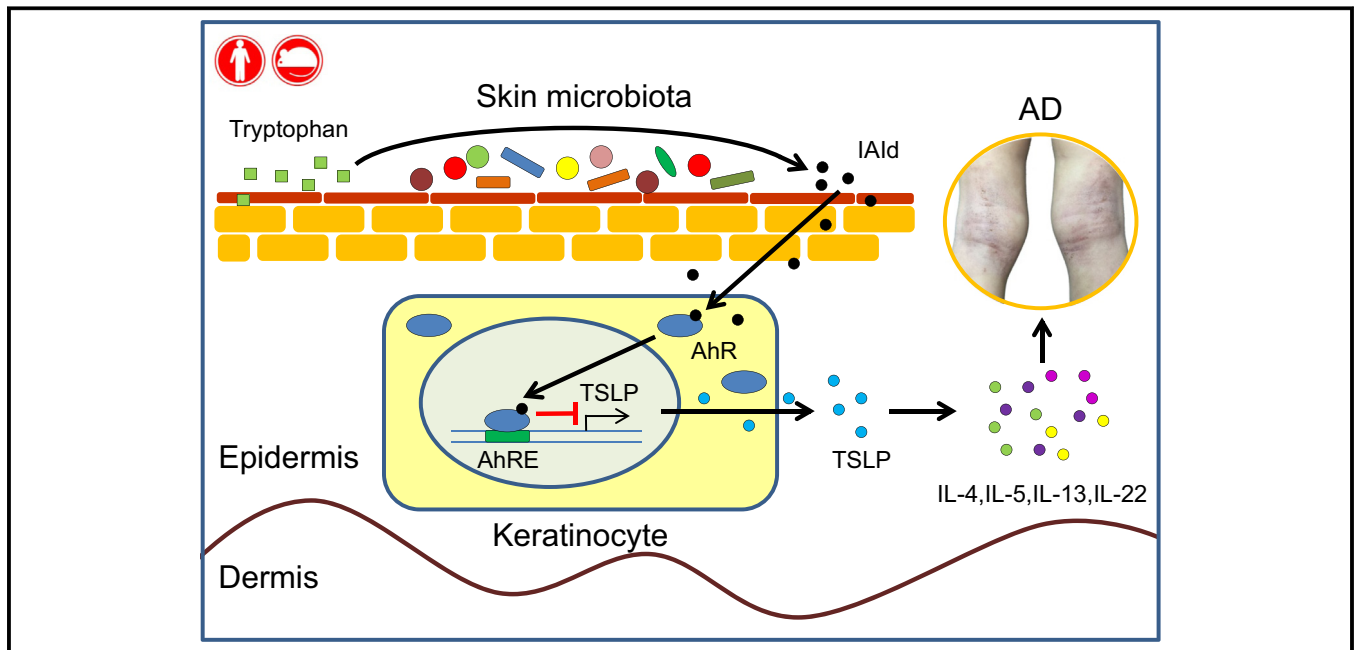


A tryptophan metabolite of the skin microbiota attenuates inflammation in patients with atopic dermatitis through the aryl hydrocarbon receptor

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GRAPHICAL ABSTRACT



Background: Previous studies have revealed significant alterations in the skin microbiota of patients with atopic dermatitis (AD) not only in diversity and composition but also in function, and the tryptophan (Trp) metabolic pathway is attenuated in the skin microbiota of patients with AD. **Objective:** We sought to assess Trp metabolites on the skin surfaces of patients with AD and to explore the function of the microbial Trp metabolites in skin inflammation in patients with AD.

Methods: A gel-patch method was developed to collect metabolites on the skin surface, which were then assessed by using liquid chromatography–tandem mass spectrometry. A mouse model of calcipotriol (MC903)–induced AD-like dermatitis was used to evaluate the effects of microbial metabolites on AD, and aryl hydrocarbon receptor (AhR)–null mice and keratinocyte cultures were used to investigate the mechanism.

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Results: Major microbial metabolites of Trp were detected on the skin surfaces of healthy subjects, and the level of indole-3-aldehyde (IAId), an indole derivative of Trp catabolism, was significantly lower in lesional and nonlesional skin of patients with AD than that of healthy subjects. IAId significantly attenuated skin inflammation in mice with MC903-induced AD-like dermatitis, and this effect was blocked by an AhR antagonist and abolished in AhR-null mice. Furthermore, IAId was found to inhibit the MC903-induced expression of thymic stromal lymphopoietin in keratinocytes *in vivo* and *in vitro*, which was mediated by binding of AhR to the thymic stromal lymphopoietin promoter.

Conclusion: IAId, a skin microbiota-derived Trp metabolite, negatively regulated skin inflammation in patients with AD, revealing that skin microbiota play a significant functional role in the pathogenesis of AD. (J Allergy Clin Immunol 2019;■■■:■■■-■■■.)

Key words: Atopic dermatitis, skin, microbiota, tryptophan, metabolites, indole-3-aldehyde, aryl hydrocarbon receptor, thymic stromal lymphopoietin

Previous studies have revealed significant alterations in the community diversity and structure of the skin microbiota in patients with atopic dermatitis (AD) compared with that in healthy subjects, as well as changes in specific microbes, such as *Staphylococcus aureus*.¹⁻⁴ However, the metabolic function of the skin microbiota in patients with AD is far less recognized, which hinders the comprehensive understanding of the functional importance of the interaction between the skin microbiota and the host in patients with AD. Microbiota-generated metabolites in the gut, such as short-chain fatty acids (SCFAs) and tryptophan (Trp) metabolites, have been demonstrated to play important roles in maturation of immune tissues and fine-tuning of inflammatory diseases, such as inflammatory bowel disease and rheumatoid arthritis.⁵⁻⁹

Compared with the gut, the skin is a desiccated, nutrient-poor, and acidic environment, and the metabolic function of the skin microbiota is very different from that of the gut.^{10,11} However, although the skin surface has a scarcity of complex fibers and carbohydrates that are required for the metabolism of SCFAs, the stratum corneum of the epidermis is enriched in amino acids derived from dead keratinocytes and broken keratin, which might provide abundant substrates for Trp metabolism by the skin microbiota.¹²

The essential amino acid Trp, an energy source for microbiota, is involved in immune modulation through various metabolites, including indole and indole derivatives.^{13,14} Indole mediates the interaction between *Escherichia coli* and mammalian hosts and tightens gut epithelial cell junctions.¹⁵ Indole-3-aldehyde (IAId), a major indole derivative, promotes IL-22 transcription and regulates intestinal inflammation, which is dependent on the aryl hydrocarbon receptor (AhR).¹⁶ Trp metabolites can also function at distant sites and regulate inflammation in the brain.¹⁷ Recently, Chng et al¹⁸ showed that the Trp metabolic pathway was attenuated in the skin microbiota of patients with AD. However, the presence of microbe-derived metabolites of Trp on the skin surface and their regulatory importance in patients with AD is not clear. Activation of AhR in keratinocytes by endogenous or exogenous ligands has been implicated in patients

Abbreviations used

AD:	Atopic dermatitis
AhR:	Aryl hydrocarbon receptor
AhRE:	Aryl hydrocarbon response element
CHS:	Contact hypersensitivity
CYP1A1:	Cytochrome P450 1A1
DMSO:	Dimethyl sulfoxide
FACS:	Fluorescence-activated cell sorting
5-HTP:	5-Hydroxytryptophan
IAA:	Indole-3-acetic acid
IAId:	Indole-3-aldehyde
IMQ:	Imiquimod
KYN:	L-kynurenine
LC-MS/MS:	Liquid chromatography–tandem mass spectrometry
OXA:	Oxazolone
SCFA:	Short-chain fatty acid
siRNA:	Small interfering RNA
Trp:	Tryptophan
TSLP:	Thymic stromal lymphopoietin

with several inflammatory skin disorders, including AD,¹⁹⁻²² and whether the microbial metabolites of Trp from the skin could also activate the AhR of keratinocytes remains to be elucidated.

In the present study, by applying a collecting method developed in house, we detected substantial amounts of the microbial metabolites of Trp on skin surfaces of healthy subjects and found that IAId levels were significantly lower in patients with AD. Furthermore, we found that IAId inhibited MC903 (calcipotriol)–induced AD-like dermatitis through its ability to suppress thymic stromal lymphopoietin (TSLP) expression through an AhR-dependent mechanism in keratinocytes. For the first time, our data revealed the presence of microbial Trp metabolites on the skin surface, which exerted a regulatory function in skin inflammation in patients with AD.

METHODS

Subjects and sample collection

The study included 19 patients with AD and 19 healthy volunteers who were matched in age and sex. Diagnosis of AD was made based on the United Kingdom criteria²³ and the SCORAD index of the patients recruited ranged from 15 to 40. Healthy subjects were defined by a lack of history of visible signs of skin damage that is indicative of AD.

The recruited subjects were instructed not to use body lotion or wash for 24 hours or topical medications for 7 days before sampling. All samples were collected in July 2016. Skin samples of patients with AD were collected from lesions in the antecubital fossae and the adjacent nonlesional forearm. Skin samples of healthy subjects were collected from the antecubital fossae.

Two methods were applied for collection of skin samples. For the gel-patch method, samples were obtained by using a gel that is usually used to pick up keratinous plugs from pilosebaceous openings (WIS, Guangzhou, China). Researchers wore sterile gloves and filled the syringe with gel in its liquid form and then painted 500 μ L of gel evenly onto a 4×4 -cm² skin surface. The gel would dry and form a membrane in a few minutes. The membrane was then peeled off with tweezers 15 minutes later and dissolved in a 15-mL conical tube with 1 mL of sterile dimethyl sulfoxide (DMSO).

For the swab method, a sterile swab moistened in sterile water was rubbed on the subject's skin (4×4 cm²) 4 times and rinsed in a 15-mL conical tube with 1 mL of sterile DMSO. The collected samples were stored at -80°C for liquid chromatography–tandem mass spectrometry (LC-MS/MS) analysis or immediately subjected to bacterial culture.

In another set of experiments, a swab was rubbed on the skin surfaces of healthy subjects and inoculated in lysogeny broth medium that was constantly shaken for 18 hours at 37°C to obtain a uniform bacterial mixture. Then 2 μ L of the mixture was added to the lysogeny broth medium supplemented with or without 0.05% Trp and incubated overnight under the same conditions. All samples were stored at –80°C until analysis.

The study was approved by the Ethics Committee of Xijing Hospital, Fourth Military Medical University, and written informed consent was obtained from the participants or their parents.

LC-MS/MS

The major metabolites of the Trp catabolism pathway were identified by using targeted metabolomics. 3-Indole ethanol (tryptophol), indole-3-lactic acid, indole-3-acetic acid (IAA), 5-hydroxytryptophan (5-HTP), L-tryptophan (Trp), quinolinic acid, L-kynurenine (KYN), and IAId were purchased from the Tokyo Chemical Industry (Tokyo, Japan), and tryptamine, indole-3-pyruvic acid, and serotonin were purchased from Sigma-Aldrich (St Louis, Mo).

All samples were analyzed by using LC-MS/MS. An aliquot of 20 μ L of the sample solution was diluted with 110 μ L of distilled water, and 20 μ L of 10 ng/mL 2-Cl-Phe was added to the diluted sample solution. The resulting samples were vortexed for 30 seconds and incubated at –20°C for 20 minutes. Next, the samples were centrifuged at 4°C (14,000g) for 10 minutes before decanting 110 μ L of the supernatants to the sample vials for subsequent ultraperformance LC-MS/MS analysis. Quantification of targeted metabolites was performed on an Ultimate 3000 system (Thermo Scientific, Waltham, Mass) by using a Hyper-sil GOLD C18 Column (2.1 $\times$ 100 mm², 1.9 μ m) coupled to a Thermo Scientific LTQ XL mass spectrometer (Thermo Scientific). The binary gradient elution system consisted of water (containing 0.1% formic acid, vol/vol) and acetonitrile (containing 0.1% formic acid, vol/vol), and separation was conducted on a C18 column. The flow rate was 0.25 mL/min, and the injection volume was set to 10 μ L. All samples were kept at 4°C during analysis. Mass spectrometric data were collected with a Thermo Scientific LTQ XL mass spectrometer equipped with an electrospray ionization source operating in the positive/negative ion mode. The source temperature was set at 120°C with a cone gas flow of 40 L/h and a desolvation gas temperature of 350°C with a gas flow of 650 L/h. The capillary voltage was set to 4000 V, and the collision energy was optimized for each standard manually. The raw MS data were analyzed by using Xcalibur software (Thermo Scientific).

