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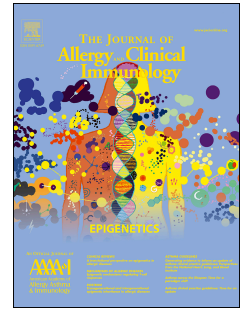
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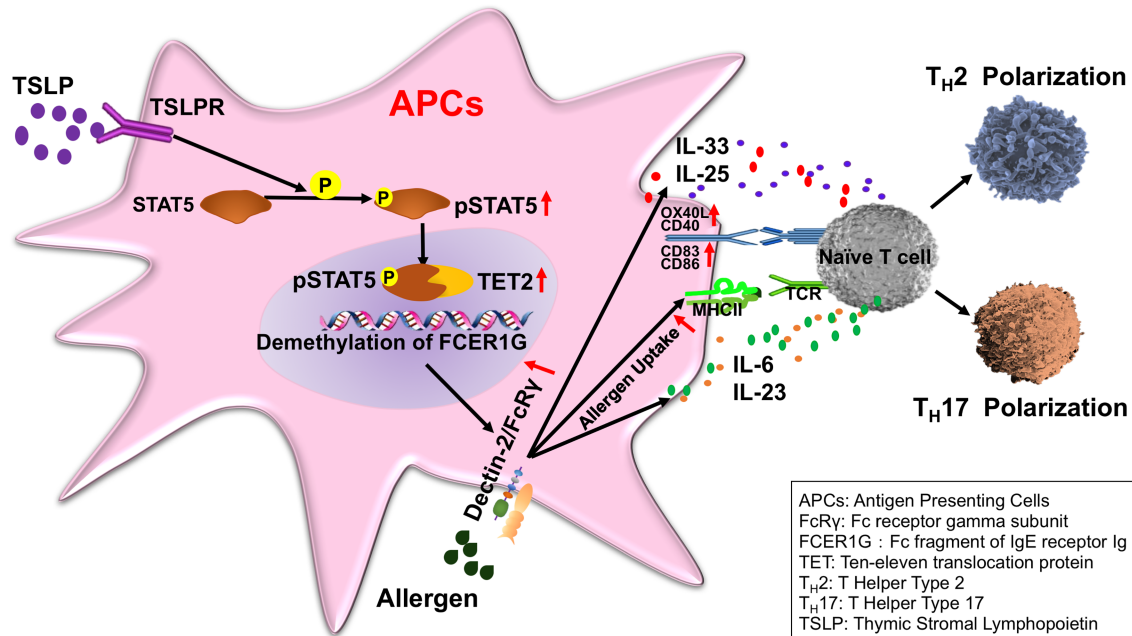
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Mechanisms of the TSLP-mediated induction of allergic TH2/TH17 polarization



**TSLP epigenetically upregulates FcR γ -related receptors on APCs and induces
T_H2/T_H17 polarization via dectin-2**

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Key message

1. TSLP epigenetically upregulates the expression of $Fc\gamma R$ and its related receptors ($Fc\epsilon R1$, CD16, and dectin-2) on APCs through the activation of pSTAT5, which recruits TET2 to induce FCER1G demethylation.

2. TSLP-induced allergic T_H2/T_H17 polarization likely depends on the dectin-2-mediated allergen sensitization and upregulation in IL-25/IL-33/IL-6/IL-23 expression.

Capsule summary

TSLP upregulates $Fc\gamma R$ -related receptors on APCs through activation of pSTAT5, which recruits TET2 to induce FCER1G demethylation, and the TSLP-induced allergic T_H2/T_H17 polarization likely depends on the dectin-2-mediated allergen sensitization and upregulation in T_H2/T_H17 -related cytokines.

Abstract

Background: Fc receptor gamma subunit (FcR γ)-related receptors expressed on antigen-presenting cells (APCs) enhance allergen sensitization and allergic inflammation. DNA demethylation of the Fc fragment of IgE receptor Ig (FCER1G) leads to FcR γ and Fc ϵ R1 overexpression on monocytes from patients with atopic dermatitis (AD).

Objective: We investigated epigenetic mechanisms underlying FCER1G demethylation and upregulation of FcR γ -related receptors on APCs and the consequent impact on allergic responses.

Methods: Effects of thymic stromal lymphopoietin (TSLP) on the expression of FcR γ and its related receptors, and methylation or hydroxymethylation of FCER1G in human monocytes were assessed. Recruitment of ten-eleven translocation protein 2 (TET2) to FCER1G by TSLP-activated phospho-signal transducer and activator of transcription 5 (pSTAT5) was evaluated. Effects of TSLP on the expression of FcR γ -related receptors and costimulatory receptors on monocyte-derived dendritic cells (MoDCs) and ability of DCs to take-up ovalbumin were analyzed. TSLP-induced T_H polarization and related cytokine production were also analyzed.

Results: pSTAT5 activation by TSLP resulted in TET2 recruitment to FCER1G, leading to FCER1G demethylation and subsequent upregulation of FcR γ -related receptors on monocytes. TSLP not only stimulated MoDC maturation but also main-

tained their allergen uptake ability likely through maintenance and upregulation of FcR γ -related receptors. Allergen sensitization and upregulation of T_H2/T_H17-related cytokines contributed to TSLP-DC-induced T_H2/T_H17 polarization. The latter was attenuated upon neutralization with a dectin-2 antibody.

Conclusions: TSLP mediated upregulation of FcR γ -related receptors on APCs through activation of pSTAT5, which recruited TET2 to induce FCER1G demethylation. TSLP-induced allergic T_H2/T_H17 polarization likely depends on dectin-2-mediated allergen sensitization and upregulation of T_H2/T_H17-related cytokines.

Keywords: Atopic dermatitis, TSLP, STAT5, TET2, DNA demethylation, FcR γ -related receptors, Dectin-2, Allergen uptake, IL-33, IL-25, T_H2/T_H17 polarization

Abbreviations

AD, Atopic dermatitis; APCs, Antigen-presenting cells; BS, Bisulfite; ChIP, Chromatin immunoprecipitation, CoIP, Co-immunoprecipitation; DC, Dendritic cell; ELISA, Enzyme-linked immunosorbent assay; FBS, fetal bovine serum; FCER1G, The high affinity IgE receptor gamma subunit gene; FcεR1, The high affinity IgE receptor; FcR γ , Fc receptor gamma subunit; FcεR γ , The high affinity IgE receptor gamma subunit; GM-CSF, Granulocyte-macrophage colony-stimulating factor; HDM, House dust mite; IDEC, Inflammatory dendritic epidermal cell; Im-DC, Im-

mature monocyte-derived dendritic cell; IFN- γ , interferon gamma; ITAM, Immuno-
 receptor tyrosine-based activation motif; MoDC, Monocyte-derived dendritic cell;
 oxBS, Oxidative bisulfite; OVA, Ovalbumin; LPS, Lipopolysaccharide; PBMC, Pe-
 ripheral blood mononuclear cell; RT-qPCR, Real time quantitative polymerase chain
 reaction; SCORAD, SCORing Atopic Dermatitis; STAT5, Signal transducer and ac-
 tivator of transcription 5; TET, Ten-eleven translocation protein; TSLP, Thymic
 stromal lymphopoietin; TNF α , Tumor necrosis factor alpha

INTRODUCTION

Atopic dermatitis (AD), one of the most common chronic inflammatory dermato-
 ses, is characterized with intense itching and recurrent eczematous lesions. AD af-
 fects up to 20% of the general population in developed countries¹. The Fc receptor
 gamma subunit (FcR γ ; also called Fc ϵ R1 γ) is a universal signaling component of
 multiple receptors that contain two immunoreceptor tyrosine-based activation motifs
 (ITAMs). The activation of FcR γ -related receptors, including Fc ϵ R1, CD16, and
 dectin-2 on antigen-presenting cells (APCs) may result in the stimulation of allergen
 sensitization and induction of allergic inflammation in patients with AD and animal
 models²⁻⁶. Our previous study demonstrated the overexpression of FcR γ and Fc ϵ R1
 on the monocytes from patients with AD upon demethylation of the FcR γ locus
 (FCER1G)⁷. However, the epigenetic mechanisms underlying the upregulation in the

demethylation of FCER1G and expression of FcR γ -related receptors in AD are unknown.

DNA methylation and demethylation in the mammalian genome are key epigenetic modifications that are dynamically regulated by DNA methyltransferases and DNA demethylases, respectively. Ten-eleven translocation proteins (TETs) catalyze the conversion of 5-methylcytosine (5mC) to 5-hydroxymethylcytosine (5hmC) to induce DNA demethylation^{8,9}. Specific transcription factors are essential for the TET-mediated induction of active demethylation of signature cytokine genes. For instance, TET2 and key transcription factors cooperatively regulate the expression of signature genes. The TET2-mediated DNA demethylation plays a crucial role in the control of the expression of signature cytokines in T_H cells¹⁰. Nonconsensus GAS motifs of signal transducer and activator of transcription 5a (STAT5a) tetramers have been observed from -328 to -290 bp sequences of the FCER1G promoter using the TRANSFAC® database^{11,12}.

