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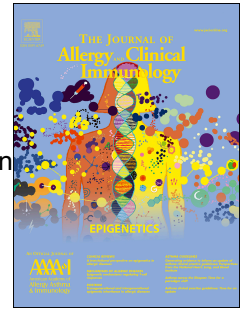
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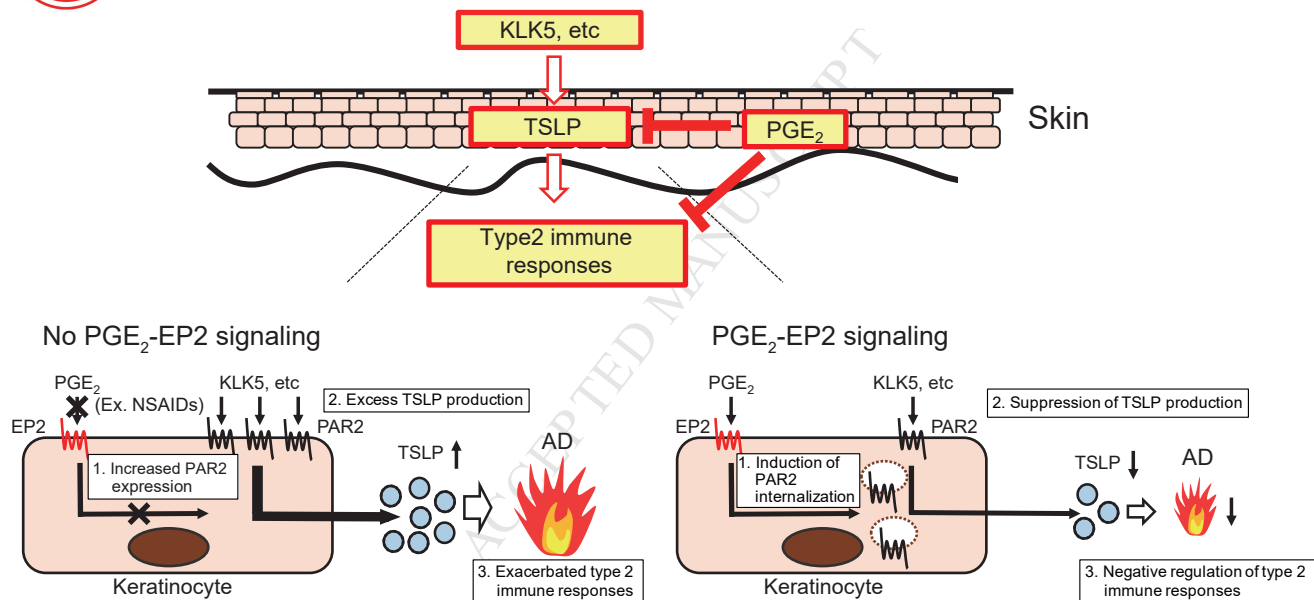
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AD, Atopic dermatitis; **KLK5**, Serine protease kallikrein 5; **NSAIDs**, Non-Steroidal Anti-Inflammatory Drugs; **PAR2**, Protease-activated receptor 2; **PGE₂**, Prostaglandin E₂; **TSLP**, Thymic stromal lymphopoietin

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Declarations of interest: none

Abstract

Background: Atopic dermatitis (AD) is a common, chronic inflammatory skin disease of type 2 immunity. Keratinocyte-derived cytokines, including thymic stromal lymphopoietin (TSLP) and IL-33, are considered to induce the development of AD. Production of prostanoids, a family of lipid mediators, is elevated in AD lesions. However, their physiological functions remain to be clarified.

Objectives: To elucidate the functions of prostanoids in the development of AD.

Methods: The roles of prostanoids were investigated in a mouse model of AD induced by repeated application of hapten, and PAM212, a keratinocyte cell line.

Results: Application of indomethacin, which blocks the synthesis of prostanoids, leads to enhanced TSLP and IL-33 production in the skin, increased serum IgE level, and exacerbation of the skin inflammation in this AD model. The skin inflammation was attenuated in TSLP receptor-deficient mice, but not in IL-33-deficient mice, and the indomethacin-enhanced type 2 immune responses were abolished in TSLP receptor-deficient mice. Indomethacin increased protease-activated receptor 2 (PAR2)-mediated TSLP production in keratinocytes *in vitro*, and prostaglandin (PG) E₂ reversed the increase of TSLP through its receptor EP2 by downregulating the surface expression of PAR2. Administration of an EP2 agonist cancelled the indomethacin-enhanced TSLP production and type 2 immune responses in the skin, while an EP2 antagonist caused an enhancement of TSLP production and type 2 immune responses in the skin.

Conclusion: PGE₂-EP2 signaling negatively regulates murine AD-like skin inflammation by suppressing TSLP expression.

Clinical implications: Stimulation of the PGE₂-EP2 pathway can be a possible novel

therapeutic strategy for the treatment of AD.

Capsule Summary

PGE₂-EP2 signaling inhibits TSLP production in the skin and acts as an endogenous regulator in type 2 immune responses in a mouse model of AD.

Key words

Prostaglandin E₂, EP2, Thymic stromal lymphopoietin, IL-33, Protease-activated receptor 2

Abbreviations

Ab: Antibody

AD: Atopic dermatitis

BM: Bone marrow

BMC: Bone marrow chimeras

cAMP: Cyclic AMP

CCL: Chemokine (C-C motif) ligand

COX: Cyclooxygenase

CRTH2: Chemoattractant receptor homologous-molecule expressed on Th2 cells

DP: Prostaglandin D₂ receptor

EP: Prostaglandin E₂ receptor

FP: Prostaglandin F₂ receptor

IL: Interleukin

ILC2: Innate lymphoid cells type 2

IP: Prostaglandin I₂ receptor

KLK5: Serine protease kallikrein 5

mAb: Monoclonal antibody

PAR2: Protease-activated receptor 2

PG: Prostaglandin

PKA: Protein kinase A

Th2: T helper 2

TP: Thromboxane A₂ receptor

TSLP: Thymic stromal lymphopoietin

TSLPR: Thymic stromal lymphopoietin receptor

Tx: Thromboxane

WT: Wild-type

Introduction

Atopic dermatitis (AD) is a chronic inflammatory skin disease that affects approximately 2-10% of adults in industrialized countries.¹ Clinical symptoms include dry, pruritic eczematous dermatitis.² Because patients with AD exhibit high serum IgE levels and an elevation of type 2 cytokines, including interleukin (IL)-4, IL-5 and IL-13, in the lesional skin,³ AD is considered an inflammatory condition mediated by type 2 immune responses. Indeed, an antibody against the IL-4 receptor, which inhibits IL-4 and IL-13 signaling, exerts therapeutic effects on AD.⁴ For the induction of type 2 immunity, several types of cells, such as T helper 2 (Th2) cells, innate lymphoid cells type 2 (ILC2) and basophils, produce type 2 cytokines.³ Keratinocyte-derived cytokines, especially thymic stromal lymphopoietin (TSLP) and IL-33, play pivotal roles in the induction and activation of these type 2 immune cells.⁵ TSLP is induced by various external stimuli, such as physical trauma and proteases.^{6, 7} For example, a serine protease kallikrein 5 (KLK5) activates protease-activated receptor 2 (PAR2) signaling in keratinocytes and induces TSLP, leading to the development of AD-like skin inflammation in mice.^{8, 9, 10} Likewise, skin-specific overexpression of IL-33 induces an AD-like inflammation in mice.¹¹ Based upon the above pathogenesis, drugs targeting these cytokine cascades are in clinical trials for the treatment of AD.^{12, 13}

Prostanoids, including prostaglandin (PG)D₂, PGE₂, PGF_{2α}, PGI₂, and thromboxane (TX)A₂, are metabolites of arachidonic acid released from membrane phospholipids, and are produced by the sequential reaction of cyclooxygenase (COX) and respective synthases in various pathophysiological conditions.¹⁴ Each prostanoid has its cognate receptor: two subtypes of the PGD receptor (DP and chemoattractant receptor

homologous-molecule expressed on Th2 cells (CRTH2)), four subtypes of the PGE receptor (EP1, EP2, EP3, and EP4), the PGF receptor (FP), the PGI receptor (IP), and the TXA receptor (TP).¹⁴ Acting in these receptors expressed on target cells, prostanoids exert a variety of pathophysiological functions as lipid mediators. The effects of receptor stimulation vary in a context-dependent manner; the stimulation sometimes results in opposing effects, such as excitatory and inhibitory outcomes, depending on receptor types and depending on the context even in the same receptor. Therefore, to understand the physiological role of prostanoids, it is essential to define the context and analyze the function of each receptor separately.

In AD lesions, an elevated levels of prostanoids, such as PGD₂ and PGE₂, have been reported.¹⁵ To date, however, the functions of prostanoids in AD has not been investigated extensively, partly because the effect of COX inhibitors, which block the synthesis of prostanoids, has been controversial in the treatment of AD. However, the recent development of agonists/antagonists for each prostanoid receptor and mice deficient in individual receptor has enabled us to dissect the role of each prostanoid receptor *in vivo* and has revealed unexpected, multiple functions of prostanoids in cutaneous immunity.¹⁶

In this study, we have investigated the role of prostanoids in the development of AD, using a mouse model of AD induced by repeated hapten application, and have identified that a blockade of PGE₂-EP2 signaling increases TSLP production in the skin and enhances type 2 immune responses. Our results suggest that PGE₂-EP2 signaling in the skin functions as an endogenous negative regulator in AD-like skin inflammation.

Methods

Animals

Female BALB/c mice were purchased from Japan SLC (Hamamatsu, Japan). TSLPR-deficient mice (BALB/c background)¹⁷ and IL-33-deficient mice¹⁸ were kindly provided by Dr. Steven Ziegler and Dr. Tomohiro Yoshimoto, respectively. All experiments were conducted on 8-12-week-old mice.

