

IL-17A as an Inducer for Th2 Immune Responses in Murine Atopic Dermatitis Models

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Atopic dermatitis (AD) is generally regarded as a type 2 helper T (Th2)-mediated inflammatory skin disease. Although the number of IL-17A-producing cells is increased in the peripheral blood and in acute skin lesion of AD patients, the role of IL-17A in the pathogenesis of AD remains unclear. To clarify this issue, we used murine AD models in an IL-17A-deficient condition. In a repeated hapten application-induced AD model, skin inflammation, IL-4 production in the draining lymph nodes (LNs), and hapten-specific IgG1 and IgE induction were suppressed in IL-17A-deficient mice. $V\gamma 4^{+}$ $\gamma\delta$ T cells in the skin-draining LNs and $V\gamma 5^{-}$ dermal $\gamma\delta$ T cells in the skin were the major sources of IL-17A. Consistently, in flaky-tail (*Flg^{fl/fl} ma/ma*) mice, spontaneous development of AD-like dermatitis and IgE induction were attenuated by IL-17A deficiency. Moreover, Th2 differentiation from naive T cells was promoted *in vitro* by the addition of IL-17A. Taken together, our results suggest that IL-17A mediates Th2-type immune responses and that IL-17A signal may be a therapeutic target of AD.

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INTRODUCTION

Skin is an essential immune organ that can elicit a variety of immune responses against foreign antigen exposure (Egawa and Kabashima, 2011). Atopic dermatitis (AD) is a pruritic chronic retractable inflammatory skin disease that is induced by a complex interaction between susceptibility genes encoding skin barrier components and stimulation by environmental antigens (Palmer *et al.*, 2006; Kabashima, 2012, 2013). AD is classically characterized by a type 2 helper T (Th2)-dominant condition, which seems to be mediated by epidermal Langerhans cells stimulated with thymic stromal lymphopoietin (TSLP) on keratinocytes (Soumelis *et al.*, 2002). TSLP also induces Th2 chemokines, including thymus- and activation-regulated chemokine/CCL17, and is involved in allergic responses, IgE production, and eosinophilia (Liu *et al.*, 2007; Nakajima *et al.*, 2012).

IL-17A is known to be involved in host defense against various pathogens. The inappropriate production of IL-17A is considered to be involved in the development of Th17-mediated autoimmune and inflammatory conditions, such as multiple sclerosis, rheumatoid arthritis, and psoriasis (Iwakura *et al.*, 2011). Consistently, the IL-17A-Th17 pathway is a good therapeutic target of psoriasis (Miossec and Kolls, 2012). IL-17A is also involved in the pathogenesis of contact hypersensitivity (CHS) responses, as the CHS response was attenuated in IL-17A-deficient (*IL-17A^{-/-}*) mice via the impaired expansion of hapten-specific $CD4^{+}$ T cells in the sensitization phase (Nakae *et al.*, 2002; Honda *et al.*, 2013). Th17 axis in AD is known to be attenuated compared with psoriasis, reflecting the model for AD as Th2/Th22 and psoriasis as a Th1/Th17-polarized disease, respectively. This is reflected in part by low levels of downstream molecules in the Th17 pathway, including key antimicrobial peptides, which is related to the increased infection rate seen in AD (Ong *et al.*, 2002).

In contrast, others and we have reported that the number of IL-17A-producing cells is increased in the peripheral blood mononuclear cells of patients with severe AD and at the acute eczematous lesions of AD in humans (Toda *et al.*, 2003; Koga *et al.*, 2008). Consistently, IL-17A was highly detected in the spontaneously developed AD-like skin lesions in flaky-tail (*Flg^{fl/fl}*) mice, which carry mutations in *filaggrin* and *matted* genes (Fallon *et al.*, 2009; Oyoshi *et al.*, 2009; Moniaga and Kabashima, 2011).

In addition to $CD4^{+}$ Th17 cells, subsets of $CD8^{+}$ T cells, $\gamma\delta$ T cells, natural killer T cells, monocytes, natural killer cells, and lymphoid tissue inducer-like cells have been shown to produce IL-17A (Cua and Tato, 2010). Among them, $\gamma\delta$ T cells

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Abbreviations: AD, atopic dermatitis; CHS, contact hypersensitivity; LN, lymph node; Th, T helper; TSLP, thymic stromal lymphopoietin

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produce IL-17A in psoriasis skin lesions in murine models and human samples (Cai *et al.*, 2011; Laggner *et al.*, 2011). In murine skin, there exist at least two populations of $\gamma\delta$ T cells: $V\gamma 5^+ \gamma\delta$ TCR^{high} $\gamma\delta$ T cells in the epidermis and $V\gamma 5^- \gamma\delta$ TCR^{mid} $\gamma\delta$ T cells in the dermis. It has recently been revealed that CCR6⁺ IL-23 receptor (IL23R)⁺ $\gamma\delta$ T cells are present in the murine dermis (Gray *et al.*, 2011), which secrete IL-17A and IL-22 by responding to IL-23 and IL-1 β (Sutton *et al.*, 2009; Cua and Tato, 2010), and that IgE induction is impaired in $\gamma\delta$ T cell-deficient mice (Strid *et al.*, 2011). Therefore, it is important to determine which cells produce IL-17A and to evaluate the role of IL-17A in the pathogenesis of AD.

RESULTS

IL-17A deficiency attenuated the development of hapten-induced AD-like skin lesions

Single hapten elicitation (hereafter, called acute CHS) is known to induce delayed-type hypersensitivity, as a murine model of human contact dermatitis (Honda *et al.*, 2013). On the other hand, repeated hapten exposure (hereafter, called chronic CHS) shifts immune responses from a typical Th1-dominated delayed-type hypersensitivity response to a chronic Th2-dominated late-phase and immediate-type hypersensitivity response, which are clinically relevant to the acute phase of AD in humans (Kitagaki *et al.*, 1997; Man *et al.*, 2008; Jin *et al.*, 2009; Hatano *et al.*, 2010). Consistently, recent studies have proposed that cutaneous exposure to hapten contributes to the induction of AD—this is called the hapten-atopy hypothesis (McFadden *et al.*, 2011). We applied this murine AD-like model to wild-type (WT) and IL-17A^{-/-} mice on BALB/c background.

In acute CHS, ear swelling 24 and 48 hours after application (i.e., a delayed-type hypersensitivity reaction) was attenuated in IL-17A^{-/-} mice, as reported previously (Nakae *et al.*, 2002); (Figure 1a, upper panel), whereas ear swelling without sensitization was comparable between WT and IL-17A^{-/-} mice. In chronic CHS, WT mice exhibited prominent ear-swelling responses 1 and 6 hours after the last hapten application, which were significantly attenuated in IL-17A^{-/-} mice (Figure 1a, lower panel). Histological examination of skin from WT mice revealed epidermal hyperplasia with inflammatory cell infiltration, which is similar to acute AD skin lesions (Figure 1b, Supplementary Table S1 online). These findings were less apparent in IL-17A^{-/-} mice (Figure 1b; Supplementary Table S1 online). The total histological score of IL-17A^{-/-} mice was significantly lower than that of WT mice (Figure 1c).