Mice and treatment

Wild-type C57BL/6 and BALB/c mice were purchased from the Vital River Company (Import Commission Agent of Charles River Laboratories, Beijing, China). AhR-null mice were purchased from the Jackson Laboratory (Bar Harbor, Me). All mice were 8 to 12 weeks old and maintained on a 12-hour light/dark cycle under specific pathogen-free conditions. Animal experiments were performed in compliance with the guidelines outlined in the “Guide for the care and use of laboratory animals” of the university. The MC903-induced AD-like dermatitis was produced by painting 2 nmol of MC903 (calcipotriol; Leo Pharma, Ballerup, Denmark) on mouse ears for 14 consecutive days.²⁴ The imiquimod (IMQ)-induced psoriasis model was prepared by topically applying 5 mg of Aldara cream (containing 5% IMQ; MEDA Pharma, Paris, France) or control cream on mouse ears for 7 consecutive days.²⁰ The oxazolone (OXA)-induced contact hypersensitivity (CHS) was prepared by sensitizing the mice with 100 μ L of 3.0% OXA (dissolved in anhydrous ethanol; Sigma-Aldrich) on the shaved abdomen and challenging them with 10 μ L of 1% OXA or ethanol on ears 5 days later.²⁵ Thirty microliters of IAId, 5-HTP, KYN, or IAA dissolved in DMSO (100 ng/mL; TCI) was painted on mouse ears 30 minutes after MC903, IMQ, or OXA application.²⁶

In experiments assessing the effects of oral IAId, 200 μ L of 10 μ g/mL IAId was fed orally to mice daily for 3 days before MC903, IMQ, or OXA application and then fed throughout the experiment. For the inhibition experiment, 20 μ L of 10 μ mol/L CH223191 (an AhR antagonist; Sigma-Aldrich) was painted on mouse ears 30 minutes before IAId application.²⁶ At the time points indicated, the full thickness of the ears was measured with a

micrometer. Scratching was monitored by counting the number of scratches for a period of 30 minutes. Skin tissue was either snap-frozen for RNA or protein extraction, fixed in formalin for histopathologic analysis, or digested to achieve single-cell suspensions for further fluorescence-activated cell sorting (FACS) analysis. Blood samples were collected for ELISA, and histology was performed in compliance with our previous articles.²⁵

Single-cell suspension preparation and flow cytometry

Mice were killed with CO₂. Skin tissues were cut into pieces and incubated in RPMI-1640 containing collagenase type IV (2 mg/mL; Worthington, Lakewood, NJ), DNase I (1 mg/mL; Sigma-Aldrich), and 2% FCS (HyClone, San Angelo, Tex) for 45 minutes at 37°C. Single-cell suspensions were incubated with anti-CD16/32 antibody to block Fc receptors and subsequently stained with fluorescent antibodies for 30 minutes on ice. Antibodies were all obtained from BioLegend (San Diego, Calif): CD4 (clone GK1.5), CD8 (clone 53-6.7), and Gr1 (clone RB6-8C5).

CountBright absolute counting beads (C36950; Molecular Probes, Life Technologies, Eugene, Ore) were used for calculation of absolute cell numbers. Dead cells were excluded by staining with 7-aminoactinomycin D before analysis. Flow cytometry was performed on the BD FACSCalibur, and data were analyzed with FlowJo 7.6.4 software (TreeStar, Ashland, Ore).

Quantitative real-time RT-PCR

Total RNA was extracted from skin tissues by using TRIzol reagent, according to the manufacturer's protocol (Invitrogen, Life Technologies, Carlsbad, Calif). The PrimeScript RT Reagent Kit (Takara, Beijing, China) was used to synthesize cDNA. mRNA levels were measured with the CFX384 Real Time PCR Detection System (Bio-Rad Laboratories, Hercules, Calif) and SYBR Premix Ex Taq II (Takara). Fold changes were normalized to those of hypoxanthine-guanine phosphoribosyltransferase. All primer sequences are shown in Table E1 in this article's Online Repository at www.jacionline.org.

Western blotting

Total protein was lysed with RIPA buffer (Beyotime, Jiangsu, China). Total protein concentrations in lysates were measured with the BCA Protein Assay Kit (Thermo Scientific, Pierce). Thirty micrograms of protein was loaded onto an SDS-PAGE gel, transferred to a polyvinylidene difluoride membrane, and probed with 1 μ g/mL rat anti-mouse TSLP mAb (clone #152614; R&D Systems, Minneapolis, Minn) or mouse anti-mouse cytochrome P450 1A1 (CYP1A1) mAb (clone b-2; Santa Cruz Biotechnology, Dallas, Tex), followed by goat anti-rat IgG secondary antibody (BioLegend) or goat anti-mouse IgG secondary antibody (BioLegend). The results were analyzed with Photoshop software (Adobe, San Jose, Calif) and normalized to β -actin.

Cell culture and treatment

Primary mouse keratinocytes were cultured, as previously described.²⁷ Cells were seeded in flat-bottom 6-well plates and cultured until they reached 80% to 90% confluence, and medium was replaced with fresh medium. After further cultivation for 24 hours, cells were stimulated with MC903 (100 ng/mL; Sigma-Aldrich)²⁸ and/or different doses of IAId (from 1 ng/mL to 10 μ g/mL) at 37°C in 5% CO₂ and incubated for another 16 hours. Next, the cells were harvested and subjected to Western blotting and quantitative real-time PCR assay.

JB6 cells, a mouse keratinocyte cell line, were purchased from the BeNa culture collection. The cells were cultured in high-glucose DMEM culture medium that contained 10% heat-inactivated FBS. AhR-specific small interfering RNA (siRNA) and control siRNA were transfected into JB6 cells with the Geneporter 2 Transfection Reagent (GTS, San Diego, Calif), according to the standard protocol. Target sequences of AhR for transient silencing were as follows: siRNA-1, 5'-GAGGGAUUAACUUCUAGAU-3'; siRNA-2, 5'-GAGGAACCUUGGGAUUGAU-3'. Control sequences were

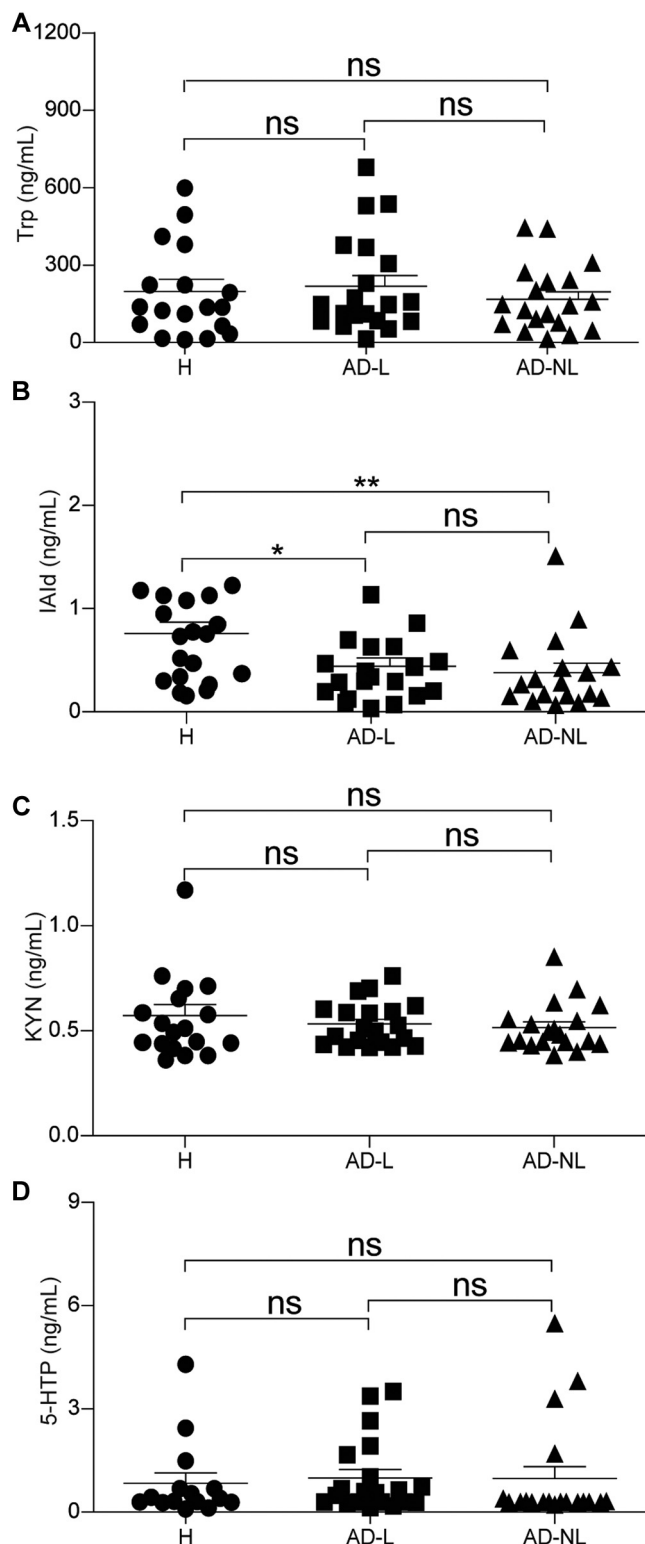


FIG 1. IAld levels were decreased on skin surfaces of patients with AD. Skin surface samples were collected from patients with AD and healthy subjects by using a gel-patch method and subjected to LC-MS/MS analysis. Concentrations of Trp (A) and the representative metabolites of Trp (B-D) in skin samples of healthy volunteers (H), lesional skin of patients with AD (AD-L), or nonlesional skin of patients with AD (AD-NL) are shown. Data are expressed as means $\pm$ SEMs. Significance was analyzed with the Kruskal-Wallis test, followed by the Dunn test ($n = 19$). * $P < .05$ and ** $P < .01$. 5-HTP, 5-Hydroxy-L-tryptophan; IAld, indole-3-carboxaldehyde; ns, not significant.

5'-UUCUCCGAACGUGUCACGU-3'. Transfected cells were treated with MC903 and IAld, as described above.

Chromatin immunoprecipitation assay

The chromatin immunoprecipitation assay was performed by using the SimpleChIP Plus Sonication Chromatin IP Kit (#56383; Cell Signaling Technology, Danvers, Mass), according to the instructions in the manual. Briefly, 1×10^7 JB6 cells were seeded in a 150-mm dish with 20 mL of medium and stimulated with IAld (100 ng/mL) or solvent control for 24 hours. The cells were fixed with 1% formaldehyde. Chromatin was sheared by means of sonication, and the supernatant was collected after centrifugation and diluted. Each sample was incubated with 5 μ g of anti-mouse AhR antibody (BML-SA210; Enzo Life Sciences, Farmingdale, NY) or isotype IgG control for 12 hours, and chromatin and antibody complexes were incubated with G proteins for 5 hours. Finally, after reversing the crosslink, DNA fragments were purified and analyzed by using SYBR Green real-time PCR. Primer sequences are shown in Table E2 in this article's Online Repository at www.jacionline.org.

Statistical analysis

For the human study, data were analyzed with the Kruskal-Wallis test followed by the Dunn test. For other data, unpaired t tests were used to compare 2 groups. Comparisons among multiple groups were performed with ANOVA, followed by the Tukey test. All data were analyzed with GraphPad Prism software (version 7.0; GraphPad Software, La Jolla, Calif). A P value of less than .05 was considered statistically significant.