Thymic stromal lymphopoietin (TSLP) is an interleukin (IL)-7-related cytokine that is mainly expressed in human epithelial cells, keratinocytes, and bronchial smooth muscle cells. The main effector cells of TSLP are dendritic cells (DCs)¹³. The role of TSLP in the induction of allergic T_H2 immune response in DCs and the pathogenesis of allergic diseases (AD, asthma, etc.) have been previously demonstrated¹⁴⁻¹⁶. The TSLP-mediated activation of STAT5 phosphorylation is critical for the development of T_H2 response in DCs¹⁷. We speculate that the TSLP-induced ac-

tivation of pSTAT5 leads to DNA demethylation and upregulated expression of FcR γ and its related receptors. Therefore, in the present study, we investigated the epigenetic mechanisms underlying the upregulation in the demethylation of FCER1G and expression of FcR γ -related receptors in AD and the consequent impact on allergic response.

METHODS

Materials and subjects

Human peripheral blood cells were collected from sex- and age-matched healthy volunteers recruited from the Second Xiangya Hospital and Changsha Blood Center. Both inpatients and outpatients with AD (n = 15) were recruited at the Second Xiangya Hospital. AD was diagnosed according to Hanifin and Rajka diagnostic criteria¹⁸, and disease activity was assessed using the SCORing Atopic Dermatitis (SCORAD) index¹⁹. Healthy control subjects (n = 15) were recruited from the medical staff at the Second Xiangya Hospital. Patients with AD and healthy controls were matched for age and sex. This study was approved by the human ethics committee of the Second Xiangya Hospital, and written informed consent was obtained from all subjects. The information about patients and control subjects is provided in Table E1.

Cell isolation and culture

Peripheral blood mononuclear cells (PBMCs) were isolated using Ficoll-Hypaque

density gradient centrifugation (GE Healthcare, Sweden, Catalog 17-1440-03). CD14⁺ monocytes and naive CD4⁺ T cells were isolated by magnetic beads (Human CD14 Monocyte Cocktail Kit, Catalog 130-050-201; Human Naive CD4⁺ T Cell Isolation Kit II, Catalog 130-094-131, Miltenyi Biotec, Germany), according to the manufacturer's instructions. The purity of the sorted cells was > 90%. Cells were cultured in Roswell Park Memorial Institute (RPMI)-1640 (Gibco, Beijing, China, Catalog C11875500BT) medium supplemented with 10% fetal bovine serum (FBS; HyClone, Guangzhou, China, Catalog 10270-106), 100 U penicillin, and 100 mg streptomycin at 37°C and 5% CO₂. In some experiments, immature monocyte-derived dendritic cells (MoDCs) were differentiated from CD14⁺ monocytes using granulocyte-macrophage colony-stimulating factor (GM-CSF; 20 ng/mL, PeproTech, Catalog 96-AF-300-03-20) and IL-4 (20 ng/mL, PeproTech, Catalog 200-04-20) for 7 days, followed by treatment with TSLP (50 ng/mL, Miltenyi Biotec, Catalog 130106270) or lipopolysaccharide (LPS; 1 µg/mL, Sigma, Catalog L4524) for 3 days. The MoDCs (immature DCs, TSLP-DCs, and LPS-DCs) were washed and replated for the subsequent ovalbumin (OVA) uptake and T_H polarization assays. In other experiments, freshly isolated CD14⁺ monocytes were stimulated with human TSLP (50 ng/mL, Miltenyi Biotec, Catalog 130106270) for 3 days. PBMCs and freshly isolated CD14⁺ monocytes were stimulated with LPS (1 µg/mL, Sigma, Catalog L4524) for 14 h.

RNA isolation and real-time quantitative polymerase chain reaction (RT-qPCR)

Total RNA was extracted from monocytes and MoDCs using the RNeasy Mini Kit (Qiagen, Germany, Catalog 74104) according to the manufacturer's instructions. qPCR was performed using a Rotor-Gene 3000 Real-time PCR instrument (Corbett Research, Australia). mRNA was amplified with SYBR Green qPCR using the One Step PrimeScript RT-PCR Kit (TaKaRa Biotech, China, Catalog D350A). Primers for target genes, including *FCER1G* (FcR γ), *FCER1A* (Fc ϵ R1), *FCG3A* (CD16), *CLEC6A* (dectin-2), *TET1*, *TET2*, *TET3*, *IL-33*, *IL-25*, and *β -actin* are listed in Table E2. Relative expression was calculated using the $2^{-\Delta CT}$ method, where $\Delta CT = (CT_{\text{Target Gene}} - CT_{\beta\text{-actin}})$.

Western blot analysis

Monocytes were lysed in radioimmunoprecipitation assay (RIPA) buffer supplemented with protease (Beyotime, China, Catalog P1009) and phosphatase inhibitors (Cell Signaling Technology, Catalog 5870). The total proteins were extracted and protein concentrations determined. The antibodies used in this study were mouse anti-Fc ϵ R1 γ (FcR γ) antibody (1:500, Santa Cruz Biotechnology, Catalog sc390222), rabbit anti-Fc ϵ R1 antibody (1:800, Proteintech, Catalog 10980-1-AP), rabbit anti-CD16 antibody (1:500, Proteintech, Catalog 16559-1-AP), mouse anti-dectin-2 antibody (1:1000, Absin, China, Catalog abs139702), mouse anti-TET1 antibody (1:1000, Sigma, Catalog SAB2700730), rabbit anti-TET2 antibody (1:1000, Sigma, Catalog SAB1304574), mouse anti-TET3 antibody (1:1000, Sigma, Catalog SAB2702210), rabbit anti-pSTAT5 antibody (1:1000, Cell Signaling Technology,

Catalog 4322T), rabbit anti-STAT5 antibody (1:1000, Absin, China, Catalog abs121050), goat anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody (1:2000, GenScript, Catalog A00915), and mouse anti- β -actin antibody (1:2000, Abcam, Catalog ab6272). Data were analyzed using a GE ImageQuant LAS 4000 mini (GE Healthcare). For quantification of FcR γ , TET1, TET2, and TET3, the protein levels were normalized to β -actin level by densitometry. For quantification of Fc ϵ R1, CD16, dectin-2, p-STAT5, and STAT5, the protein levels were normalized to GAPDH level by densitometry. Images were cropped for presentation.

Bisulfite and oxidative bisulfite amplicon sequencing (BS and oxBS)

Approximately 200 ng of genomic DNA was purified using magnetic beads supplied with the TrueMethyl Seq Kit (CEGX), as per the manufacturer's instructions. After purification, the product was divided into two equal parts for oxBS and BS reactions. BS treatment of genomic DNA was performed as previously described⁷. The oxidation reaction was conducted in a reaction volume of 25 μ L with 1 μ L of Oxidant Solution (CEGX) for each oxBS reaction, and the products were incubated at 40°C for 30 min in a thermocycler with the lid heated to 57°C. The reaction mixture was centrifuged at 14,000 $\times g$ for 10 min, and the supernatant was transferred to a new 0.2 mL PCR tube for BS treatment. The purified genomic DNA for BS reaction was directly subjected to BS conversion. After BS conversion, BS and oxBS products were used as templates for PCR amplification. PCR products were used for library construction, and the final libraries were quantified with an Agilent 2100 Bio-

analyzer (Agilent Technologies) and real-time PCR assay and then sequenced by Illumina HiSeq. The protocol of 5-methylcytosine and 5-hydroxymethylcytosine oxBS sequencing are described in detail by Michael J. Booth et al^{20, 21}. Two PCR primers were used for the FCER1G promoter fragment and the primers FCER1G-2 and FCER1G-3 are listed in Table E2.

Chromatin immunoprecipitation (ChIP)-qPCR

ChIP assays were performed with a ChIP kit (Millipore, Sigma-Aldrich, Catalog 17-371). Briefly, formaldehyde was used to crosslink the DNA and proteins in the cell. The chromatin was fractured using ultrasound to an interrupted length between 200 and 1,000 bp. Target protein-specific antibodies were used for chromatin immunoprecipitation. Protein A/G agarose was used for the isolation of the immunoprecipitation complex, and the immunoprecipitated complex was eluted after the removal of nonspecifically bound chromatin. Reverse crosslinking was performed at 65°C, and the DNA was purified with phenol-chloroform extraction and ethanol precipitation. The resulting DNA was amplified with real-time PCR. A rabbit anti-STAT5 antibody (Abcam, catalog ab235934) and rabbit IgG, polyclonal (Abcam, catalog ab171870) isotype control were used. The eluted DNA fragments were analyzed with qPCR. The PCR primers used for human *FCER1G* gene (*FCER1G-4*) are listed in Table E2.