To evaluate the extent of induction of Th2 in IL-17A^{-/-} mice, we analyzed cytokine mRNA expression levels of the skin and draining lymph nodes (LNs) by quantitative PCR analysis 6 hours after the last elicitation of chronic CHS and in the steady state (indicated as control). Consistent with the observation that IL-17A-producing cells are increased in human AD (Toda *et al.*, 2003; Koga *et al.*, 2008), the IL-17A expression levels of the skin and draining LNs were elevated after repeated hapten exposures (Figure 2a). The IL-4 mRNA expression levels of the skin and draining LNs in IL-17A^{-/-} mice were significantly decreased when compared with those

of WT mice (Figure 2a). On the other hand, the IFN- γ , IL-10, and IL-22 mRNA expression levels of the skin and draining LNs did not show any significant differences between WT and IL-17A^{-/-} mice (Figure 2a).

It is known that IgE and IgG1 are Th2-dependent Igs and that IgG2a is a Th1-dependent Ig, respectively (Snapper *et al.*, 1988a, b). Serum 4-ethoxymethylene-2-phenyloxazolin-5-one-specific IgE, IgG1, and IgG2a levels in IL-17A^{-/-} mice after chronic CHS were significantly lower than those in WT mice (Figure 2b). These data suggest that IL-17A is involved in the development of both acute CHS (Th1) and chronic CHS (Th2) models.

Th2 chemokine expression levels and IL-4-producing cells were attenuated by IL-17A deficiency

TSLP and CCL17/thymus- and activation-regulated chemokine have a pivotal role in Th2-mediated allergic response. We therefore analyzed mRNA expression levels of these genes 6 hours after the last elicitation of chronic CHS. TSLP and CCL17/thymus- and activation-regulated chemokine mRNA levels in the ear skin were increased in WT mice. After chronic CHS, the mRNA levels of TSLP and CCL17/thymus- and activation-regulated chemokine in IL-17A^{-/-} mice were significantly lower than those in WT mice by 70 and 30%, respectively (Figure 3a and b). The above findings suggest that Th2 induction was impaired in IL-17A^{-/-} mice. As such, we focused on the IL-4 induction via IL-17A signaling.

Consistent with the mRNA analysis (Figure 2a), the number of IL-4⁺ cells was increased in chronic CHS by intracellular cytokine staining, and the number of IL-4⁺ cells in IL-17A^{-/-} mice was significantly lower than that in WT mice (Figure 3c). In addition, the number of CD4⁺ IL-4⁺ Th2 cells in IL-17A^{-/-} mice was significantly lower than that in WT mice (Figure 3d). It is known that naive T cells express IL-17R (Ishigame *et al.*, 2009); therefore, there is a possibility that IL-17A may be involved in IL-4-producing Th2-cell differentiation. To dissect the direct action of IL-17A in Th2 differentiation, we incubated naive CD4⁺ T cells under a Th2-skewing condition in the presence or absence of IL-17A. Under the Th2-skewing condition, the number of IL-4-producing cells in CD4⁺ T cells was increased by IL-17A under different anti-CD3/CD28 stimulation conditions (Figure 3e). The above findings suggest that IL-17A may contribute to Th2 differentiation in the draining LN.

$\gamma\delta$ T cells are the main producer of IL-17A

We examined the enhanced IL-17A mRNA expression after repeated hapten application as above (Figure 2a). Next, we confirmed this phenomenon using an intracellular cytokine staining assay. The numbers of IL-17A-producing cells in the skin-draining LNs (Figure 4a) and in the lesional skin (Figure 4b) were increased after repeated hapten application. The main producer of IL-17A in the skin-draining LNs was $V\gamma 4^+ \gamma\delta$ TCR⁺ T cells (Figure 4a). In the skin, there exist at least two $\gamma\delta$ T-cell subsets: $V\gamma 5^+$ epidermal $\gamma\delta$ T cells, and $V\gamma 5^-$ dermal $\gamma\delta$ T cells (Mabuchi *et al.*, 2011). After repeated hapten application, the number of CD45⁺ IL-17A⁺ cells was increased in the lesional skin, and the main producer of

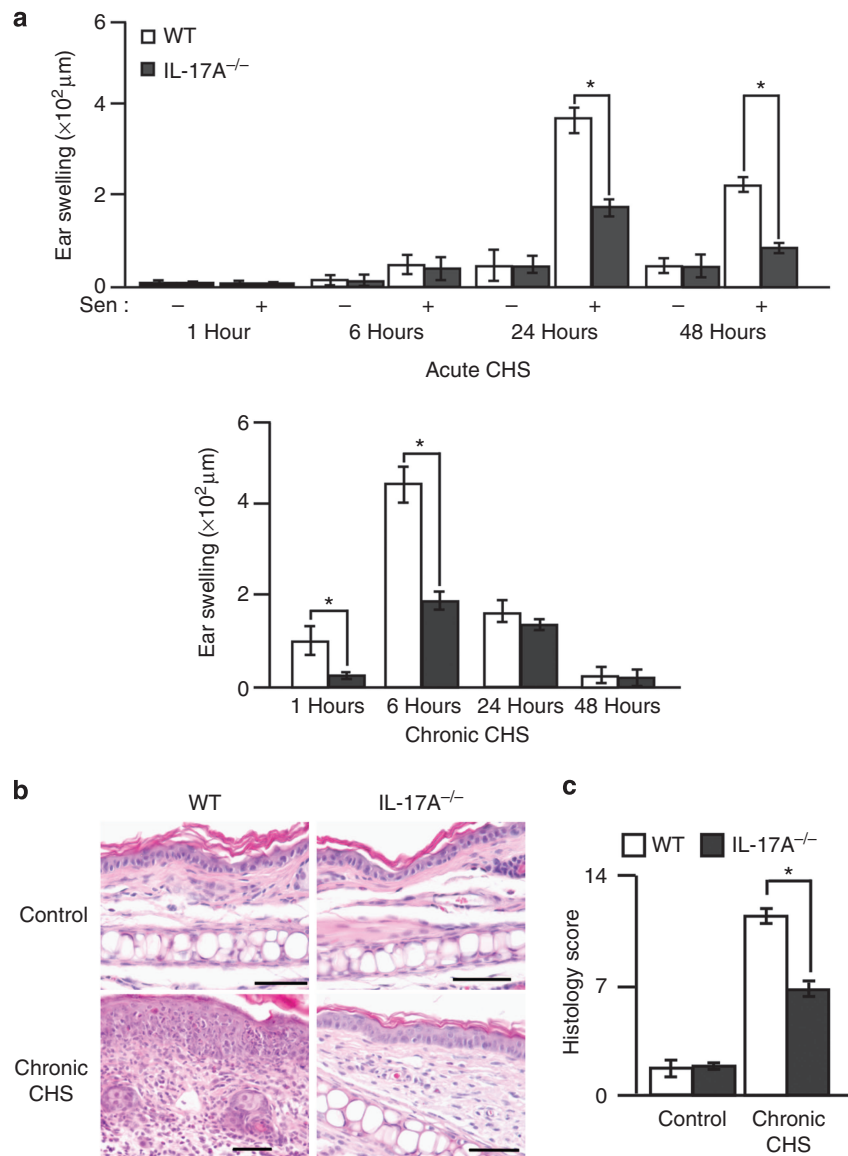


Figure 1. IL-17A deficiency attenuated the development of hapten-induced atopic dermatitis (AD)-like skin lesions. (a) Time course of antigen-specific ear-swelling responses of wild-type (WT) mice and IL-17A^{-/-} mice after the last elicitation with 4-ethoxymethylene-2-phenyloxazolin-5-one (OX) in acute (upper) and chronic contact hypersensitivity (CHS; lower). In acute CHS, ear swelling of mice with (+) or without (-) sensitization (sens) was shown. (b) Histology (hematoxylin and eosin staining) of the ear skin of WT or IL-17A^{-/-} mice 6 hours after the last application with OX in chronic CHS or untreated mice (control). Bar = 100 μm . (c) Total histology score. * $P < 0.05$ ($n = 5$ mice per group).