Reagents

Indomethacin was purchased from Sigma-Aldrich (St. Louis, MO). Alexa Fluor 546-conjugated anti-mouse IgG antibody (Ab) was purchased from Invitrogen (Carlsbad, CA). A rabbit anti-mouse PAR2 antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). A PAR2 agonist (SLIGRL-NH₂) was purchased from Abcam (Cambridge, MA). Oxazolone was purchased from Wako Pure Chemical Industries (Osaka, Japan). FITC-conjugated anti-CD45 monoclonal Ab (mAb), PE-conjugated anti-IL-4 mAb, and PE-Cy7-conjugated anti-TCR β mAb were purchased from eBioscience (San Diego, CA). PGD₂, PGE₂, PGF₂ α , PGI₂, and a TxA₂ agonist (I-BOP)¹⁴ were purchased from Cayman Chemical (Ann Arbor, MI). A Protein kinase A (PKA) inhibitor H-89 was purchased from Sigma-Aldrich. An EP1 antagonist (ONO-8713)¹⁹, an EP2 antagonist (ONO-AE8-116)²⁰, an EP3 antagonist (ONO-AE3-240)²¹, an EP4 antagonist (ONO-AE3-208)²², and an EP2 agonist (ONO-AE1-259-01)^{23, 24} were kindly provided from Ono Pharmaceuticals (Osaka, Japan). The specificity of each EP receptor agonist/antagonist is described in each reference.

An AD model by repeated hapten elicitation

Mice were sensitized and were repeatedly elicited on the same skin site with hapten, as previously described²⁵ with some modification. Briefly, BALB/c mice were sensitized by topical application of 20 μ l of 1% oxazolone in ethanol to both ears 30 min after topical application of 0.2 mg/ml of indomethacin or vehicle (acetone). Seven days after the sensitization, the same treatments were repeatedly applied to the ears in 2 d intervals for a total of 8 times. Ear thickness was measured for each mouse before hapten application and 1, 6, and 24 h after hapten application using a thickness gauge (Teclok, Nagano, Japan). For EP2 agonist/antagonist treatment, 20 μ l of an EP2 agonist (ONO-AE1-259-01) (200 μ M) and an EP2 antagonist (ONO-AE8-116) (500 μ M) in ethanol was topically applied to both ears 30 min before the application of oxazolone. Ethanol was used as a vehicle.

Histology and immunostaining

For histological examination, ears were excised at 24 h after the final elicitation fixed in 10% formaldehyde, and embedded in paraffin. Sections of 5 μ m thickness were prepared and stained with hematoxylin and eosin. For the immunostaining of PAR2 in PAM212 cells, cells were plated on coated coverslips, fixed for 15 min with 3.7% formalin (Wako Pure Chemical Industries) and permeabilized with 0.1% Triton-X (Sigma-Aldrich) in PBS for 7 min at room temperature. Next, slides were incubated with anti-mouse PAR2 Ab for 1 h and then stained with Alexa Fluor 546-conjugated anti-mouse IgG (Invitrogen) for 30 min at room temperature.

Flow cytometry

One piece of skin was taken by 8-mm punch biopsy from the center of each ear skin, and

the whole sample was used for flow cytometry analysis. For the preparation of single cell suspensions from skin, ears were removed 24 h after final elicitation, split into dorsal and ventral halves, and the cartilage was removed. The ear skin was incubated for 30 min in cRPMI (RPMI 1640, Sigma-Aldrich, containing 10% heat-inactivated FCS (Invitrogen), 50 μ M 2-mercaptoethanol, 2 mM L-glutamine, 25 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid, 1 mM nonessential amino acids, 1 mM sodium pyruvate, 100 units/ml penicillin, and 100 μ g/ml streptomycin) containing 2 mg/ml collagenase II (Worthington Biochemical., Freehold, NJ) and 100 μ g/ml DNase I (Sigma-Aldrich). The cell suspensions were filtered with a 40- μ m cell strainer and stained with the indicated Abs. These cells were subjected to flow cytometry (Fortessa, BD Biosciences), and the data were analyzed with FlowJo software (TreeStar, San Carlos, CA).

Keratinocyte culture

For keratinocyte culture, PAM212 cells, a spontaneously transformed cell line derived from BALB/c keratinocytes,²⁶ and normal human epidermal keratinocyte (Kurabo Industries, Osaka, Japan) were used. Cells were seeded onto 24-well plates at a density of 1×10^5 cells per well, cultured in cDMEM (DMEM containing 10% FCS, 10 mM HEPES, 2 mM L-glutamine, 100 units/ml penicillin, and 100 μ g/ml streptomycin) (Invitrogen)) at 37°C under 5% CO₂ with the indicated combination of reagents for 24 h, and the supernatant was extracted for the measurement of TSLP by ELISA. The concentration of each reagent is as follows: PAR2 agonist (SLIGRL-NH₂) (100 μ M), indomethacin (1 μ M), prostanoids (PGD₂, PGE₂, PGF₂ α , PGI₂) (10 μ M), TP agonist (I-BOP) (10 μ M), EP1 antagonist (ONO-8713) (20 μ M), EP2 antagonist (ONO-AE8-116) (20 μ M), an EP3

antagonist (ONO-AE3-240) (20 μ M), an EP4 antagonists (ONO-AE3-208) or EP2 agonist (ONO-AE1-259-01) (20 μ M).

ELISA

Total serum IgE and TSLP in culture supernatant were measured by using a mouse IgE ELISA kit (Bethyl Laboratories, Montgomery, TX) and a mouse and a human TSLP Quantikine ELISA Kit (R&D Systems, Minneapolis, MN), respectively, in accordance with the manufacturer's protocol. Absorbance was measured at a wavelength of 450 nm with a microplate reader (Bio-Rad, Hercules, CA).

Quantitative real-time PCR analysis

Ear skin was taken 24 h after the 1st elicitation, and total RNA from the skin was extracted using the Trizol RNA extraction kit (Invitrogen, San Diego, CA). cDNA was reverse transcribed from the total RNA samples using the Prime Script RT reagent kit (Takara Bio, Otsu, Japan). Quantitative real-time PCR was performed by monitoring the synthesis of dsDNA during the various PCR cycles using SYBR Green I (Takara Bio) and a Light Cycler real-time PCR apparatus (Roche, Basel, Switzerland) according to the manufacturer's instructions. The primer sequences were as follows: *Klk5*, 5'-ATG GGC AAT GGC TAC CCT G-3' (forward), 5'-GTT CGG TTC CAG AGG GGT T-3' (reverse); *Tslp*, 5'-TTC ACT CCC CGA CAA AAC ATT T-3' (forward), 5'-AGT CCT CGA TTT GCT CGA ACT-3' (reverse); *Ccl17*, 5'-TAC CAT GAG GTC ACT TCA GAT GC-3' (forward), 5'-GCA CTC TCG GCC TAC ATT GG-3' (reverse); *Ccl22*, 5'-AGG TCC CTA TGG TGC CAA TGT-3' (forward), 5'-CGG CAG GAT TTT GAG GTC CA-3' (reverse); *Il33*, 5'-TCC AAC TCC AAG ATT TCC CCG-3' (forward),

5'-CAT GCA GTA GAC ATG GCA GAA-3' (reverse); *Ptges*, 5'- GGA TGC GCT GAA ACG TGG A-3' (forward), 5'-CAG GAA TGA GTA CAC GAA GCC-3' (reverse); *Gapdh*, 5'-AGG TCG GTG TGA ACG GAT TTG-3' (forward), 5'-GGG GTC GTT GAT GGC AAC A-3' (reverse). All primers were obtained from Greiner Japan (Tokyo, Japan). For each sample, triplicate test reactions and a control reaction lacking reverse transcriptase were analyzed for expression of the genes, and results were normalized to those of the housekeeping *Gapdh* mRNA.

Immunoblotting

Phosphorylated PAR2 was detected by Phos-tag SDS-PAGE (Wako Pure Chemical Industries) according to the manufacturer's protocol with some modifications. Briefly, cells were lysed directly in their 10-cm plates with ice cold lysis buffer. Proteins from the total cell lysate were separated by 6% SDS-PAGE gel containing Phos-tag, transferred to a nitrocellulose membrane, and blotted for phosphorylated PAR2 using anti-mouse PAR2 mAb.

Generation of bone marrow chimera mice

To generate bone marrow chimera mice, recipient WT BALB/c underwent 9 Gy total body irradiation and were injected with bone marrow cells (1×10^6 cells/body) from WT or TSLPR-deficient mice (BALB/c background) intravenously through the tail vein.

Microarray data analysis

Expression data for 14 atopic dermatitis patients were obtained from a public data set deposited in the National Center for Biotechnology Information (NCBI) Gene Expression

Omnibus (GEO) database (GEO accession no. GSE120721). Patient and sample information were described previously.²⁷

Statistical analysis

All statistical analyses were carried out using GraphPad Prism 6.05 (GraphPad Prism Software Inc., La Jolla, CA). Student's *t*-test was used to calculate significant differences between two groups. Statistical significance between multiple groups was analyzed by using the Tukey-Kramer multiple comparisons test. Statistical correlation was evaluated by a nonparametric Spearman correlation test. All *P*-values less than 0.05 were considered statistically significant.

Study approval

The murine studies were conducted with the approval of and in accordance with the Guidelines for Animal Experiments of the Kyoto University Graduate School of Medicine. The human study protocol was approved by the Institutional Review Boards of the Kyoto University Graduate School of Medicine.

Results

A blockade of prostanoid synthesis exacerbates skin inflammation in a mouse model of AD

To investigate prostanoids in the development of AD-like skin inflammation, we first tested the effect of a COX inhibitor, indomethacin, on skin inflammation in a mouse model of AD. We used an AD model of repeated hapten applications, which results in a shift from Th1-mediated cutaneous inflammation to Th2-mediated cutaneous inflammation, mimicking human AD.^{25, 28} Oxazolone was used as a hapten and repeatedly applied to the ear skin with or without topical indomethacin application; the ear swelling response, the number of IL-4 producing cells in the skin, and serum IgE levels were examined as the indicators of a type 2 immune response. A single elicitation with hapten induces contact hypersensitivity, which is a representative of Th1-type delayed-type hypersensitivity. The initial Th1-type delayed-type hypersensitivity, as determined by the ear swelling response at the 24 h time point, was not significantly modified by indomethacin application. However, the ear swelling response after the 3rd hapten application was significantly enhanced by indomethacin treatment, which was repeatedly observed until the final hapten application (**Fig 1A**, and **see Fig E1 in the Online repository**). Consistently, histological examination revealed increased epithelial hyperplasia, dermal edema and infiltration of inflammatory cells, including eosinophils and lymphocytes, into the dermis in indomethacin-treated mice (**Fig 1B**). The serum IgE levels and the frequency and the number of IL-4-producing CD45⁺TCR β ⁺ cells in the skin were also significantly enhanced by indomethacin treatment (**Fig 1C-E**, **see Fig E2 and E3 in the Online repository**). These findings suggest the existence of endogenous prostanoids that play regulatory roles in the development of type 2 immune responses in

the skin.