Co-immunoprecipitation (Co-IP) assays

Co-IP assays were performed with the Dynabeads Protein G Immunoprecipitation

Kit (Novex, Catalog 10007D) according to the manufacturer's instructions. First, we used RIPA lysis buffer supplemented with protease and phosphatase inhibitors to collect the freshly treated cells and extract proteins (TSLP-treated monocytes). We obtained a new Ab-bait-target complex by adding rabbit anti-STAT5 capture antibody (Abcam, catalog ab36153) to the lysate; rabbit polyclonal IgG (Abcam, catalog ab171870) was used as an isotype control. The capture antibody was used for the immobilization of proteins on the beads (Life Technology). The unbound proteins were removed by a series of washes. The bait-protein complexes were finally eluted from the beads and dissociated by boiling in sodium dodecyl sulfate (SDS). The endpoint of the assay was the determination of the presence or absence of the target protein by western blotting. Rabbit anti-pSTAT5 (Abcam, catalog ab32364) and rabbit anti-TET2 (Abcam, catalog ab94580) antibodies were used.

MoDC OVA uptake assay

We used a fluorescein isothiocyanate (FITC)-NH₂ protein labeling kit (Dojindo, Japan, LK01) to label OVA according to the manufacturer's instructions. The MoDCs (1×10^6 immature DCs, TSLP-DCs, and LPS-DCs) were incubated with FITC-labeled OVA (1 mg/mL) at 37°C and 5% CO₂ for 6 h in the dark. As controls, the same concentrations of MoDCs were incubated without the FITC-labeled OVA for 6 h at 37°C. The MoDCs were washed thrice and analyzed for the uptake of FITC-labeled OVA with fluorescence microscopy and flow cytometry.

OVA-pulsed T_H polarization assay

MoDCs (immature DCs, TSLP-DCs, and LPS-DCs) were cocultured with autologous CD4⁺ naive T cells at a 1:5-1:10 ratio in the presence or absence of OVA (1 mg/mL, Sigma, Catalog A5503) for 7 days. T_H polarization was detected with flow cytometry. Cytokines in supernatant, including IL-33, IL-31, IL-10, IL-25, IL-23, IL-6, and tumor necrosis factor (TNF)- α , were detected with an ultrasensitive multi-factor electrochemical luminescence kit (Meso Scale Discovery, Catalog K15067L-1). The levels of IL-4, IL-17A, interferon (IFN)- γ , and IL-22 in the supernatant were analyzed using enzyme-linked immunosorbent assay (ELISA) kits. All procedures were carried out according to the manufacturer's instructions.

ELISA

The levels of IL-4, IL-17A, IFN- γ (DAKEWE, China, Catalog 1110402, 1111702, 1110002), and IL-22 (Abcam, ab216170) in the coculture supernatant and the level of TSLP (R&D, DTSLP0) in the sera of patients with AD were analyzed using ELISA kits. All measurements were performed in duplicates. The standards were processed using a gradient dilution and used for the preparation of a standard curve. The absorbance values of the samples were substituted into the standard curve equation to determine the concentrations of corresponding samples.

Neutralization of dectin-2 on MoDCs

MoDCs (TSLP-DCs and immature DCs) were incubated with anti-dectin-2 antibody (1 mg/mL, InvivoGen, Catalog mabg-hdect2) or IgG control (InvivoGen, Catalog mabg2a-ctrlm) at 37°C and 5% CO₂ for 1 h. About 1×10^5 cells were added to

1 mg/mL of the neutralizing antibody and processed according to the instructions indicated in the OVA-pulsed T_H polarization assay manual.

Flow cytometric analysis

Cytokines and cell surface markers were detected using FACS Canto II (BD Biosciences). For cell surface marker detection, cell suspensions were incubated with target surface marker antibodies for 30 min at 4°C, washed twice with 2 mL of phosphate-buffered saline (PBS)/bovine serum albumin (BSA), and centrifuged at 400 ×g for 5 min. For cytokine detection, Phorbol 12-Myristate 13-Acetate (PMA) and ionomycin with GolgiPlug (BD Biosciences, Catalog 555028) were used to stimulate cells for 4 h. For intracellular staining, the cells were fixed and permeabilized with Cytofix/Cytoperm (BD Biosciences, Catalog 555028) and stained with fluorescent antibodies for additional 30 min in the dark at 4°C. Antibodies used for flow cytometric analysis are listed in Table E3. Data were analyzed using the FlowJo software (Tree Star). Dead cells were excluded from the analysis, and the gates were generated using isotype antibody controls.

Statistical analysis

Data were expressed as the mean ± standard error of mean (SEM). Paired and unpaired Student's *t*-tests were used. Statistical analysis was performed with GraphPad Prism, version 7 (GraphPad Software Inc., California, USA) (**p* < 0.05; ***p* < 0.01; ****p* < 0.001).

RESULTS

TSLP upregulates the expression of FcR γ and FcR γ -related receptors on human monocytes

We first assessed the effects of TSLP on the expression levels of FcR γ and its related receptors on monocytes. As shown in Fig 1, the 3-day treatment of normal human CD14⁺ monocytes with TSLP resulted in a significant elevation in the expression levels of FcR γ mRNA and protein as well as its receptors Fc ϵ R1, CD16, and dectin-2 (Fig 1, A-D).

The upregulation of Fc ϵ R1 and CD16 expression on the monocytes of patients with AD may promote their differentiation into Fc ϵ R1⁺⁺ inflammatory dendritic epidermal cells (IDECs) in AD lesions^{2,22}. We detected significant upregulation of dectin-2 expression on peripheral blood CD14⁺ monocytes from 15 patients with AD (9.21% $\pm$ 1.07%) and 15 healthy controls (3.71% $\pm$ 0.46%) using flow cytometry (Fig 1, E). However, we failed to observe any correlation between serum TSLP levels (105.6 $\pm$ 24.41 pg/mL, n = 15) and dectin-2 expression on the monocytes from patients with AD (p = 0.723).

TSLP induces demethylation of the FCER1G promoter on human monocytes

As demethylation of FCER1G was shown to upregulate FcR γ and its related receptor Fc ϵ R1 on the monocytes of patients with AD⁷, we investigated whether TSLP induces demethylation of FCER1G promoter in human monocytes. DNA methylation levels significantly decreased at eight CpG sites (-428, -426, -392, -375, -358,

–349, –341, and –333 bp) on the FCER1G promoter region in the TSLP-treated group as compared with that in the control group (Fig 2, A-B, $34.00\% \pm 3.70\%$ versus $52.13\% \pm 2.45\%$). In contrast, DNA hydroxymethylation levels dramatically increased in the TSLP-treated group at eight CpG sites as compared with that in the control group (Fig 2, C-D, $10.80\% \pm 2.02\%$ versus $5.10\% \pm 0.88\%$).

TSLP induces FCER1G demethylation through pSTAT5, which recruits TET2 to the FCER1G promoter

DNA demethylases, TETs, regulate active DNA demethylation. Previous studies have shown that TET2 participates in active DNA demethylation involved in monocyte differentiation²³ and that the TET2-induced active demethylation of signature cytokine genes relies on specific transcription factors¹⁰. However, we failed to observe any significant differences in either TET1-3 protein or mRNA level between TSLP-treated and control monocytes in western blot analysis and qPCR (Fig 2, E-F). In contrast, the activation of pSTAT5 (Fig 2, G) and the interaction between pSTAT5 and TET2 (Fig 2, H) and FCER1G promoters (Fig 2, I) were observed in TSLP-treated monocytes. Together, we identified a novel epigenetic mechanism, wherein TSLP induced FCER1G demethylation through pSTAT5, which recruited TET2 to the FCER1G promoter in human monocytes.