IL-17A was $\text{V}\gamma 5^-$ dermal $\gamma\delta$ T cells (Figure 4b). Next, we analyzed single-cell suspensions from the epidermis. We detected major histocompatibility complex class II⁺ IL-17A-producing cells in the epidermis, after repeated application, and the majority of them were $\text{V}\gamma 5^-$ $\gamma\delta$ T cells (Figure 5a and b). This result suggests that IL-17A-producing dermal $\gamma\delta$ T cells migrated into the epidermis after repeated hapten application.

IL-17A deficiency led to impaired Th2 induction in *Flg^{fl}* mice
Finally, we sought to evaluate the role of IL-17A in another murine AD-like model, *Flg^{fl}* mice, which mutated in *filaggrin* and *matted* genes. *Tmem79* (*ma/ma*) mutation is responsible for the spontaneous dermatitis phenotype in *Flg^{fl}* mice (Sasaki *et al.*, 2013; Saunders *et al.*, 2013), and deficiency of filaggrin

gene alone does not induce spontaneous dermatitis (Kawasaki *et al.*, 2012). The *Flg^{fl}* mice exhibited Th17-dominant milieu in the skin and skin-draining LNs (Fallon *et al.*, 2009; Oyoshi *et al.*, 2009; Moniaga and Kabashima, 2011). We crossed *Flg^{fl}* mice with IL-17A^{-/-} mice on B6 background to evaluate the role of IL-17A in the development of spontaneous AD-like skin lesions in *Flg^{fl}* mice. Clinical appearance was mild in IL-17A^{-/-} *Flg^{fl}* mice when compared with *Flg^{fl}* mice at the age of 20 weeks (Figure 6a, Supplementary Table S3 online). Consistently, the histological score of IL-17A^{-/-} *Flg^{fl}* mice was lower than that of IL-17A^{+/+} *Flg^{fl}* mice (Figure 6b; Supplementary Figure S2 online; Supplementary Table S2 online). We measured serum Th2-type Ig (IgE and IgG1) and Th1-type Ig (IgG2c) levels, and found that IL-17A deficiency

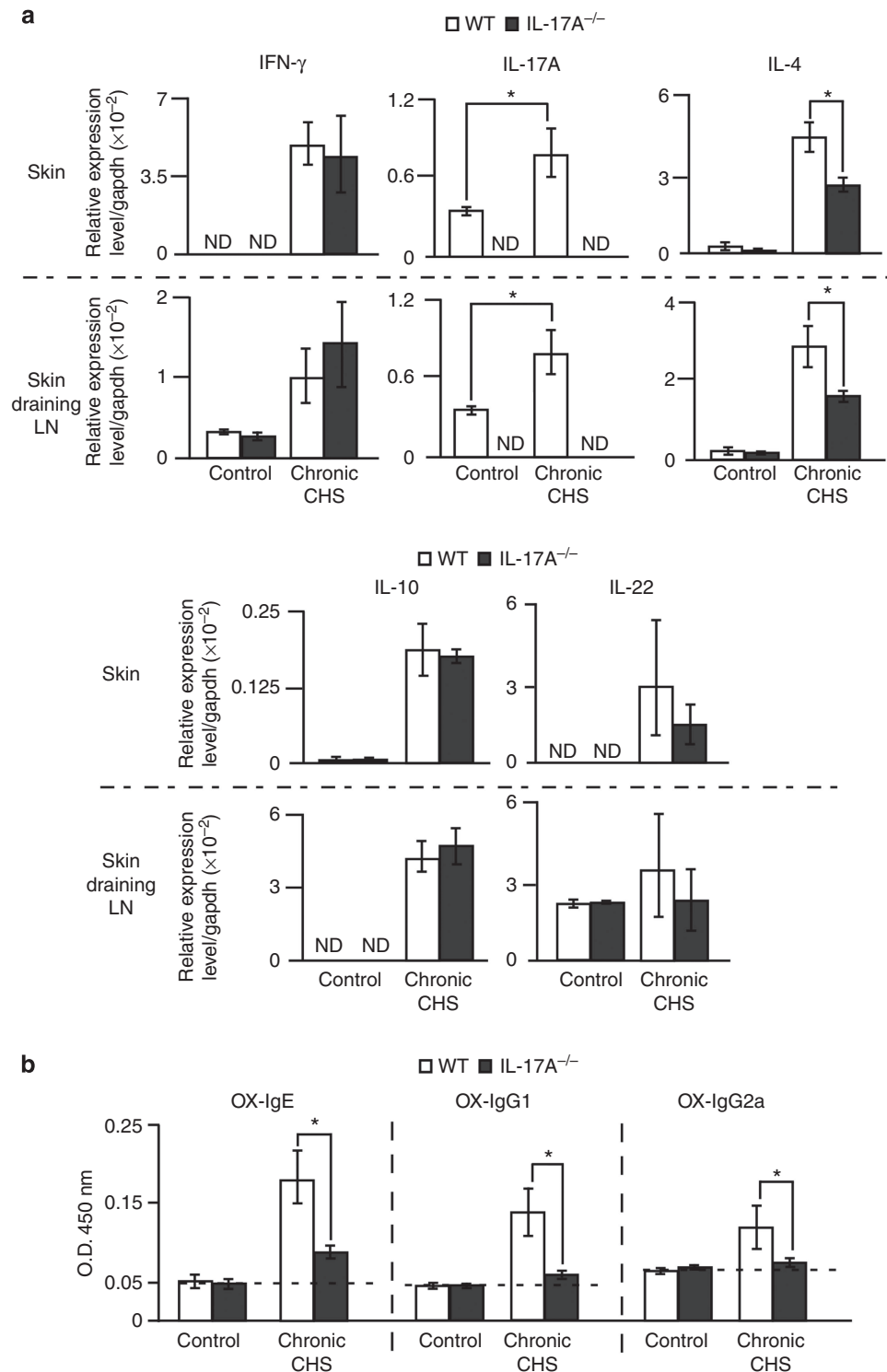


Figure 2. Cytokine expression levels and antigen-specific Ig production. (a) mRNA expression levels in the skin and skin-draining lymph nodes (LNs) 6 hours after the last elicitation of chronic contact hypersensitivity (CHS) or untreated mice (control). (b) Serum 4-ethoxymethylene-2-phenyloxazolin-5-one (OX)-specific IgE, IgG1, and IgG2a levels, as determined by ELISA. Optical density value for IgE, IgG1, and IgG2a levels were measured at a wavelength of 450 nm. * $P < 0.05$ ($n = 5$ mice per group). WT, wild type.

led to impaired IgE production in *Flg^{fl}* mice (Figure 6c), whereas the levels of IgG1 and IgG2c were comparable between these two groups (Figure 6c).