RESULTS

IAld levels were decreased in lesional and nonlesional skin of patients with AD

The metagenomic study by Chng et al¹⁸ and our unpublished data showed that the Trp metabolic pathway of the skin microbiota was attenuated in patients with AD. Thus we set out to assess the microbial metabolites of Trp on the skin surfaces of patients with AD using targeted metabolomics analyses. First, we developed a gel-patch method to collect metabolites on the skin surfaces of healthy subjects, and the samples were subjected to LC-MS/MS analysis. Both the swab and gel-patch methods uncovered a substantial amount of Trp and Trp metabolites on the skin surface, and the gel-patch method yielded a much higher level of metabolites than the swab method (see Fig E1 in this article's Online Repository at www.jacionline.org). Next, we compared level of Trp metabolites on skin surfaces between patients with AD and healthy subjects. Among the metabolites investigated, IAld levels on the surface of both lesional and nonlesional skin of patients with AD were significantly lower than those of the healthy subjects, as shown in Fig 1. However, there were no differences between patients with AD and healthy control subjects in levels of the other major Trp metabolites, including KYN, 5-HTP, IAA, indole-3-lactic acid, tryptamine, tryptophol, quinolinic acid, serotonin, and indole-3-pyruvic acid (Fig 1 and see Fig E2 in this article's Online Repository at www.jacionline.org). An increased level of IAld was also detected in the culture broth that contained Trp after inoculation with skin swabs from healthy subjects, indicating that the skin microbiota could metabolize Trp and produce IAld (see Fig E3 in this article's Online Repository at www.jacionline.org). These data demonstrated that there were microbial metabolites of Trp on the skin surface. Additionally, levels of IAld but not those of other metabolites were decreased on the skin surfaces of patients with

AD, indicating that IAId might be involved in skin inflammation in patients with AD.

Topical IAId attenuated the MC903-induced AD-like dermatitis in mice

To explore the function of IAId in skin inflammation in patients with AD, we used a mouse model of MC903-induced AD-like dermatitis. IAId was topically painted on mouse ears 30 minutes after application with MC903 for 14 consecutive days. The ear thickness of the mice treated with both MC903 and IAId was significantly lower than that of those treated with MC903 at multiple time points, and there were considerably fewer visible scales and less redness in ears treated with MC903 plus IAId (Fig 2, A and B). Hematoxylin and eosin staining demonstrated decreased epidermal thickness and less inflammatory cell infiltration in the ears of mice in the MC903 plus IAId group (Fig 2, C and D), and the itch score and levels of total serum IgE were also much lower in mice in the MC903 plus IAId group than those in the MC903 group (Fig 2, E and F). However, KYN, 5-HTP, or IAA, the major metabolites of Trp, did not have any effects on the MC903-induced skin inflammation (see Fig E4, A-E, in this article's Online Repository at www.jacionline.org), although previous studies showed that some of the metabolites had inhibitory effects in various inflammatory conditions.¹³⁻¹⁵ The interference of DMSO, the dissolvent of IAId, on the effect of MC903 was excluded because there was no difference between the mice treated with MC903 and those treated with MC903 plus DMSO (see Fig E4, F-J).

We also assessed the effects of IAId on mouse models of IMQ-induced psoriatic dermatitis or OXA-induced CHS. The results showed that consecutive application with topical IAId had no effects on the IMQ-induced psoriatic dermatitis, and topical application with IAId during the sensitization and challenge stage did not affect the skin inflammation of CHS (see Fig E5, A-D, in this article's Online Repository at www.jacionline.org). Oral application with IAId showed similar results (see Fig E5, E-H). Collectively, our data demonstrated that topical application with IAId specifically attenuated the skin inflammation of AD-like dermatitis induced by MC903.

Topical IAId alleviated inflammatory cell infiltration in mice with MC903-induced dermatitis

Next, we explored the effects of topical application with IAId on inflammatory cell infiltration in MC903-induced AD-like dermatitis. Ear tissues of the lesional skin of mice were digested by using collagenase IV, and a single-cell suspension was prepared, stained with antibodies, and subjected to FACS analysis. CountBright absolute counting beads were used for calculation of absolute cell numbers, and the gating approaches are shown in Fig E6 in this article's Online Repository at www.jacionline.org. The results showed that there were considerably fewer CD4⁺ T cells or Gr1⁺ cells in the lesional skin of mice in the MC903 plus IAId group than those in the MC903 group (Fig 3, A and B). There was no difference in the number of CD8⁺ T cells between the 2 groups (Fig 3, A and B). Quantitative real-time PCR results demonstrated that expression of the T_H2 cytokine transcripts (IL-4, IL-5, IL-6, IL-13, and TSLP) and IL-22 was significantly lower in the MC903 plus IAId group than that in the MC903 group (Fig 3, C). Decreased expression of

TSLP was further confirmed by using Western blotting analysis (Fig 3, D). The expression of IL-17A, IL-23p19, IL-23p40, or INF- γ did not show a difference between the MC903 plus IAId group and MC903 groups (see Fig E7 in this article's Online Repository at www.jacionline.org). These data demonstrated that topical application with IAId inhibited infiltration of inflammatory cells and expression of T_H2 cytokines and IL-22 in skin inflammation induced by MC903.

AhR was required for the inhibitory effects of IAId

Next, we explored whether AhR, a receptor for indole derivatives, mediated the effects of IAId in MC903-induced dermatitis. CH223191, a known AhR antagonist, was painted onto the mouse ears 30 minutes before application of IAId. Results showed that application of CH223191 significantly abrogated the inhibitory effects of IAId on several parameters of MC903-induced inflammation. The redness, scales, and ear swelling were significantly more severe in mice treated with CH223191 plus IAId and MC903 than those in mice treated with IAId and MC903 (Fig 4, A and B). Mice treated with CH223191 plus IAId and MC903 also showed more frequent scratching, a higher level of total serum IgE, and increased expression of TSLP in skin lesions compared with mice treated with IAId and MC903 (Fig 4, C-E). Similar results were found in mice orally administrated CH223191 (see Fig E8 in this article's Online Repository at www.jacionline.org).

AhR-null mice were used and the effect of IAId was assessed to further confirm the dependence of the effect of IAId on AhR. Although similar to that in wild-type mice, MC903 application induced apparent inflammation in the ears of AhR-null mice (Fig 4, F-J), and there was no difference in ear thickness, scratching counts, IgE levels, or production of T_H2 cytokines (TSLP, IL-4, IL-5, and IL-13) in lesional skin between AhR-null mice treated with MC903 and those treated with MC903 plus IAId (Fig 4, F-J, and see Fig E9 in this article's Online Repository at www.jacionline.org). These results indicated that the absence of AhR abolished the IAId-mediated attenuation of skin inflammation in mice with MC903-induced dermatitis. Collectively, our data revealed that IAId exerted its effects through activation of AhR in regulation of MC903-induced dermatitis.

IAId inhibited TSLP production in keratinocytes through activation of AhR

TSLP is highly expressed in keratinocytes of the lesional skin of patients with AD²⁹ and is an important cytokine of T_H2 inflammation in MC903-induced AD-like dermatitis, which initiates aberrant immune responses. Because application with IAId almost completely abolished the inflammation of MC903-induced dermatitis and significantly decreased TSLP levels, we hypothesized that IAId might regulate TSLP production in keratinocytes. We cultured primary keratinocytes from the skin of neonatal mice and first tested the toxic effects of IAId on keratinocytes in culture. We found that any concentration of less than 10 μ g/mL did not affect keratinocyte growth (data not shown). We then found that supplementation with MC903 resulted in increased production of TSLP in primary keratinocytes at both the mRNA and protein levels, which was abrogated when IAId was added at an appropriate concentration (Fig 5, A and C). When AhR

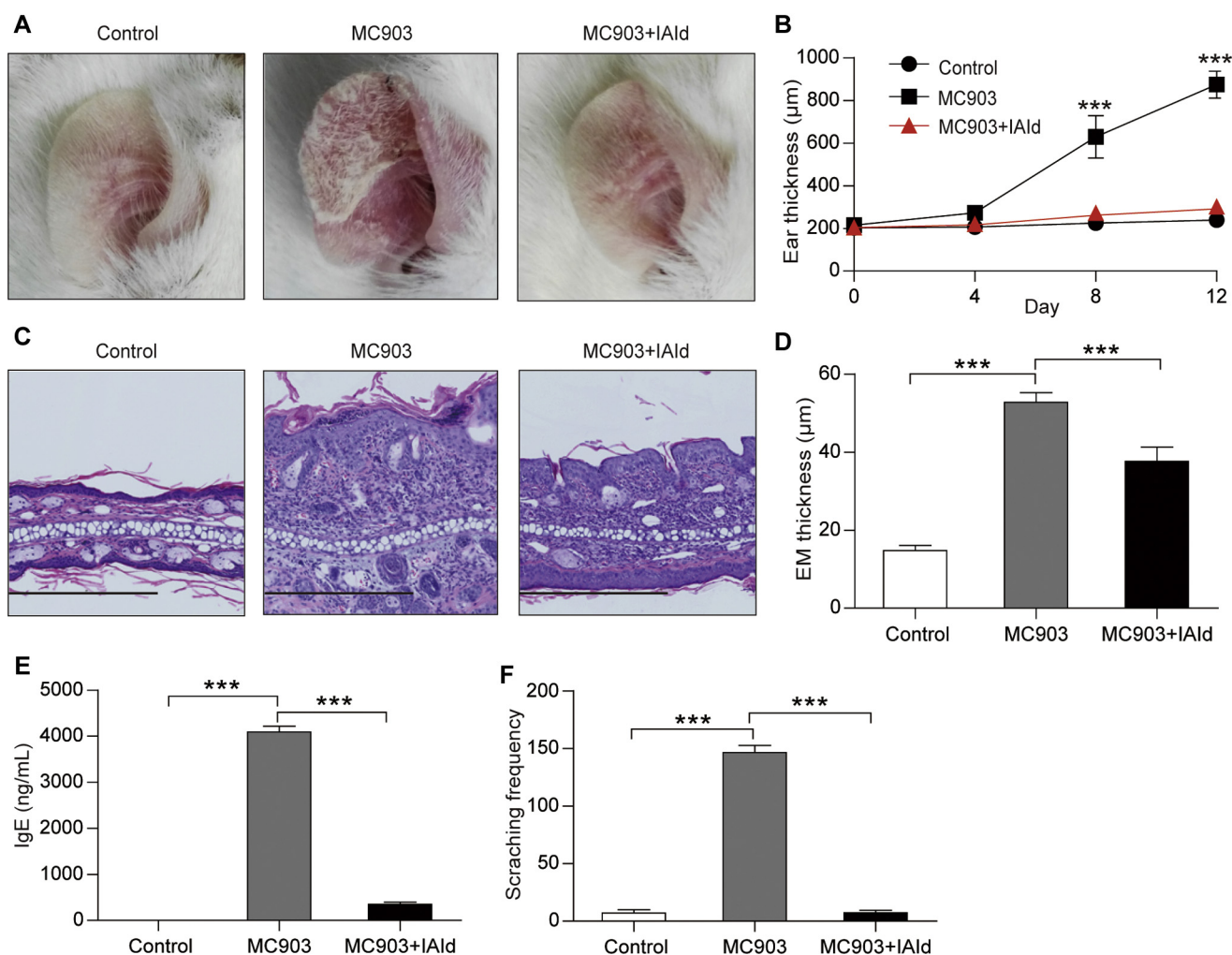


FIG 2. Topical IAId attenuated MC903-induced AD-like dermatitis of mice. BALB/c mice had MC903 or MC903 plus IAId painted onto their ears for 14 days. **A**, Gross appearance of ears at day 14. **B**, Dynamic changes in ear thickness at the indicated time points. **C**, Hematoxylin and eosin staining of ear sections at day 14. Original magnification $\times 100$. Scale bar = 200 μm . **D**, Thickness of the epidermis (EM) in ear sections under high-power magnification. **E**, ELISA results showing concentrations of total serum IgE. **F**, Scratching counts on day 14 over a 30-minute duration. Data are representative of 3 independent experiments and expressed as means $\pm$ SEMs. Significance was analyzed with ANOVA, followed by the Tukey test ($n = 3-5$). *** $P < .001$.

expression was silenced in the mouse keratinocyte cell line JB6 by using AhR-specific siRNA, the increased production of TSLP on MC903 treatment was attenuated (see Fig E10 in this article's Online Repository at www.jacionline.org). Furthermore, treatment of keratinocytes with IAId significantly induced expression of CYP1A1, an AhR-targeting gene, at both the mRNA and protein levels in a dose-dependent manner; 1 $\mu\text{g/mL}$ of IAId exhibited the most significant effect (Fig 5, B and C). Silencing of AhR in JB6 cells attenuated the IAId-induced expression of CYP1A1 (see Fig E10). These data suggested that IAId could activate the AhR signaling pathway and inhibit TSLP production.