Because TSLP and IL-33 are suggested to play pivotal roles in the induction of a type 2 immune response in the skin,^{7, 29} we next examined the expression levels of these cytokines in the skin. After the 1st elicitation, TSLP and IL-33, and their downstream molecules, such as Th2 chemokines CCL17 and CCL22, were induced in the skin. The mRNA levels of these cytokines were further promoted by topical indomethacin treatment (**Fig 2**). On the other hand, the mRNA level of KLK5, which is known to induce TSLP expression in keratinocytes, was similar in both groups. These results suggest that prostanoids regulate the production of TSLP and IL-33, and suppress the induction of type 2 immune responses in the skin.

Prostanoids facilitate type 2 immune responses through the TSLP receptor signaling pathway

We next examined whether TSLP and IL-33 are involved in the development of type 2 immunity in this AD model using TSLP receptor-deficient (TSLPR^{-/-})¹⁷ and IL-33-deficient (IL-33^{-/-}) mice.¹⁸ The ear swelling response after repeated hapten application in TSLPR^{-/-} mice was significantly less than that of wild-type (WT) mice (**see Fig E4 in the Online repository**), which is consistent with a previous report.³⁰ On the other hand, IL-33^{-/-} mice exhibited an equivalent ear swelling response compared with WT mice (**see Fig E4 in the Online repository**). These results suggest that TSLP, rather than IL-33, mediates the induction of type 2 immune responses in this AD model.

TSLP is abundantly produced by keratinocytes in the skin, especially in human and mouse AD,^{7, 31} and acts on inflammatory cells such as Th2 cells, basophils, Langerhans cells, and ILC2 to promote type 2 immunity.³¹⁻³⁵ To examine whether enhanced type 2

immune responses induced by the loss of prostanoids are mediated through TSLPR signaling, we created bone marrow (BM) chimeras of irradiated WT mice reconstituted with either WT- or TSLPR^{-/-}-BM and tested the effect of indomethacin on the type 2 immune response in these chimeras. The type 1 immune response induced by a single elicitation with hapten was similar in both groups (**Fig 3A, left**); however, the ear swelling of the type 2 immune response induced by repeated elicitation with hapten was almost completely abrogated in chimeras reconstituted with TSLPR^{-/-}-BM, in which bone marrow-derived immune cells, such as Th2 cells and basophils, lack TSLPR (**Fig 3A, right**). Notably, the exacerbation by indomethacin was also diminished in chimeras reconstituted with TSLPR^{-/-}-BM. Consistently, the increase of serum total IgE levels and the number and the frequency of IL-4-producing CD45⁺TCR β ⁺ cells in the skin after indomethacin treatment were abolished in the chimeras (**Fig 3B, C, see Fig E5 in the Online repository**). These findings suggest that the enhanced type 2 immune responses induced by the loss of prostanoids depend on TSLP-TSLPR-signaling in radiosensitive immune cells.

PGE₂-EP2 signaling plays regulatory roles in TSLP production in keratinocytes

Based on the above results, we hypothesized that some prostanoids could suppress TSLP production in the skin. To test this hypothesis, we evaluated the effect of indomethacin or prostanoids on TSLP production in keratinocytes using PAM212 cells.²⁶ We stimulated PAM212 cells with a PAR2 agonist (SLIGRL-NH₂), a key inducer of TSLP,⁸ and examined the effect of indomethacin and prostanoids (PGD₂, PGE₂, PGF₂ α , and PGI₂, and a TXA₂ receptor agonist, I-BOP) on the production of TSLP. The TSLP production in PAM212 cells induced by the PAR2 agonist was enhanced by the addition of

indomethacin (**Fig 4A**), while it was markedly reduced by the addition of PGE₂ but not by other prostanoids (**Fig 4A**). On the contrary, PGE₂ has no additional inhibitory effect on TSLP production in the absence of indomethacin. These findings suggest that endogenous PGE₂ produced by keratinocytes regulates the induction of TSLP in keratinocytes.

PGE₂ exerts its physiological roles through four types of receptor (EP1, EP2, EP3 and EP4).¹⁴ To identify which PGE₂ receptor signaling is involved in the indomethacin-induced TSLP production in keratinocytes, we next tested antagonists against each PGE receptor on TSLP production. Among these antagonists, only an EP2 antagonist²⁰ mimicked the effect of indomethacin (**Fig 4B**). Conversely, an EP2 agonist^{23, 24} completely abolished the enhancing effect of indomethacin on TSLP production (**Fig 4B**). In addition, the inhibitory effects of PGE₂ and EP2 agonist on TSLP production were cancelled in the presence of EP2 antagonist. Furthermore, EP2 agonist has no additional inhibitory effect on TSLP production in the absence of indomethacin. These results indicate that the endogenous PGE₂-EP2 signaling pathway regulates PAR2-mediated TSLP production in keratinocytes.

PGE₂-EP2 signaling induces internalization of PAR2 on keratinocytes

The expression of PAR2 on the cell surface is critical to elicit its functions via PAR2.^{36, 37} Therefore, we examined the effect of an EP2 agonist on the surface expression of PAR2 in PAM212 cells. Flow cytometric analysis revealed that the surface expression, not the total amount of PAR2, was significantly downregulated in cells treated with the EP2 agonist (see **Fig E6 A-C in the Online repository**). Because the PGE₂-EP2 signaling pathway exerts its function through the activation of the cyclic AMP (cAMP)-PKA

pathway, we examined the effect of PKA inhibitor H-89 on the effect of the EP2 agonist. H-89 completely abrogated the inhibitory effect of the EP2 agonist on PAR2 expression (see Fig E6 A-C in the Online repository). Moreover, the EP2 agonist promoted PAR2 phosphorylation (see Fig E6D in the Online repository), which is necessary to induce clathrin-dependent receptor internalization of PAR2.³⁸ Consistently, immunohistochemical analysis revealed that the EP2 agonist increased the dot-like subcellular signals of PAR2, suggesting the presence of intracytoplasmic vesicles and/or Golgi pool containing PAR2,⁸ which were decreased by the further addition of H-89 (see Fig E6E in the Online repository). These results suggest that the PGE₂-EP2 signaling pathway induces PAR2 internalization in a cAMP-PKA-dependent manner.

PGE₂-EP2 signaling regulates the development of mouse AD-like skin inflammation *in vivo*

Having identified the effect of PGE₂-EP2 signaling on PAR2-mediated TSLP production in keratinocytes *in vitro*, we next evaluated the involvement of PGE₂-EP2 signaling in TSLP expression in the skin and the subsequent development of type 2 immune responses after repeated hapten elicitation. Application of hapten markedly induced the gene expression levels of PGE synthase (*Ptges*) in ear skin (see Fig E7 in the Online repository). Mice topically treated with an EP2 antagonist induced higher mRNA expression levels of TSLP and Th2 chemokines (CCL17 and CCL22) in the skin, while topical application of an EP2 agonist abolished the increase of mRNA expression levels induced by indomethacin (Fig 5A). Consistently, ear swelling, the number of IL-4-producing CD45⁺TCRβ⁺ cells in the skin, and total serum IgE levels were increased by an EP2 antagonist treatment, and an EP2 agonist reversed the increase induced by

indomethacin (**Fig 5B-D**) On the contrary, mice treated with the EP2 agonist without indomethacin did not exhibit suppressed phenotypes in all of the above parameters compared with vehicle-treated mice (**Fig 5A-D**, and **see Fig E8A in the Online repository**), suggesting that endogenously produced PGE₂ have exerted its maximum inhibitory effects through EP2. Furthermore, the number of IFN- γ -producing CD45⁺TCR β ⁺ cells was unchanged by indomethacin or an EP2 agonist/antagonist treatment (**see Fig E8B and C in the Online repository**), suggesting that an interference with PGE₂-EP2 axis does not affect Th1/Th2 balance in our model.

Finally, to ensure the human relevance, we examined the mRNA expressions of *TSLP*, *PTGER2* (a human EP2 receptor), and *PTGES* in human AD lesions, using a public microarray data set.²⁷ Consistent with our results, a negative correlation was observed between *TSLP* and *PTGER2* mRNA levels (**see Fig E9A in the Online Repository**), suggesting that EP2 may regulate TSLP expression also in human AD. Furthermore, we confirmed that indomethacin and EP2 antagonist facilitated PAR2 agonist-induced TSLP production by normal human epidermal keratinocytes, and the effects of indomethacin was completely cancelled by the EP2 agonist (**see Fig E9B in the Online Repository**).

Taken together, these results suggest that the PGE₂-EP2 signaling pathway is an endogenous regulator for TSLP production in the skin potentially via regulating PAR2 expression and attenuates the development of AD-like skin inflammation.

Discussion

Although an elevated production of PGE₂ in AD lesions has previously been reported,^{15, 39} its physiological functions *in vivo*, especially those on keratinocytes, have been largely unknown. Here, using a mouse model of AD, we showed that a blockade of PGE₂-EP2 signaling leads to the exacerbation of AD-like skin inflammation, an increase in IL-4 producing cells in the skin, and an elevation of serum IgE levels. The enhanced type 2 immune responses induced by the blockade of PGE₂-EP2 signaling were mediated through the upregulation of TSLP in the skin. Stimulation of PGE₂-EP2 signaling downregulated the expression of TSLP in keratinocytes *in vitro*. These results suggest that PGE₂-EP2 signaling acts as an endogenous regulator of AD-like skin inflammation by regulating TSLP expression.

It has been reported that COX-2-deficient mice or mice that have been administered COX-2 inhibitors exhibit enhanced type 2 immune responses in a mouse model of AD, such as elevated serum IgE levels and IL-4 production in the skin,⁴⁰ suggesting the existence of prostanoids that play protective roles in the induction of type 2 immune responses in the skin. However, which prostanoid and how it regulates type 2 immune responses in the skin have remained unclear. Our current results raise the possibility that PGE₂ plays protective roles in type 2 immune responses in the skin through EP2. On the other hand, the proinflammatory actions of PGD₂ in AD-like skin inflammation have been proposed by several previous studies.^{41, 42} For example, mice lacking CRTH2, a PGD₂ receptor, exhibited reduced inflammation in a model of AD.⁴¹ These results together with our findings suggest that prostanoids play both pro- and anti-inflammatory roles in AD, depending on the prostanoids and their receptors. These data may explain why COX inhibitors, which block the synthesis of all prostanoids, do not necessarily

inhibit or exacerbate the development of AD in clinical practice.