To further characterize the effect of IL-17A deficiency in *Flg^{fl}* mice, we analyzed the compositions of LN and spleen cells by means of flow cytometry. Although IL-17A deficiency

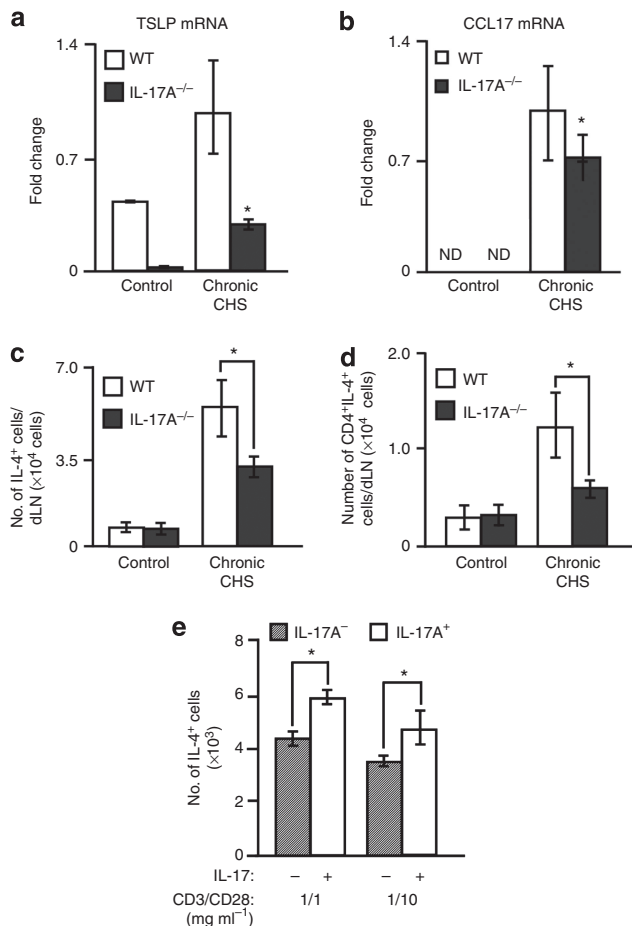


Figure 3. IL-17A deficiency attenuated type 2 helper T (Th2) chemokine expression and IL-4 induction. (a, b) Thymic stromal lymphopoietin (TSLP) and CCL17/thymus- and activation-regulated chemokines (TARC) mRNA expression levels of the ears 6 hours after the last elicitation of chronic contact hypersensitivity (CHS) or untreated mice (control) were normalized to *GAPDH* mRNA in the same sample. Results are presented as fold change relative to wild-type (WT) in chronic CHS, taken as one. (c, d) The number of total IL-4-producing cells (c) and IL-4-producing CD4⁺ T cells (d) in the draining lymph nodes (LNs). (e) *In vitro* type 2 helper T (Th2) differentiation assay. The number of IL-4⁺ cells per well is shown. **P* < 0.05.

did not attenuate spleen cells in B6 and *Flg^{fl}* mice, the total cell numbers of LN cells and cell counts and frequencies of CD4⁺ and CD8⁺ T-cell subsets were significantly decreased in IL-17A^{-/-} *Flg^{fl}* mice (Supplementary Figure S3a and b online).

We then prepared single-cell suspensions from LNs and stimulated them with phorbol 12-myristate 13-acetate/ionomycin for 4 hours to analyze the number of IL-4-producing CD4⁺ T cells. The number of IL-4-producing CD4⁺ T cells in IL-17A^{-/-} *Flg^{fl}* mice was significantly decreased compared with IL-17A^{+/+} *Flg^{fl}* mice (Figure 6d). These results suggest that IL-17A facilitates Th2 induction in *Flg^{fl}* mice. In our study, we observed that the IgE induction was attenuated in murine AD-like models. It has been reported that IL-17A promotes IgE production in B cells in humans *in vitro* (Doreau *et al.*, 2009; Milovanovic *et al.*, 2010). Consistently, IL-17A marginally promoted IgE production in B cells in a concentration-

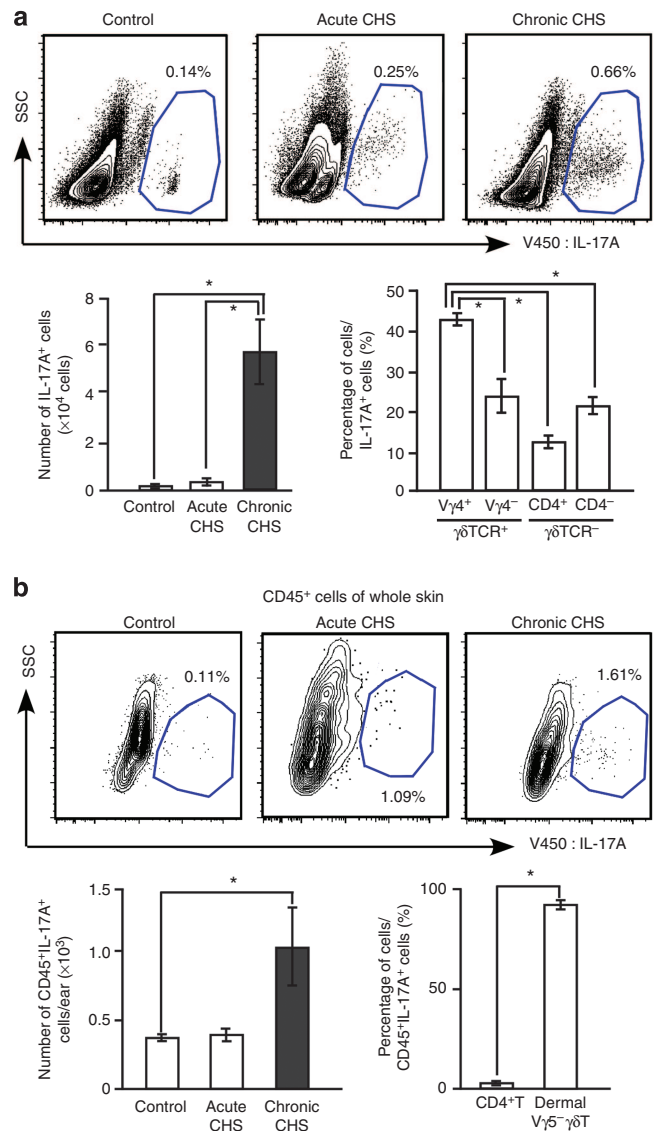


Figure 4. γδ T cells are the main producers of IL-17A. (a, b) Single-cell suspensions were collected 6 hours after the last elicitation of chronic contact hypersensitivity (CHS) for intracellular IL-17A staining. The number of IL-17A-producing cells in skin-draining lymph nodes (LNs; a) and CD45⁺ IL-17A⁺ cells in the lesional skin (epidermis and dermis) (b). The percentages of Vγ4⁺ or Vγ4⁺ γδ TCR⁺ cells and CD4⁺ or CD4⁺ γδ TCR⁺ cells (a) and CD4⁺ and dermal Vγ5⁺ γδ T cells (b) among IL-17A-producing cells (right lower panel). **P* < 0.05 (*n* = 5 per group).

dependent manner under indicated anti-CD40 antibody and recombinant murine IL-4 concentrations (Figure 6e).

