 to 80% confluency. Cells were co-transfected with the pCL-Eco viral packaging

plasmid and plasmid DNA encoding *Cyp11a1*, or control *luc* shRNA using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Two days post-transfection, the virus-containing supernatant was collected and used to transfect cells.

Retroviral transduction of Th2 cells was performed as previously described (27). Sorted $CD4^+$ T cells were cultured under Th2 cell differentiation conditions as previously reported (24). Cells were transduced with retroviruses (control *luc* shRNA or *Cyp11a1* shRNA) by centrifugation in the presence of 8 μg/ml polybrene (Sigma). Cells were expanded and analyzed on day 5.

Supplementary Figure Legends

Figure E1. Decreased mast cell infiltration in the intestinal wall of PE/PE mice treated with AMG. Intestinal mucosa mast cells were quantified in jejunum using chloroacetate esterase staining. Representative sections of (A) PBS/PE/vehicle mice; (B) PE/PE/vehicle mice; (C) PE/PE/AMG (5 mg/kg) mice; (D) PE/PE/AMG (10 mg/kg) mice; and (E) PE/PE/AMG (20 mg/kg) mice. Magnification x400. Bar=100 μM.

Figure E2. Decreased numbers of goblet cells in intestinal epithelium of sensitized and challenged mice treated with AMG. Goblet cells were identified by PAS staining 24 hrs after the last challenge. Representative sections of (A) PBS/PE/vehicle mice, (B) PE/PE/vehicle mice, (C) PE/PE/AMG (5 mg/kg) mice, (D) PE/PE/AMG (10 mg/kg) mice, and (E) PE/PE/AMG (20 mg/kg) mice. Magnification x200. Bar=100 μM.

Figure E3. Treatment with AMG had no effect on serum immunoglobulin production in peanut sensitized and challenged mice. Serum levels of peanut-specific IgE, IgG1, and IgG2a were assessed by ELISA 24 hrs after the last challenge and expressed as optical density of diluted serum as described in Methods. Results were obtained from 3 individual experiments with 4 mice per group. #$P<0.001$, n.s. not significant.

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

Figure E5. Dose-dependent inhibitory effects of AMG on cytokine production in *in vitro* polarized Th cells. Cytokine levels in supernatants of cultured CD4 T cells treated with different doses of the inhibitor or vehicle under Th1, Th2, and Th17 polarizing conditions. *P<0.05, n.s.: not significant.

Figure E6. Cell viability in the presence of different concentrations of AMG in *in vitro* cultures of polarized Th cells. Results were obtained from 3 individual experiments. *P<0.05.

References

E1. Wang M, Takeda K, Shiraishi Y, Okamoto M, Dakhama A, Joetham A, et al. Peanut-induced intestinal allergy is mediated through a mast cell-IgE-FcepsilonRI-IL-13 pathway. J Allergy Clin Immunol 2010;126:306-16.

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E3. Ashino S, Wakita D, Shiohama Y, Iwakura Y, Chamoto K, Ohkuri T, et al. A T(h)17-polarized cell population that has infiltrated the lung requires cells that convert to IFN-{gamma} production in order to induce airway hyperresponsiveness. Intl Immunol 2010;22:503-13.