To date, the involvement of PGE₂ on cutaneous immune responses has mostly been discussed in the context of T cell differentiation and dendritic cell (DC) function.^{16,43} For instance, PGE₂ facilitates Th1 differentiation through EP1 on T cells.⁴⁴ PGE₂ also acts on cutaneous DCs through EP4 and promotes their migration from the skin to draining lymph nodes, leading to the facilitation of Th1 immune responses.⁴⁵ Such immune-modulatory functions of PGE₂ were exerted through the direct actions of PGE₂ on target immune cells. Our results, on the other hand, indicate that PGE₂ negatively regulates cutaneous immune responses through the regulation of keratinocyte functions via EP2, which subsequently leads to impaired Th2 immune responses. PGE₂ may thus regulate cutaneous immune responses in various sites by independent mechanisms in a context dependent manner.

Various factors, such as cytokines and proteases, have been shown to induce TSLP in the skin,^{6,7} however, the factors inhibiting the expression of TSLP are less understood. We have demonstrated that the PGE₂-EP2 signaling pathway regulates TSLP expression in the skin. Although the precise regulatory mechanisms *in vivo* remain unclear, inducing the internalization and subsequent desensitization of PAR2 is a potential mechanism, based upon our observation that the stimulation of EP2 signaling induced phosphorylation and internalization of PAR2 in a keratinocyte cell line *in vitro*, which is consistent with the previous finding that PGE₂ induces PAR2 internalization in a renal cell line.⁴⁶ The stimulation of PAR2 signaling induces TSLP expression in the skin and the subsequent development of AD-like skin inflammation in mice.⁴⁷ Therefore, we hypothesize that the regulation of PAR2 expression is a potential mechanism by which PGE₂-EP2 signaling regulates TSLP expression.

The TSLP inhibitors for AD are still undergoing clinical trials^{12, 48} and the actual significance of TSLP in human AD remains unclear. However, previous animal studies indicate the importance of TSLP in the induction of AD-like skin inflammation through the activation of Th2 cells and ILC2.^{10, 33, 34} Because our results suggest that PGE₂ works as an endogenous negative regulator for TSLP expression in the skin, a blockade of PGE₂-EP2 signaling by the use of COX inhibitors might break this regulatory system and lead to the upregulation of TSLP, which may cause excess activation of type 2 immune cells in the skin. Furthermore, it has been reported that PGE₂ directly suppresses ILC2 functions through EP2/EP4,⁴⁹ suggesting that regulation of ILC2 functions may also be involved in the regulatory mechanisms of PGE₂-EP2 signaling in our model. In addition, the regulatory roles of the PGE₂-EP2 pathway have been suggested in other allergic disease models, such as asthma⁵⁰ and chronic rhinosinusitis.⁵¹ Therefore, from the perspective of preventing allergies, COX inhibitors, especially topical COX inhibitors, should be used cautiously. In this study, we have identified novel roles of PGE₂-EP2 signaling as an endogenous negative regulator in AD-like skin inflammation in mice. In a certain condition where endogenous PGE₂ is not sufficiently produced, the stimulation of the PGE₂-EP2 pathway may become a possible novel therapeutic strategy for the treatment of AD.

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Figure legend**Fig 1. Exacerbation of type 2 immune responses in the skin by a cyclooxygenase (COX) inhibitor**

(A) Ear thickness change at the first elicitation (left) and the 8th elicitation (right) with or without a COX inhibitor (indomethacin) (B) Histology of ear skin 24 h after the 8th elicitation. The skin sections were stained with hematoxylin and eosin (original magnification $\times 10$, scale bar, 200 μm). The black square in each picture indicates the area of high magnification shown in the lower left corner (original magnification $\times 200$, scale bar, 50 μm). (C) Total IgE level in serum before and after the third elicitation. (D, E) Flow cytometry analysis of IL-4-producing cells in the skin 24 h after the 8th elicitation. Representative flow cytometry plots are shown in (D), and the number of IL-4-producing cells per ear are shown in (E). The numbers in (D) indicate the percentage of IL-4-producing cells in $\text{CD45}^+\text{TCR}\beta^+$ cells. The results are expressed as the mean $\pm$ standard deviation (SD). Each dot represents an individual mouse. *, $P < 0.05$. Data are representative of three independent experiments.

Fig 2. The mRNA expression levels in the skin 24 h after the 1st elicitation.

Expression levels of *Tslp*, *Il33*, *Ccl17*, *Ccl22*, and *Klk5* mRNA were analyzed by quantitative real-time PCR. The results are expressed as the mean $\pm$ SD. Each dot represents an individual mouse. *, $P < 0.05$. Data are representative of three independent experiments.

Fig 3. TSLP receptor signaling mediates the exacerbation of type 2 immune responses by indomethacin

(A) Ear thickness change at the first elicitation (left) and the 8th elicitation (right) in bone marrow chimeras (BMC). WT mice were reconstituted with either WT-BM or TSLPR-deficient-BM. (B) Total IgE in the serum of BMC before and after the third elicitation. (C) The number of IL-4-producing cells in the skin 24 h after the 8th elicitation. The results are expressed as the mean $\pm$ SD. Each dot represents an individual mouse. *, $P < 0.05$. Data are representative of three independent experiments.

Fig 4. PGE₂-EP2 signaling regulates TSLP production in keratinocytes, PAM212

(A) Effect of indomethacin and prostanoids on TSLP production in cultured keratinocytes (PAM212). PAM212 cells were stimulated with the indicated combinations of a PAR2 agonist (100 μ M), indomethacin (1 μ M), prostanoids or agonists for prostanoid receptors (PGD₂, PGE₂, PGF₂ α , PGI₂, and TP agonist (I-BOP), each 10 μ M) for 24 h. The amount of TSLP in culture supernatant was measured by ELISA. (B) Effects of PGE₂ receptor antagonists or EP2 agonist on TSLP production in keratinocytes. PAM212 cells were stimulated with the indicated combinations of PAR2 agonist (100 μ M), indomethacin (1 μ M), EP1 antagonist (ONO-8713) (20 μ M), EP2 antagonist (ONO-AE8-116) (20 μ M), an EP3 antagonist (ONO-AE3-240) (20 μ M), an EP4 antagonists (ONO-AE3-208) or EP2 agonist (ONO-AE1-259-01) (20 μ M) for 24 h. The results are expressed as the mean $\pm$ SD. Each dot represents an individual sample. *, $P < 0.05$. Data are representative of three independent experiments.

Fig 5. PGE₂-EP2 signaling regulates type 2 immune responses in the skin

(A) The mRNA expression levels of *Tslp*, *Ccl17*, and *Ccl22* in the skin 24 h after the 1st elicitation with the indicated combination of indomethacin, EP2 antagonist, or EP2

agonist. (B) The ear swelling response after the 8th elicitation. (C) Total IgE levels in serum before and after the 3rd elicitation. (D) The number of IL-4-producing cells in the skin after the 8th elicitation. The results are expressed as the mean $\pm$ SD. Each dot represents an individual mouse. *, $P < 0.05$. Data are representative of three independent experiments.

