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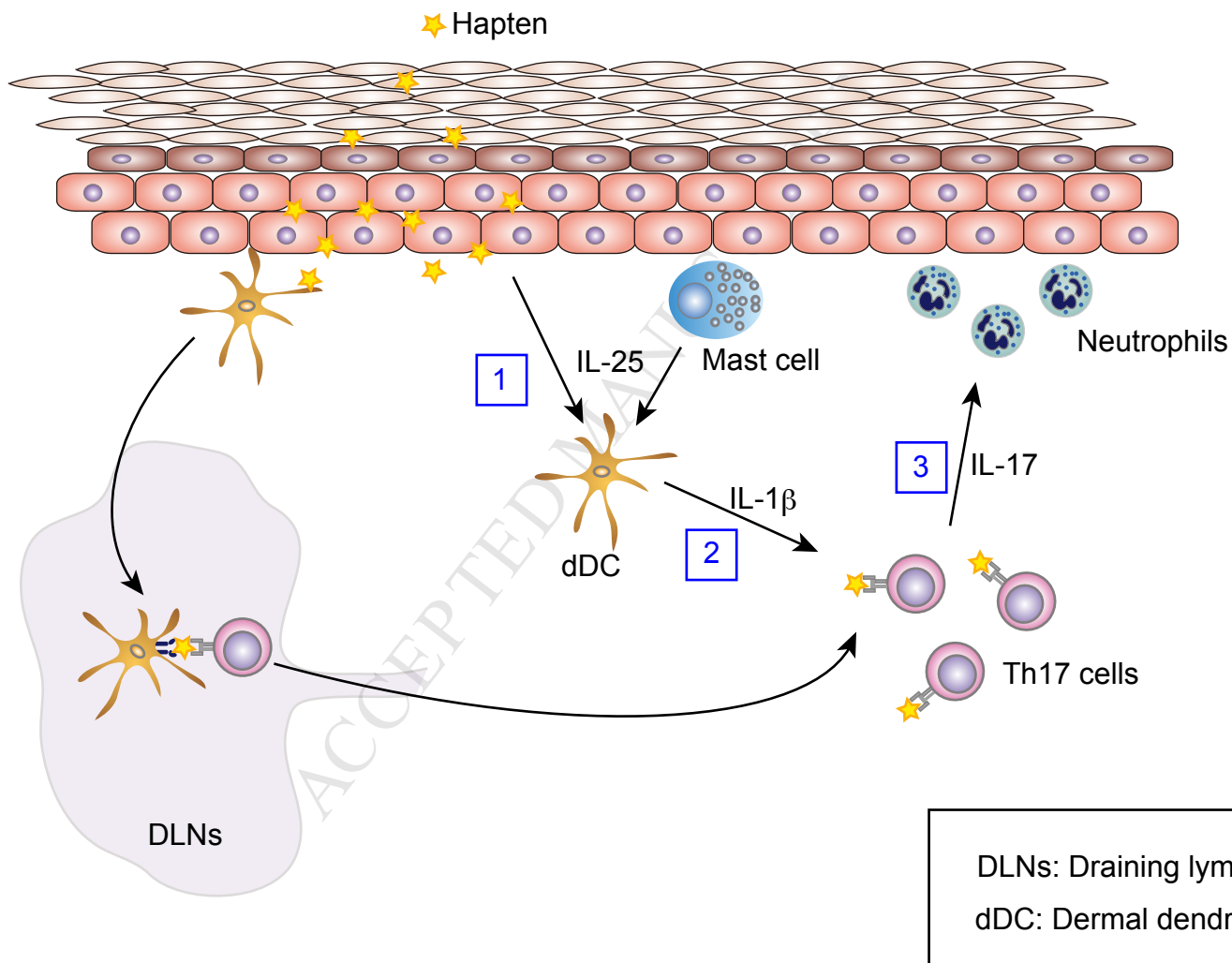
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# IL-25 enhances Th17-mediated contact dermatitis by promoting IL-1 $\beta$ production from dermal dendritic cells



# **IL-25 enhances Th17 cell-mediated contact dermatitis by promoting IL-1 $\beta$ production by dermal dendritic cells**

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**Abstract**

**Background:** As well as thymic stromal lymphopoietin (TSLP) and IL-33, IL-25 is known to induce Th2 cytokine production by various cell types—including Th2 cells, Th9 cells, invariant NKT cells and group 2 innate lymphoid cells—involved in Th2-type immune responses. Since both Th2-type and Th17-type cells/cytokines are crucial for contact hypersensitivity (CHS), IL-25 may contribute to this by enhancing Th2-type immune responses. However, the precise role of IL-25 in the pathogenesis of FITC-induced CHS is poorly understood.

**Objective:** We investigated the contribution of IL-25 to CHS using *Il25<sup>-/-</sup>* mice.

**Methods:** CHS was evaluated by measurement of ear skin thickness in mice after FITC-painting. Skin dendritic cell (DC) migration, hapten-specific Th cell differentiation and detection of IL-1 $\beta$ -producing cells were determined by flow cytometry, ELISA and immunohistochemistry, respectively.

**Results:** In contrast to TSLP, we found that IL-25 was not essential for skin DC migration or hapten-specific Th cell differentiation in the sensitization phase of CHS. Unexpectedly, mast cell- and non-immune cell-derived IL-25 was important for hapten-specific Th17 cell-, rather than Th2 cell-, mediated inflammation in the elicitation phase of CHS by enhancing Th17-related, but not Th2-related, cytokines in the skin. In particular, IL-1 $\beta$  produced by dermal DCs in response to IL-25 was crucial for hapten-specific Th17 cell activation, contributing to induction of local inflammation in the elicitation phase of CHS.

**Conclusion:** Our results identify a novel IL-25 inflammatory pathway involved in induction of Th17, but not Th2, cell-mediated CHS. IL-25 neutralization may be a

potential approach for treatment of CHS.

**Key messages:**

- IL-25 is not essential for skin DC migration and hapten-specific Th cell differentiation in the sensitization phase of CHS.
- IL-25, which is produced by mast cells and non-immune cells in the skin, stimulates dermal dendritic cells to produce IL-1 $\beta$  and thereby contributes to activation of Th17 cells, but not Th2 cells, in the elicitation phase of CHS.
- IL-25 may be a potential therapeutic target for CHS.

**Capsule summary:** (34 > 35 words)

This study is the first to show a novel IL-25 inflammatory pathway: IL-25 induces IL-1 $\beta$  production by dermal dendritic cells, followed by induction of Th17 cell–, but not Th2 cell–, mediated CHS.

**Key words:** Interleukin-25, interleukin-33, thymic stromal lymphopoietin, contact hypersensitivity, gene deficient-mice; Th2; Th17

**Abbreviations used:**

CHS: contact hypersensitivity

DC: dendritic cell

EPO: eosinophil peroxidase

MC: mast cell

MPO: myeloperoxidase

96 LN: lymph node

97 TSLP: thymic stromal lymphopoietin

98

## 99 **Introduction**

100 IL-25 (also called IL-17E), which is a member of the IL-17 family of cytokines, is  
 101 preferentially produced by epithelial cells and such immune cells as  
 102 macrophages, mast cells, basophils, eosinophils and T cells.<sup>1, 2</sup> IL-25 induces  
 103 Th2 cytokine production by various types of cells, including Th2 cells, Th9 cells,  
 104 invariant NKT cells and group 2 innate lymphoid cells,<sup>3, 4</sup> leading to eosinophilia in  
 105 the lung and gut and increased serum IgG1 and IgE levels.<sup>5</sup> Moreover, IL-25 can  
 106 induce Th2 cell differentiation and activation,<sup>6, 7</sup> suggesting its involvement in  
 107 Th2-type immune responses such as in nematode infection and allergic disorders.  
 108 Indeed, IL-25 was crucial for host defense against *Trichuris muris*,<sup>8</sup>  
 109 *Nippostrongylus brasiliensis*<sup>9</sup> and *Trichinella spiralis*,<sup>10</sup> and for development of  
 110 allergic airway inflammation<sup>11, 12</sup> in studies using IL-25-deficient (*IL25<sup>-/-</sup>*) mice  
 111 and/or mice treated with anti-IL-25–neutralizing Ab. On the other hand, IL-25 can  
 112 inhibit Th17 cell differentiation dependent on IL-13, contributing to suppression of  
 113 Th17-mediated autoimmune diseases.<sup>13, 14</sup> These observations suggest that  
 114 IL-25 is a potent enhancer of Th2-type immunity, but a suppressor of Th1 and  
 115 Th17-type immunity.

116 Contact hypersensitivity (CHS) is a T cell-mediated cutaneous allergic  
 117 response. IL-25 was elevated in keratinocytes from inflamed skin of patients with  
 118 contact dermatitis as well as atopic dermatitis and psoriasis.<sup>15, 16</sup> Based on  
 119 studies using gene-deficient mice, Th2- and Th17-cytokines are thought to be

involved in the development of CHS.<sup>17, 18</sup> Therefore, IL-25 may be involved in CHS induction by enhancing Th2-type, but suppressing Th17-type, immune responses. However, the precise role of IL-25 in the pathogenesis of CHS is poorly understood. Thus, in the present study, we used *Il25*<sup>-/-</sup> mice to investigate the contribution of IL-25 to CHS.