TSLP stimulates MoDC maturation while maintaining the allergen uptake ability likely through the maintenance and upregulation of FcR γ -related receptors

To investigate whether TSLP regulates the expression of FcR γ -related receptors on DCs, immature MoDCs were obtained by the treatment of normal human CD14⁺ monocytes with both GM-CSF and IL-4 for 7 days, followed by incubation with TSLP or LPS for 3 days. The expression of costimulatory receptor molecules (CD83, CD86, CD40, and OX40L) and FcR γ -related receptors (Fc ϵ RI, CD16, and dectin-2) on DCs was detected with flow cytometry. As shown in Fig 3, A-C, both TSLP and LPS stimulated MoDC maturation, as evident from the increase in the expression of CD83, CD86, CD40, and OX40L. TSLP treatment maintained the high level expression of dectin-2 and upregulated the expression of CD16 and Fc ϵ RI on DCs, although the expression level of Fc ϵ RI was low. In contrast, LPS treatment, either combined with or without TSLP treatment, resulted in the downregulation in the expression of dectin-2 on DCs (Fig E1, Fig 3 B,C). It is well known that mature DCs exhibit strong costimulatory and T-cell activation abilities but decreased antigen uptake and processing abilities as compared with immature DCs²⁴. We investigated whether mature TSLP-DCs retain their allergen uptake ability through the maintenance and upregulation of FcR γ -related receptors using FITC-labeled OVA. Our results showed that mature TSLP-DCs and immature DCs showed a comparable ability to take-up OVA, while mature LPS-DCs exhibited a poor ability to take-up OVA (Fig 3, D-G). These results imply that TSLP not only stimulated MoDC maturation but also maintained their allergen uptake ability, likely through the maintenance and upregulation of FcR γ -related receptors.

TSLP-induced T_H2/T_H17 polarization likely depends on the dectin-2-mediated allergen sensitization and signaling pathway

TSLP directly induces the pathogenic T_H2 response in allergic diseases¹⁴⁻¹⁶; however, two basic mechanisms, including the effects of TSLP on allergen sensitization and the T_H2 -polarizing cytokine milieu produced by DCs independent of IL-4, are unclear²⁵. To investigate these mechanisms, we co-incubated mature TSLP-DCs or LPS-DCs and immature MoDCs with autologous $CD4^+$ naive T cells in the presence or absence of OVA for 7 days. Surprisingly, the treatment of TSLP-DCs with OVA induced T_H2/T_H17 polarization, as evident from the increase in the population of IL-4⁺/IL-17A⁺ T cells and upregulation in IL-4/IL-17A protein levels in the coculture supernatant (Fig 4, A-E). However, T_H1/T_H22 polarization was not different among the treatment groups (Fig E2). T_H2 -related inflammatory cytokines, including IL-33, IL-25, and IL-31, as well as T_H17 -related inflammatory cytokines, IL-6 and IL-23, were significantly upregulated in the coculture supernatant of TSLP-DCs pulsed with OVA as compared with the supernatant from the LPS-DCs and immature MoDCs (Fig 4, E). Moreover, the expression levels of these cytokines were higher in the OVA-treated cells than in the non-OVA-treated cells (Fig 4, E), suggesting that allergen stimuli and sensitization are important for the TSLP-DC-mediated secretion of T_H2/T_H17 -related cytokines. Interestingly, the induction of T_H2/T_H17 polarization (Fig 5, A-D) and secretion of T_H2/T_H17 -related cytokines (Fig 5, E) were attenuated after the neutralization of dectin-2 on MoDCs

using a dectin-2-specific antibody. We also found that OVA stimulation significantly upregulated the expression of *IL-25* and *IL-33* mRNAs in TSLP-DCs, and this effect was attenuated after dectin-2 neutralization (Fig E3, A). In contrast, the neutralization of dectin-2 had no effect on the expression of costimulatory molecules (CD86, CD83, CD40, and OX40L) on the surface of OVA-stimulated TSLP-DCs (Fig E3, B-C). Consistent with the TSLP-DC-induced $\text{TNF-}\alpha^{++}\text{IL-10}^{-}$ inflammatory $\text{T}_\text{H}2$ cell differentiation, we observed a decrease in the secretion of $\text{TNF-}\alpha$ mainly from $\text{T}_\text{H}2$ cells after neutralization of dectin-2 expression on MoDCs (Fig 5, E), while low-level secretion of IL-10 and IFN- γ was detected in all groups (Fig 4, E, Fig 5, E, and Fig E2, E). Together these data show that the TSLP-induced OVA-specific $\text{T}_\text{H}2/\text{T}_\text{H}17$ polarization was likely dependent on the dectin-2-mediated OVA sensitization and $\text{T}_\text{H}2/\text{T}_\text{H}17$ -related cytokine upregulation.

DISCUSSION

We have previously demonstrated that demethylation of FCER1G leads to the overexpression of $\text{FcR}\gamma$ and its related receptor $\text{Fc}\epsilon\text{R1}$ on the monocytes from patients with AD⁷. In the present study, we found that TSLP induced FCER1G promoter demethylation and upregulated the expression of $\text{FcR}\gamma$ and $\text{FcR}\gamma$ -related receptors ($\text{Fc}\epsilon\text{R1}$, CD16, and dectin-2) on human monocytes. We subsequently identified the intrinsic epigenetic mechanism underlying the TSLP-mediated induction of FCER1G demethylation through the activation of pSTAT5, which recruits TET2 to the FCER1G promoter in human monocytes.

TSLP initiates signaling through the establishment of a ternary complex with its specific receptor, TSLPR, and then with IL-7R α in the cells co-expressing TSLPR and IL-7R α ²⁶. Borriello F, et al. recently emphasized the distinct phenotypic, functional, and transcriptomic profiles of TSLPR-positive monocytes as compared with TSLPR-negative monocytes, and revealed the enrichment of a subset of CD14⁺ CD1c⁺ monocytes²⁷. We speculate that TSLP upregulates the expression of FcR γ and its related receptors on TSLPR-positive monocytes. Further analysis of the effects of TSLP on monocytes using purified TSLPR-positive monocytes is warranted.

FcR γ -related receptors on APCs promote allergen sensitization and allergic inflammation in allergic diseases^{4, 5, 28}. As a characteristic feature, Langerhans cells and IDECs bearing Fc ϵ R1 in patients with AD are thought to take-up allergens through IgE and induce T-cell-mediated allergic reactions^{1, 2}. Although TSLP significantly upregulated the expression of Fc ϵ R1 on monocytes, it also promoted low-level expression of Fc ϵ R1 on MoDCs, suggesting that the surface expression of Fc ϵ RI on DCs may be regulated by a complex “atopic environment.” For instance, IgE retains the surface expression of Fc ϵ RI on atopic Fc ϵ R1⁺ MoDCs³.

The percentage of CD14⁺CD16⁺ intermediate monocytes, characterized with high levels of antigen presentation and costimulatory molecules that stimulate T cell proliferation, was significantly elevated in patients with AD and asthma^{22, 29}. Signaling through Fc γ RIII (CD16) on DCs upon stimulation with allergen-specific IgG immune complexes may promote and exacerbate allergic T_H2 responses^{4, 5, 30, 31}. In the

present study, we demonstrated that TSLP significantly upregulated the expression of CD16 on CD14⁺ monocytes and MoDCs. MoDCs may promote T_H2 responses induced by allergen-specific IgG immune complexes. We found that LPS upregulated the expression of CD16 on MoDCs, partly explaining the reason underlying the augmentation in the ability to promote Th2 responses upon signaling via FcγRIII (CD16) in conjunction with TLR4 stimulation^{30, 31}.

Regulation of T_H polarization by DCs involves at least three central signaling pathways, namely antigen uptake and presentation, costimulatory factor signaling, and immediate secretion of cytokines by DCs to regulate T_H polarization²⁴. TSLP has been shown to directly induce pathogenic T_H2 response in allergic diseases¹⁴⁻¹⁶. The TSLP-activated DCs (TSLP-DCs) preferentially express the costimulatory factor OX40L without the production of IL-12 and concomitantly produce chemokine (C-C motif) ligand 17 (CCL17). In addition, TSLP-DCs expressing OX40L strongly prime naive CD4⁺ T cells to differentiate into TNF-α⁺⁺IL-10⁻ inflammatory T_H2 cells³². However, the effects of TSLP on allergen uptake and the immediate cytokine milieu regulating T_H2 polarization remain to be elucidated²⁵.

Antigen uptake by DCs is mediated via three independent mechanisms, namely, macropinocytosis, phagocytosis, and receptor-mediated endocytosis³³. In receptor-mediated endocytosis, C-type lectin receptors (i.e., langerin, DC-SIGN, dectin-2, and mannose receptors) are the most prevalent antigen uptake receptors³³. In general, inflammatory mediators induce terminal differentiation of immature DCs into mature

immunogenic DCs. This process is associated with reduced uptake of antigens, impaired antigen processing activity, and downregulation of the expression of antigen uptake receptors. Furthermore, mature DCs showed a strong costimulatory and T cell activation capacity²⁴. We identified that TSLP induced DC maturation while maintaining their ability to take-up allergens to levels comparable with those observed with immature DCs, possibly through the maintenance or upregulation of dectin-2 and other antigen receptor expression. In other words, TSLP-activated DCs are special DCs that exhibit the ability to take-up allergens and strongly stimulate T cell activation.