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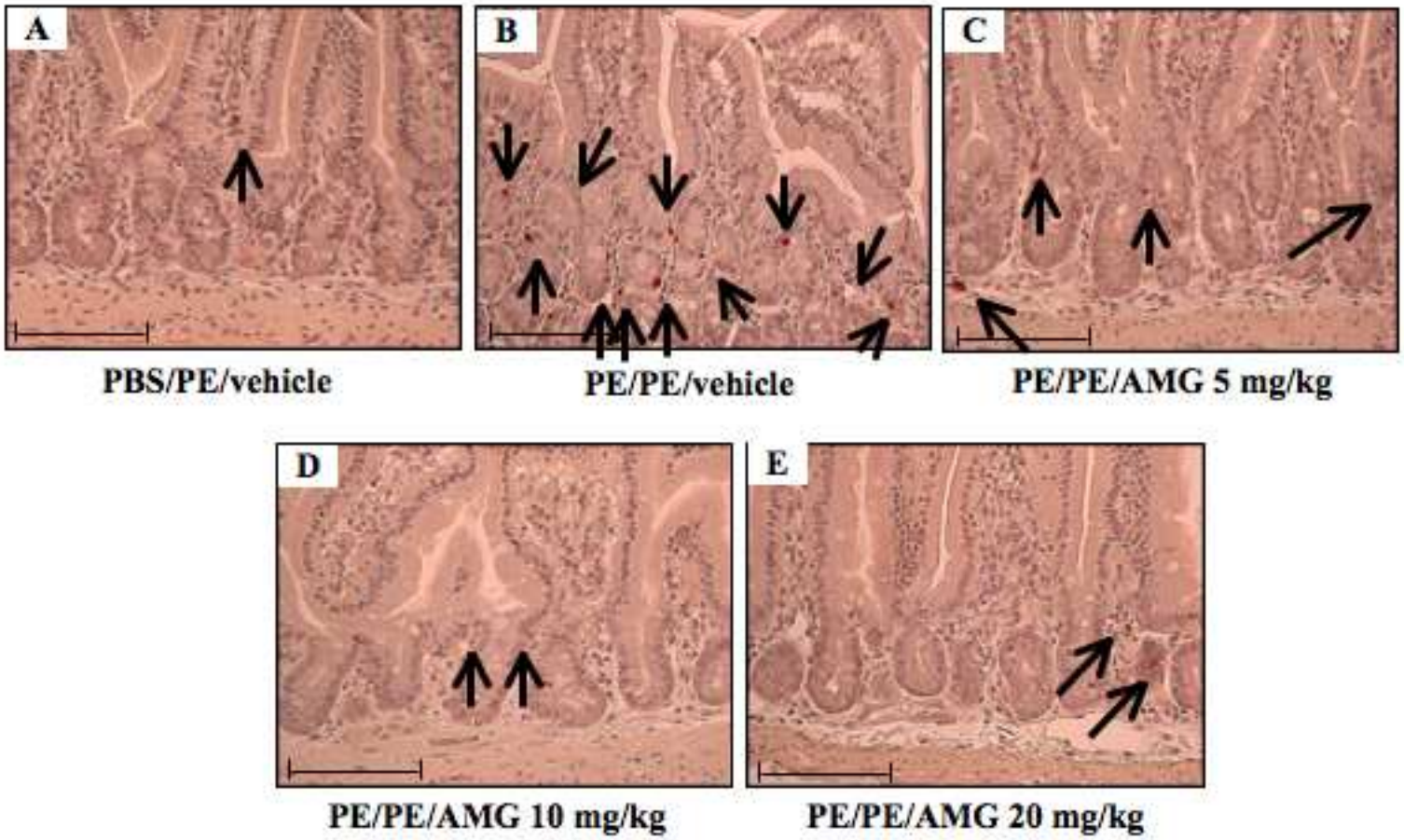
Figure E1