Based on bioinformatics analysis, we found that there were 4 aryl hydrocarbon response elements (AhREs) in the promoter region of the *TSLP* gene, as shown in Fig 5, D, and Table E2. A chromatin immunoprecipitation assay was then conducted to assess binding of AhR to the specific sequence of the TSLP

promoter in JB6 cells. The results showed that on IAId treatment, there was apparent binding of AhR to both AhRE2 and AhRE4 in the promoter region of the *TSLP* gene (Fig 5, E). Collectively, our data revealed that after activation with IAId, AhR bound to the promoter region of TSLP and inhibited its expression in keratinocytes.

Oral IAId alleviated skin inflammation in mice with MC903-induced AD-like dermatitis

We also explored the effects of oral IAId on MC903-induced AD-like dermatitis. Mice were orally administered IAId along with topical application of MC903. The results showed that the mice that received oral IAId showed considerably milder ear inflammation than the mice that received vehicle controls, as evidenced by morphology, ear thickness, and total IgE level (Fig 6, A-D). Additionally, quantitative real-time PCR results

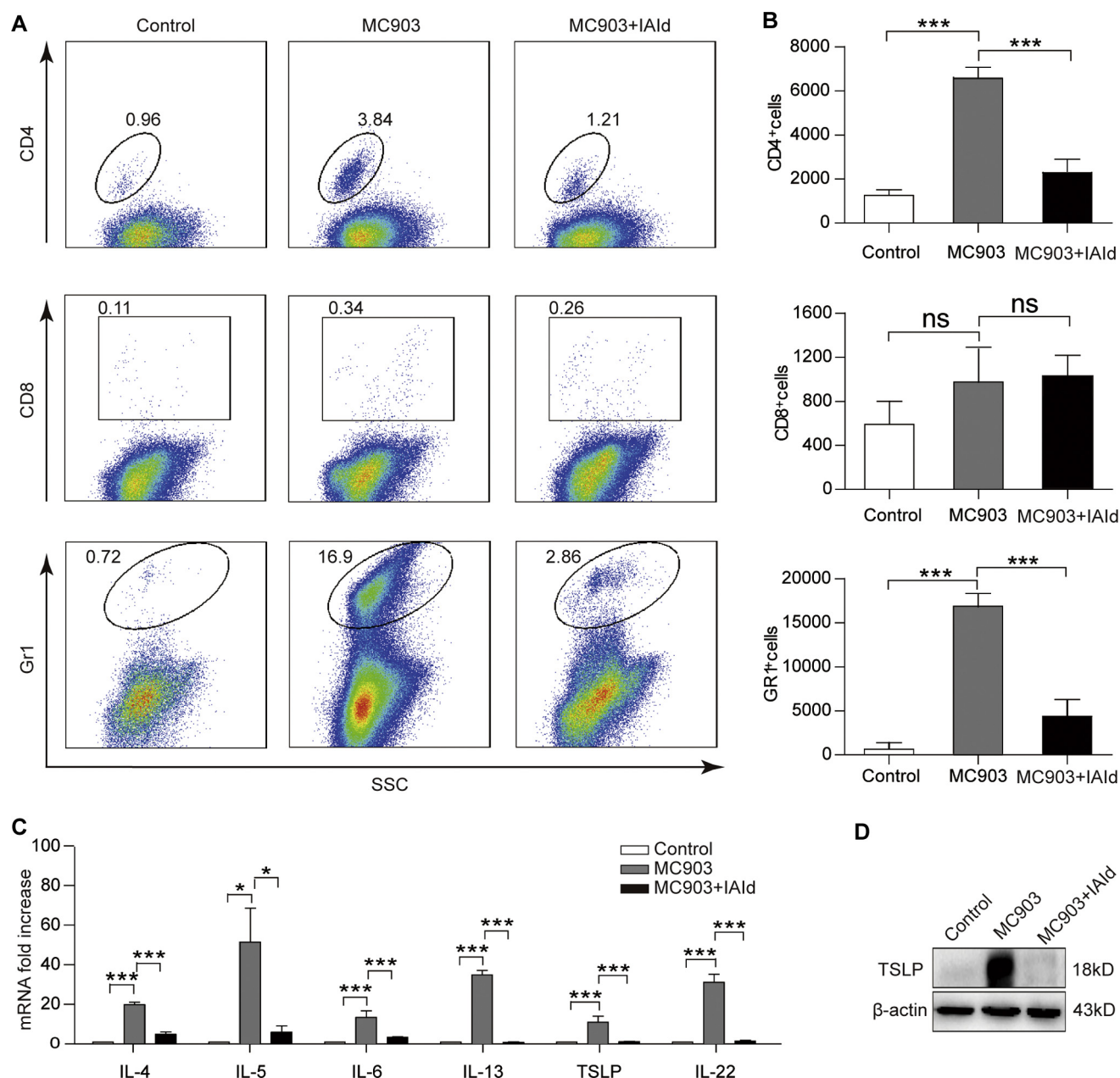


FIG 3. Topical IAId alleviated inflammatory cell infiltration in mice with MC903-induced dermatitis. **A**, Single-cell suspensions of the ears were prepared, stained with antibodies, and analyzed through FACS. SSC, Side scatter. **B**, Absolute numbers of indicated cell populations. **C**, Quantitative real-time results showing levels of mRNA expression of various cytokines in the ears on day 14. **D**, Western blotting result showing expression of TSLP protein. Data are representative of 3 independent experiments and expressed as means $\pm$ SEMs. Significance was analyzed with ANOVA, followed by the Tukey test ($n = 3-5$). * $P < .05$, ** $P < .01$, and *** $P < .001$. ns, Not significant.

showed decreased expression of IL-6, IL-13, and TSLP in IAId-treated mice (Fig 6, E-I). These data demonstrated that the oral route for IAId administration also alleviated skin inflammation in mice with MC903-induced AD-like dermatitis.

DISCUSSION

Interactions between the microbiota and the host are mediated mainly through 2 arms, microbial surface antigens and microbial

metabolites,^{6,30} the latter of which have been attracting extensive interest during the past several years. Studies have revealed important roles of gut microbial metabolites under both physiologic and pathologic conditions^{6,31,32}; however, the metabolites of the skin microbiota are far less recognized. AD is the most well-studied skin disease among studies on skin microbiota, with an emphasis placed mostly on the structure and diversity of the microbial community.¹⁻³ A previous study indicated that the Trp metabolism pathway is attenuated in the skin microbiome

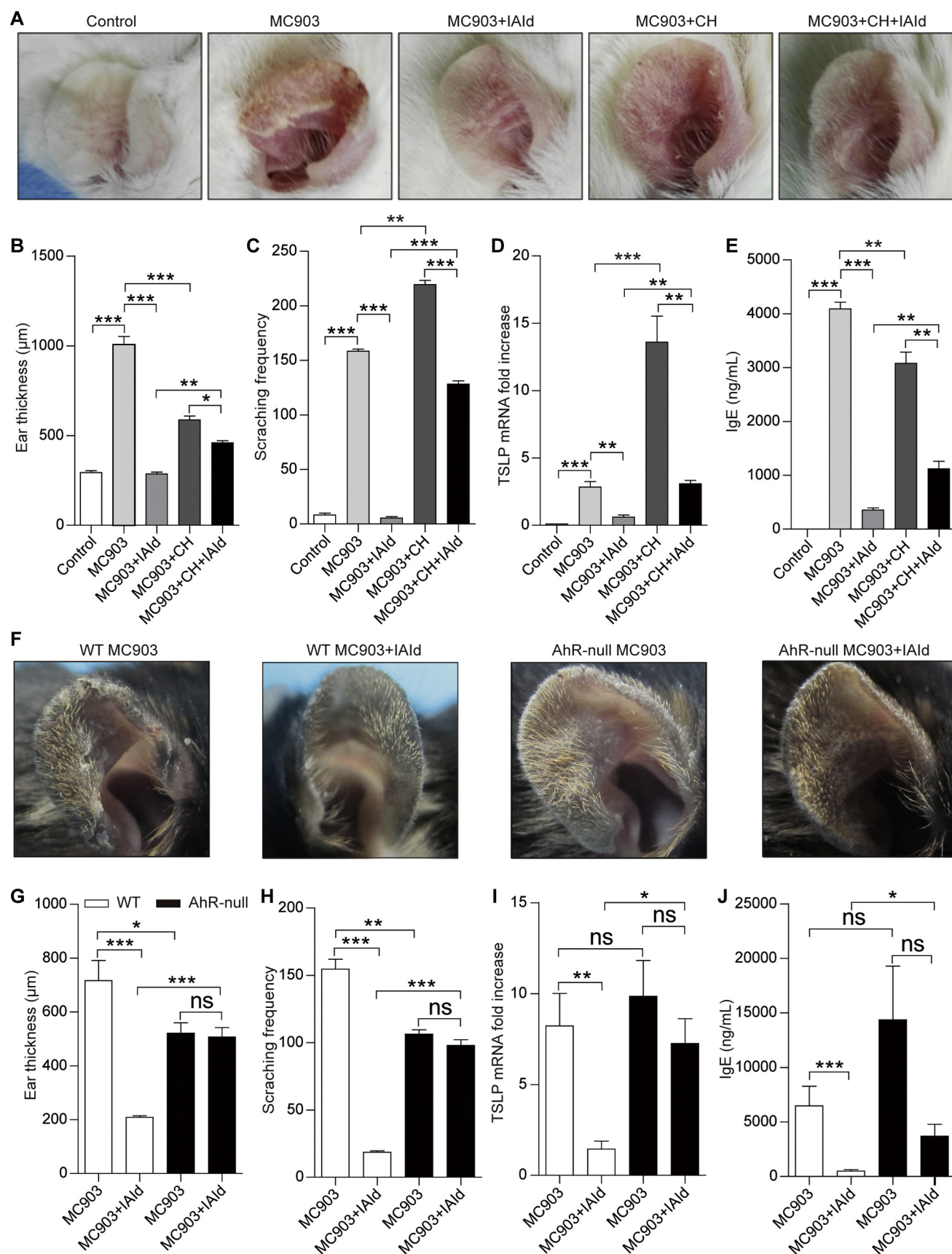


FIG 4. AhR was required for the inhibitory effects of IAld. **A-E**, CH223191 was applied to the ears of mice along with MC903, IAld, or both for 14 days. Gross appearance of the ears (Fig 4, A), ear thickness (Fig 4, B), scratching frequency (Fig 4, C), TSLP expression (Fig 4, D), and total serum IgE level (Fig 4, E) are shown.