DISCUSSION

The roles of IL-17A in the induction of Th2 and the development of AD remain to be elucidated. In this study, we sought to clarify this issue using two AD-like murine models: a repeated hapten application-induced chronic CHS model and *Flg^{fl}* mice (Kitagaki *et al.*, 1997; Spergel *et al.*, 1998; Moniaga *et al.*, 2010). In the chronic CHS model, IL-17A-producing cells were increased in the lesional skin and draining LNs. The skin inflammation and IL-4 production

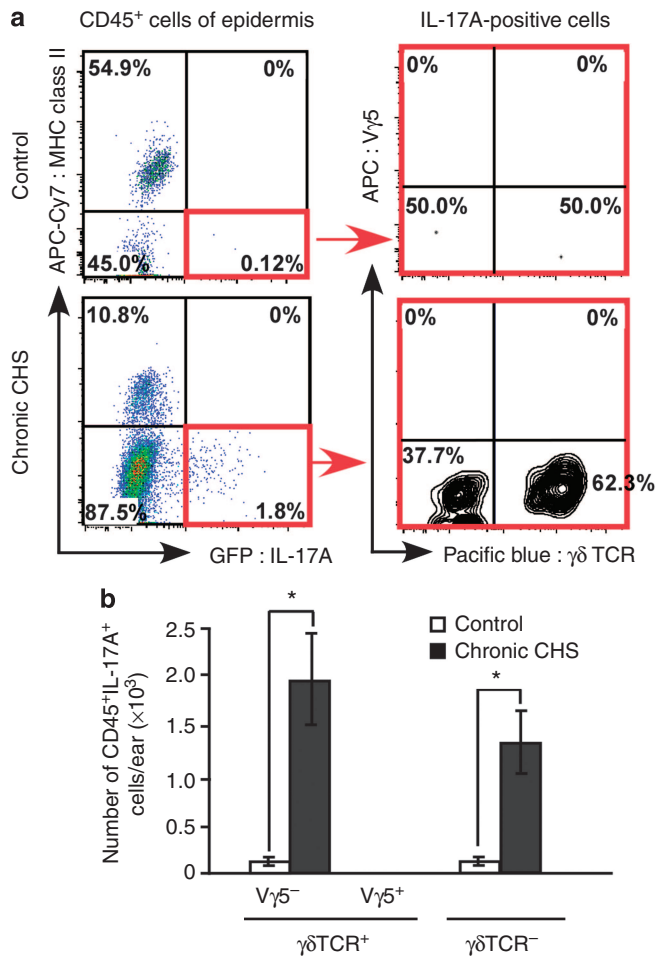


Figure 5. Dermal Vγ5⁻ γδ T cells were detected in the epidermis after chronic contact hypersensitivity (CHS). (a) Single epidermal cell suspensions from the lesional skin of IL-17A enhanced green fluorescent protein (eGFP) reporter mice were collected 6 hours after the last elicitation of chronic CHS. FACS plots of IL-17A-positive cells (surrounded with red line, left panel) and characterization of IL-17A-positive cells (right panel). (b) **P* < 0.05 (*n* = 5 per group).

were suppressed by IL-17A deficiency. Consistently, in *Flg^{fl}* mice, AD-like dermatitis was attenuated by depletion of IL-17A. *In vitro* Th2 differentiation from naive T cells was enhanced by the addition of IL-17A. IL-17A deficiency decreased TSLP and CCL17 expressions in chronic CHS, which may contribute to the induction of Th2 indirectly. These findings suggest that IL-17A mediates the development of AD. The findings may be consistent with a recent report that IL-17A-producing Th2 cells represent the key pathogenic Th2 cells that promote the exacerbation of allergic asthma in humans (Wang *et al.*, 2010).

On the other hand, IL-4 has been considered to inhibit IL-17A production potentially (Eyerich *et al.*, 2009). Consistently, IL-17A-positive cells infiltrated into the lesions of AD more attenuated in chronic lesions than in acute lesions (Koga *et al.*, 2008). In addition, Th17 axis in AD is rather attenuated compared with psoriasis in humans. Therefore, although IL-17A seems to promote Th2, IL-4 as a Th2 cytokine may suppress IL-17A production as a feedback mechanism.

Intriguingly, the immune responses of IL-17A^{-/-} mice are impaired in Th1-based acute CHS and Th2-based AD models (as shown in this study). Therefore, we speculate that IL-17A works as the enhancer of both Th1 and Th2 differentiation depending on the cytokine milieu, although it does not decide the direction of differentiation by itself. We therefore suppose that IL-17A may not promote Th2-dominated milieu under pure Th17 conditions in established Th17 diseases.

The effect of IL-17A on IgE induction is another issue to be addressed. We demonstrated that IL-17A deficiency decreased Th2-type IgE production in *Flg^{fl}* mice, whereas IL-17A deficiency decreased both Th1- and Th2-type Ig production in the chronic CHS model. This difference may stem from the differences of these models. *Flg^{fl}* mice exhibited Th2/Th17 conditions without induction of Th1, whereas the chronic CHS model exhibited Th2 conditions by shifting from Th1 conditions upon repeated hapten application. The mechanisms of IgE production and class switching from IgG1 to IgE remain unclear. The patients of classical-type hyper-IgE syndrome due to dominant-negative mutations in the *STAT3* (signal transducer and activator of transcription-3) gene exhibit high concentrations of IgE, as contrasted with normal serum levels of all other Igs (IgG, IgA, IgM, and IgD) and IgG subclasses (Minegishi, 2009). Moreover, a recent study demonstrated that *STAT3* expression on Th2 cells regulates class switch from IgG1 to IgE *in vitro* and *in vivo* (Mari *et al.*, 2013). Therefore, the dependency of *STAT3* for the induction of IgE may vary between the chronic hapten application-induced AD model and *Flg^{fl}* mice, which may explain the different phenotypes between these two groups.

Given that this study was conducted in mice, there are limitations when translating the findings to humans. These points may include the following: (1) we used the mouse model rather than human AD skin or blood samples; (2) we applied the hapten-induced murine AD-like model, which may induce nonspecific immune activation of several inflammatory axes as reported (He *et al.*, 2008; Jin *et al.*, 2009), and it might be difficult to draw conclusions in humans from this model; (3) in humans, the functional role for γδ T cells remains unclear in terms of IL-17 production; it has not been demonstrated in humans unlike mice. We agree that there still remain limitations in this study, but our findings suggest that IL-17A mediates Th2-type immune responses in the perspective of the development of AD, and that IL-17A signal may be a therapeutic target of AD.