Fig 1

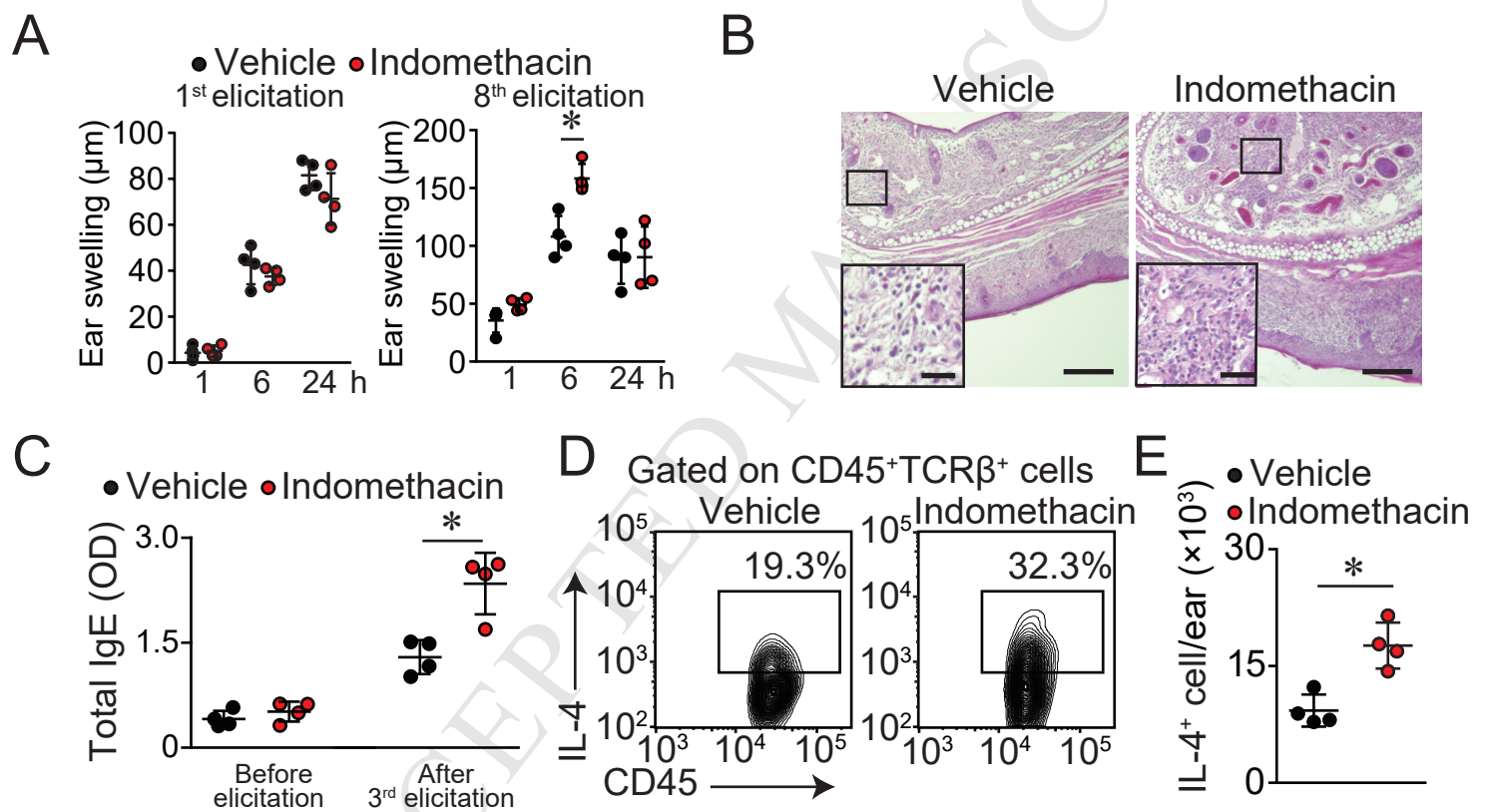


Fig 2

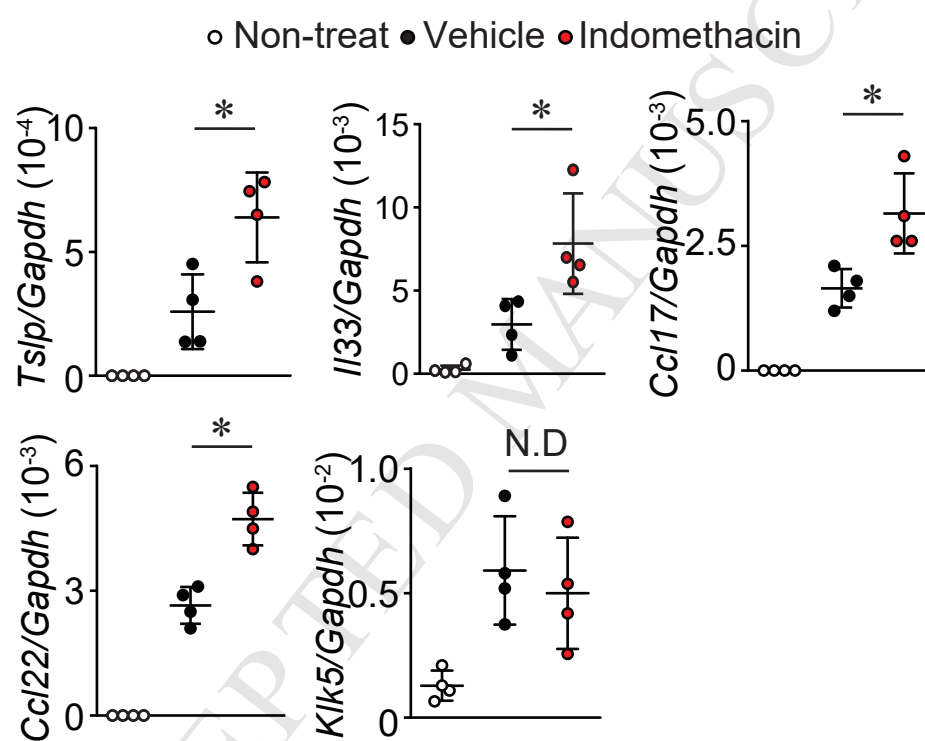


Fig 3

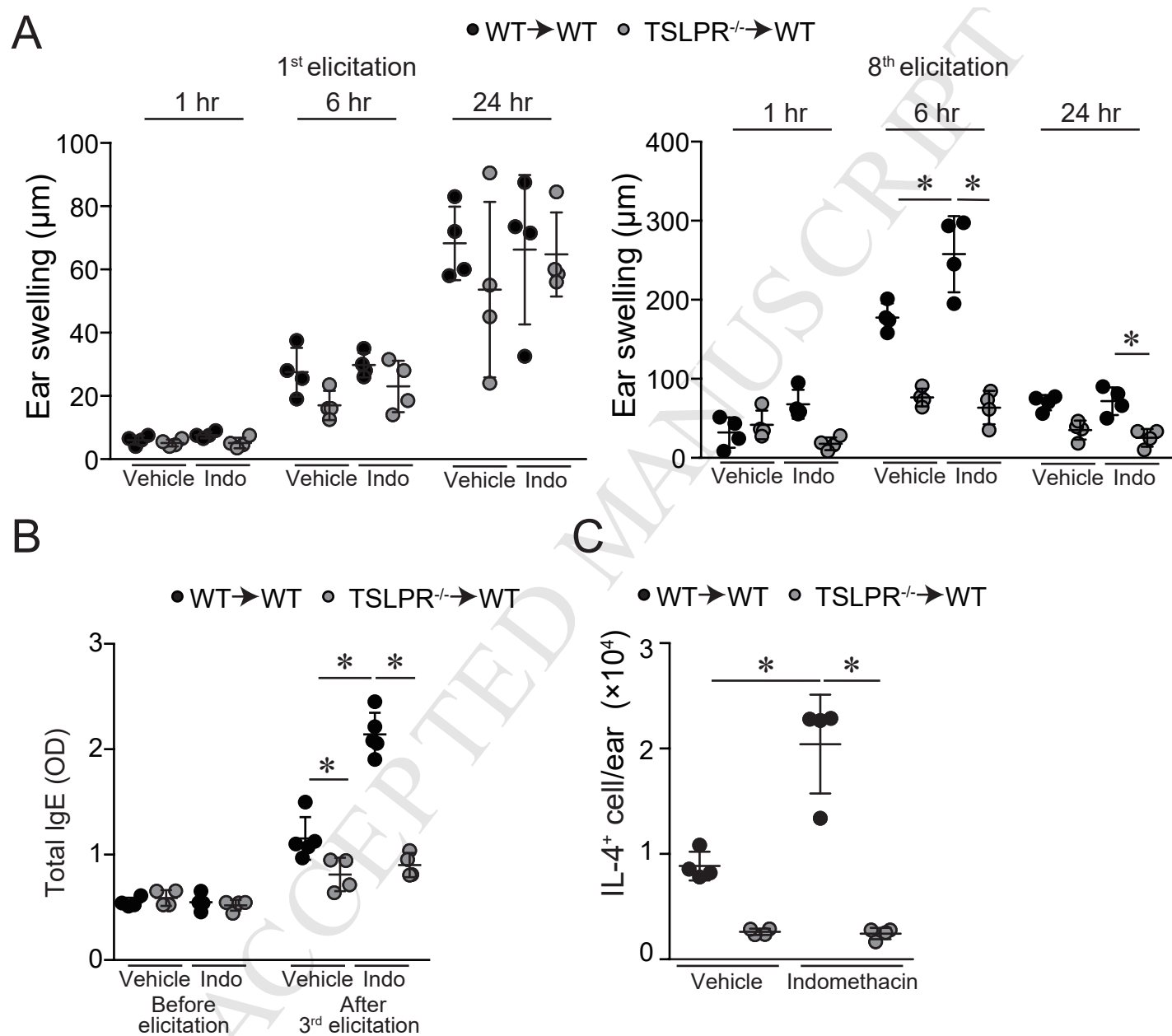


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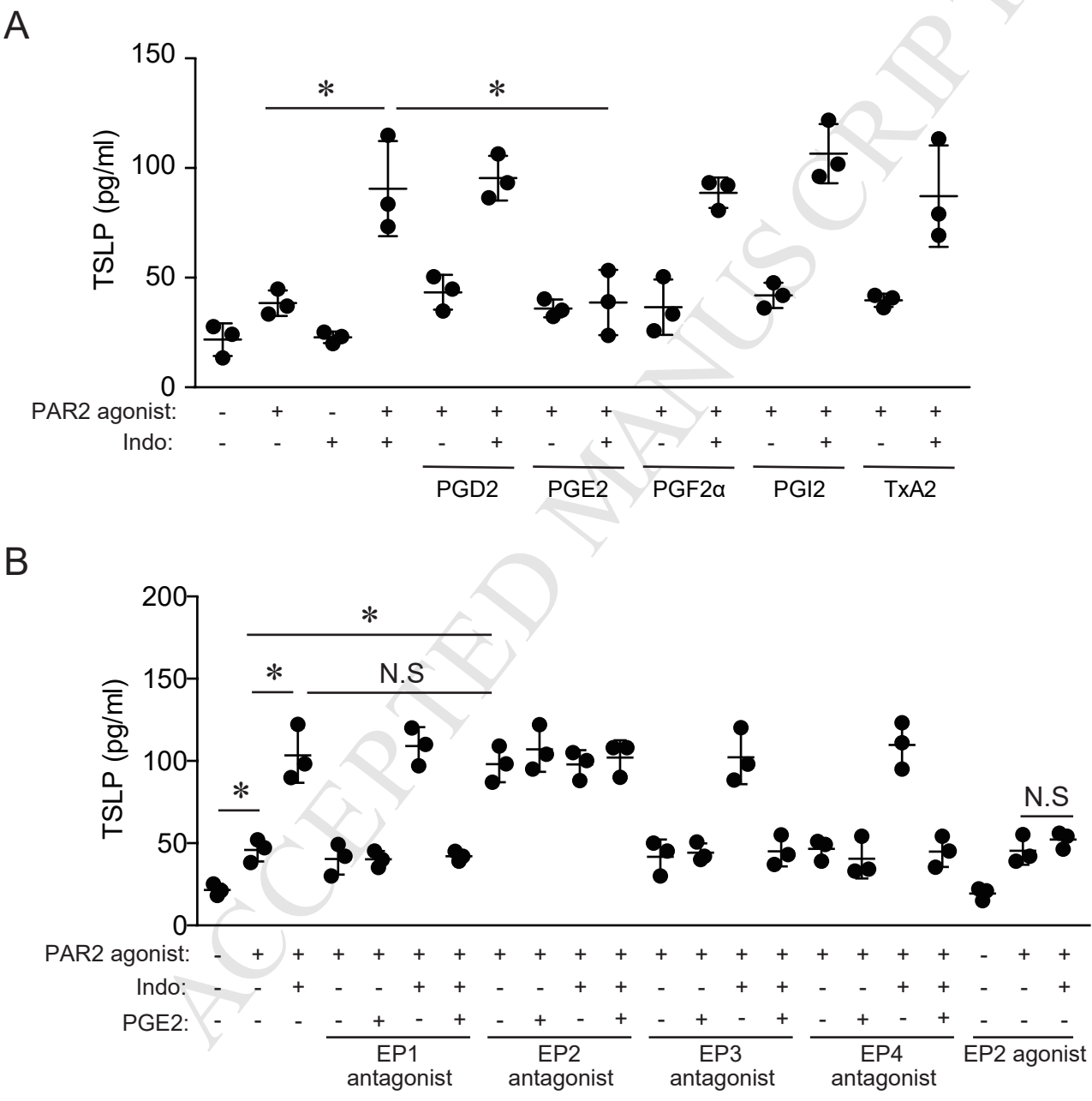