A
B
C
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D
E
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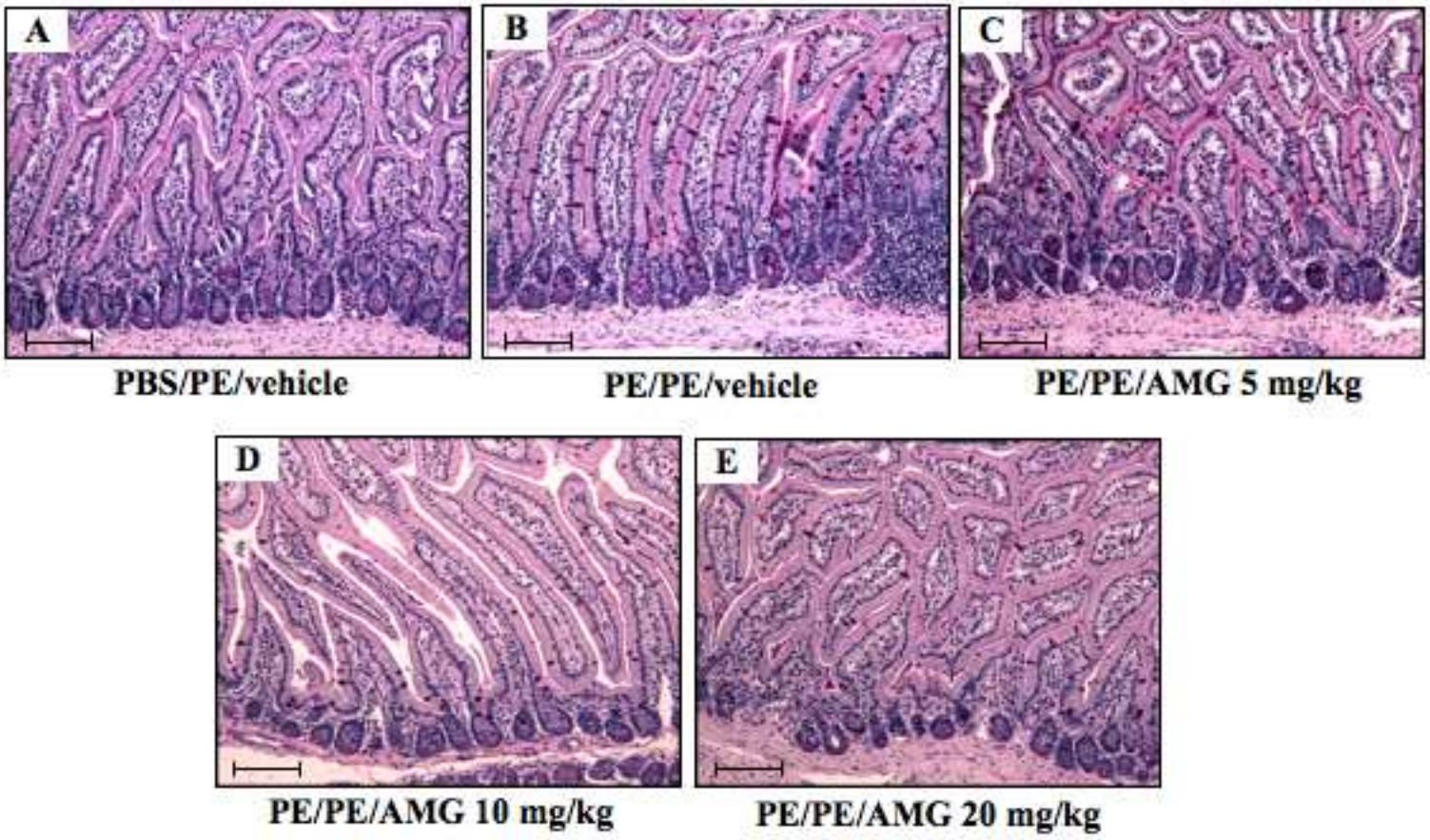
PE/PE/AMG 20 mg/kg