MATERIALS AND METHODS

Mice

C57BL/6NCrSlc (B6) and BALB/c mice were purchased from Japan SLC (Shizuoka, Japan). IL-17A^{-/-} mice on B6 and BALB/c genetic backgrounds were generated (Nakae *et al.*, 2002). Flaky-tail mice (STOCK a/a ma ft/ma ft); *Flg^{fl}* mice; Fallon *et al.*, 2009; Moniaga *et al.*, 2010) were backcrossed more than six generations onto B6 mice background. IL-17A enhanced green fluorescent protein reporter mice were kindly provided by Dr Masato Kubo. Seven- to 12-week-old female mice that were bred in specific pathogen-free facilities at Kyoto University were used for all experiments, which were approved

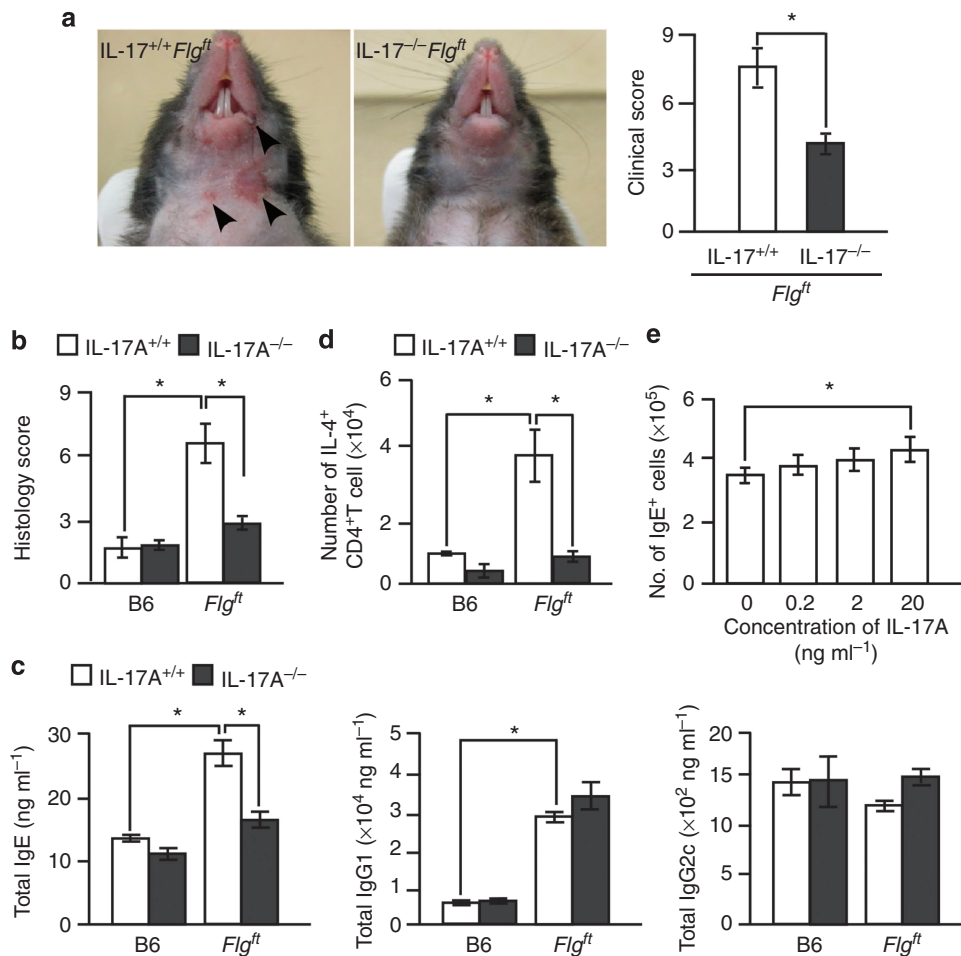


Figure 6. IL-17A deficiency attenuated cutaneous inflammation and type 2 helper T (Th2) induction in *Flg^{fl}* mice. (a, b) Clinical findings and total clinical scores (a) and histology scores (b) of IL-17^{+/+} and IL-17^{-/-} *Flg^{fl}* mice. Arrows indicate erosion of the skin. (c) Serum IgE, IgG1, and IgG2c levels. (d) The number of IL-4-producing CD4⁺ T cells. (e) Class switch recombination assay. The number of IgE-producing splenic CD19⁺ B220⁺ cells in various concentrations of IL-17A. **P* < 0.05 (*n* = 5 per group).

by the Institutional Animal Care and Use Committee of Kyoto University Graduate School of Medicine.

Acute and chronic CHS

The acute CHS reaction to hapten is an animal model of human allergic contact dermatitis (Honda *et al.*, 2013). For sensitization, the shaved abdominal skin was applied with 25 μ l of 3% 4-ethoxymethylene-2-phenyloxazolin-5-one (Wako, Osaka, Japan) solution in ethanol (day 0). For acute CHS induction, 5 days after the initial abdominal application when sensitization was established, 20 μ l of 0.6% 4-ethoxymethylene-2-phenyloxazolin-5-one solution was applied to the ear (Supplementary Figure S1a online). For chronic CHS induction, 4-ethoxymethylene-2-phenyloxazolin-5-one solution was applied to the ear every other day until day 13 (Supplementary Figure S1b online). Ear thicknesses were measured using a micrometer before (0h) and 1, 6, 24, and 48h after elicitation. The differences between the indicated time points and 0h were referred to as ear swelling. Immediate-type, late-phase, and delayed-type hypersensitivity reactions were manifested by ear-swelling responses at 1, 6, and 24h after the last hapten application, respectively. Histology was obtained 6h after the last hapten application (Supplementary Figure S1b online).

Quantitative reverse transcription-PCR analysis

We collected ears and cervical LNs 6 hours after the last elicitation to analyze cytokine mRNA expression levels. Total RNA was isolated with RNeasy kits and DNase I (Qiagen, Hilden, Germany). Complementary DNA was reverse transcribed using the Prime Script RT reagent kit (Takara Bio, Otsu, Japan). Quantitative PCR analysis was performed as reported previously (Nakajima *et al.*, 2010). All primers were obtained from Greiner Japan (Tokyo, Japan). The primer sequences were as follows: IFN- γ , 5'-GAA CTG GCA AAA GGA TGG TGA-3' (forward), 5'-TGT GGG TTG TTG ACC TCA AAC-3' (reverse); IL-4, 5'-GGT CTC AAC CCC CAG CTA GT-3' (forward), 5'-GCC GAT GAT CTC TCT CAA GTG AT-3' (reverse); IL-17A, 5'-CTC CAG AAG GCC CTC AGA CTA C-3' (forward), 5'-GGG TCT TCA TTG CGG TGG-3' (reverse); IL-10, 5'-GCT CTT ACT GAC TGG CAT GAG-3' (forward), 5'-CGC AGC TCT AGG AGC ATG TG-3' (reverse); CCL17, 5'-CAG GGA TGC CAT CGT GTT TCT-3' (forward), and 5'-GGT CAC AGG CCG TTT TAT GTT-3' (reverse). For each sample, triplicate test reactions and a control reaction lacking reverse transcriptase were analyzed for expression of the genes, and the results were normalized to those of "housekeeping" glyceraldehyde-3-phosphate dehydrogenase mRNA.