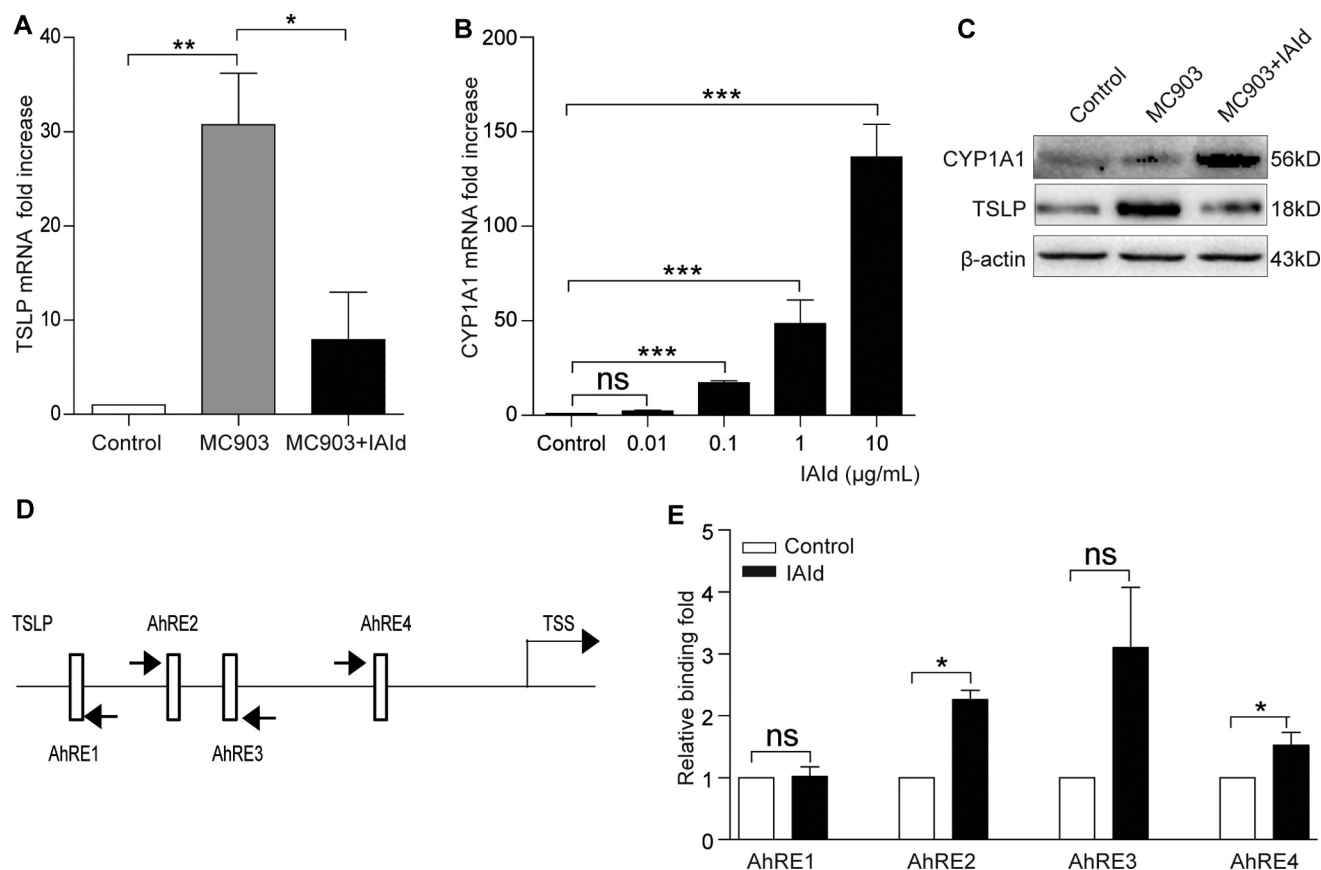


FIG 5. IAld inhibited production of TSLP in keratinocytes through AhR activation. **A**, Primary mouse keratinocytes in culture were treated with MC903, IAld, or both, and expression of *TSLP* mRNA was assessed by using quantitative real-time PCR. **B**, Primary mouse keratinocytes in culture were treated with different concentrations of IAld, and *CYP1A1* mRNA expression was assessed by using quantitative real-time PCR. **C**, Western blotting showing expression of CYP1A1 and TSLP proteins in keratinocytes after treatment with MC903, IAld, or both. **D**, Schematic showing the 4 AhREs in the promoter region of the *TSLP* gene. **E**, JB6 cells were treated with IAld in culture and subjected to the chromatin immunoprecipitation assay to assess binding of AhR to the promoter region of *TSLP*. Data are representative of at least 3 independent experiments and expressed as means $\pm$ SEMs. Significance was analyzed with ANOVA, followed by the Tukey test (Fig 5, A and B) or unpaired *t* tests (Fig 5, E; *n* = 3). **P* < .05, ***P* < .01, and ****P* < .001. ns, Not significant.

of patients with AD.¹⁸ In the present study we detected substantial amounts of microbiota-derived Trp metabolites on the skin surfaces of healthy subjects and patients with AD, and the level of IAld was significantly lower in patients with AD than in healthy control subjects. Interestingly, topical application of IAld alleviated skin inflammation in a mouse model of AD-like dermatitis, in part through its ability to inhibit production of TSLP in keratinocytes in an AhR-dependent fashion. To our knowledge, this is the first study to characterize the microbial metabolites of the skin, revealing that Trp metabolites that are derived from the skin microbiota play a regulatory function in skin inflammation.

In contrast to the humid nutritious environment of the gut, the skin surface is a desiccated and nutrient-poor location^{10,11}; however, there are plenty of amino acids, including Trp, on the skin surface (Fig 1 and see Fig E1) that may be mainly derived from the stratum corneum.¹² Accordingly, a substantial amount of

the microbial metabolites of Trp was detected on the skin surface (Fig 1 and see Figs E1 and E2). Levels of Trp metabolites on the skin surface appeared lower than those in the gut¹⁶; however, considering the dry environment of the skin and the low efficiency of the collection method, the amount of metabolites in contact with keratinocytes should be much greater and comparable with that in the gut. Indole and indole derivatives are mainly catabolized by the microbiota, and 5-HTP and KYN are produced by both the microbiota and the host cells.^{13,14,16} Thus the decreased level of IAld, but not 5-HTP and KYN, on the skin surfaces of patients with AD indicates that Trp metabolism by the skin microbiota is attenuated, which is consistent with the metagenomics results obtained by Chng et al¹⁸ and with our unpublished data.

The success of the detection of metabolites on the skin surface is mainly due to the use of our newly developed gel-patch method. The gel dissolved lipids and metabolites when in its liquid form,

F-J, Ears of AhR-null mice were topically painted with MC903, IAld, or both for 14 days. Gross appearance of the ears (Fig 4, F), ear thickness (Fig 4, G), scratching frequency (Fig 4, H), TSLP expression (Fig 4, I), and total serum IgE level (Fig 4, J) are shown. Data are representative of 3 independent experiments and expressed as means $\pm$ SEMs. Significance was analyzed with ANOVA, followed by the Tukey test (Fig 4, B-E) or unpaired *t* tests (Fig 4, G-J) (*n* = 3-5). **P* < .05, ***P* < .01, and ****P* < .001. ns, Not significant.

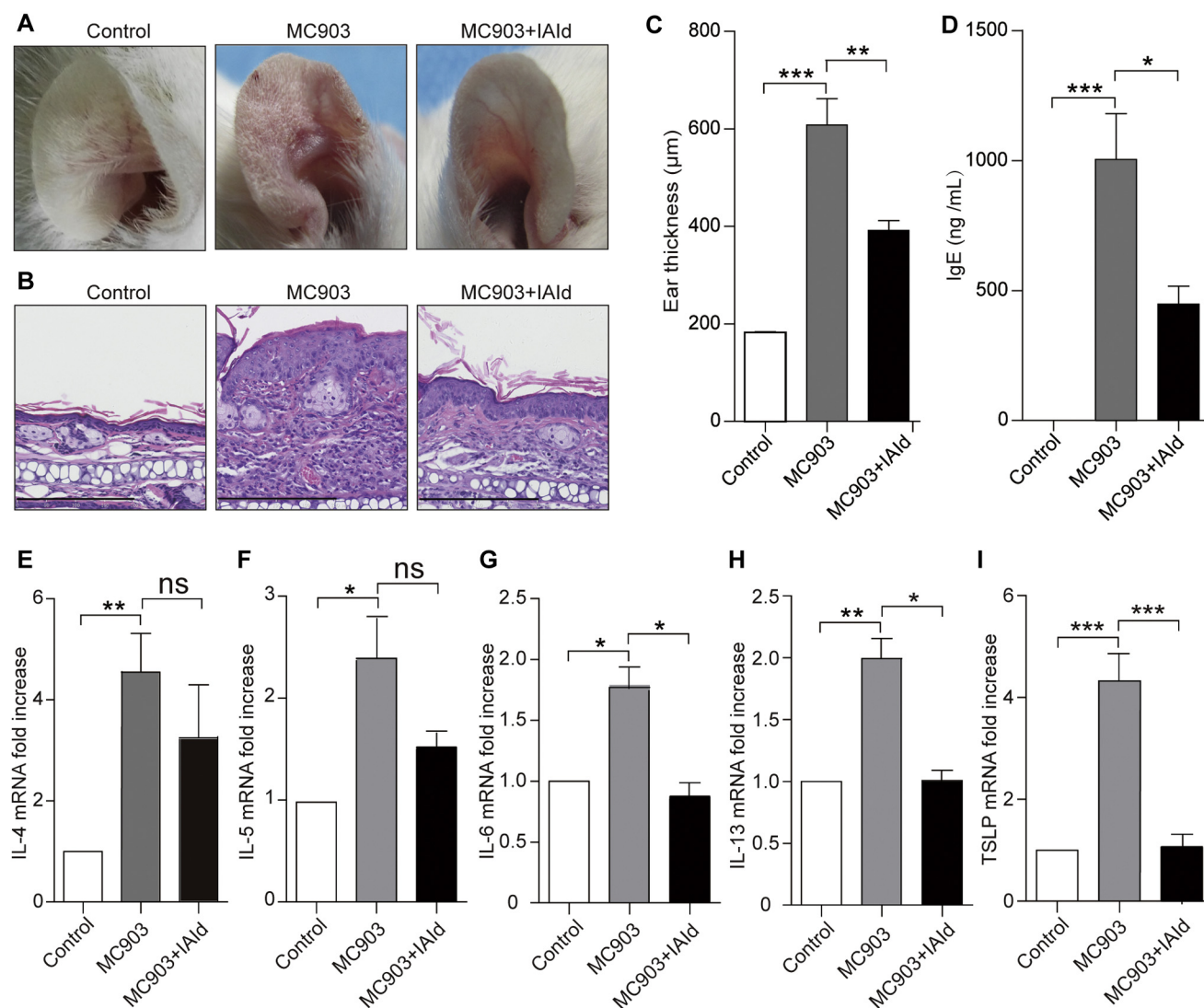


FIG 6. Oral IAld alleviated skin inflammation in mice with MC903-induced AD-like dermatitis. Mice were orally administered IAld along with MC903 skin application. Gross appearance (**A**), hematoxylin and eosin staining (**B**), ear thickness (**C**), total serum IgE levels (**D**), and mRNA levels of various cytokines (**E-I**) of the treated mice are shown. Data are representative of 3 independent experiments and expressed as means $\pm$ SEMs. Significance was analyzed with ANOVA, followed by the Tukey test ($n = 3-5$). * $P < .05$, ** $P < .01$, and *** $P < .001$. ns, Not significant.

and it removed the stratum corneum of the epidermis and a part of the keratinous plug when concreting. Thus this method was able to collect a considerably higher amount of metabolites than the swab method (see Fig E1).

Compared with the other metabolites investigated, only IAld was significantly decreased on the skin surfaces of patients with AD. In addition to the microbiota-dependent Trp metabolism pathway and the limitation of collection efficiency that were discussed above, the possible reason for the exclusive decrease in IAld levels also includes the specific attenuation in IAld metabolism. We are now pursuing studies on the metagenome of the skin microbiota of patients with AD to explore the function of the enzyme for the metabolism of IAld. In the present study we focused only on Trp metabolism because there is no fiber on the skin surface that is needed for the metabolism of SCFAs. However, we cannot exclude the possibility that there are

metabolites other than the Trp metabolic pathway, such as SCFAs, long-chain fatty acids, bile acid, polysaccharides, amino acid, and vitamins. In the future a global assessment of metabolites on the skin surfaces of healthy subjects and patients with AD should be carried out.