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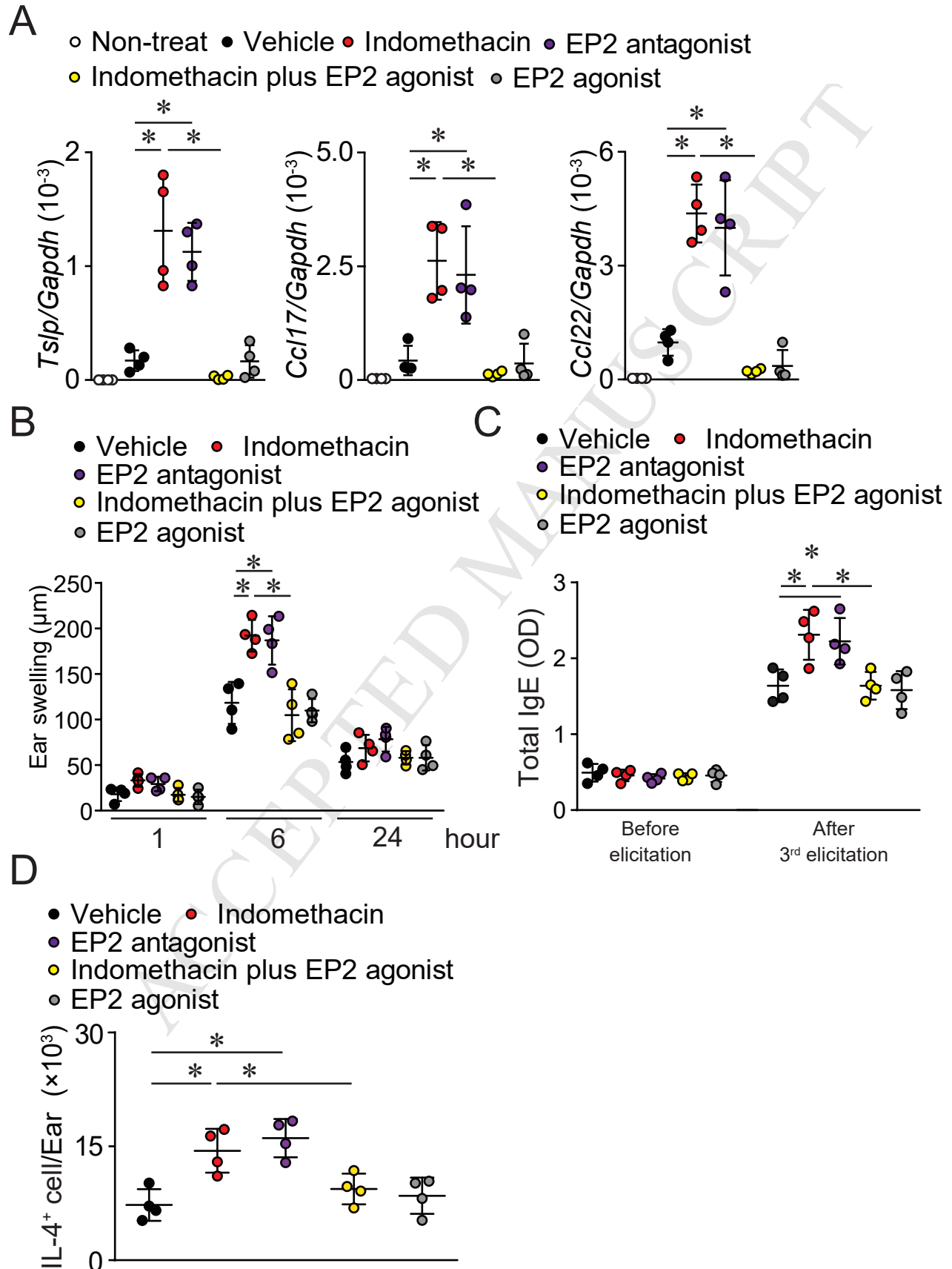


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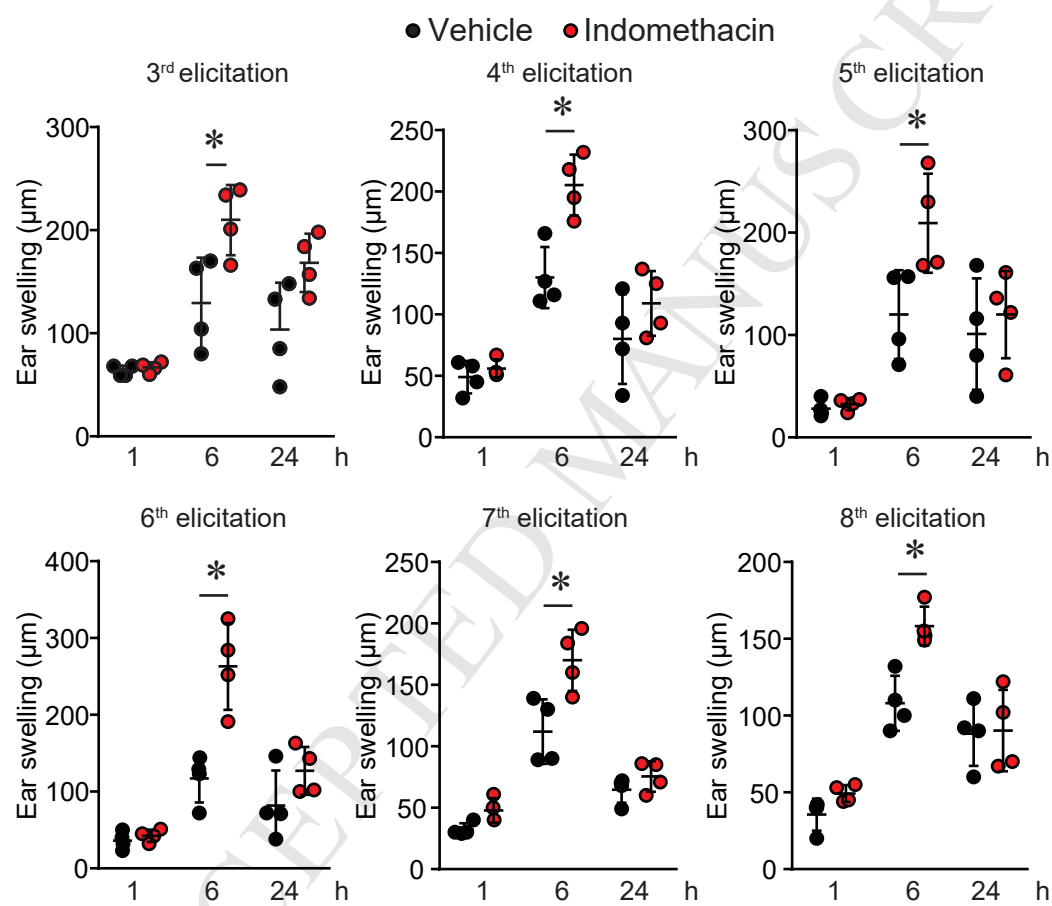


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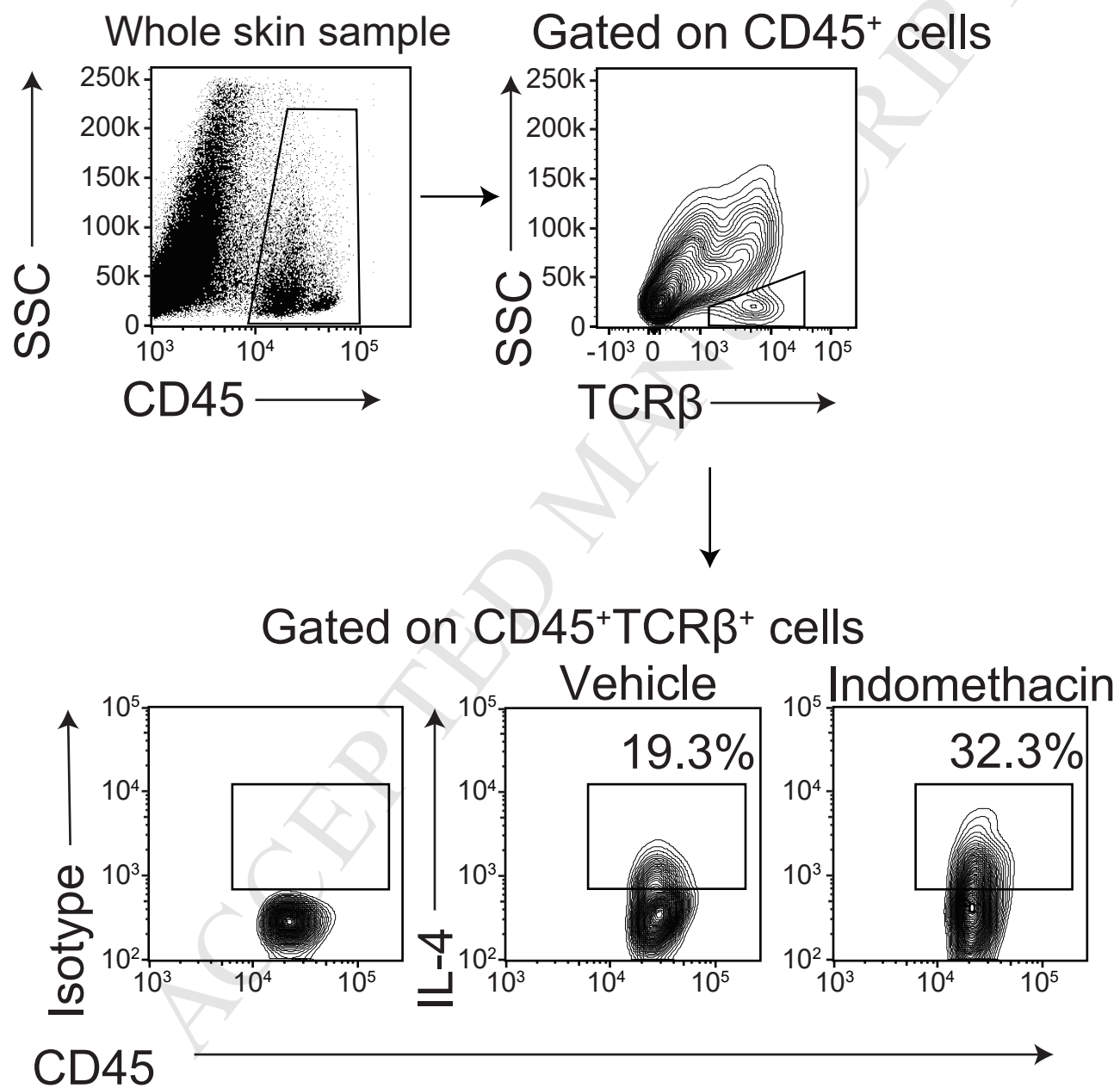