Consistent with the results of a previous study with cocultures of DCs and autologous CD4⁺ T cells, we found that TSLP alone was a weak inducer of T_H2 responses; however, DCs coprimed with TSLP and allergen synergistically amplified T_H2 responses³⁴. We observed that allergen stimulation and sensitization were important for the TSLP-DC-mediated secretion of T_H2-related cytokines (IL-33 and IL-25) and T_H17-related cytokines (IL-6 and IL-23) and consequently induce allergen-specific T_H2/T_H17 polarization. Two recent studies in mouse models demonstrated that the house dust mite (HDM)-stimulated dectin-2 signaling pathway directly upregulated the production of T_H2-related IL-33 and T_H17-related IL-6 and IL-23 by DCs, leading to the development of the T_H2/T_H17-type allergic response^{4, 6}. In the present study, we found that TSLP stimulation maintained the high-level expression of dectin-2 on mature TSLP-DCs. The neutralization of dectin-2 on human DCs resulted in

the attenuation of the effects of TSLP, such as the allergen stimulation of DCs to produce T_H2/T_H17 -related cytokines and induction of T_H2/T_H17 polarization. These results show that the TSLP-induced allergic T_H2/T_H17 polarization was likely dependent on the dectin-2-mediated allergen sensitization and upregulation in the expression of T_H2/T_H17 -related cytokines.

Our study has several limitations. First, although statistically significant, the differences between the experimental and control groups were small. Whether the small change had any biomedical effect is still unknown, and verification of in vivo and in vitro physiological functions is essential in the future. Second, we did not analyze the effects of TSLP on monocytes using purified TSLPR-positive monocytes, thereby necessitating further studies.

In conclusion, the present study reveals a novel epigenetic mechanism underlying the pathogenesis of AD. TSLP upregulates the expression of FcR γ -related receptors on APCs through pSTAT5, which recruits TET2 for the induction of FCER1G demethylation. The direct TSLP-induced allergic T_H2/T_H17 responses likely depend on the dectin-2-mediated allergen sensitization and upregulation in the expression of IL-25/IL-33/IL-6/IL-23. Thus, we have elucidated the molecular mechanisms underlying the TSLP-mediated direct induction of allergic T_H2/T_H17 responses and suggest dectin-2 as a novel therapeutic target for patients with AD.

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FIG 1. TSLP upregulates the expression of FcR γ and FcR γ -related receptors on human monocytes.

Human normal CD14⁺ monocytes were treated with or without TSLP for 3 days. The mRNA (A) and protein (B-C) levels of FcR γ and FcR γ -related receptors in TSLP-treated monocytes (n = 3) and control cells (n = 3) were assessed with RT-qPCR and western blotting, respectively. Flow cytometry was used to analyze the expression of FcR γ -related receptors on monocytes (D-E). (F) The proportion of dectin-2⁺CD14⁺/CD14⁺ cells was quantified in PBMCs from patients with AD (n = 15) and healthy controls (n = 15) using flow cytometry. Data are expressed as the mean $\pm$ SEM. Paired and unpaired Student's *t*-tests were used to determine statistical significance. **p* < 0.05, ***p* < 0.01, and ****p* < 0.001. RT-qPCR, real-time quantitative polymerase chain reaction .

FIG 2. TSLP induces demethylation of the FCER1G promoter through pSTAT5, which recruits TET2 to the FCER1G promoter on monocytes.

Normal human CD14⁺ monocytes were treated with or without TSLP for 3 days. DNA methylation (A-B) and hydroxylation (C-D) levels in TSLP-treated (n = 3) and control (n = 3) cells at eight CpG sites on the FCER1G promoter were detected with BS and oxBS sequencing. TET1-3 protein (E) and mRNA (F) levels in TSLP-treated monocytes (n = 3) and control cells (n = 3) were determined by western blotting and RT-qPCR, respectively. (G) Total STAT5 and pSTAT5 proteins in TSLP-treated monocytes and control cells were determined with western blotting. (H) Recruitment of TET2 by TSLP-activated pSTAT5 in TSLP-treated monocytes was detected with Co-IP. (I) Binding of pSTAT5 to the FCER1G promoter was assessed in TSLP-treated monocytes using ChIP-qPCR. Data are expressed as the mean $\pm$ SEM. Paired and unpaired Student's *t*-tests were used to determine the significance in sub-figures B, D, F and I. ns, not significant, **p* < 0.05, ***p* < 0.01.

FIG 3. TSLP stimulates MoDC maturation while maintaining their allergen uptake ability likely through the maintenance and upregulation of FcR γ -related receptors.

Immature MoDCs generated from human CD14⁺ monocytes were treated with GM-CSF (20 ng/mL) and IL-4 (20 ng/mL) for 7 days, followed by treatment with medium alone, TSLP (50 ng/mL), or LPS (1 μ g/mL) for 3 days. The expression levels of costimulatory molecules (CD86, CD83, CD40, and OX40L) and FcR γ -related receptors (Fc ϵ R1, CD16, and dectin-2) in MoDCs were detected with flow cytometry (A-C). The uptake of FITC-OVA by MoDCs was assessed using fluorescence microscopy (D-E) and flow cytometry (F-H). Data are expressed as the mean $\pm$ SEM. n = 3. Unpaired Student's *t*-test was used to determine statistical significance. ns, not significant. **p* < 0.05, ***p* < 0.01.

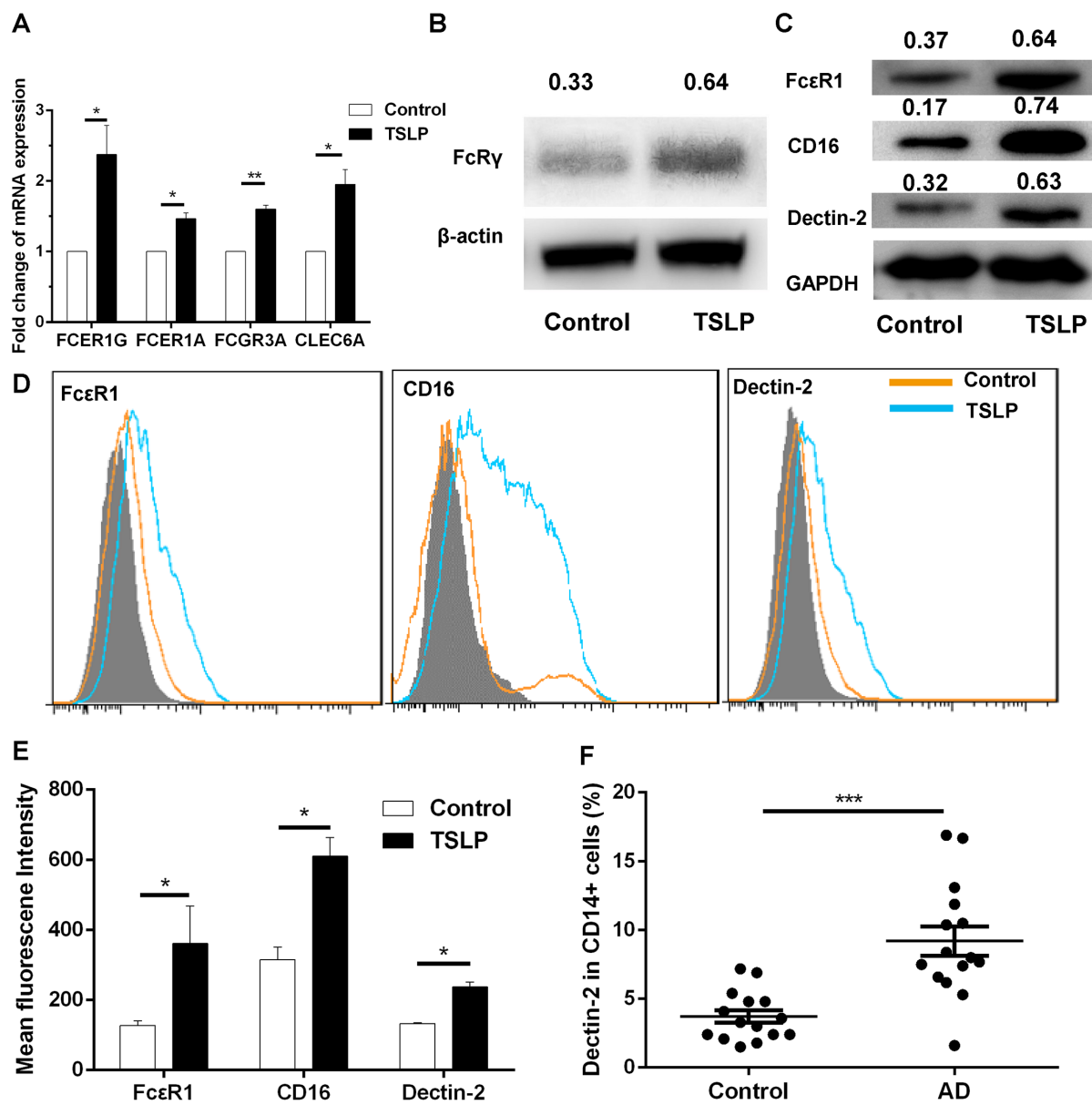
FIG 4. TSLP induces polarization of OVA-treated T_H2/T_H17 cells and secretion of T_H2/T_H17-related cytokines.