An increasing body of evidence has revealed the important role of microbial metabolites of Trp in fine-tuning host defense and microbiota homeostasis.³³⁻³⁶ For example, IAld from the gut microbiota balances mucosal reactivity through IL-22 and provides colonization resistance to *Candida albicans*¹⁶; the microbial metabolites of Trp can modulate astrocyte activity and central nervous system inflammation.¹⁷ Oral poly- γ -glutamate, which is naturally produced by a few gram-positive bacteria, including *Staphylococcus* and *Bacillus* species, ameliorates AD-like dermatitis in Nc/Nga mice by suppressing the T_H2-biased immune response.³⁷ In the present study we demonstrated that IAld

from the skin microbiota played a regulatory role in controlling skin inflammation in the setting of AD-like dermatitis (Figs 2 and 3), revealing the function of the skin microbial metabolites of Trp for the first time. IAId is an important indole derivative of Trp catabolism and has been reported to be produced by *Lactobacillus reuteri* in the stomach.¹⁶ Our unpublished data, which were obtained from analyzing the 16S ribosomal RNA sequence of the skin microbiota, showed that the abundance of the genus *Lactobacillus* was decreased in the skin microbiota of patients with AD, which was consistent with decreased levels of IAId on the skin surfaces of patients with AD. In a recent report Rosas-Salazar et al³⁸ demonstrate that nasopharyngeal *Lactobacillus* species is associated with a reduced risk of childhood wheezing illness after acute respiratory syncytial virus infection in infancy. Lactobacilli have also been implicated in many probiotics, which show preventive effects on atopic disorders.⁴ Lactobacilli are the major components of the vaginal microflora, and birth by means of cesarean section instead of through the vagina has been reported to be related with increased risk of AD.^{39,40} Whether Trp metabolism is involved in the beneficial effects of lactobacilli in allergic patients remains to be explored further. We cultured skin swabs in culture medium containing Trp and found that IAId levels were significantly increased; however, it remains to be explored which bacteria are the major source of IAId. AhR has been shown to be involved in homeostasis of the skin under both physiologic and pathologic conditions. Binding of AhR by external and/or internal ligands initiates translocation of AhR to the promoter region of the target gene and induces various effects in a dose- and cell type-dependent manner. An endogenous ligand of AhR, 6-formylindolo(3,2-b)carbazole, mediates UV-induced photooxidative stress¹⁹; 6-formylindolo(3,2-b)carbazole also inhibits skin inflammation in patients with IMQ-induced psoriatic dermatitis.²⁰ Exogenous ligands of AhR, such as coal tar or polycyclic aromatic hydrocarbons from air pollution, promote the recovery of skin barrier dysfunction in patients with AD²¹ or promote the skin inflammation of AD,²² respectively. Our findings revealed for the first time that microbial metabolites on the skin surface could also activate AhR in keratinocytes and regulate skin inflammation. Specifically, we found that IAId, the Trp metabolite of the skin microbiota, activated AhR and attenuated AD-like dermatitis by inhibiting TSLP production in keratinocytes (Fig 4). We further uncovered that AhR could bind to the promoter region of TSLP on stimulation with IAId (Fig 5); this revealed a novel mechanism of AhR in the pathogenesis of AD. The inhibitory effect of IAId on TSLP production could explain the specific IAId-mediated attenuation of MC903-induced AD-like dermatitis but not IMQ-induced psoriatic dermatitis or OXA-induced CHS (see Fig E5). Thus the skin commensal-derived IAId can maintain the immune homeostasis of the skin by regulating the expression of TSLP in healthy subjects; however, under the conditions of AD-induced skin inflammation, the decreased IAId levels associated with the aberrant skin microbiota can lose control of TSLP expression, which might subsequently promote skin inflammation in patients with AD.

Our data suggest that metabolites produced by the skin microbiota were able to regulate skin homeostasis, providing an experimental basis for developing a safer and more effective strategies for the treatment of AD. The strategies for the treatment of AD using microbial metabolites can include the following: development of topical or systemic drugs from the metabolites, supplementation with specific microbes that produce “good”

metabolites, and modulation of the skin microbiota for promotion of production of specific metabolites. Future work should pay more attention to the metabolism of the skin microbiota, which would accelerate the translational study in the field of skin microbiota.

We thank Professor Shau-Ku Huang for helpful suggestions and reviewing the manuscript.

Key messages

- Levels of microbial metabolites of Trp metabolism on skin surfaces of patients with AD were lower than those of healthy subjects.
- IAId significantly attenuated skin inflammation in a mouse model of MC903-induced AD-like dermatitis.
- IAId inhibited TSLP expression in keratinocytes in an AhR-dependent manner.

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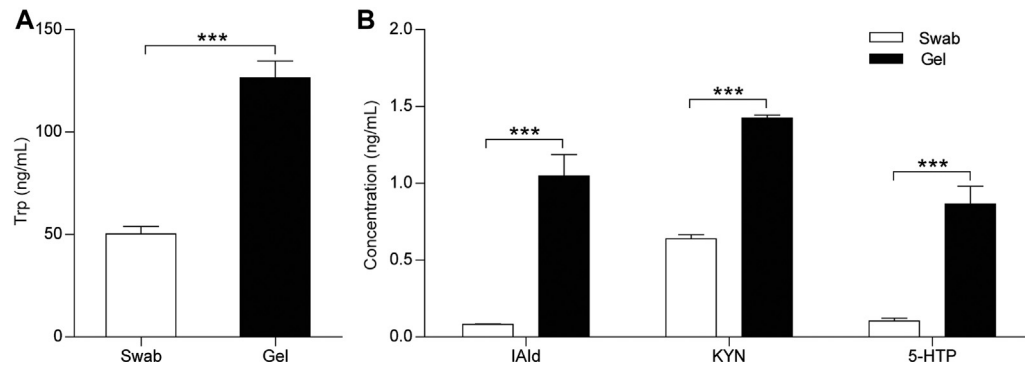


FIG E1. Comparison of the 2 methods for collection of Trp metabolites on the skin surface. Skin surface samples were collected from healthy subjects by using the swab or gel-patch method and subjected to LC-MS/MS analysis. **A**, Trp. **B**, IAld, KYN, and 5-HTP. Data are representative of 3 independent experiments and expressed as means $\pm$ SEMs. Significance was analyzed with unpaired *t* tests ($n = 3$). *** $P < .001$. 5-HTP, 5-Hydroxy-L-tryptophan; IAld, indole-3-carboxaldehyde.

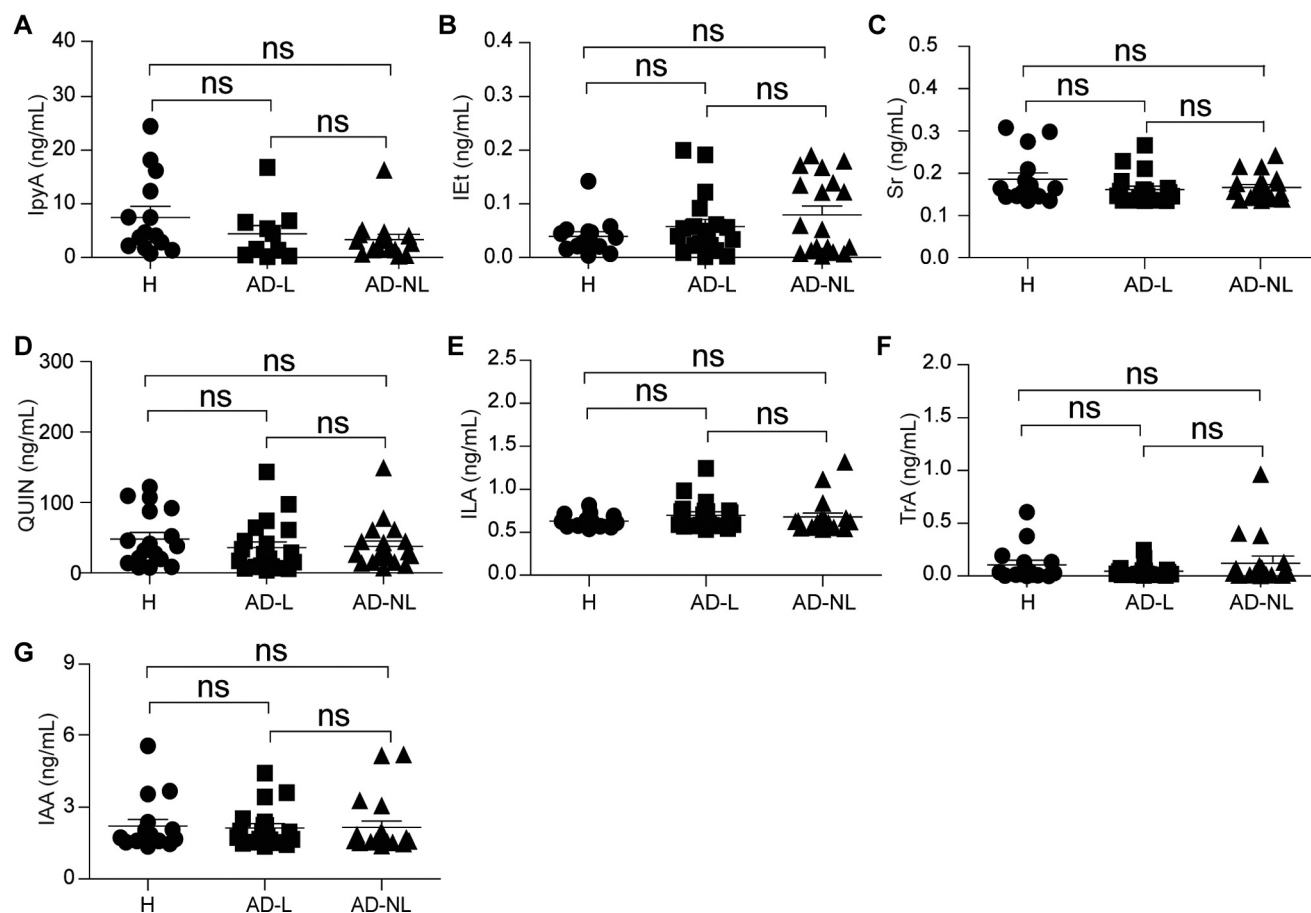


FIG E2. Levels of the major metabolites of Trp on the skin surface. Skin surface samples were collected from patients with AD and healthy subjects by using gel-patch methods and subjected to LC-MS/MS analysis. Concentrations of the major metabolites of Trp in skin samples of healthy volunteers (*H*), lesional skin of patients with AD (*AD-L*), or nonlesional skin of patients with AD (*AD-NL*) were shown. Data are expressed as means $\pm$ SEMs. Significance was analyzed with the Kruskal-Wallis test, followed by the Dunn test ($n = 19$). *IEt*, 3-Indole ethanol; *ILA*, indole-3-lactic acid; *IpyA*, Indole-3-pyruvic acid; *QUIN*, quinolinic acid; *Sr*, serotonin; *TrA*, tryptamine.

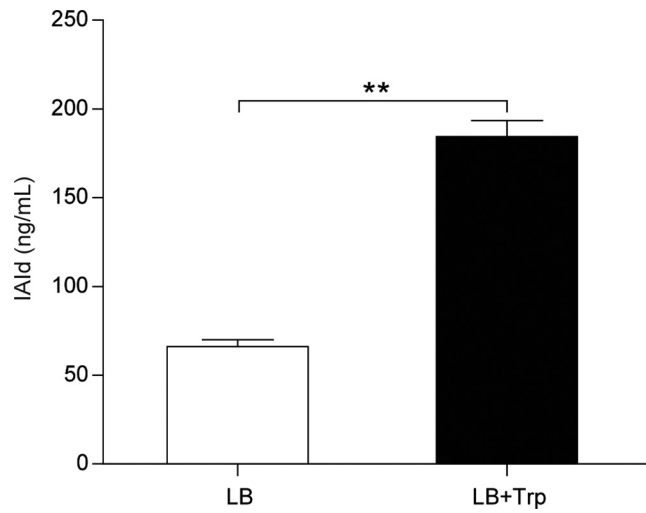


FIG E3. Concentration of IAld in culture broth. LB medium was inoculated with the skin swab in the presence or not of 0.05% Trp, and supernatants were analyzed by using LC-MS/MS. Data are representative of 3 independent experiments and expressed as means $\pm$ SEMs. Significance was analyzed with unpaired *t* tests (*n* = 3). ***P* < .01. IAld, Indole-3-carboxaldehyde; LB, lysogeny broth.