## METHODS

### Mice

Wild-type (C57BL/6J, C57BL/6N and BALB/cA) mice were purchased from Japan SLC, Inc. *Ifng*<sup>-/-</sup>, *Il17a*<sup>-/-</sup>, *Il1a*<sup>-/-</sup>, *Il1b*<sup>-/-</sup>, *Il1a*<sup>-/-</sup>*Il1b*<sup>-/-</sup> and *Il1r1*<sup>-/-</sup> mice, *Rorc*<sup>-/-</sup> mice, and *Rag2*<sup>-/-</sup> mice on the C57BL/6J background were kindly provided by Drs. Yoichiro Iwakura (Tokyo University of Science, Tokyo, Japan), Ichiro Taniuchi (RIKEN, Yokohama, Japan), and Hiromitsu Nakauchi (The University of Tokyo, Tokyo, Japan), respectively. *Stat6*<sup>-/-</sup> mice on the BALB/c background were kindly provided by Dr. Masato Kubo (Tokyo University of Science, Tokyo, Japan). *Il25*<sup>-/-19</sup> and *Il33*<sup>-/-20</sup> mice on the C57BL/6N background were generated as described previously. *Kit*<sup>W-sh/W-sh</sup> mice on the C57BL/6N background were obtained from Sankyo Labo Service Corporation (Tsukuba, Japan).

*Tslp*<sup>-/-</sup> mice (Accession No. CDB0777K:

<http://www2.clst.riken.jp/arg/mutant%20mice%20list.html>) were generated as follows: A BAC clone, which contains the translational start site of *Tslp* gene, was obtained from BACPAC Resources (<https://bacpac.chori.org>). The targeting vector was constructed as described at the website of RIKEN (Kobe, Japan; <http://www2.clst.riken.jp/arg/protocol.html>). Briefly, the region

(ACAGCATGGGTGACTATGGGCTGTGCAGGGACTGGGAAGGGGTGGTGAG  
 GGCTGATAC) between the first exon, which contains the initiation codon of *Tslp*,  
 and the second exon was replaced with a cassette consisting of the mutated  
*Aequorea* green fluorescent protein (*GFP*) gene and the *neomycin resistance*  
 gene (*neo*<sup>r</sup>), flanked by loxP sequences. The targeting vector was electroporated  
 into C57BL/6N ES cells (HK3i).<sup>21</sup> Male chimeric mice were obtained from three  
 distinct targeted clones and mated with C57BL/6N female mice. Genotyping of  
 mice was performed by PCR using the following primers: TSLP common  
 (5'-GATCAGGAAGACTCCACGTTC-3'), TSLP WT  
 (5'-TCAGGTCATGAAAAATTATGTT-3') and TSLP KO  
 (5'-CACCTCGGCGCGGGTCTTGTA-3'). The TSLP common and TSLP WT  
 primers were used for detection of wild-type alleles (~350 bp), and the TSLP  
 common and TSLP KO primers were used for detection of mutant alleles (~430  
 bp). Detailed information regarding the *Tslp*<sup>-/-</sup> mice (Accession No. CDB0777K) is  
 available at <http://www2.clst.riken.jp/arg/mutant%20mice%20list.html>. All mice  
 were housed in a specific-pathogen-free environment at The Institute of Medical  
 Science, The University of Tokyo. The animal protocol for experiments was  
 approved by the Institutional Review Board of the Institute (A11-28), and all  
 experiments were conducted according to the ethical and safety guidelines of the  
 Institute.

### **Contact hypersensitivity (CHS)**

FITC-induced CHS and DNFB-induced CHS were induced as described  
 elsewhere.<sup>20, 22</sup>

### **Skin DC migration**

FITC-induced DC migration was performed as described elsewhere.<sup>20, 22</sup>

### **FITC-specific LN cell responses**

FITC-specific LN cell responses were examined as described elsewhere.<sup>20, 22</sup>

### **Measurement of myeloperoxidase and eosinophil peroxidase activities**

The levels of myeloperoxidase (MPO) and eosinophil peroxidase (EPO) activities in tissues were examined as described elsewhere.<sup>20</sup>

### **Histology**

Twenty-four hours after epicutaneous challenge with FITC or vehicle, the ear skin was harvested, fixed in Carnoy's fluid and embedded in paraffin. Sections were prepared and stained with hematoxylin-eosin.

### **IL-25 injection**

Five µg of recombinant mouse IL-25/IL-17E (R&D Systems) in 20 µl of PBS (left ear) or 20 µl of PBS alone (right ear) was injected intradermally to naïve C57BL/6N wild-type mice or *IL25*<sup>-/-</sup> mice.

### **Quantitative PCR**

Quantitative real-time PCR was performed with THUNDERBIRD SYBR qPCR Mix (Toyobo) and a CFX384<sup>TM</sup> Real-Time System (Bio-Rad). The relative gene expression was determined against GAPDH gene expression. PCR primers were

designed as shown in Table E1.

## **Generation of bone marrow cell-derived mast cells, macrophages and dendritic cells**

Bone marrow (BM) cells were collected from femora and tibiae of mice. Bone marrow cell-derived cultured mast cells (BMCMCs), macrophages (BMM $\phi$ ) and dendritic cells (BMDCs) were generated by culture of BM cells from mice as described elsewhere.<sup>22, 23</sup>

## **Statistical analysis**

Data show the mean  $\pm$  SEM. Differences were evaluated by the two-tailed Student's t test and considered significant at a P value of less than 0.05.

The detailed methods are presented in the Methods section of this article's Online Repository at [www.jacionline.org](http://www.jacionline.org).

## **RESULTS**

### **IL-25 is involved in CHS without affecting hapten-sensitization**

FITC-induced CHS was significantly reduced in *Stat6*<sup>-/-</sup>, *Il17a*<sup>-/-</sup> and *rorc*<sup>-/-</sup> mice, but not *Ifng*<sup>-/-</sup> mice, compared with wild-type mice (Fig 1, A), indicating that Th2- and Th17-, but not Th1-, cytokines/cells are required for the responses. Like IL-25, IL-33 and TSLP are potent inducers of Th2 cytokines.<sup>1, 2</sup> Consistent with the findings of previous reports using TSLP receptor-deficient (*Crlf2*<sup>-/-</sup>) or *Il33*<sup>-/-</sup> mice,<sup>20, 24</sup> FITC-induced CHS was significantly decreased in *Tslp*<sup>-/-</sup> mice, which

were newly generated (Fig E1), but not *Il33*<sup>-/-</sup> mice, compared with wild-type mice (Fig 1, B). Moreover, we found that the thickness of ear skin, the levels of myeloperoxidase and eosinophil peroxidase activities, and skin inflammation (based on histological analysis) were significantly decreased in *Il25*<sup>-/-</sup> mice compared with wild-type mice (Fig 1, B, C and Fig E2). mRNA expression for IL-1 $\beta$  and CCL22, but not for other tested cytokines, including IL-4 and IL-13, and chemokines, was reduced in the skin of *Il25*<sup>-/-</sup> mice compared with wild-type mice 24 hours after FITC challenge (Fig 1, D). mRNA expression for both IL-17A and IL-25 was below the limit of detection in the setting (data not shown). These observations suggest that IL-25 is involved in development of FITC-induced CHS. Likewise, DNFB-induced CHS was significantly decreased in *Il25*<sup>-/-</sup> mice as well as *Tslp*<sup>-/-</sup>, *Stat6*<sup>-/-</sup>, *Il17a*<sup>-/-</sup> and *rorc*<sup>-/-</sup> mice, but not *Ifng*<sup>-/-</sup> mice (FIG E3).

*Crlf2*<sup>-/-</sup> mice showed reduced migration of DCs from the skin to draining LNs following impairment of hapten-specific Th2 cell differentiation in the sensitization phase of FITC-induced CHS.<sup>24</sup> Here, *Il25*<sup>-/-</sup> mice showed migration of DCs from the skin to draining LNs 24 hours after epicutaneous treatment with FITC (Fig 2, A, B), FITC-specific LN cell proliferation and -IFN- $\gamma$ , IL-4, IL-5 and IL-17 production that were comparable to in wild-type mice (Fig 2, C, D). Consistent with this, development of CHS in non-sensitized *Rag2*<sup>-/-</sup> mice engrafted with LN cells from FITC-sensitized *Il25*<sup>-/-</sup> mice (*Il25*<sup>-/-</sup> LN cells  $\rightarrow$  *Rag2*<sup>-/-</sup> mice) was equivalent to that in non-sensitized *Rag2*<sup>-/-</sup> mice engrafted with LN cells from FITC-sensitized wild-type mice (WT LN cells  $\rightarrow$  *Rag2*<sup>-/-</sup> mice) after epicutaneous treatment with FITC (Fig 2, E). These observations suggest that IL-25 is not essential for skin DC activation and differentiation of hapten-specific Th cell subsets, including Th2

and Th17 cells, in the sensitization phase of FITC-induced CHS. In addition, T cell-derived IL-25 was not essential for development of FITC-induced CHS, although IL-25 is expressed by Th2 cells<sup>5</sup> and cecal patch T cells.<sup>8</sup>

### **IL-25 is involved in induction of local inflammation in the elicitation phase of CHS**

IL-25 may be important for induction of local inflammation in the elicitation phase of CHS. The reduced CHS in IL-25-deficient mice was restored when rIL-25 was administered before challenge (Fig E4), but not before sensitization (data not shown). Indeed, development of CHS in non-sensitized *Rag2*<sup>-/-</sup> *Il25*<sup>-/-</sup> mice engrafted with LN cells from FITC-sensitized wild-type mice (WT LN cells → *Rag2*<sup>-/-</sup> *Il25*<sup>-/-</sup> mice) was significantly suppressed in comparison with in similarly engrafted non-sensitized *Rag2*<sup>-/-</sup> mice (WT LN cells → *Rag2*<sup>-/-</sup> mice) after epicutaneous FITC treatment (Fig 2, F). This indicates that IL-25 is required for induction of local inflammation in the elicitation phase of CHS.