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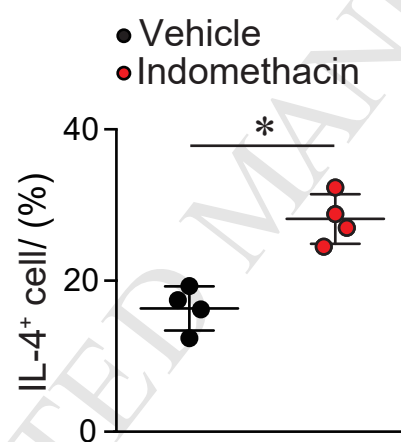


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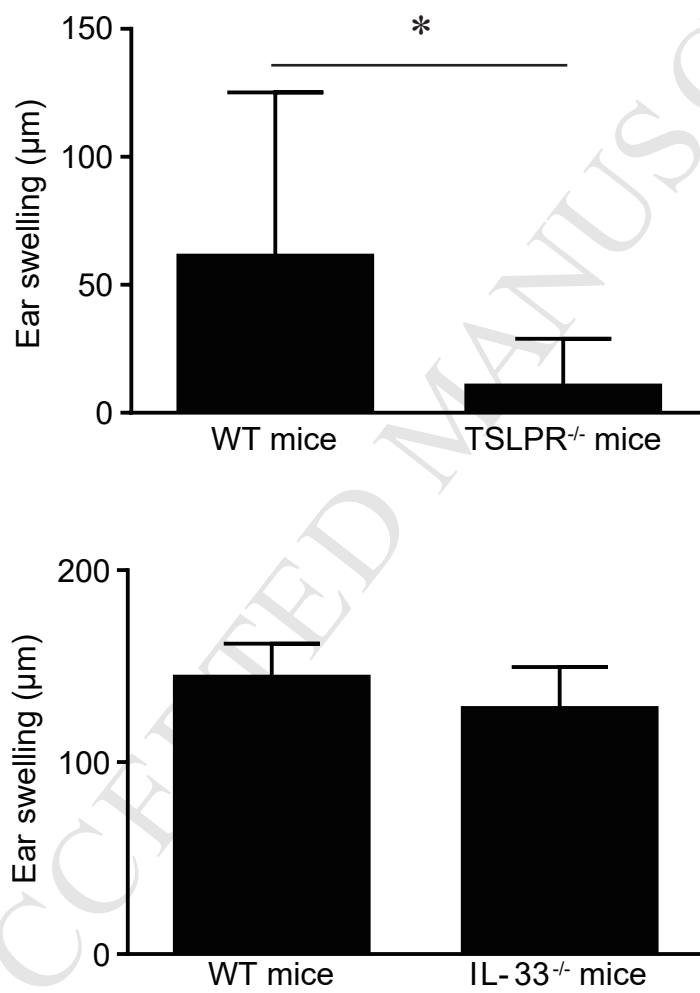


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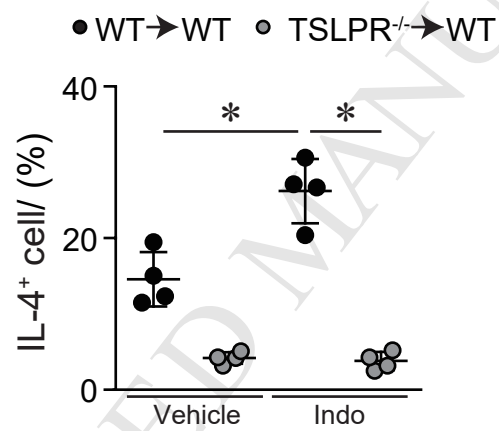


Fig E6

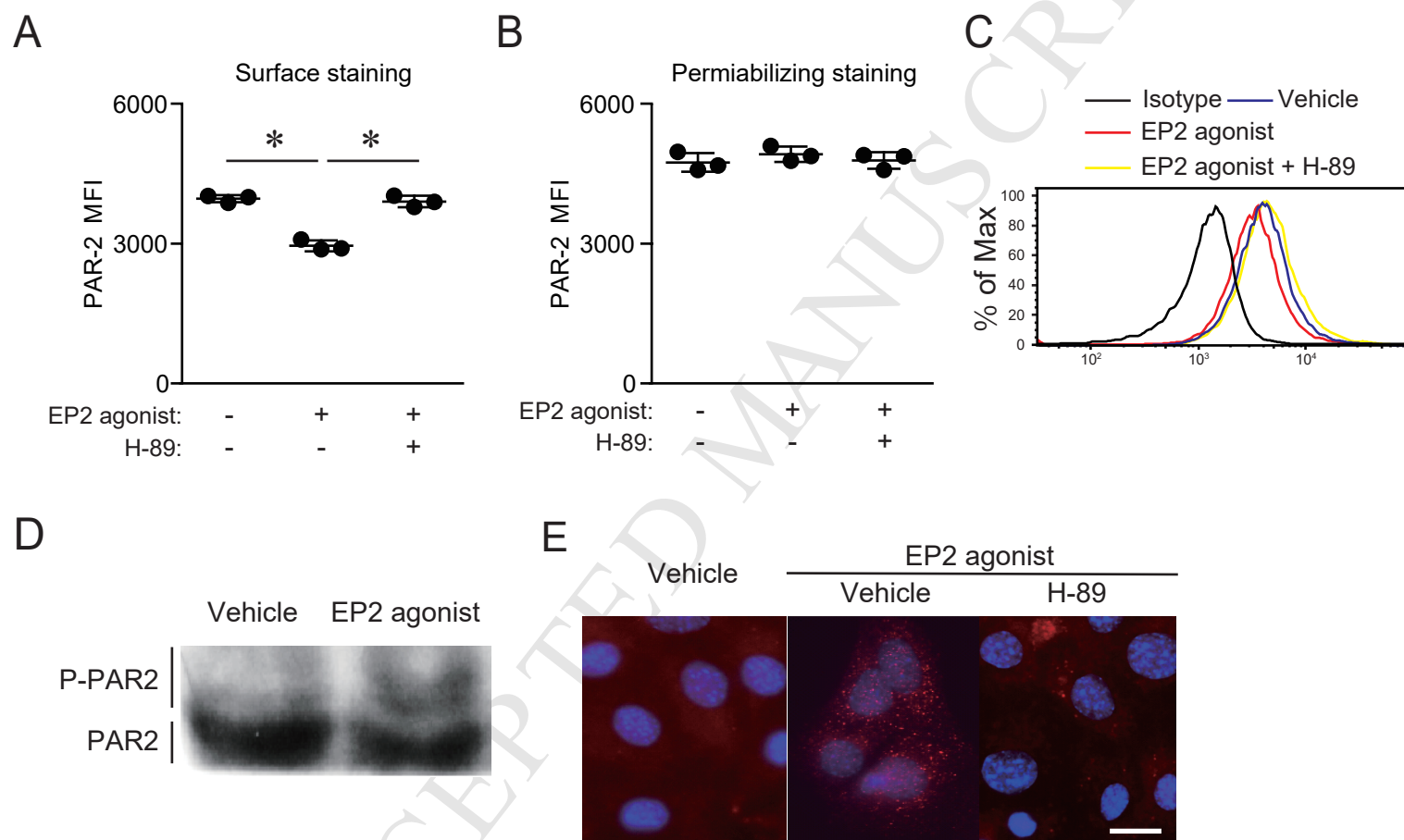


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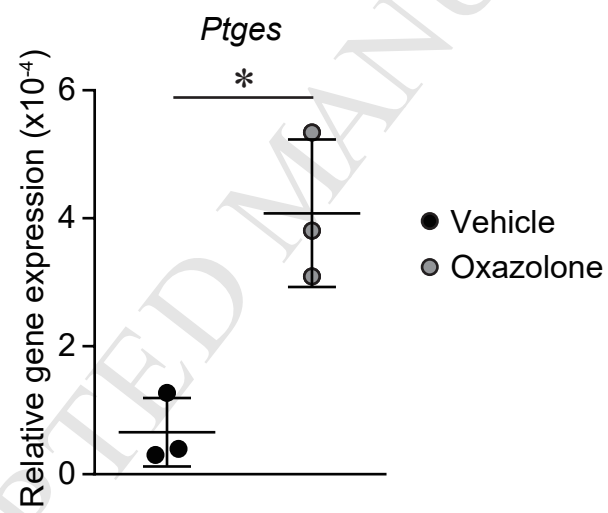