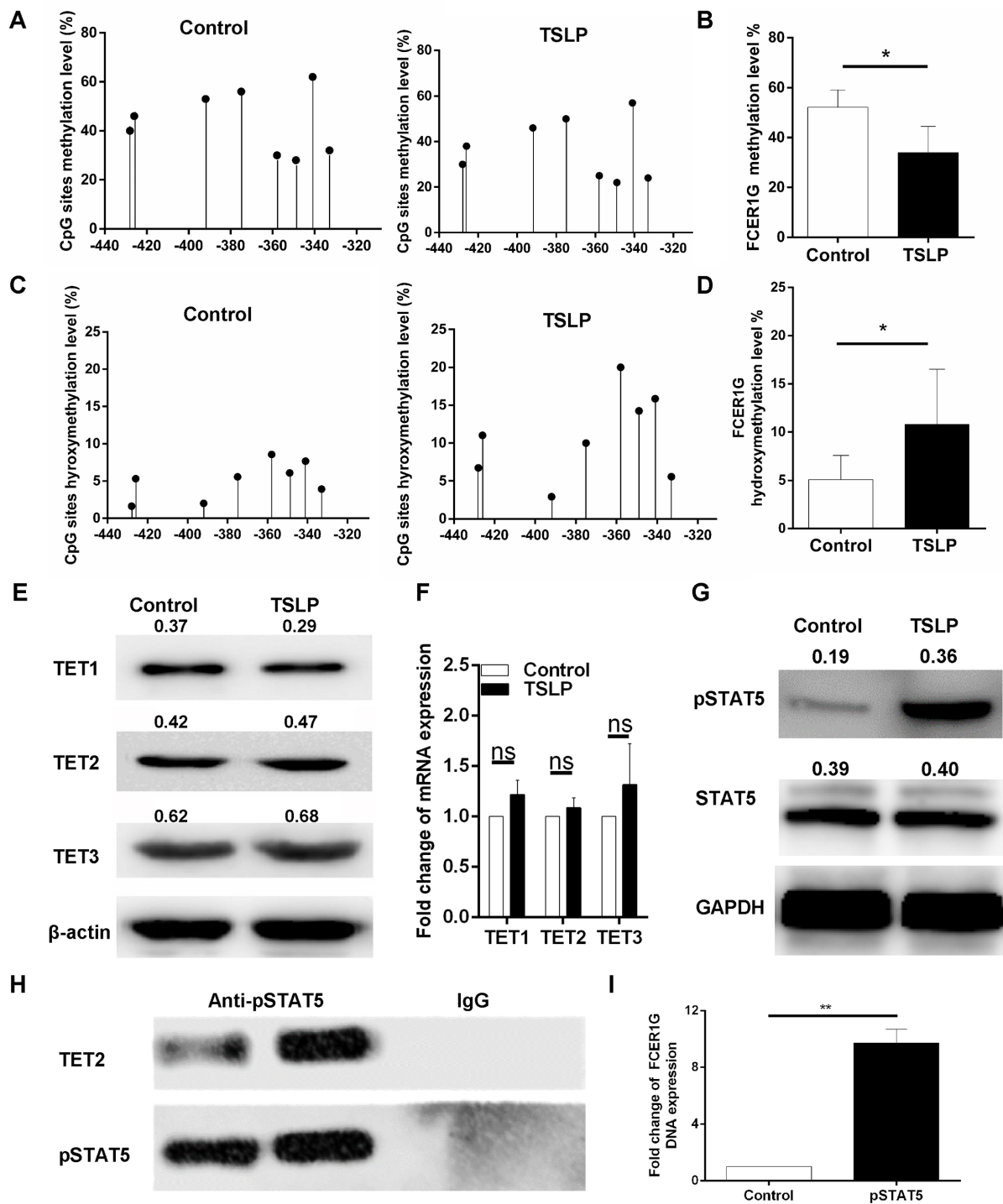
Mature MoDCs treated with either TSLP (50 ng/mL) or LPS (1 µg/mL) and immature MoDCs co-incubated with CD4⁺ naive T cells were treated with or without OVA for 7 days. Levels of the intracellular cytokines IL-4 (A-B) and IL-17A (C-D) in T_H cells were assessed with flow cytometry. (E) Levels of T_H2/T_H17-related cytokines (IL-33, IL-25, IL-10, IL-31, IL-6, IL-23, and TNF-α) in the coculture supernatant were measured using an ultrasensitive multifactor electrochemical luminescence kit. The levels of IL-4 and IL-17A in the coculture supernatant were measured with ELISA kits. Data are expressed as the mean ± SEM, n = 3. Unpaired Student's *t*-test was used to determine statistical significance. ns, not significant.*p < 0.05,**p < 0.01.

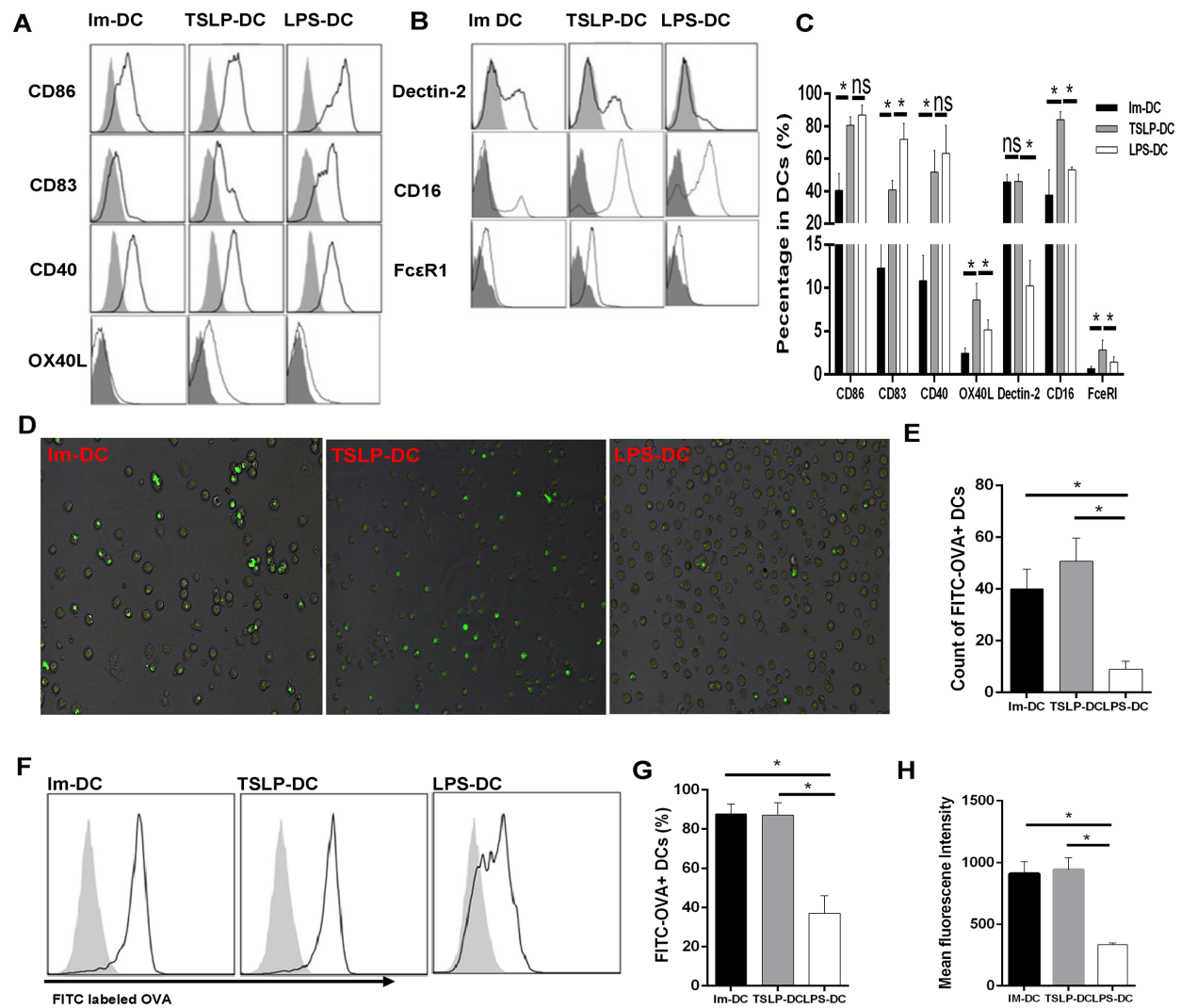
FIG 5. TSLP-induced direct T_H2/T_H17 polarization likely depends on the dec-
tin-2-mediated allergen sensitization and signaling pathways.