qPCR detected *Il25* mRNA in CD45-negative epidermal cells (mainly keratinocytes) and CD45-positive and -negative dermal cells from the skin of naïve and FITC-challenged wild-type mice (Fig E5), but IL-25 proteins were below the limit of detection in immunohistochemical analysis (data not shown). To identify the producers of IL-25, we performed bone marrow cell transfer analysis. FITC-induced CHS was reduced in *Il25*<sup>-/-</sup> mice engrafted with bone marrow (BM) cells from wild-type mice (WT BM cells → *Il25*<sup>-/-</sup> mice) compared with wild-type mice engrafted with BM cells from wild-type mice (WT BM cells → WT mice). This suggests that IL-25 derived from BM cell-derived immune cells is important for

development of FITC-induced CHS (Fig 2, G). Meanwhile, FITC-induced CHS was similarly reduced in *IL25<sup>-/-</sup>* BM cells → WT mice and WT BM cells → *IL25<sup>-/-</sup>* mice compared with wild-type BM cells → wild-type mice. This suggests that IL-25 derived from non-immune cells is also involved in development of FITC-induced CHS (Fig 2, G).

Mast cells (MCs), which are crucial effector cells in FITC-induced CHS,<sup>22, 25</sup> are a potential producer of IL-25.<sup>26</sup> Indeed, IL-25 proteins were detected in cell lysates, but not culture supernatants (data not shown), of MCs, but not macrophages and dendritic cells, derived from BM cells (Fig E5). Consistent with our previous report,<sup>22</sup> *Kit<sup>W-sh/W-sh</sup>* mice exhibited significantly impaired development of FITC-induced CHS compared with wild-type mice (Fig 2, H). That impairment was abolished by engraftment with wild-type MCs (WT MCs → *Kit<sup>W-sh/W-sh</sup>* mice)(Fig 2, H). On the other hand, engraftment with *IL25<sup>-/-</sup>* MCs (*IL25<sup>-/-</sup>* MCs → *Kit<sup>W-sh/W-sh</sup>* mice) partially, but not completely, abolished the impairment (Fig 2, H). These observations suggest that IL-25 produced by MCs and other types of cells, including non-immune cells, and/or other factors derived from MCs is important for development of FITC-induced CHS

### **IL-25-IL-1 $\beta$ axis is an important pathway for Th17 cell-mediated CHS**

Systemic administration of rIL-25 resulted in increased expression of such Th2-type cytokines as IL-4, IL-5 and IL-13 in various tissues.<sup>5</sup> However, mRNA expression for Th2-type and -related cytokines such as IL-4, IL-13, IL-33 and TSLP was not increased in the skin after intradermal injection of rIL-25 (Fig 3, A).

Interestingly, in the setting, expression of IL-1 $\beta$ , IL-6 and TNF, which enhance Th17 cell differentiation and activation,<sup>18</sup> was markedly increased, while IL-1 $\alpha$ , IL-21 and IL-23 were partially, but not significantly, upregulated (Fig 3, A). Th2 cells and Th17 cells express, respectively, CCR3 and CCR4<sup>27</sup>, and CCR4, CCR6 and CXCR3.<sup>28, 29</sup> rIL-25 injection also increased mRNA expression for CCR4 ligands (CCR17 and CCL22), a CCR6 ligand (CCL20) and a CXCR3 ligand (CXCL10) in the skin (Fig 3, A). mRNA expression for both IL-17A and IL-25 was below the limit of detection in the setting (data not shown).

To elucidate the role of IL-25 in Th2 or Th17 cell activation in the skin, we performed FITC-responsive Th2 cell or Th17 cell engraftment. Development of CHS was aggravated, rather than suppressed, in *IL25*<sup>-/-</sup> mice engrafted with FITC-responsive Th2 cells (Th2 cells  $\rightarrow$  *IL25*<sup>-/-</sup> mice) in comparison with similarly engrafted wild-type mice (Th2 cells  $\rightarrow$  WT mice) (Fig 3, B). Conversely, CHS was significantly suppressed in *IL25*<sup>-/-</sup> mice engrafted with FITC-responsive Th17 cells (Th17 cells  $\rightarrow$  *IL25*<sup>-/-</sup> mice) compared with similarly engrafted wild-type mice (Th17 cells  $\rightarrow$  WT mice), after epicutaneous FITC treatment (Fig 3, C). These findings suggest that IL-25 induces local skin inflammation by activating Th17 cells, but not Th2 cells, during FITC-induced CHS.

rIL25 injection resulted in augmented mRNA expression for such Th17-related cytokines as IL-1 $\beta$ , IL-6 and TNF in the skin (Fig 3, A), while mRNA expression for IL-1 $\beta$ , but not for other tested cytokines, including IL-1 $\alpha$ , IL-6 and TNF, or chemokines, was reduced in the skin of *IL25*<sup>-/-</sup> mice compared with wild-type mice during FITC-induced CHS (Fig 1, D). Thus, IL-25-induced IL-1 $\beta$

may contribute to induction of Th17 cell-mediated local skin inflammation during FITC-induced CHS. Indeed, predominantly IL-1 $\beta$ <sup>+</sup> cells were observed in the dermis 24 hours after FITC challenge (Fig 3, D). Likewise, IL-17RB, which is a component of IL-25R, was also expressed on CD11c<sup>+</sup> cells in the dermis 24 hours after FITC challenge (Fig E6). In addition, after intradermal rIL-25 injection, IL-1 $\beta$ <sup>+</sup> cells were observed in the dermis, and they co-expressed CD11c (Fig 3, E), suggesting that CD11c<sup>+</sup> dermal DCs are major producers of IL-1 $\beta$  in response to IL-25. However, IL-1 $\beta$  was not essential for induction of CHS by oxazolone and TNCB in a study using *Il1b*<sup>-/-</sup> mice.<sup>30-32</sup> Indeed, FITC-induced CHS was significantly attenuated in *Il1a*<sup>-/-</sup>*Il1b*<sup>-/-</sup> mice, but not *Il1a*<sup>-/-</sup> mice or *Il1b*<sup>-/-</sup> mice, compared with wild-type mice (Fig 4, A). Since both IL-1 $\alpha$  and IL-1 $\beta$  bind to the same receptor, these observations suggest that both are required for optimal FITC induction of CHS through synergism.

As shown in Fig 1D, mRNA for IL-1 $\beta$ , but not IL-1 $\alpha$ , was increased in skin from wild-type mice during FITC-induced CHS. And mRNA for IL-1 $\beta$  was significantly decreased in skin from IL-25-deficient mice. As shown in Fig 3A, mRNA for IL-1 $\beta$ , but not IL-1 $\alpha$ , was induced by injection of IL-25 into the skin. These findings suggest that IL-25 can induce IL-1 $\beta$ , but not IL-1 $\alpha$ , in the skin. Therefore, we next investigated the effect of IL-1 $\beta$  on the reduced CHS responses seen in *Il25*<sup>-/-</sup> mice. The reduced CHS was restored by administration of rIL-1 $\beta$  (Fig 4, B). Consistent with Fig 3, C, ear skin swelling was significantly reduced in Th17 cells  $\rightarrow$  *Il25*<sup>-/-</sup> mice compared with Th17 cells  $\rightarrow$  wild-type mice (Fig 4, B, Untreated group). Since PBS injection resulted in edema, the ear skin

thickness in the PBS-injected group was increased compared with the untreated group, even after treatment with vehicle alone (Fig 4, B, PBS-injected group). On the other hand, the reduced ear skin swelling seen in the Th17 cells  $\rightarrow$  *Il25*<sup>-/-</sup> mice was restored to the level observed in Th17 cells  $\rightarrow$  wild-type mice following intradermal injection of rIL-1 $\beta$  (Fig 4, B, rIL-1 $\beta$ -injected group). These observations suggest that IL-1 $\beta$  is important for induction of Th17 cell-mediated local skin inflammation during FITC-induced CHS. Next, to elucidate whether IL-1 $\beta$  activates Th17 cells directly, we used Th17 cells derived from mice deficient in IL-1R1, which is a crucial adapter molecule for signal transduction of IL-1 receptor. However, the reduced response seen in *Il1r1*<sup>-/-</sup> Th17 cells  $\rightarrow$  wild-type mice was not reversed by intradermal IL-1 $\beta$  injection (Fig E7), indicating that IL-1 $\beta$  activates Th17 cells directly in the setting.