Figure E2


A
B
C
PBS/PE/vehicle
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D
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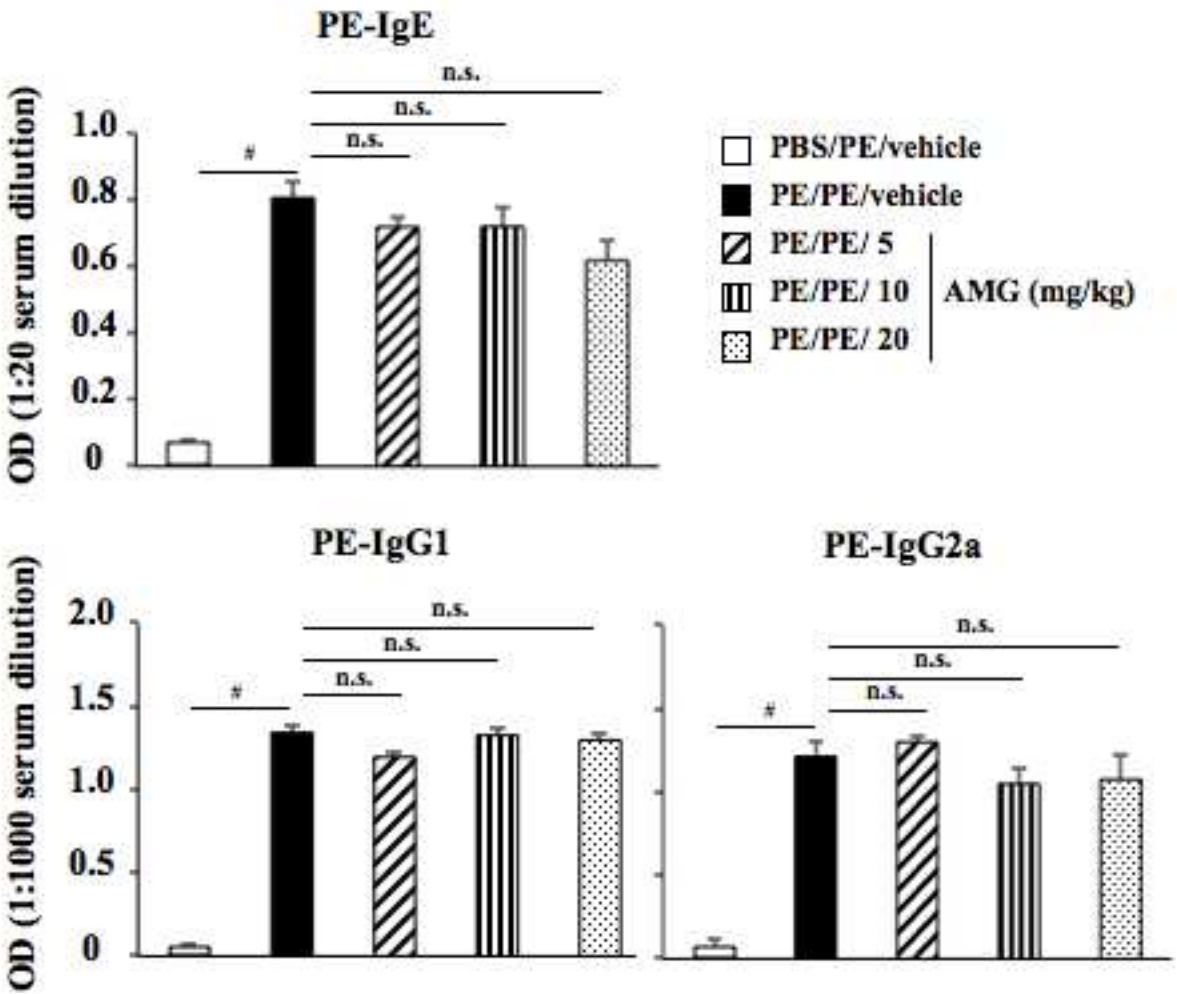
Figure E3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0.6
0.4
0.2
0
#
n.s.
n.s.
n.s.
PBS/PE/vehicle
PE/PE/vehicle
PE/PE/ 5
PE/PE/ 10
PE/PE/ 20
AMG (mg/kg)
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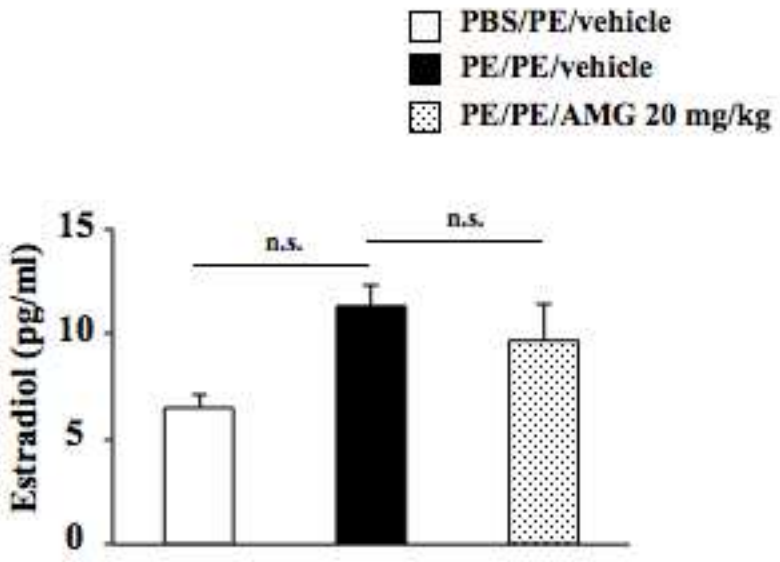
1.0
0.5
0
PE-IgG2a

Figure E4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10
5
0
n.s.
n.s.