Fig E8

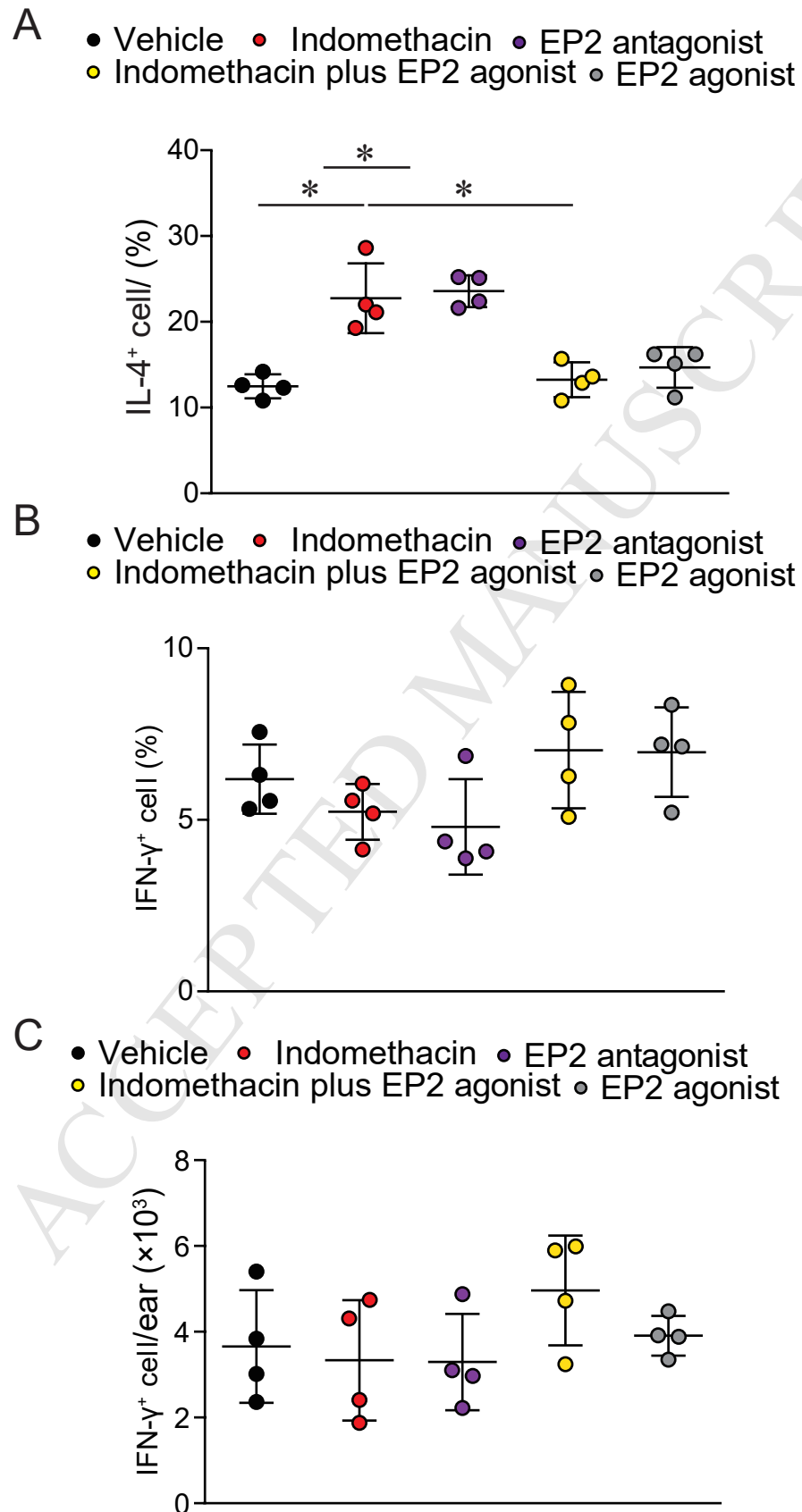
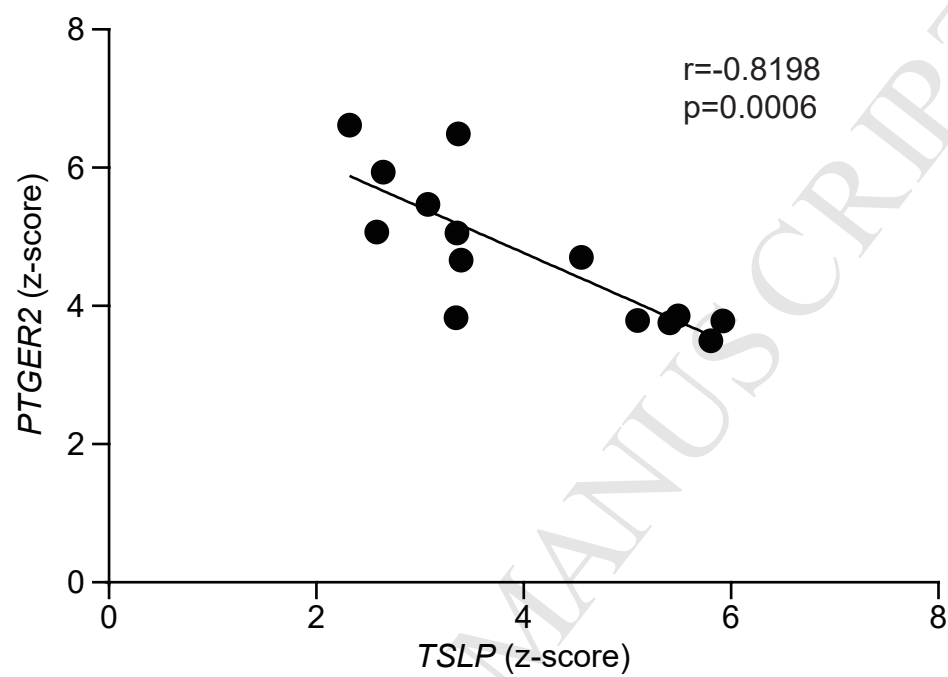


Fig E9

A



B

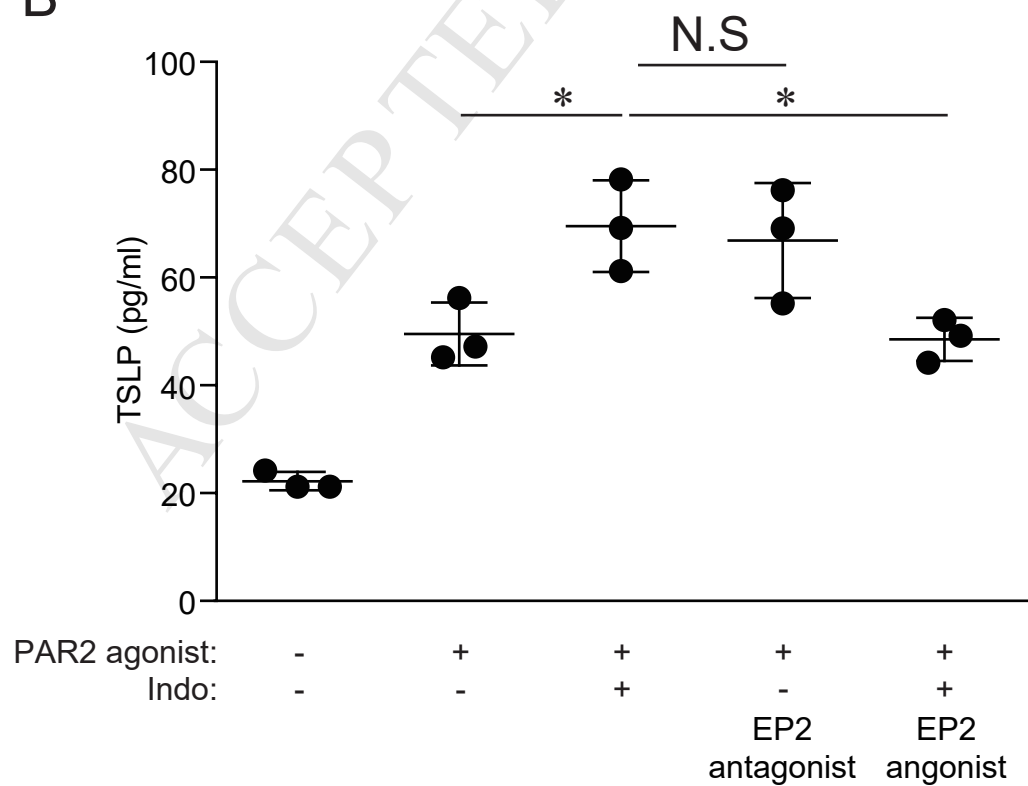


Figure legend**Fig E1. Ear swelling responses in repeated hapten application treated with either vehicle or indomethacin**

Ear thickness change from the 3rd to 8th elicitation. The results are expressed as the mean $\pm$ standard deviation (SD). Each dot represents an individual sample. Data are representative of at least three independent experiments. *, $P < 0.05$.

Fig E2. Gating strategy for IL-4⁺CD45⁺TCR β ⁺ cells

Single cell suspensions from skin were stained with anti-CD45 antibody, anti-TCR β antibody, and were subjected to intracellular staining with anti-IL-4 antibody or its isotype control.

Fig E3. The frequency of IL-4⁺CD45⁺TCR β ⁺ cells in the skin at the 8th elicitation in mice treated with or without indomethacin

The percentage of IL-4-producing CD45⁺TCR β ⁺ cells. The results are expressed as the mean $\pm$ SD. Each dot represents an individual sample. Data are representative of three independent experiments. *, $P < 0.05$.

Fig E4. Ear swelling responses in repeated hapten elicitation are attenuated in TSLPR-deficient (TSLPR^{-/-}) mice

Ear swelling responses in TSLPR^{-/-} mice and IL-33-deficient (IL-33^{-/-}) mice 6 h after the 3rd elicitation ($n = 7$). The results are expressed as the mean $\pm$ SD. *, $P < 0.05$.

Fig E5. The frequency of IL-4⁺CD45⁺TCR β ⁺ cells in the skin at the 8th elicitation in bone marrow chimeric mice treated with or without indomethacin

The percentage of IL-4-producing cells in CD45⁺TCR β ⁺ cells. WT mice were

reconstituted with either WT-BM (WT→WT) or TSLPR-deficient-BM (TSLPR^{-/-}→WT). The results are expressed as the mean ± SD. Each dot represents an individual sample. *, P < 0.05.

Fig E6. PGE₂-EP2 signaling regulates the surface expression of PAR2 in keratinocytes, PAM212

(A-C) Flow cytometric analysis of surface (A) or both surface and intracellular expression (B) of PAR2 in PAM212 cells treated with an EP2 agonist (20 μM) and/or H-89 (3 μM). (C) A representative histogram of flowcytometry analysis in (A). (D) Immunoblot analysis for phosphorylated PAR2. PAM212 cells were stimulated with either vehicle or an EP2 agonist (20 μM) for 30 min, and subjected to immunoblot analysis. Phosphorylated PAR2 was separated by Phos-tag-containing SDS-PAGE gel. (E) Immunohistochemistry of PAR2 (red signals) in PAM212 cells treated with an EP2 agonist and/or H-89. The results are expressed as the mean ± SD. Each dot represents an individual sample. *, P < 0.05.

Fig E7. The mRNA expression level of *Ptges* in the skin

The mRNA expression level of *Ptges* in the skin 24 h after the 1st elicitation with oxazolone. Expression of *Ptges* mRNA was analyzed by quantitative real-time PCR. The results are expressed as the mean ± SD. Each dot represents an individual mouse. *, P < 0.05.

Fig E8. The frequency and the number of IL-4⁺ or IFN-γ⁺ CD45⁺TCRβ⁺ cells in the skin at the 8th elicitation in mice treated with or without EP2 agonist or antagonist

(A) The percentage of IL-4-producing cells in CD45⁺TCRβ⁺ cells. (B, C) The

percentage (B) and the number (C) of IFN- γ -producing cells in CD45⁺TCR β ⁺ cells. The results are expressed as the mean $\pm$ SD. Each dot represents an individual sample. *, $P < 0.05$.

Fig E9. The mRNA expression levels of TSLP and PTGER2 in AD lesions, and TSLP production from normal human epidermal keratinocytes (nHEK)

(A) The correlation between TSLP and PTGER2 mRNA expressions (Z Score) in the lesional skin taken from AD patients was analyzed. Data were obtained from a public data set (GEO accession no. GSE120721). (B) Effects of indomethacin, EP2 agonist and EP2 antagonist on TSLP production in nHEK. nHEK were stimulated with the indicated combinations of PAR2 agonist (100 μ M), indomethacin (1 μ M), EP2 antagonist (ONO-AE8-116) (20 μ M), and EP2 agonist (ONO-AE1-259-01) (20 μ M) for 24 h, and the supernatant was subjected to an ELISA analysis for TSLP. The results are expressed as the mean $\pm$ SD. Each dot represents an individual sample. *, $P < 0.05$.