## Discussion

IL-25, IL-33 and TSLP, which are produced mainly by epithelial cells, have redundant roles in certain immune responses<sup>2</sup>. In addition, excessive/inappropriate production of these cytokines is considered to be involved in the development of allergic disorders such as atopic dermatitis and asthma.<sup>33</sup> Increased expression of IL-33 was observed in specimens from patients with contact dermatitis<sup>34</sup> as well as atopic dermatitis and asthma.<sup>33</sup> However, IL-33 was not essential for development of CHS in studies using *Il33*<sup>-/-</sup> mice.<sup>20</sup>

TSLP levels were also increased in specimens from patients with atopic

dermatitis and asthma,<sup>33</sup> but not in skin lesions from patients with nickel-induced contact dermatitis or disseminated lupus erythematosus.<sup>35</sup> Nevertheless, development of CHS was significantly reduced in *Crlf2*<sup>-/-</sup> mice<sup>24</sup> and *Tslp*<sup>-/-</sup> mice (Fig 1, B), suggesting that TSLP is crucial for induction of CHS. Regarding this, TSLP expression was increased in mouse skin after hapten sensitization<sup>24</sup> but not after challenge (Fig 1, D), suggesting that TSLP may be important for immune responses in the sensitization phase, but not the challenge phase, of CHS. In support of this notion, it is known that TSLP is required for migration of DCs from the skin to draining LNs following development of hapten-specific Th2 cell differentiation in the sensitization phase of CHS.<sup>24</sup>

Although IL-25 expression was also increased in specimens from patients with contact dermatitis,<sup>15</sup> the role of IL-25 in the development of CHS has not been elucidated in the mouse model. In the present study, we clearly demonstrated that IL-25 is important for the development of FITC-induced and DNFB-induced CHS, and its roles were different from those of TSLP in the setting. As noted above, TSLP is crucial for DC function and hapten-specific Th2 cell differentiation in the sensitization phase of CHS.<sup>24</sup> On the other hand, we demonstrated that IL-25 was not essential for either DC function or Th2 cell differentiation in the sensitization phase of FITC-induced CHS. In addition, although TSLP was involved in Th2 cell activation during CHS,<sup>24</sup> we found—unexpectedly—that IL-25 induced local inflammation via Th17 cell activation, but not Th2 cell activation, in the elicitation phase of FITC-induced CHS. It is generally thought that IL-17 and IL-25 predominantly induce neutrophilia and eosinophilia, respectively.<sup>5, 36</sup> However, neutrophilia was

enhanced in mice overexpressing IL-25,<sup>36-38</sup> suggesting IL-25's involvement in induction of both Th2-cytokine associated eosinophilia and Th17-cytokine associated neutrophilia. In support of this notion, we found for the first time that IL-25 can induce such Th17-related cytokines as IL-1 $\beta$ , IL-6 and TNF in the skin (Fig 3, A). Notably, we demonstrated that IL-25 is crucial for Th17 cell activation via production of IL-1 $\beta$  by dermal DCs during FITC-induced CHS (Figs 3, 4).

IL-25 was reported to be produced by various types of immune cells such as T cells, macrophages, mast cells, eosinophils and basophils, and non-immune cells such as epithelial cells and endothelial cells, in humans and/or mice.<sup>39</sup> As far as we tested, IL-25-positive signals were hardly detectable (below the limit of detection) by immunohistochemical analysis (data not shown). However, IL-25 mRNA expression or IL-25 protein was detected in keratinocytes, dermal CD45<sup>+</sup> and CD45<sup>-</sup> cells or in cell lysates, but not culture supernatants, of mast cells, but not macrophages or DCs, respectively (Fig E5). In addition, we demonstrated that mast cell- and epithelial cell-, but not T cell-, derived IL-25 is important for development of FITC-induced CHS by adoptive BM cell, MC and LN cell transfer studies (Fig 2, E-H). In the skin, IL-17RB, a component of IL-25R, is expressed on macrophages, eosinophils, neutrophils, mast cells, ILC2 and keratinocytes.<sup>15, 40, 41</sup> We showed that IL-17RB-positive signals were detected in CD11c<sup>+</sup>, but not tryptase<sup>+</sup>, CD3<sup>+</sup>, F4/80<sup>+</sup> or Gr1<sup>+</sup>, cells (data not shown) in the dermis of the skin during FITC-induced CHS (Fig E6). These observations suggest that mast cell- and epithelial cell-derived IL-25 stimulates IL-17RB-expressing CD11c<sup>+</sup> dermal DCs to induce IL-1 $\beta$  secretion *in vivo*. We investigated whether BMDCs, which were generated by culture of BM cells in the presence of GM-CSF or GM-CSF +

IL-4, could produce IL-1 $\beta$  in response to IL-25 *in vitro*. However, they did not express IL-17RB, and could not produce IL-1 $\beta$  in response to IL-25 (data not shown), suggesting that IL-17RB-expressing CD11c<sup>+</sup> dermal DCs are a different population from GM-CSF–induced or GM-CSF + IL-4–induced BMDCs.

Taken all together, our findings improve our understanding of the molecular mechanisms involved in development of CHS, and suggest that neutralization of IL-25 may be a potential therapeutic approach for CHS.

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## Figure Legends

### FIG 1. IL-25 is crucial for FITC-induced CHS.

Twenty-four hours after challenge with FITC, the ear skin thickness of mice was measured, and ear skin tissues were collected.

**A**, Ear skin thickness in wild-type (WT; n=10-12), *Ifng*<sup>-/-</sup> (n=8), *Il17a*<sup>-/-</sup> (n=10), *Rorc*<sup>-/-</sup> (n=8) and *Stat6*<sup>-/-</sup> (n=8) mice.

**B**, Ear skin thickness in WT (n=5-10), *Il25*<sup>-/-</sup> (n=10), *Il33*<sup>-/-</sup> (n=5), and *Tslp*<sup>-/-</sup> (n=5) mice.

**C**, Sections of ear skin from WT and *Il25*<sup>-/-</sup> mice in (B) were stained with hematoxylin and eosin. Representative data are shown.

**D**, mRNA expression levels of cytokines and chemokines in the ear skin of WT and *Il25*<sup>-/-</sup> mice in (B); determined by quantitative PCR.

The data show the mean + SEM. Results are representative of similar results that were obtained in 2-3 independent experiments. \*p<0.01 vs. WT.

### FIG 2. IL-25 is not essential for sensitization, but is required for elicitation of CHS.

**A, B**, The proportion of FITC-positive cells among 7-aminoactinomycin D-negative MHC class II<sup>hi</sup> CD11c<sup>+</sup> cells in draining LNs from wild-type (WT; n=10) and *Il25*<sup>-/-</sup> (n=10) mice 24 hours after sensitization with FITC or vehicle alone; determined by flow cytometry. Representative flow cytometric data are shown. Shaded area = LNs obtained from the vehicle-treated left ear (lower number [%]), and solid lines = LNs obtained from the FITC-treated right ear (upper number [%]).

**C, D**, LN cells from FITC-sensitized mice were cultured in the presence and absence of 40 µg/ml FITC for 72 hours. Incorporation of [3H]-thymidine (**C**) and the levels of IFN-γ, IL-4, IL-5 and IL-17 in the culture supernatants (**D**). Wild-type (WT; n=9) and *Il25*<sup>-/-</sup> (n=9) mice.

**E-H**, Twenty-four hours after epicutaneous treatment with FITC or vehicle alone, the mice were examined for an increase in ear thickness. LN cells from FITC-sensitized WT mice or FITC-sensitized *Il25*<sup>-/-</sup> mice were intravenously injected into naïve *Rag2*<sup>-/-</sup> mice (WT LN cells → *Rag2*<sup>-/-</sup> mice [n=15]; *Il25*<sup>-/-</sup> LN cells → *Rag2*<sup>-/-</sup> mice [n=15]) (**E**). LN cells from FITC-sensitized WT mice were intravenously injected into naïve *Rag2*<sup>-/-</sup> mice (WT LN cells → *Rag2*<sup>-/-</sup> mice; n=10) or naïve *Rag2*<sup>-/-</sup> *Il25*<sup>-/-</sup> mice (WT LN cells → *Rag2*<sup>-/-</sup> *Il25*<sup>-/-</sup> mice; n=9) (**F**). Bone marrow (BM) cells from WT mice or *Il25*<sup>-/-</sup> mice were intravenously injected into irradiated naïve WT mice (WT BM cells → WT mice [n=15]; *Il25*<sup>-/-</sup> BM cells → WT mice [n=13]) or *Il25*<sup>-/-</sup> mice (WT BM cells → *Il25*<sup>-/-</sup> mice [n=15]; *Il25*<sup>-/-</sup> BM cells → *Il25*<sup>-/-</sup> mice [n=13]) (**G**). Bone marrow cell-derived cultured mast cells (MCs) from wild-type (WT) or *Il25*<sup>-/-</sup> mice were intradermally injected into the ear skin of mast cell-deficient *Kit*<sup>W-sh/W-sh</sup> mice (WTMCs → *Kit*<sup>W-sh/W-sh</sup> mice [n=15]; *Il25*<sup>-/-</sup> MCs → *Kit*<sup>W-sh/W-sh</sup> mice [n=15]) (**H**). The data show the mean + SEM (**B-H**). Results are representative of similar results that were obtained in 2 independent experiments. \*p<0.05 vs. WT LN cells → WT mice (**F**), WT BM cells → WT mice (**G**) and WT mice (**H**); and †p<0.05 vs. WT BM cells → *Il25*<sup>-/-</sup> mice or *Il25*<sup>-/-</sup> BM cells → WT mice (**G**) and *Il25*<sup>-/-</sup> MCs → *Kit*<sup>W-sh/W-sh</sup> mice (**H**).

**FIG 3. IL-25 enhances Th17 cell–, but not Th2 cell–, mediated inflammation during FITC-induced CHS.**

**A**, Six hours after intradermal injection of recombinant mouse IL-25 and PBS into the ear skin of wild-type mice (n=5), the skin was collected. The expression levels of mRNA in the skin were determined by quantitative PCR.