Figure E5

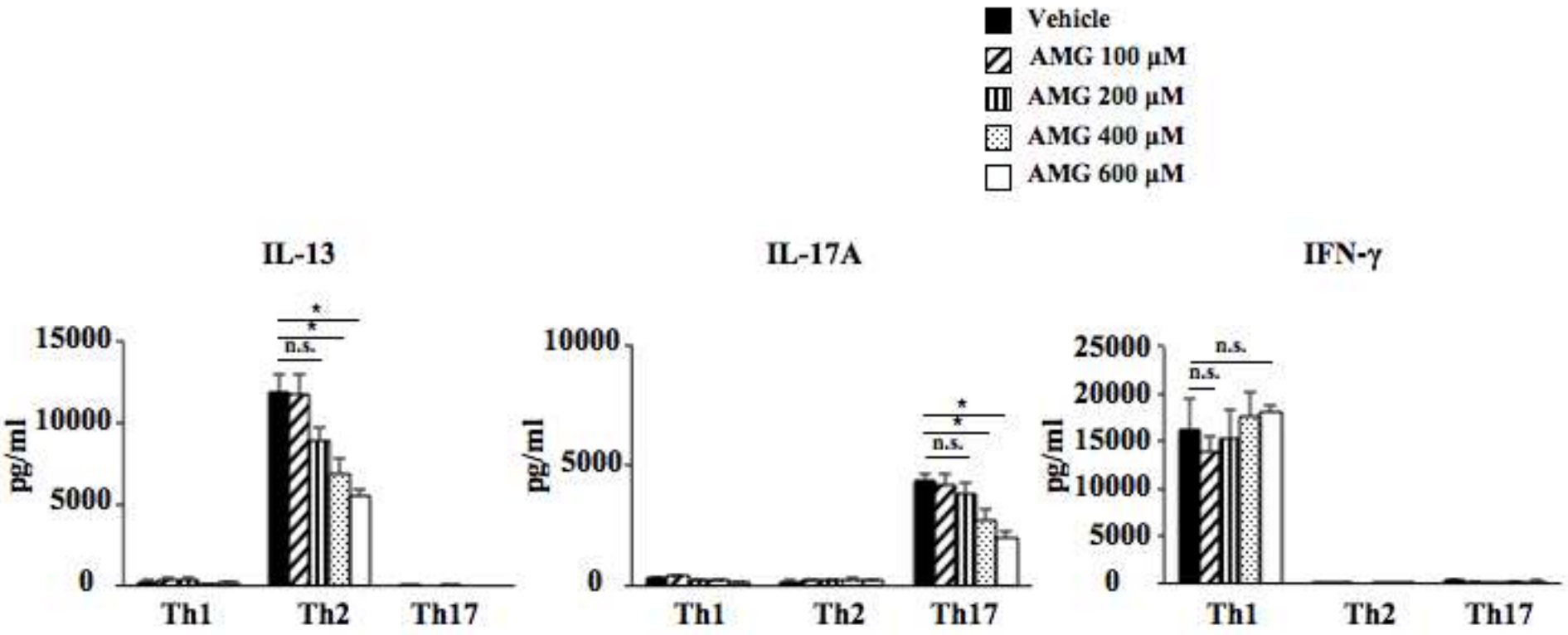
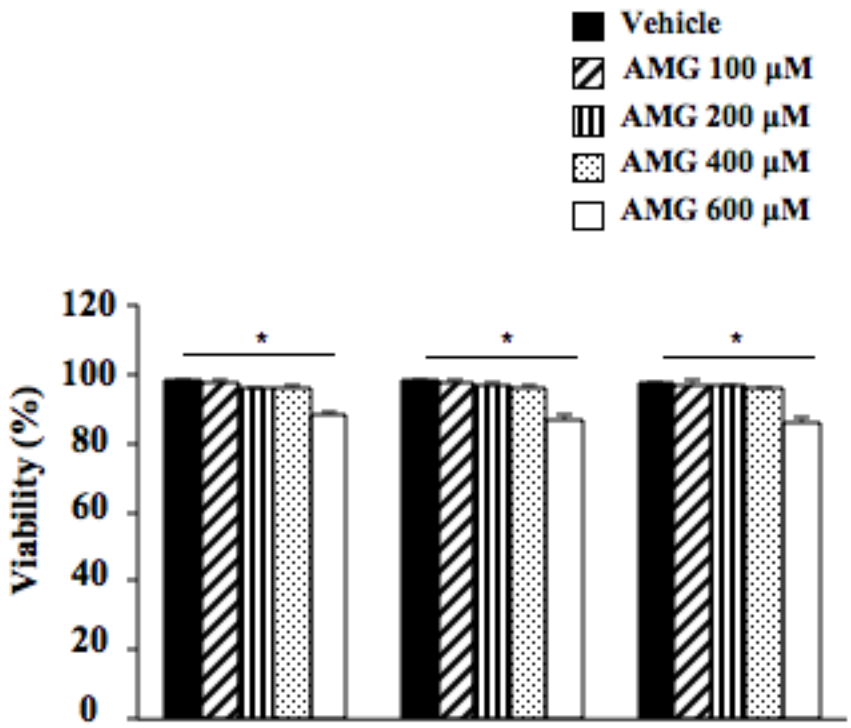

Vehicle
AMG 100 μM
AMG 200 μM
AMG 400 μM
AMG 600 μM
IL-13
IL-17A
IFN-γ
pg/ml
15000
10000
5000
0
25000
20000
n.s.
*
Th1
Th2
Th17

Figure E6


Vehicle
AMG 100 µM
AMG 200 µM
AMG 400 µM
AMG 600 µM
Viability (%)
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100
80
60
40
20
0
*
*
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