Th2 differentiation assay

Naive CD4⁺ T cells were sorted with the autoMACS separator (Miltenyi Biotec, Bergisch Gladbach, Germany); the purity was >95%. For an *in vitro* Th2 differentiation assay, BALB/c naive CD4⁺ CD62L⁺ T cells were stimulated for 2 days with plate-bound anti-CD3 (1 µg ml⁻¹) and plate-bound anti-CD28 (1 or 10 µg ml⁻¹) in the presence of 10 ng ml⁻¹ IL-4, 10 µg ml⁻¹ anti-IFN-γ Ab, and 20 ng ml⁻¹ IL-2. Forty-eight hours after the primary stimulation, cells were washed and cultured for 72 hours under the Th2-skewing conditions without anti-CD3 or anti-CD28 Ab stimulation.

Class switch recombination assay *in vitro*

Splenocyte single-cell suspensions were isolated by CD19⁺ selection via magnetic columns (Miltenyi Biotec). CD19⁺ cells (5 × 10⁵) were cultured in flat-bottomed 24-well plates and stimulated with 1 µg ml⁻¹ of anti-CD40 antibody (BD Bioscience, San Diego, CA) plus 50 ng ml⁻¹ of recombinant mouse IL-4 (R&D Systems, Minneapolis, MN) for 6 days to induce class switching to IgE. IgE-producing cells were analyzed by flow cytometry.

Statistical analysis

Unless otherwise indicated, data are presented as the means ± SD and are representative of three independent experiments. *P*-values were calculated according to the two-tailed *t*-test and the Mann–Whitney *U*-test for independent samples and the Wilcoxon test for related samples. *P*-values < 0.05 are considered to be significant and are marked by an asterisk in the figures.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at <http://www.nature.com/jid>

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IL-17A as an inducer for Th2 immune responses in murine atopic dermatitis models

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SUPPLEMENTAL MATERIALS

Cell culture, reagents, antibodies, flow cytometry, and intracellular staining

Complete RPMI (cRPMI) culture medium consisting of RPMI 1640 (Invitrogen, Carlsbad, CA, USA) containing 10% heat-inactivated fetal calf serum, 5×10^{-5} 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 µg/mL streptomycin, was used, unless otherwise indicated.

For whole skin cell suspensions, ear skin sheets were cut into small pieces with scissors in cRPMI medium and incubated for 25 min at 37°C and 5% CO₂ in type II collagenase (Sigma, St Louis, MO). The cell suspensions were filtered through a 40 µm cell strainer (BD Bioscience, San Diego, CA, USA).

For epidermal cell suspensions, ear skin sheets were floated on 0.25% trypsin ethylenediaminetetraacetic acid (EDTA) (Gibco/Invitrogen) at 37°C and 5% CO₂. The epidermis was separated from the dermis in RPMI medium supplemented with 2% fetal

calf serum. The isolated epidermis was filtered through a 40 µm cell strainer.

We purchased OX from Wako (Osaka, Japan). Fluorochrome-conjugated antibodies to CD4, CD8, CD45, γδ TCR, vγ4, vγ5, IL-4, and IL-17A were purchased from eBioscience Inc. (San Diego, CA, USA).

For intracellular cytokine staining, cells were stimulated for 4 h with 50 ng/ml PMA and 1 µM ionomycin in the presence of GolgiStop™ (BD Biosciences). After washing, cells were fixed and permeabilized with cytofix/cytoperm buffer (BD Biosciences).

Cells were analyzed using the FACS LSR Fortessa flow cytometric system (BD Biosciences) and FlowJo software (TreeStar, Ashland, OR, USA).

Histology

For histological examination, tissues were fixed with 10% formalin in phosphate buffered saline, and then embedded in paraffin. Sections with a thickness of 5 µm were prepared and subjected to staining with hematoxylin and eosin. The histological findings were evaluated as reported previously (Nakajima *et al.*, 2010). Briefly, responses were graded as follows: 0, no response; 1, minimal response; 2, mild response; 3, moderate response; and 4, marked response. The total histology score was calculated as the sum of scores, including inflammation, neutrophil, mononuclear cells, edema, and epithelial hyperplasia.

Measurement of OX-specific serum IgE, IgG1, and IgG2a levels

Total serum IgE levels were measured using a mouse IgE enzyme-linked immunosorbent assay (ELISA) kit (Bethyl Laboratories, Montgomery, TX, USA). To measure OX-specific

Ig levels. Plates were coated and incubated with 10 µg/ml ovalbumin conjugated 2-phenyl-5-oxazolone (Biosearch Technologies, CA, USA) diluted with coating buffer overnight at 4°C. After a blocking period of 1 hour, 50 µl of diluted serum was added into each well and incubated for 2 h at room temperature. Fifty microliters of affinity-purified antibody for IgE, IgG1, and IgG2a diluted with sample conjugate was added into each well and incubated for 1 h at room temperature. Anti-mouse IgE, IgG1, and IgG2a-horseradish peroxidase conjugate (1:15,000; 50 µL) was used to conjugate the antigen–antibody complex for 60 min at room temperature. Absorbance was measured at 450 nm. The difference between the sample absorbance and the mean of negative control absorbance was taken as the result.

SUPPLEMENTAL TABLES

Table S1.

	Control		Chronic CHS	
	WT	IL-17 ^{-/-}	WT	IL-17 ^{-/-}
Inflammation	0.50±0.29	0.33±0.22	2.33±0.22	1.58±0.08
Neutrophils	0.00±0.00	0.33±0.08	2.50±0.29	1.42±0.08
Mononuclear cells	0.75±0.00	0.92±0.08	2.08±0.08	1.58±0.08
Edema	0.25±0.14	0.17±0.17	1.58±0.08	0.92±0.08
Epithelial hyperplasia	0.25±0.14	0.17±0.08	3.42±0.22	1.33±0.08

61

62 The histological findings were scored by inflammation, neutrophil infiltration, mononuclear
 63 cell infiltration, edema, and epithelial hyperplasia. Data are presented as means $\pm$ standard
 64 deviation (SD) (n = 5 per group). *, $p < 0.05$..

65

66 Table S2.

	B6		<i>Flg^{fl}</i>	
	IL-17 ^{+/+}	IL-17 ^{-/-}	IL-17 ^{+/+}	IL-17 ^{-/-}
Inflammation	0.50 $\pm$ 0.29	0.33 $\pm$ 0.22	1.25 $\pm$ 0.25	0.33 $\pm$ 0.22
Neutrophils	0.00 $\pm$ 0.00	0.33 $\pm$ 0.08	0.92 $\pm$ 0.17	0.58 $\pm$ 0.08
Mononuclear cells	0.75 $\pm$ 0.00	0.92 $\pm$ 0.08	1.08 $\pm$ 0.08	0.92 $\pm$ 0.08
Edema	0.25 $\pm$ 0.14	0.17 $\pm$ 0.17	0.75 $\pm$ 0.29	0.25 $\pm$ 0.14
Epithelial hyperplasia	0.25 $\pm$ 0.14	0.17 $\pm$ 0.08	2.75 $\pm$ 0.25	0.42 $\pm$ 0.17

67

68 The histological findings of IL-17A^{+/+} mice and IL-17A^{-/-} mice on B6 and *Flg^{fl}*
 69 backgrounds in the steady state were scored by inflammation, neutrophil infiltration,
 70 mononuclear cell infiltration, edema, and epithelial hyperplasia. Data are presented as mean
 71 $\pm$ SD (n = 5 per group). *, $p < 0.05$.