**B, C**, Twenty-four hours after epicutaneous challenge with FITC or vehicle alone, the ear thickness in naïve wild-type (WT) mice injected with FITC-responsive IL-4<sup>+</sup> Th2 cells (Th2 cells → WT mice; n=14) and *Il25*<sup>-/-</sup> mice injected with FITC-responsive IL-4<sup>+</sup> Th2 cells (Th2 cells → *Il25*<sup>-/-</sup> mice; n=15) (**B**) or in naïve WT mice injected with FITC-responsive IL-17<sup>+</sup> Th17 cells (Th17 cells → WT mice; n=8) and naïve *Il25*<sup>-/-</sup> mice injected with FITC-responsive IL-17<sup>+</sup> Th17 cells (Th17 cells → *Il25*<sup>-/-</sup> mice; n=8) (**C**) was examined.

**D**, Twenty-four hours after epicutaneous challenge with FITC, the ear skin was collected. IL-1 $\beta$  expression in sections of ear skin was detected by immunohistochemistry. Arrowheads = representative IL-1 $\beta$ <sup>+</sup> cells.  $\times 40$ .

**E**, Twenty-four hours after IL-25 injection, the ear skin was collected. IL-1 $\beta$  or CD11c expression in sections of the ear skin was detected by immunohistochemistry. Arrowheads = representative IL-1 $\beta$ <sup>+</sup> CD11c<sup>+</sup> cells.  $\times 40$ .

The data show the mean + SEM (A-C). Results are representative of similar results that were obtained in 2 independent experiments. \*p<0.05 vs. the PBS-injected group (**A**), Th2 cells → WT mice (**B**), or Th17 cells → WT mice (**C**).

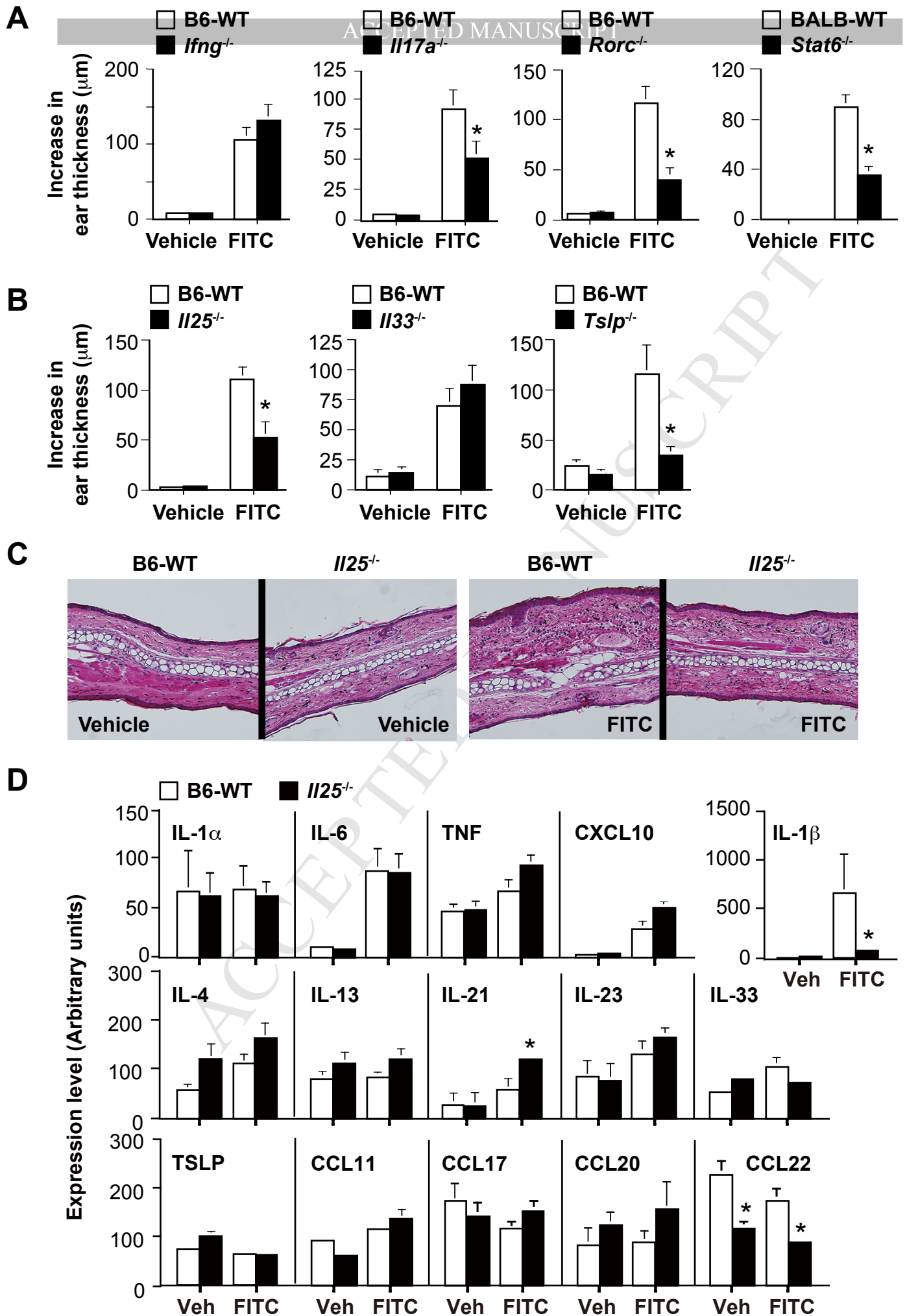
**FIG 4. IL-1 $\beta$  is crucial for Th17 cell–mediated skin inflammation during**