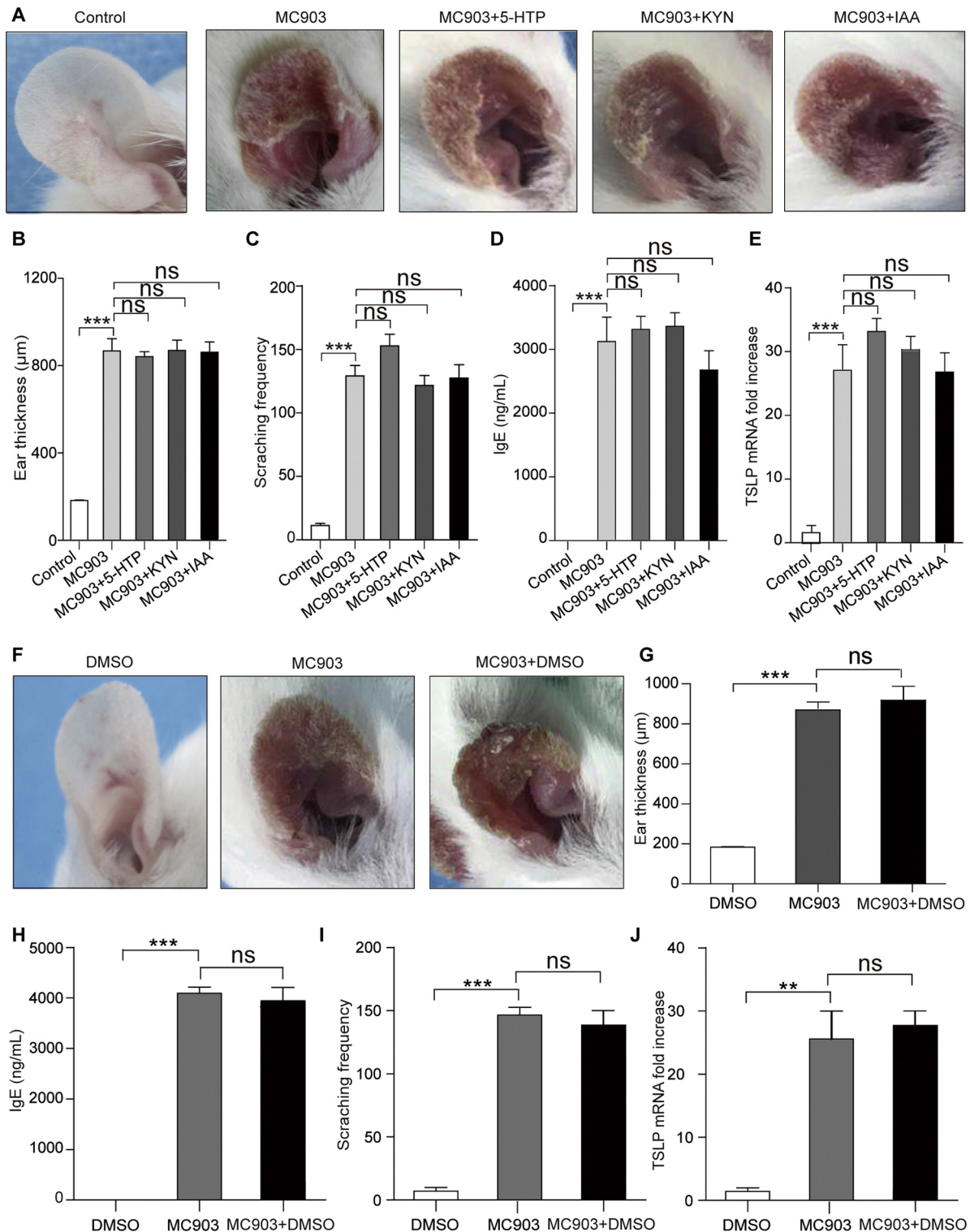


FIG E4. Effects of Trp metabolites on MC903-induced AD-like dermatitis. **A-E**, Thirty microliters of 100 ng/mL 5-HTP, KYN, or IAA was topically applied to mouse ears 30 minutes after application of MC903 daily for 14 days. Gross appearance of the ears (Fig E4, **A**), ear thickness (Fig E4, **B**), scratching frequency (Fig E4, **C**), IgE levels (Fig E4, **D**), and mRNA levels of TSLP (Fig E4, **E**) were shown. **F-J**, Effect of DMSO on MC903-induced inflammation was analyzed. Gross appearance (Fig E4, **F**), ear thickness (Fig E4, **G**), scratching frequency (Fig E4, **H**), level of IgE (Fig E4, **I**), and mRNA level of TSLP (Fig E4, **J**) at day 14 were shown. Data are representative of 2 independent experiments and expressed as means $\pm$ SEMs. Significance was analyzed with the unpaired *t* test (*n* = 3-5). ***P* < .01 and ****P* < .001. 5-HTP, 5-Hydroxy-L-tryptophan; ns, not significant.

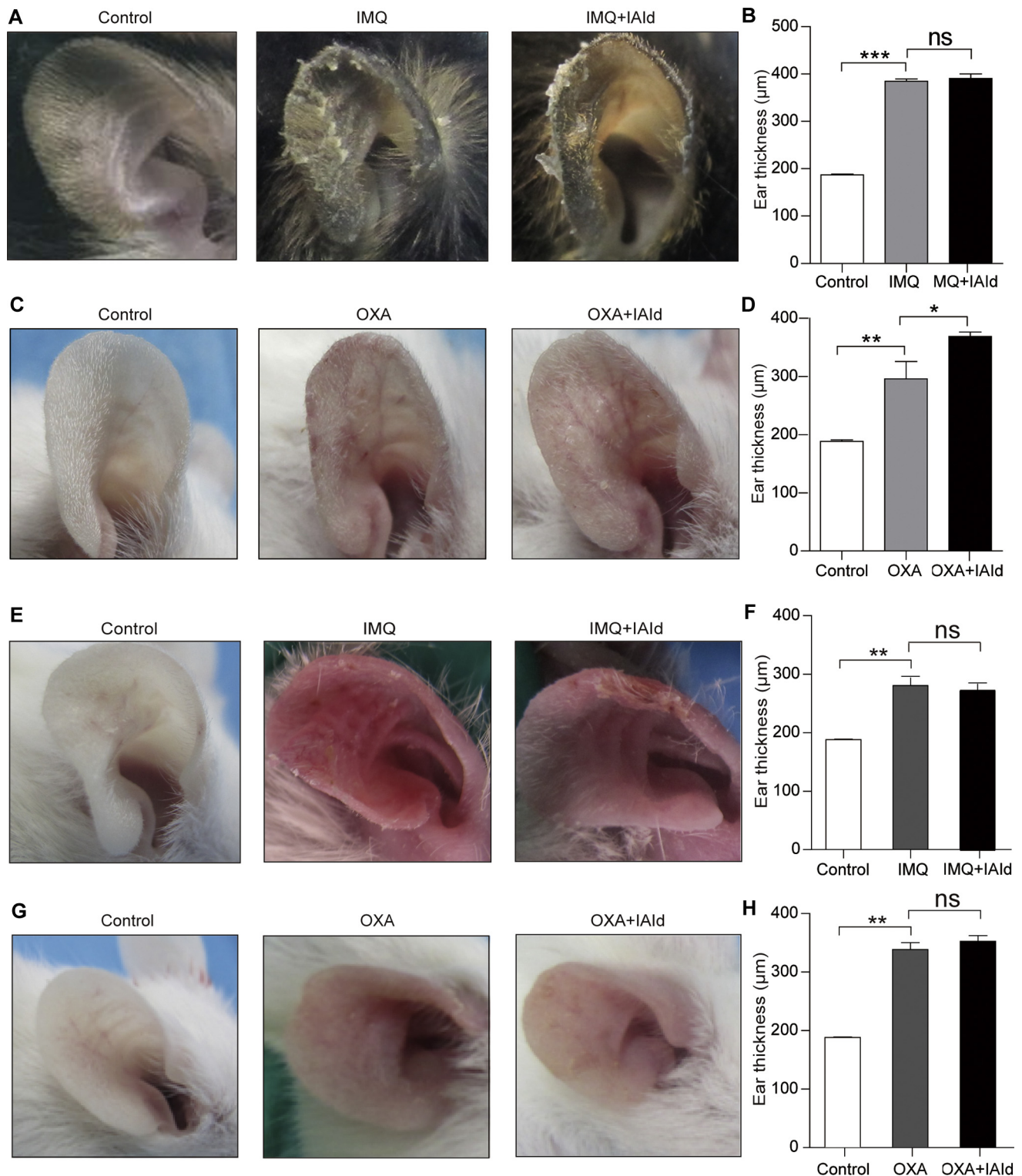


FIG E5. Effects of IAld on IMQ- or OXA-induced skin inflammation in mice. **A** and **B**, IMQ was painted on the ears of mice to produce psoriasis-like inflammation, and IAld or solvent control was applied along with application of IMQ. Gross appearance (Fig E5, **A**) and ear thickness (Fig E5, **B**) are shown. **C** and **D**, Mice were sensitized and challenged with OXA along with application of IAld. Gross appearance (Fig E5, **C**) and ear thickness (Fig E5, **D**) were shown. **E** and **F**, Mice were orally administered IAld and topically treated with IMQ, as described in Fig E5, **A** and **B**. Gross appearance (Fig E5, **E**) and ear thickness (Fig E5, **F**) were shown. **G** and **H**, Mice were orally administered IAld and sensitized and challenged with OXA, as described in Fig E5, **C** and **D**. Gross appearance (Fig E5, **G**) and ear thickness (Fig E5, **H**) were shown. Data are representative of 3 independent experiments and expressed as means $\pm$ SEMs. One-way ANOVA with the Tukey test was used to calculate the statistical significance of the experimental data ($n = 3-5$). * $P < .05$, ** $P < .01$, and *** $P < .001$. ns, Not significant.

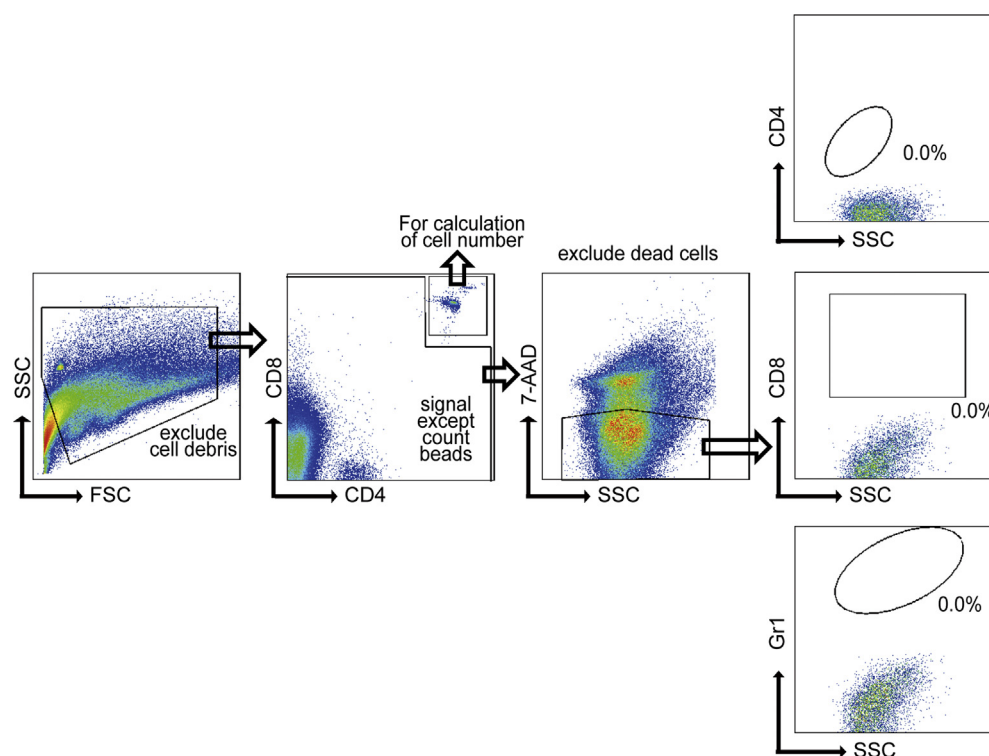


FIG E6. Gating strategy for flow cytometry. Single-cell suspensions were prepared from the ears of mice by means of digestion with collagenase type IV and DNase I and stained with anti-CD4, anti-CD8, or anti-Gr1 antibodies. CountBright absolute counting beads were used for calculation of absolute cell numbers. Dead cells were identified by staining with 7-aminoactinomycin D. Cell events were first gated by using forward scatter (FSC) and side scatter (SSC) plots to exclude debris. Then counting beads were identified by fluorescent plots, and cell concentrations and cell numbers were calculated. 7-Aminoactinomycin D-negative live cells were subjected to analysis of CD4, CD8, or Gr1 expression.