72

73 Table S3.

	IL-17 ^{+/+} <i>Flg^{fl}</i>	IL-17 ^{-/-} <i>Flg^{fl}</i>
Pruritus	2.00 $\pm$ 0.41	1.5 $\pm$ 0.29

Erythema	2.75 ± 0.25	1.50 ± 0.29
Erosion	0.50 ± 0.29	0.00 ± 0.00
Edema	1.50 ± 0.29	0.75 ± 0.25
Scaling	0.75 ± 0.25	0.25 ± 0.25

74

75 Clinical findings were designated as the sum of individual scores, graded as 0 (none), 1
 76 (mild), 2 (moderate), and 3 (severe), for the symptoms of pruritus, erythema, edema,
 77 erosion, and scaling. We evaluated scratching behavior objectively using the
 78 SCLABA-Real system (Noveltec, Kobe, Japan) as the manufacturer's protocol (Nie *et al.*,
 79 2009). Data are presented as mean $\pm$ SD (n = 4 per group).

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82 SUPPLEMENTAL REFERENCES

83

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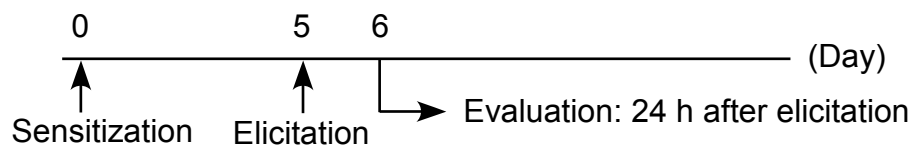
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(a) Protocol for acute CHS



(b) Protocol for chronic CHS

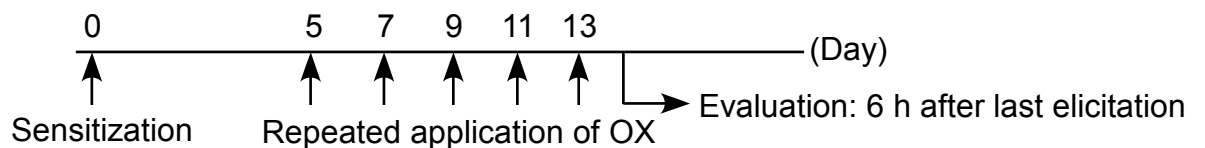


Figure S1. Protocol for acute and chronic CHS. Mice were sensitized with hapten application on the shaved abdominal skin on day 0. (A) Acute CHS. Five days after sensitization, mice were challenged with same hapten applying to the both sides of ear. (B) For chronic CHS induction, hapten was repeatedly applied to the ear every other day until day 13. Samples for histology and flow cytometry were obtained 6 h after last elicitation. Ear thickness was measured at 1, 6, 24, and 48 h after hapten application.

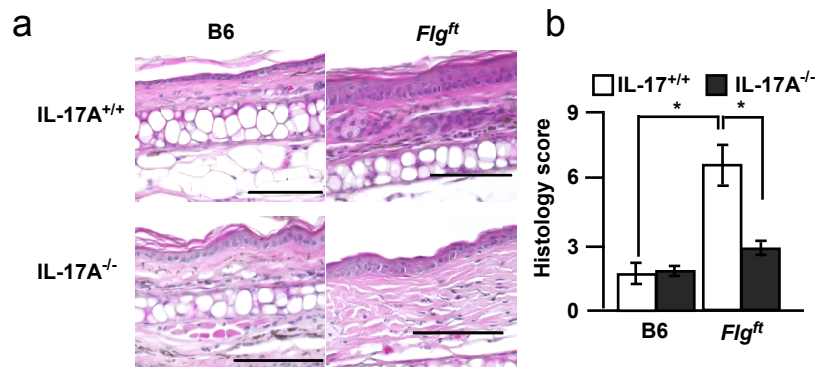


Figure S2. H&E staining of the ear skin of IL-17A^{+/+} mice and IL-17A^{-/-} mice on B6 and *Flg^{fl}* background in steady state (original magnification x400). Scale bar, 100 μ m. (B) The histological findings were scored by inflammation, neutrophil infiltration, mononuclear cell infiltration, edema and epithelial hyperplasia. Data are presented as means $\pm$ SD (n = 5). **P* < 0.05.

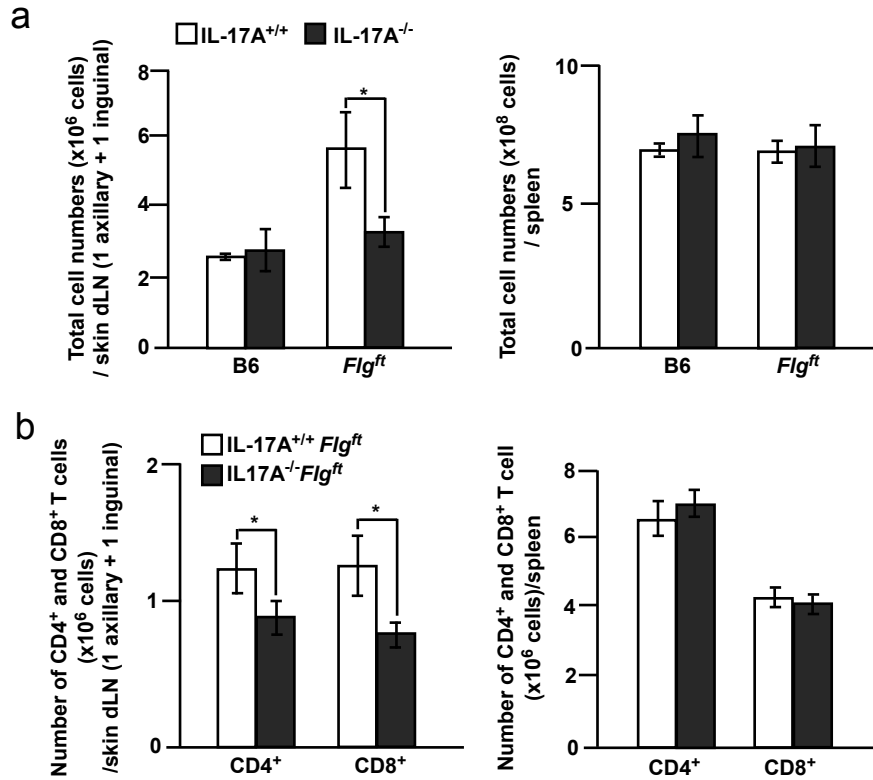


Figure S3. Cell numbers of IL-17A^{+/+} and IL-17A^{-/-} mice on B6 and *Flg^{ft}* background in a steady state. Skin-draining inguinal and axillary LN cells of IL-17A^{+/+} and IL-17A^{-/-} on B6 and *Flg^{ft}* background were collected and analyzed by flow cytometry (n=5 per group, age and sex matched). (A) Total cell numbers of skin draining LNs (left) and spleen (right). (B) The number of CD4⁺ cells and CD8⁺ cells in the skin-draining LN (left) and spleen (right) of IL-17A^{+/+} and IL-17A^{-/-} mice on *Flg^{ft}* background. * $P < 0.05$.