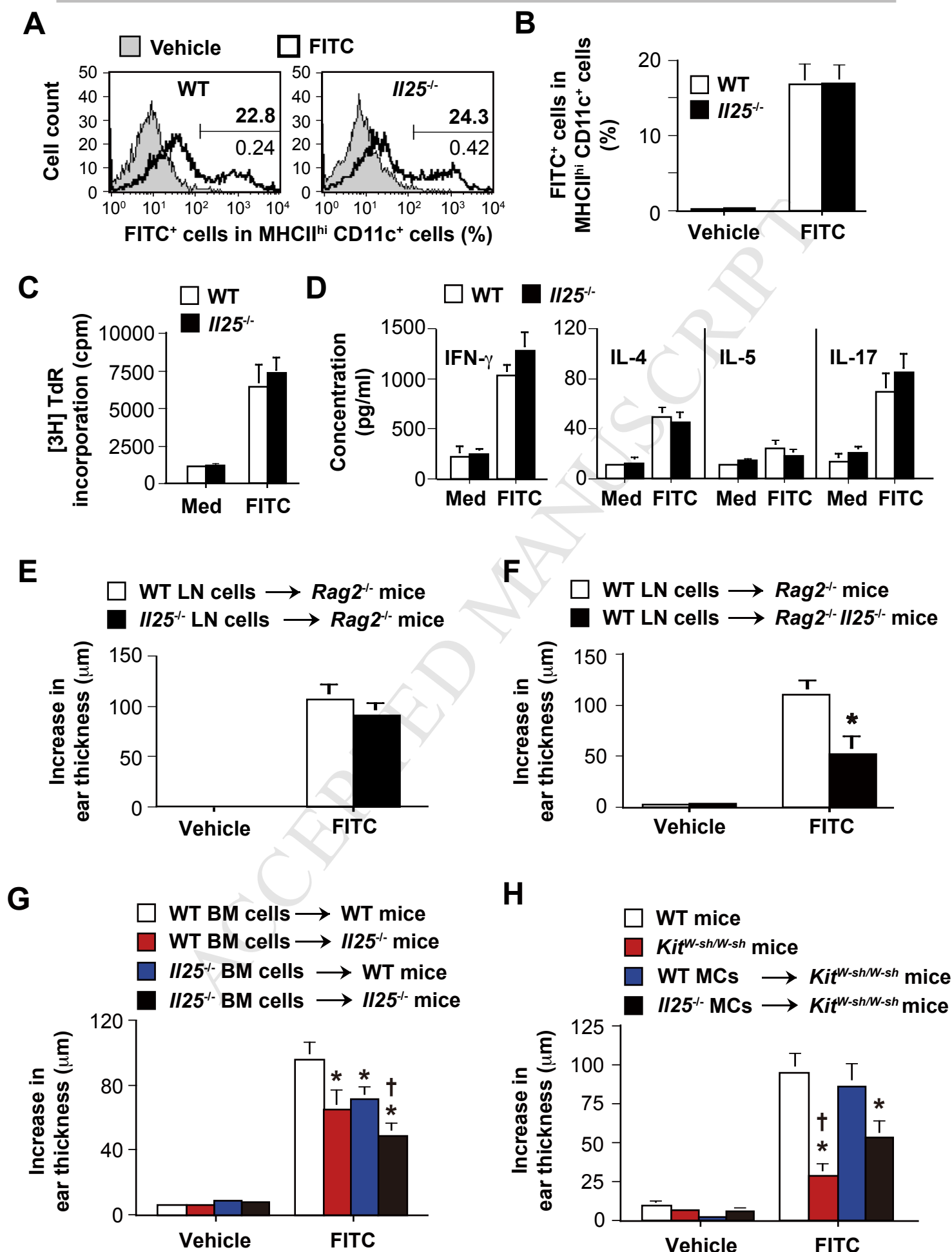
**FITC-induced CHS.**

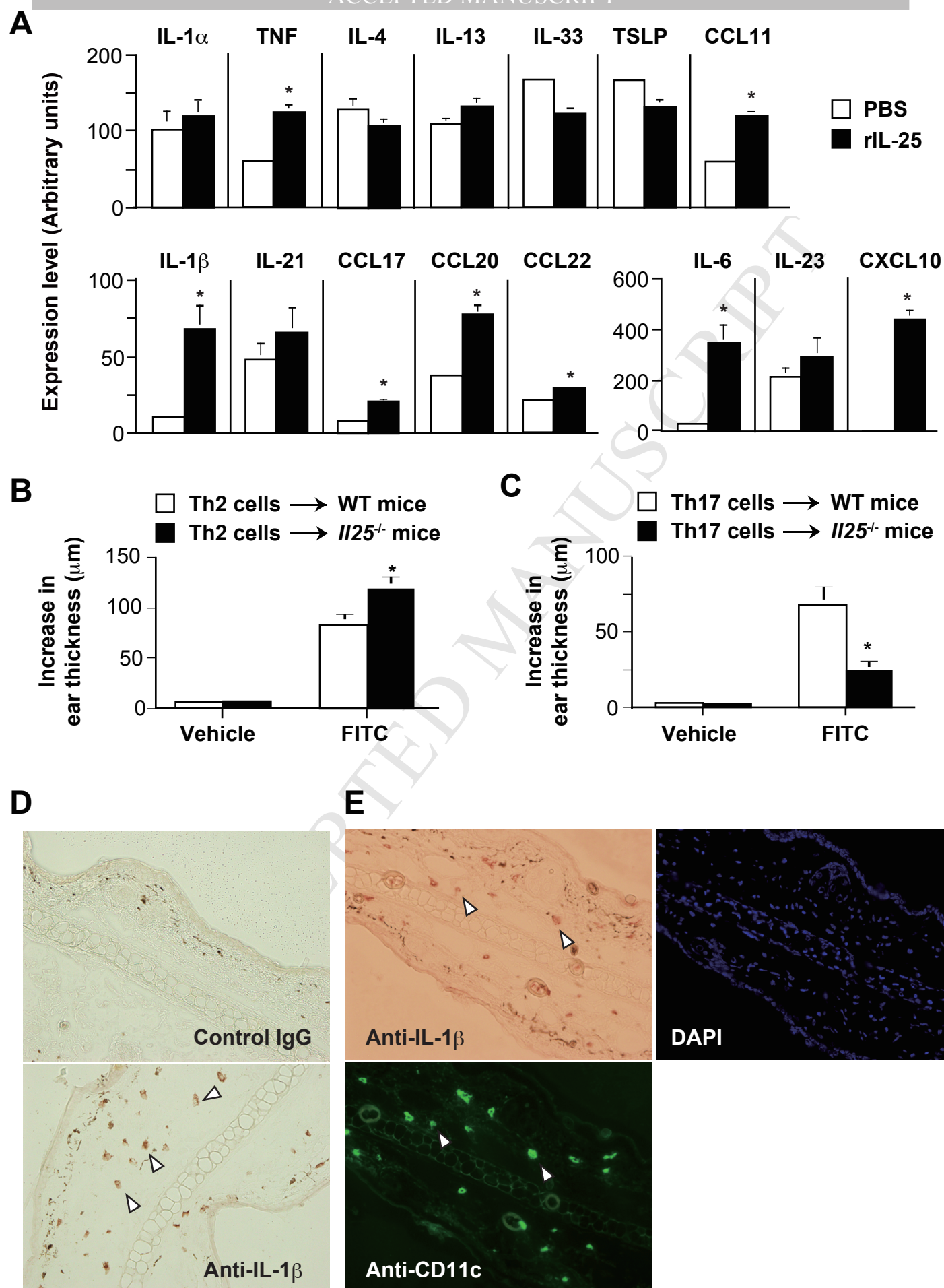
**A,** Twenty-four hours after epicutaneous challenge with FITC or vehicle alone, the ear thickness in wild-type (WT) mice (n=17), *Il1a*<sup>-/-</sup> mice (n=10), *Il1b*<sup>-/-</sup> mice (n=25) and *Il1a*<sup>-/-</sup>*Il1b*<sup>-/-</sup> mice (n=11) was examined.

**B, C,** Twenty-four hours after epicutaneous challenge with FITC or vehicle alone, with and without intradermal injection of PBS or rmIL-1 $\beta$ , the ear thickness in WT mice (n=10) and *Il25*<sup>-/-</sup> mice (n=10) (**B**) or the ear thickness in naïve WT mice injected with FITC-responsive IL-17<sup>+</sup> Th17 cells (Th17 cells  $\rightarrow$  WT mice; n=6-8) and naïve *Il25*<sup>-/-</sup> mice injected with FITC-responsive IL-17<sup>+</sup> Th17 cells (Th17 cells  $\rightarrow$  *Il25*<sup>-/-</sup> mice; n=6-8) (**C**) was examined. The data show the mean + SEM.

Results are representative of similar results that were obtained in 2 independent experiments. \*p<0.05 vs. WT mice (**A, B**), or Th17 cells  $\rightarrow$  WT mice (**C**).

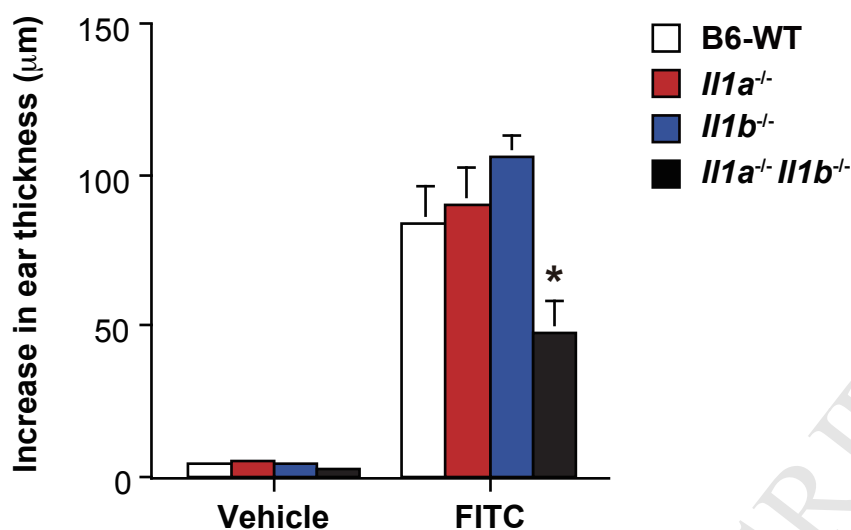
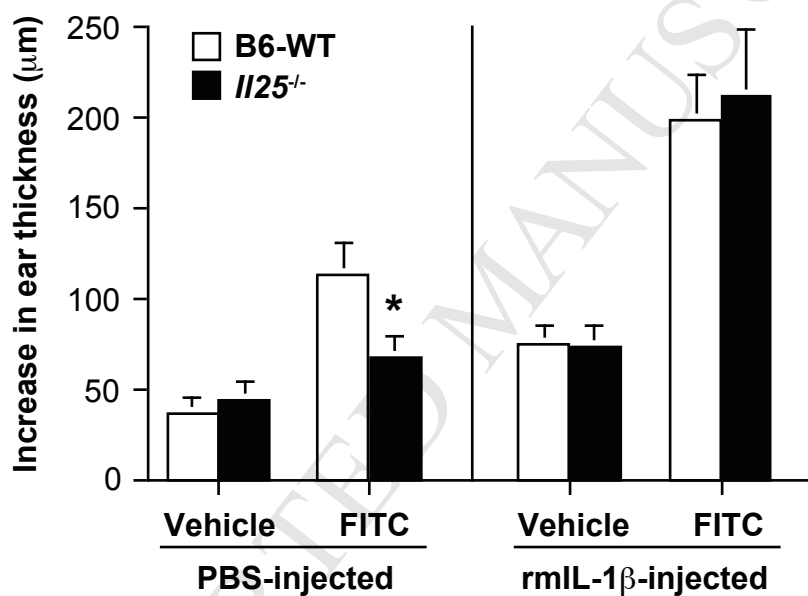
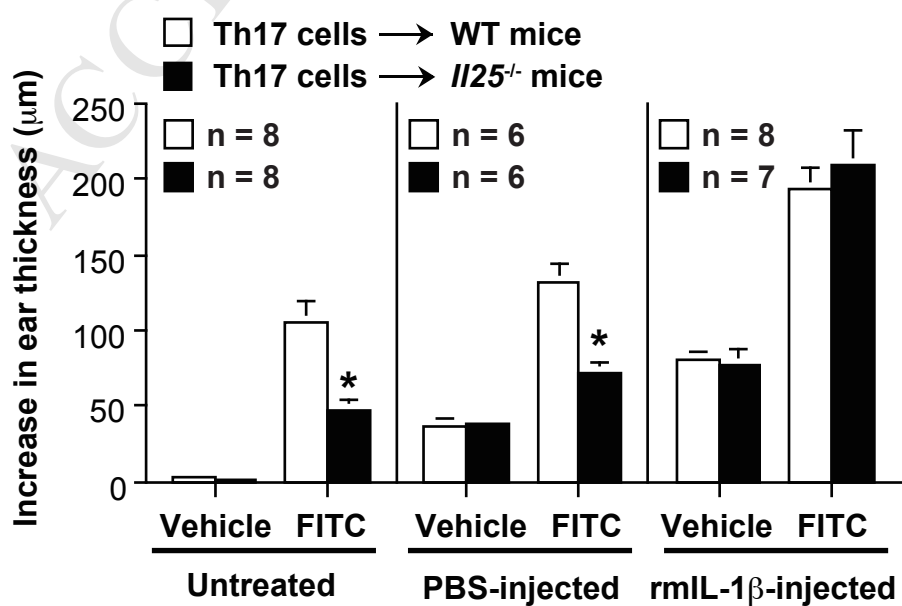