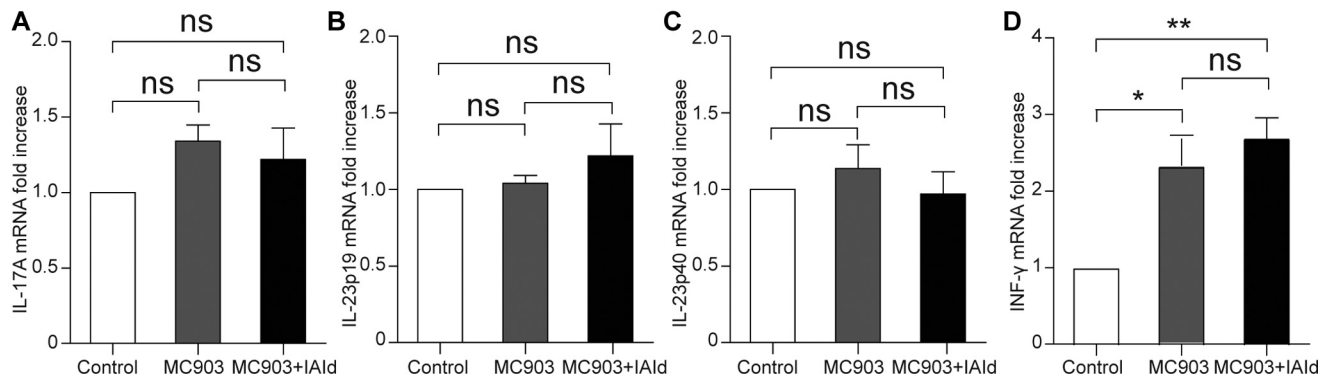


FIG E7. Effects of IAld on expression of various cytokines in the setting of MC903-induced dermatitis. BALB/c mice were painted onto the ears with MC903, MC903 plus IAld, or vehicle control for 14 days. Various cytokines in mouse ears were analyzed by using quantitative real-time PCR. mRNA levels of IL-17A (A), IL-23p19 (B), IL-23p40 (C), and INF- γ (D) were shown. Data are representative of 3 independent experiments and expressed as means $\pm$ SEMs. Significance was analyzed with ANOVA, followed by the Tukey test ($n = 3-5$). * $P < .05$ and ** $P < .01$. ns, Not significant.

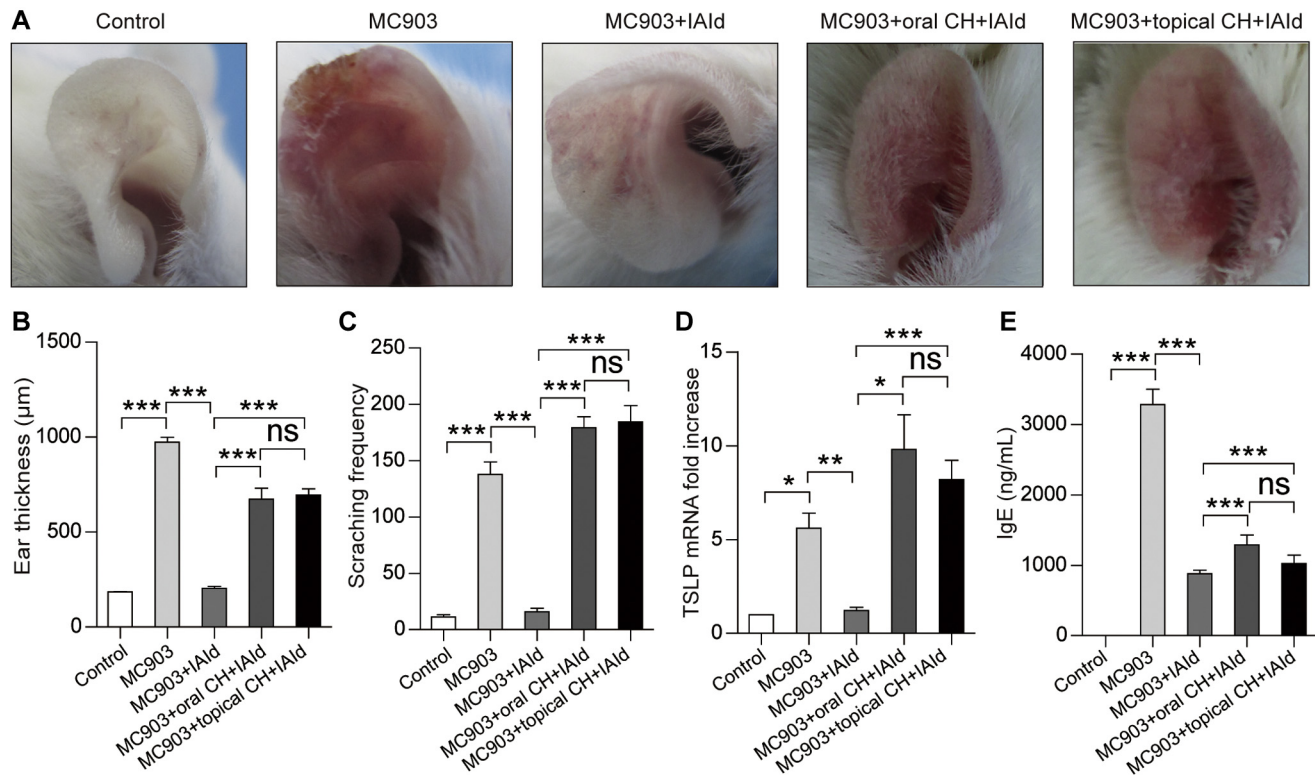


FIG E8. Effects of oral CH223191 on MC903-induced AD-like dermatitis. CH223191 was either topically or orally administrated to the mice along with MC903, IAld, or both for 14 days. Gross appearance of the ears (**A**), ear thickness (**B**), scratching frequency (**C**), mRNA level of TSLP (**D**), and total serum IgE level (**E**) were shown. Data are representative of 3 independent experiments and expressed as means $\pm$ SEMs. Significance was analyzed with ANOVA, followed by the Tukey test ($n = 3-5$). * $P < .05$, ** $P < .01$, and *** $P < .001$. ns, Not significant.

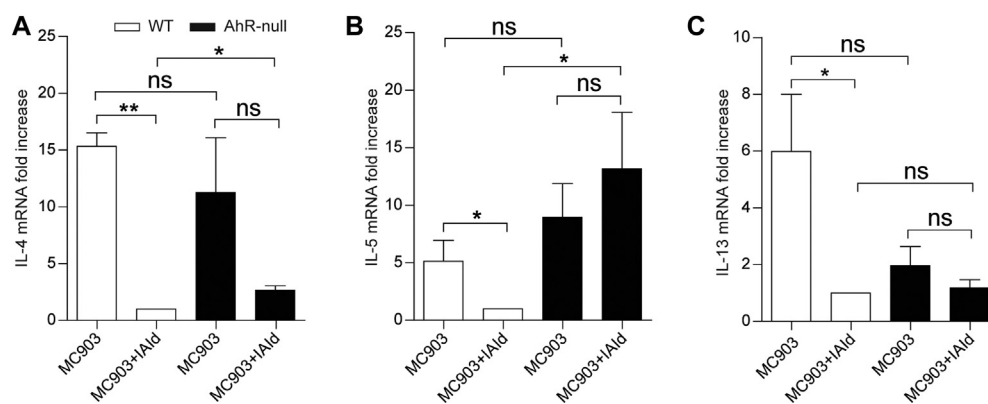


FIG E9. Expression of T_H2 -type cytokines in the setting of MC903-induced dermatitis in AhR-null mice. AhR-null mice had MC903 or MC903 plus IAld painted onto their ears for 14 days. Results of quantitative real-time PCR for mRNA levels of IL-4 (**A**), IL-5 (**B**), and IL-13 (**C**) are shown. Data are representative of 3 independent experiments and expressed as means $\pm$ SEMs. The unpaired *t* test was used to calculate the statistical significance of 2 different groups (*n* = 3-5). **P* < .05 and ***P* < .01. *ns*, Not significant.

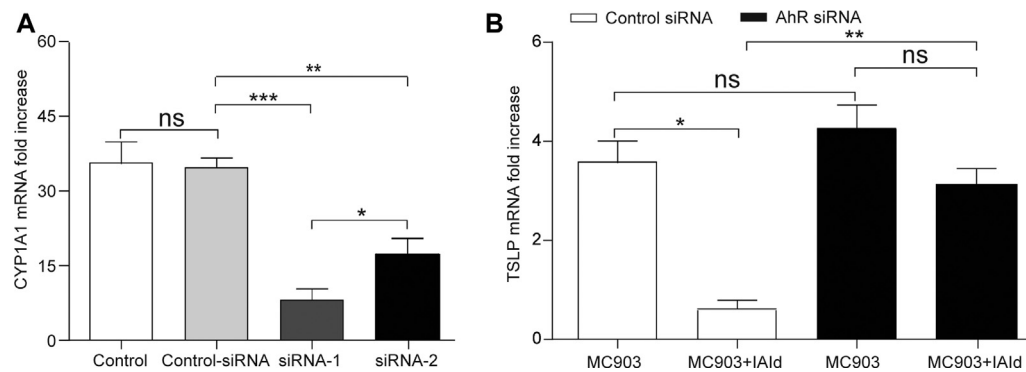


FIG E10. Effects of IAld on production of TSLP in JB6 cells after AhR knockdown. JB6 cells were transfected with AhR-specific or control siRNA, and then cells were treated with IAld, MC903, or both. Expression of CYP1A1 (**A**) and TSLP (**B**) is shown. Data are representative of 3 independent experiments and expressed as means $\pm$ SEMs. Unpaired *t* tests were used to calculate the statistical significance of 2 different groups (*n* = 3). **P* < .05, ***P* < .01, and ****P* < .001. *ns*, Not significant.

TABLE E1. Primers for quantitative real-time PCR of various cytokines

IL-4	Forward	5'-CCCCAGCTAGTTGTCATCCTG-3'
	Reverse	5'-CAAGTGATTTTGTGCGCATCCG-3'
IL-5	Forward	5'-GCAATGAGACGATGAGGCTTC-3'
	Reverse	5'-GCCCTGAAAGATTTCTCCAATG-3'
IL-6	Forward	5'-TGCAAGAGACTTCCATCCAGT-3'
	Reverse	5'-CTGCAAGTGCATCATCGTTGT-3'
IL-13	Forward	5'-GCCAAGATCTGTGTCTCTCCC-3'
	Reverse	5'-CACTCCATACCATGCTGCCG-3'
TSLP	Forward	5'-CCCCGACAAAACATTTGCCC-3'
	Reverse	5'-TGCCATTTCTGAGTACCGTC-3'
Hprt	Forward	5'-TGGATACAGGCCAGACTTTG-3'
	Reverse	5'-GATTCAACTTGCGCTCATCTTA-3'
IL-22	Forward	5'-ATGAGTTTTTCCCTTATGGGGAC-3'
	Reverse	5'-GCTGGAAGTTGGACACCTCAA-3'

TABLE E2. AhREs in the TSLP promoter region

	Location	Sequence	Strand
AhRE1	−1826 to −1845	ctgatgtcacgccagatga	−
AhRE2	−1487 to −1506	tggctcagcgtggtctgtg	+
AhRE3	−1381 to −1400	gctctgacacgcaaaagtg	−
AhRE4	−751 to −770	attaaaggcgtgcgccacc	+