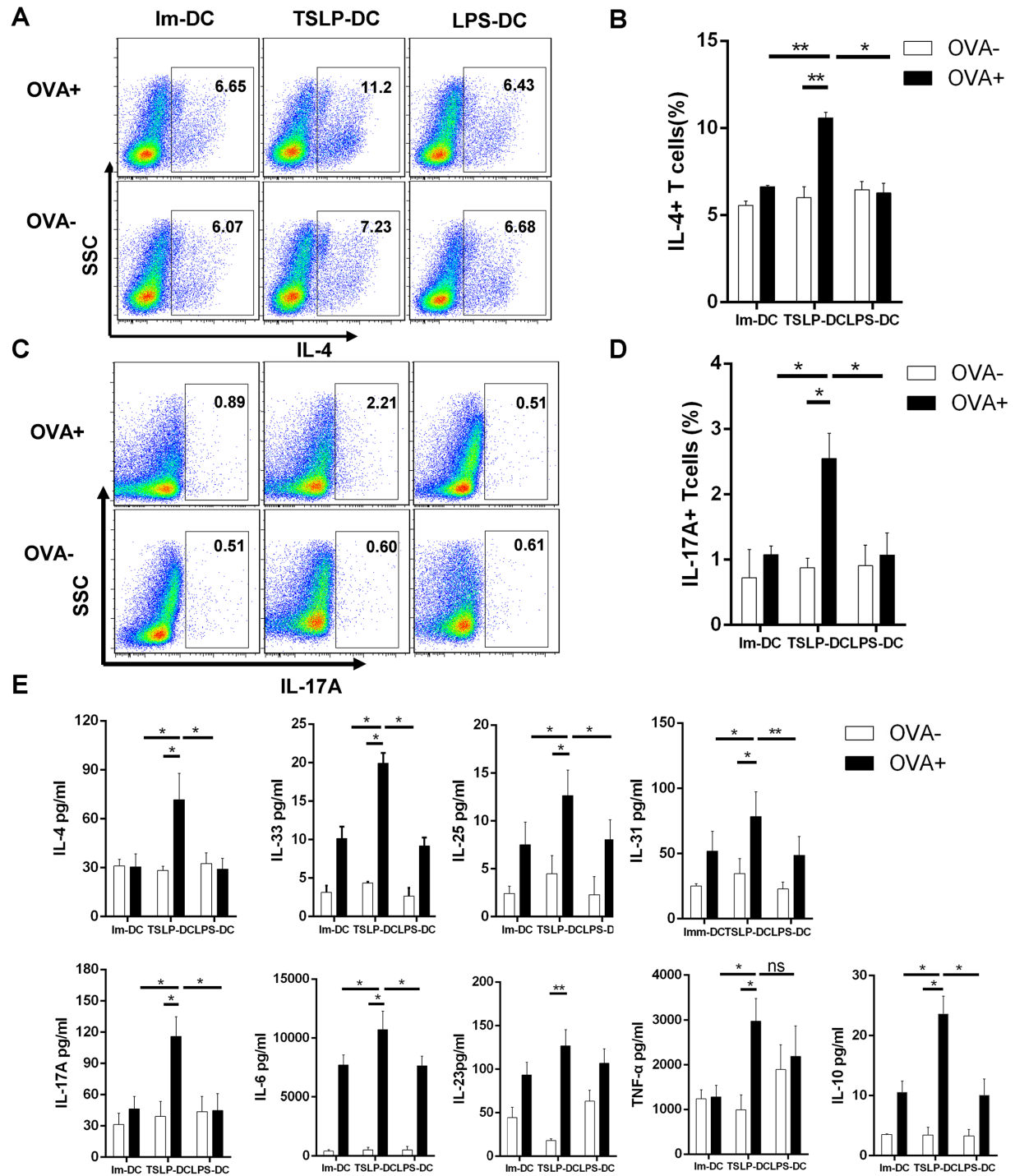
Mature TSLP-DCs and immature MoDCs were incubated with either a neutralizing antibody (1 mg/mL) or IgG control for 1 h, followed by coincubation with naive $CD4^+$ T cells treated with or without OVA for 7 days. Expression levels of intracellular cytokines IL-4 (A-B) and IL-17A (C-D) in T_H cells were assessed by flow cytometry. (E) Levels of T_H2/T_H17 -related cytokines (IL-33, IL-25, IL-10, IL-31, IL-6, IL-23, and TNF- α) in coculture supernatant were measured using an ultrasensitive multifactor electrochemical luminescence kit. Levels of IL-4 and IL-17A in coculture supernatants were measured with ELISA kits. Data are expressed as the mean $\pm$ SEM, n = 3. Unpaired Student's *t*-test was used to determine statistical significance. **p* < 0.05, ***p* < 0.01.

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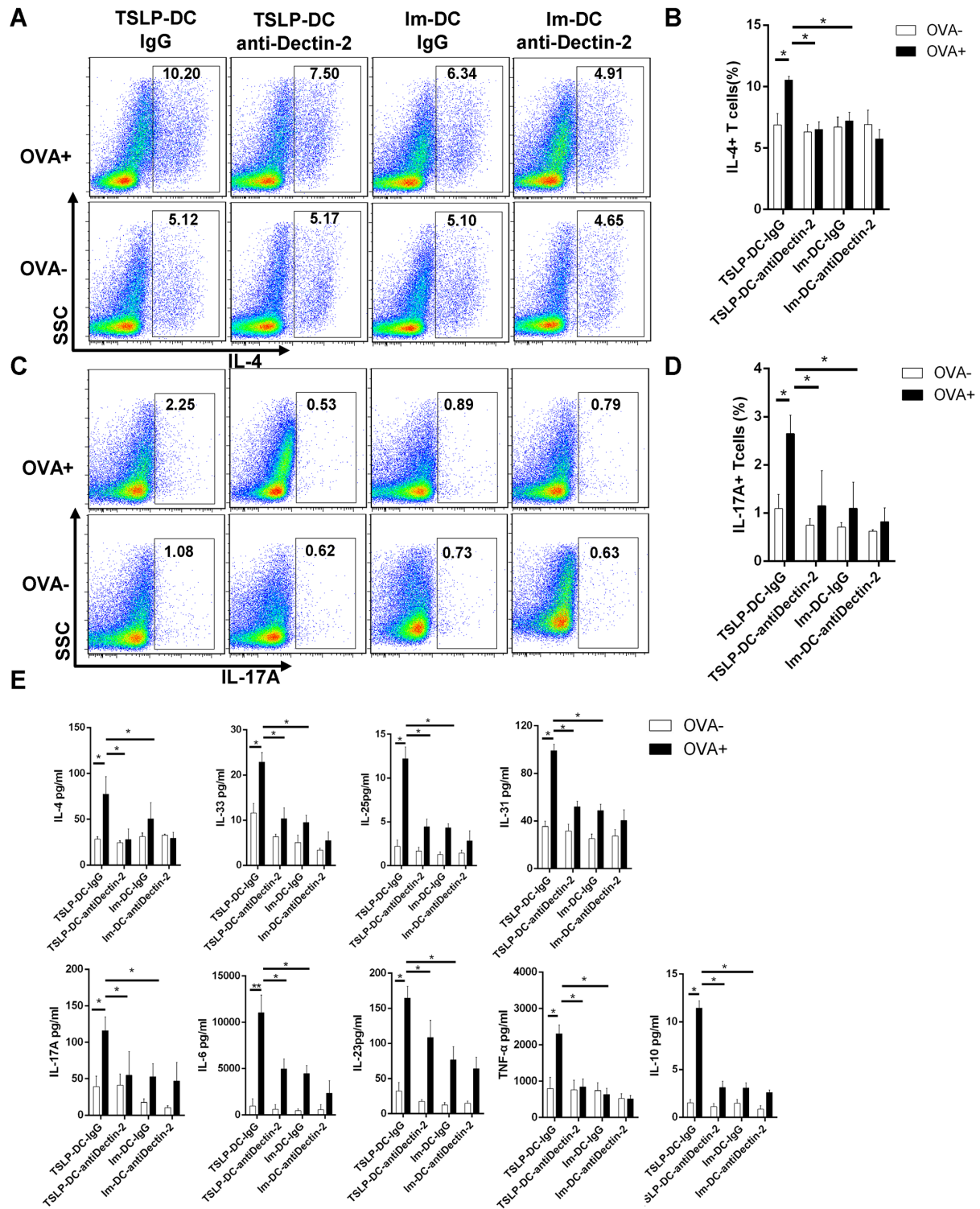


FIG E1. DCs stimulated with both TSLP and LPS show downregulated expression of FcR γ -related receptors.