**A**

ACCEPTED MANUSCRIPT

**B****C**

## Online Repository Materials

### METHODS

#### Contact hypersensitivity (CHS)

For sensitization to FITC, two days after shaving the back hair with clippers, the skin was painted with 200  $\mu$ l of a 0.5% (w/v) FITC isomer I (SIGMA) solution in a mixture of acetone and dibutylphthalate (1:1). Five days after the sensitization with FITC, the animals' ears were painted with 40  $\mu$ l of the above sensitizing solution (the left ear, 20  $\mu$ l to each surface) and 40  $\mu$ l of the vehicle alone (the right ear, 20  $\mu$ l to each surface). For sensitization to DNFB, two days after shaving the back hair with clippers, the skin was painted with 25  $\mu$ l of a 0.5% (w/v) DNFB (SIGMA) solution in a mixture of acetone and olive oil (4:1). Five days after the sensitization with DNFB, the animals' ears were painted with 20  $\mu$ l of a 0.2% (w/v) DNFB solution in a mixture of acetone and olive oil (4:1) and 20  $\mu$ l of the vehicle alone. Ear thickness was measured before and after FITC or DNFB challenge using an engineer's caliper (Ozaki) by an investigator who was blinded to the mouse genotypes.

#### Skin DC migration

Mice were epicutaneously treated with 40  $\mu$ l of the above FITC sensitizing solution (the left ear, 20  $\mu$ l to each surface) and the vehicle alone (the right ear, 20  $\mu$ l to each surface). Twenty-four hours later, submaxillary lymph nodes (LNs) were separately collected from both the FITC-treated left and vehicle-treated right ears. LN single-cell suspensions were prepared and incubated with

anti-CD16/CD32 mAb (2.4G2; BD Biosciences) on ice for 15 min for FcR blocking. The cells were then incubated with PE-anti-mouse CD11c mAb (N418; eBioscience) and APC-anti-mouse I-A/I-E mAb (M5/114.15.2; eBioscience). The proportion of FITC<sup>+</sup> cells among 7-aminoactinomycin D-negative, MHC class II<sup>hi</sup>, CD11c<sup>+</sup> cells was determined using a FACS Canto II (BD Biosciences).

### **FITC-specific LN cell responses**

Mice were sensitized by painting 2.0% FITC on both the left and right ears (20  $\mu$ l on one surface of each ear). Six days later, submaxillary LNs were collected, and single-cell suspensions were prepared. The LN cells were cultured in the presence and absence of 40  $\mu$ g/ml FITC at 37°C for 72 hours. FITC-specific LN cell proliferative responses were determined by pulsing with 0.25  $\mu$ Ci [<sup>3</sup>H]-labeled thymidine for 6 hours. Cytokine levels in the culture supernatants of FITC-specific LN cells were determined with mouse IFN- $\gamma$ , IL-4, IL-10 and IL-17 ELISA kits obtained from BD Biosciences or eBioscience.

### **Measurement of myeloperoxidase and eosinophil peroxidase activities**

Tissues were homogenized in a 0.5% cetyltrimethylammonium chloride solution (SIGMA). The homogenates were centrifuged, and the supernatants were collected. Total protein levels in the tissue supernatants were measured with a Bio-Rad DC protein assay kit (Bio-Rad Laboratories, Hercules, CA). Recombinant human MPO and EPO (Calbiochem, Darmstadt, Germany) were used as standard proteins. The MPO and EPO activities per mg of total protein in tissue homogenates were calculated.

### Quantitative PCR

Twenty-four hours after epicutaneous challenge with FITC or vehicle, or 6 hours after rIL-25 or PBS injection, the ear skin was collected and homogenized. Total RNA in the homogenates was isolated using ISOGEN (NIPPON GENE) and RNeasy Mini Kit (Qiagen). Using the isolated RNA, cDNA was synthesized by RT-PCR with an iScript cDNA Synthesis Kit (Bio-Rad). Quantitative real-time PCR was performed with THUNDERBIRD SYBR qPCR Mix (Toyobo) and a CFX384™ Real-Time System (Bio-Rad). The relative gene expression was determined against GAPDH gene expression. PCR primers were designed as shown in Table E1.

For detection of IL25 mRNA, quantitative real-time PCR was performed as above. Then, the solution for quantitative real-time PCR was loaded onto agarose gels, and the PCR products were purified from the gels. Then a 2<sup>nd</sup> round of quantitative real-time PCR was performed using the purified PCR products.

### Immunohistochemistry

Twenty-four hours after epicutaneous challenge with FITC or after rIL-25 injection, the ear skin was frozen in OCT compound. For detection of IL-1 $\beta$ <sup>+</sup> cells, sections were fixed in 4% PFA at r.t. for 15 min and then washed three times in PBS. For inactivation of intrinsic peroxidases, the sections were serially incubated in 70% ethanol at r.t. for 5 min, 100% ethanol at r.t. for 5 min two times, 0.3% H<sub>2</sub>O<sub>2</sub> in methanol at r.t. for 30 min, 70% ethanol at r.t. for 5 min, distilled water at r.t. for 5

min and finally PBS at r.t. for 5 min. Then the sections were incubated in 0.1% BSA in PBS for blocking at r.t. for 1 hour and stained with rabbit anti-mouse IL-1 $\beta$  Ab (H-153; Santa Cruz Biotechnology) or rabbit IgG (a control for anti-mouse IL-1 $\beta$  Ab; Santa Cruz Biotechnology) at 4°C overnight, or FITC-conjugated anti-mouse CD11c mAb (N418; eBioscience) at r.t. for 1 hour. After washing with PBS, the sections were stained with HRP-conjugated goat anti-rabbit IgG (H+L) at r.t. for 1 hour. IL-1 $\beta$ <sup>+</sup> cells were then visualized by addition of 3,3'-diaminobenzidine (DAB) substrate liquid (DakoCytomation).

For detection of IL-17RB<sup>+</sup> cells, the sections were fixed in 4% PFA at r.t. for 10 min and then washed twice in PBS. Then the sections were incubated in Blocking One Histo solution (06349-64; Nacalai Tesque, Inc.) at r.t. for 10 min. After washing in PBS containing 0.1% Tween 20, the sections were incubated in avidin blocking solution (Avidin/Biotin Blocking Kit; Vector Laboratories) at r.t. for 15 min, and then in biotin blocking solution (Avidin/Biotin Blocking Kit; Vector Laboratories) at r.t. for 15 min. Next, the sections were incubated with primary Abs (rabbit anti-mouse CD3 Ab [ab5690; Abcam], hamster anti-mouse CD11c Ab [ab33484; Abcam], rat anti-mouse F4/80 [MCA497GA; Bio-Rad], rat anti-mouse Ly-6G/6C Ab [ab2557; Abcam] and rabbit anti-mouse tryptase Ab [ab134932; Abcam] and biotin-conjugated goat anti-mouse IL-17RB Ab [BAF1040; R&D Systems]) at 4°C overnight. After washing, the sections were incubated with secondary Abs or reagents (Alexa Fluor<sup>®</sup> 594-conjugated donkey anti-rabbit Ab [ab150064; Abcam], Alexa Fluor<sup>®</sup> 594-conjugated donkey anti-rat Ab [ab150156; Abcam], Alexa Fluor<sup>®</sup> 594-conjugated goat anti-hamster Ab [A21113; Thermo Fisher Scientific], and Alexa Fluor<sup>®</sup> 647-conjugated streptavidin [S21374;

Thermo Fisher Scientific]). Nuclei were stained with DAPI (R37606; Thermo Fisher Scientific). Images were acquired using a fluorescent microscope (BZX-700, KEYENCE) and analyzed with a software (BZ-analysis application, KEYENCE).

### Cell transfer

For LN cell transfer, wild-type or *Il25*<sup>-/-</sup> mice were epicutaneously sensitized with FITC as described above. Five days after sensitization, draining LNs were collected, and LN cells ( $2 \times 10^7$  cells) were injected intravenously into naïve *Rag2*<sup>-/-</sup> or *Rag2*<sup>-/-</sup> *Il25*<sup>-/-</sup> mice. For BM transfer, BM cells ( $2 \times 10^7$  cells) were injected intravenously into irradiated naïve wild-type or *Il25*<sup>-/-</sup> mice. Twenty-four hours after LN cell transfer, the ear skin was treated with 0.5% FITC or the vehicle.

For MC transfer, BMCMCs ( $1 \times 10^6$  cells) were injected intradermally to the ears of *Kit*<sup>W-sh/W-sh</sup> mice. Two months after MC transfer, the mice were sensitized and challenged with FITC as described above.