Immature MoDCs generated from human CD14⁺ monocytes were treated with GM-CSF (20 ng/mL) and IL-4 (20 ng/mL) for 7 days, followed by treatment with medium alone, TSLP (50 ng/mL), or both TSLP (50 ng/mL) and LPS (1 μ g/mL) for 3 days. The expression levels of costimulatory molecules (CD86, CD83, and CD40) and FcR γ -related receptors (Fc ϵ R1, CD16, and dectin-2) in MoDCs were detected with flow cytometry (A-C). Data are expressed as the mean $\pm$ SEM, n = 3. Unpaired Student's *t*-test was used to determine significance. ns, not significant. *p < 0.05, **p < 0.01.

FIG E2. TSLP fails to induce OVA-specific T_H1/T_H22 polarization.

Mature MoDCs treated with either TSLP (50 ng/mL) or LPS (1 µg/mL) and immature MoDCs co-incubated with CD4⁺ naive T cells were treated with or without OVA for 7 days. The expression levels of intracellular cytokines IFN-γ (A-B) and IL-22 (C-D) in T_H cells were measured with flow cytometry. (E) Levels of IFN-γ and IL-22 in coculture supernatants were measured with ELISA kits. Data are expressed as the mean ± SEM, n = 3. Paired Student's *t*-test (B, D, E) was used to determine the significance. ns, not significant.

FIG E3. Effects of neutralization of dectin-2 on the expression of IL-33 and IL-25 and costimulatory molecules in OVA-stimulated TSLP-DCs.

Mature MoDCs treated with TSLP (50 ng/mL) and immature MoDCs were incubated with either a neutralizing antibody (1 mg/mL) or IgG control for 1 h, followed by treatment with OVA for 3 days. The mRNA levels of *IL-25* and *IL-33* in MoDCs were detected with RT-qPCR (A). Expression of costimulatory molecules (CD86, CD83, CD40, and OX40L) was detected with flow cytometry (B-C). Data are expressed as the mean $\pm$ SEM, $n = 3$. Paired Student's *t*-test was used to determine significance. ns, not significant. * $p < 0.05$, ** $p < 0.01$.

Graphical Abstract. Schematic diagram of the molecular mechanisms underlying the TSLP-mediated induction of allergic T_H2/T_H17 polarization.

Direct TSLP induction of allergic T_H2/T_H17 polarization involves several signals as follows: 1) TSLP-activated DCs (TSLP-DCs) preferentially express costimulatory factor OX40L without IL-12 production. OX40L strongly primes naive CD4⁺ T cells to differentiate into TNFα⁺⁺IL-10⁻ inflammatory T_H2 cells³²; 2) TSLP-DCs maintain high levels of FcRγ-related receptors such as dectin-2 through p-STAT5, which recruits TET2 to mediate FCER1G demethylation and eventually enhances allergen uptake and sensitization. Allergen-stimulated dectin-2/FcRγ signaling results in the production of the T_H2/T_H17 cytokine milieu (IL-33/IL-25 and IL-6/IL-23) and induction of T_H2/T_H17 polarization. 3) The recruitment of T_H cells requires activation of STAT6 by TSLP for the induction of CCL17 secretion by DCs.

Table E1. Demographics and medications for patients and control subjects

Sample no.	Age/gender	SCORAD	System medications
P1	28/F	37.5	Antihistamine
P2	23/F	31.4	Medical herbs
P3	16/M	20.1	None
P4	13/F	29.9	None
P5	15/M	39.8	Antihistamine
P6	16/F	35.9	None
P7	38/M	34.6	None
P8	19/F	37.2	Antihistamine
P9	25/F	34.2	None
P10	16/F	23.6	None
P11	27/M	33.6	Antihistamine
P12	29/F	32.4	None
P13	26/M	29.5	None
P14	23/M	23.2	None
P15	28/M	34.6	Antihistamine
C1	20/F	-	-
C2	20/M	-	-
C3	28/F	-	-
C4	24/M	-	-
C5	28/F	-	-
C6	21/F	-	-
C7	20/F	-	-
C8	24/F	-	-
C9	34/M	-	-
C10	20/F	-	-
C11	21/M	-	-
C12	23/F	-	-
C13	25/M	-	-
C14	22/F	-	-
C15	27/F	-	-

Table E2. Gene-specific primer sets used in this study

Gene name	Prime name	Sequence (5'-3')
FCER1G	FCER1G-1-F	GCTCTGCTATATCCTGGATACCATC
	FCER1G-1-R	AGTCTCGTAAGTCTCCTGGTTCCTG
	FCER1G-2-F	TTAAATTAAGATTGGGGTAAAGTTG
	FCER1G-2-R	TAACATTACAAACATACACCACCAC
	FCER1G-3-F	GTGGTGGTGTATGTTTGTATGTTAGT
	FCER1G-3-R	CCTAACCCACAAAATAAAAAAAGTTT
	FCER1G-4-F	AGCTGGCCCTACCCCTATAA
	FCER1G-4-R	TTAGCTGGAGTTGGGAATGG
FCER1A	FCER1A -F	CAGTTGAAGACAGTGGAACCTAC
	FCER1A -R	TGACCTGCTGCTGAGTTGAG
FCGR3A	FCGR3A -F	GGAGAGTACAGGTGCCAGACAA
	FCGR3A -R	CCTCAGGTGAATAGGGTCTTCC
CLEC6A	CLEC6A -F	CTGGGCAACATCTTTAGGGAGAGAGG
	CLEC6A -R	GAATGGAACAAAGAGAGGAAGGGTGC
TET1	TET1-F	ACCACTTTTGCTACCGACTTG
	TET1-R	GGCTGTTCTTTCTGTTCTTGC
TET2	TET2-F	GCAGTGCTAATGCCTAATGG
	TET2-R	GAGGTATGCGATGGGTGAGT
TET3	TET3-F	GGACCAGCATAACCTCTACAAT
	TET3-R	TCTCCTCGCTACCAAATCAT
IL-33	IL-33-F	GACTCCTCCGAACACAGAGC
	IL-33-R	CCCAGCTTGAAACACAAGGC
IL-25	IL-25-F	CCAGGTGGTTGCATTCTTGG
	IL-25-R	TGGCTGTAGGTGTGGGTTCC
β-actin	β -actin-F	GCACCACA CCTTCTACAATGAGC
	β -actin-R	GGATAGCACAGCTGGATAGCAAC
GAPDH	GAPDH-F	CTTTGGTATCGTGAAGGACTC
	GAPDH-R	CTTTGGTATCGTGAAGGACTC

Table E3. Antibodies for Flow Cytometric Analysis.

Antibody.	Company	Species	CatalogNo.
FITC anti-CD14	BioLegend	Human	325607
PE anti-Dectin-2	R&D	Human	MAB3114
APC anti-FcεRIα	BioLegend	Human	334622
PE/Cy7anti-CD16	BioLegend	Human	360707
APC anti-FcεRIα	Miltenyi	Human	130-095-980
PE anti-CD40	BioLegend	Human	313006
PE/Cy7anti-CD86	BioLegend	Human	305421
PE/Cy7anti-CD83	BioLegend	Human	305326
PE anti-OX40L	BioLegend	Human	326308
PE/Cy7 anti-IL-22	eBiosciences	Human	25-7229-42
APC anti-IL-17A	BioLegend	Human	512334
PerCP-Cy5.5 anti-IFN-γ	eBiosciences	Human	45-7319-42
APC anti-IL-4	BD Biosciences	Human	560671
PerCP/Cy5.5 Mouse IgG1, κ Isotype Ctrl	BioLegend	Human	400149
PE Mouse IgG1, κ Isotype Control	BD Biosciences	Human	556650
FITC Mouse IgG1κ Isotype Control	eBioscience	Human	11-4714-41
APC Mouse IgG2b, κ Isotype Control	Miltenyi	Human	130-098-890
FITC Mouse IgG1, κ Isotype Ctrl	BioLegend	Human	400110
APC Mouse IgG1, κ Isotype Ctrl	BioLegend	Human	400122
PE/Cy7 Mouse IgG1, κ Isotype Ctrl	BioLegend	Human	400125
PE Mouse IgG1, κ Isotype Ctrl	BioLegend	Human	400112
PE-Cyanine7 Mouse IgG1,κ Isotype Ctrl	eBioscience	Human	25-4714-42
PerCP-Cyanine5.5Mouse IgG1, κ Isotype Ctrl	eBioscience	Human	45-4714-82
PE Mouse IgG1, κ Isotype Ctrl	BD Biosciences	Human	400114