For FITC-responsive Th2 cell or Th17 cell transfer, LN cells were collected from FITC-sensitized mice as described above. They were then cultured for 5 days with 40 µg/ml FITC (FITC Isomer I; SIGMA) in the presence of recombinant mouse IL-4 (40 ng/ml; Peprotech), anti-mouse IFN-γ mAb (40 µg/ml; XMG1.2; eBioscience) and anti-mouse IL-12 mAb (40 µg/ml; C18.2; eBioscience) to generate Th2 cells, or in the presence of recombinant mouse IL-1β (10 ng/ml; Peprotech), IL-6 (20 ng/ml; Peprotech), IL-23 (10 ng/ml; R&D Systems), TNF (10

ng/ml; Peprotech), recombinant human TGF- $\beta$ 1 (5 ng/ml; Peprotech), anti-mouse IL-4 mAb (40  $\mu$ g/ml; 11B11; eBioscience) and anti-mouse IFN- $\gamma$  mAb (40  $\mu$ g/ml; XMG1.2; eBioscience) to generate Th17 cells. Then IL-4<sup>+</sup> Th2 cells or IL-17<sup>+</sup> Th17 cells were enriched by using a Mouse IL-4 or IL-17 Secretion Assay Kit (Milteny Biotec) according to the manufacturer's protocol. ELISA confirmed that the Th2 cells produced IL-4, but not IFN- $\gamma$  or IL-17, and that the Th17 cells produced IL-17, but not IFN- $\gamma$  or IL-4 (data not shown). The FITC-responsive Th2 cells or Th17 cells ( $5 \times 10^5$  cells) were injected intravenously into naïve wild-type or *Il25*<sup>-/-</sup> mice. Twenty-four hours later, the animals' ears were painted with 0.5% FITC or vehicle.

### **IL-1 $\beta$ injection**

C57BL/6N wild-type mice were injected intravenously with FITC-responsive wild-type or *Il1r1*<sup>-/-</sup> Th17 cells derived as described above. Twenty-four hours later, both ears were injected intradermally with 20  $\mu$ l of PBS containing 5  $\mu$ g of recombinant mouse IL-1 $\beta$  or 20  $\mu$ l of PBS. After IL-1 $\beta$  or PBS injection, the ear skin was painted with 0.5% FITC or vehicle.

### **Isolation of skin cells**

Ears were split into dorsal and ventral halves and incubated in 0.25% trypsin/EDTA solution (Sigma-Aldrich) at 37°C for 45 min. Then the epidermal sheets were separated from the dermal sheets using a forceps. For isolation of epidermal cells, epidermal sheets were dissociated in HBSS solution using a

GentleMACS Dissociator (Miltenyi Biotec). For isolation of dermal cells, dermal sheets were minced and incubated in PBS supplemented with 1% BSA, 400 units/ml collagenase type 2 (Worthington), 1000 units/ml hyaluronidase type 4-S (Sigma-Aldrich) and 50 units/ml DNase I (Roche) at 37°C for 60 min, followed by dissociation using a GentleMACS Dissociator. The dissociated sheets were passed through a nylon filter (70 µm; Corning), and epidermal and dermal cell suspensions were collected, respectively. The cells were then incubated with anti-CD16/CD32 mAb (2.4G2; BD Biosciences) at 4°C for 15 min for FcR blocking, followed by incubation with biotinylated anti-mouse CD45 (30-F11; BioLegend) at 4°C for 15 min. After washing, the cells were incubated with streptavidin-beads (Miltenyi Biotec) at 4°C for 15 min, and the CD45-positive and CD45-negative cell fractions were separated by MACS (Miltenyi Biotec), respectively.

## Figure E Legends

### FIG E1. Generation of *Tslp*<sup>-/-</sup> mice

**A.** TSLP gene targeting strategy. The first exon containing a translational start codon was replaced with a tandemly arrayed promoter-less GFP gene and a floxed neomycin resistance gene (Neor) (targeted allele).

**B.** Southern blot analysis of genomic DNA obtained from wild-type or mutant ES cells. The DNA probes used for Southern blot analysis are shown in (A). By digestion of genomic DNA with Bam HI, the probes detected endogenous wild-type (WT; 14.1 kbp) and/or targeted (MT; 11.4 kbp) fragments.

**FIG E2. The levels of EPO and MPO activities in ear tissue homogenates.**

The levels of EPO and MPO activities in ear tissue homogenates at 24 hours after FITC and vehicle challenge. Data show the mean + SEM. \* $p < 0.05$  vs. WT.

**FIG E3. IL-25 is crucial for DNFB-induced CHS.**

Twenty-four hours after challenge with DNFB, the ear skin thickness of mice was measured.

**A**, Ear skin thickness in wild-type (WT;  $n=10-12$ ), *Ifng*<sup>-/-</sup> ( $n=10$ ), *Il17a*<sup>-/-</sup> ( $n=10$ ), *Rorc*<sup>-/-</sup> ( $n=10$ ) and *Stat6*<sup>-/-</sup> ( $n=15$ ) mice.

**B**, Ear skin thickness in WT ( $n=5-12$ ), *Il25*<sup>-/-</sup> ( $n=10$ ), *Il33*<sup>-/-</sup> ( $n=5$ ), and *Tslp*<sup>-/-</sup> ( $n=5$ ) mice.

The data show the mean + SEM. Results are representative of similar results that were obtained in 2-3 independent experiments. \* $p < 0.01$  vs. WT.

**FIG E4. The reduced CHS in *Il25*<sup>-/-</sup> mice was restored by injection of rIL-25 before challenge.**

Twenty-four hours after epicutaneous challenge with FITC or vehicle alone, with and without intradermal injection of PBS or rIL-25, the ear thickness in naïve WT mice ( $n=10$ ) and *Il25*<sup>-/-</sup> mice ( $n=10$ ) was examined. The data show the mean + SEM. Results are representative of similar results that were obtained in 2 independent experiments. \* $p < 0.05$  vs. WT mice.

**FIG E5. Expression of IL-25.**

**A**, The ear skins from naïve wild-type mice and from wild-type mice 24 h after

FITC challenge were collected. Then, epidermis and dermis were separated. CD45<sup>+</sup> and CD45<sup>-</sup> cells from the epidermis and dermis were purified by MACS. mRNA was isolated from these cells, and the expression levels of IL25 mRNA were quantified by qPCR. Data are representative results in 2 independent experiments.

**B**, Bone marrow cell-derived mast cells (BMCMCs), macrophages (BMM $\phi$ ) and dendritic cells (BMDCs) were cultured in the presence and absence (Medium alone; Med) of PMA + Ionomycin (P+I) or LPS. Then, cell lysates were prepared. The concentration of IL-25 in cell lysates was determined by ELISA. The data show the mean + SEM (n=3).

**FIG E6. IL-17RB expression on dermal DCs.**

Twenty-four hours after epicutaneous challenge with FITC, the ear skin was collected. IL-17RB expression in sections of the ear skin was detected by immunohistochemistry. Arrowheads = representative IL-17RB<sup>+</sup> CD11c<sup>+</sup> cells.

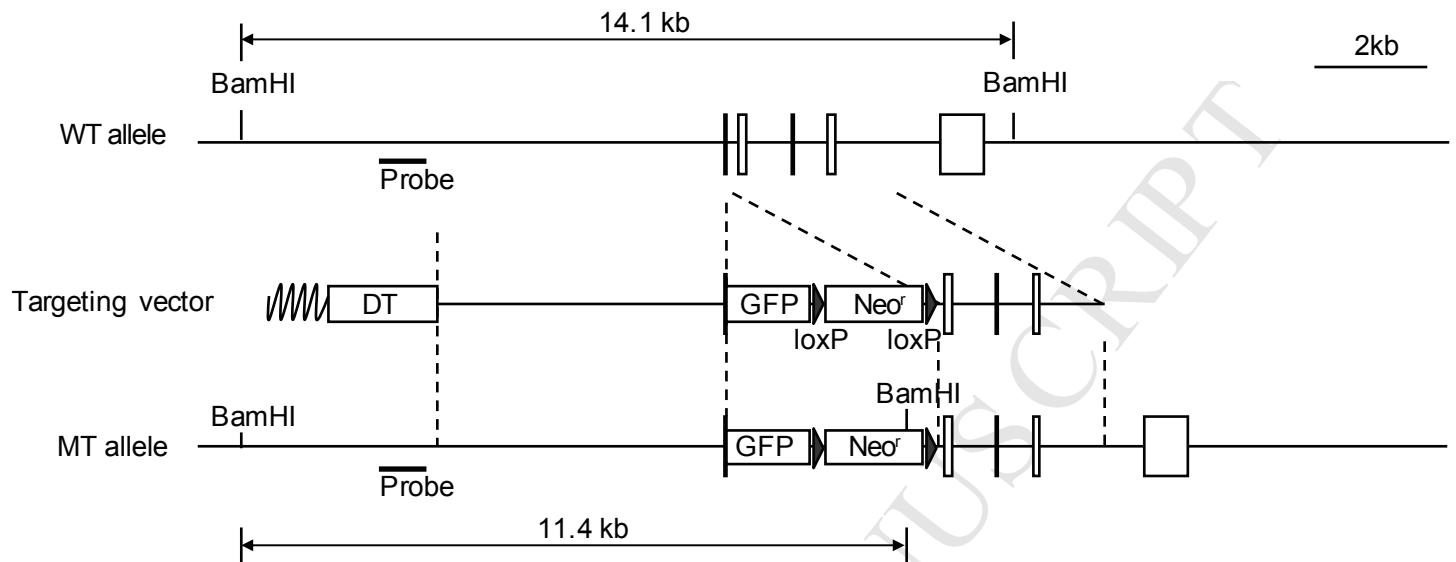
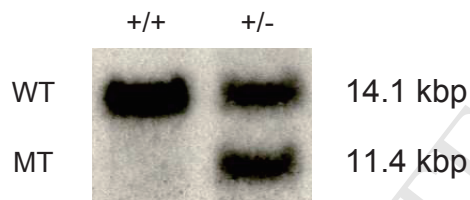
**FIG E7. IL-1 $\beta$  is crucial for Th17 cell-mediated skin inflammation during FITC-induced CHS.**

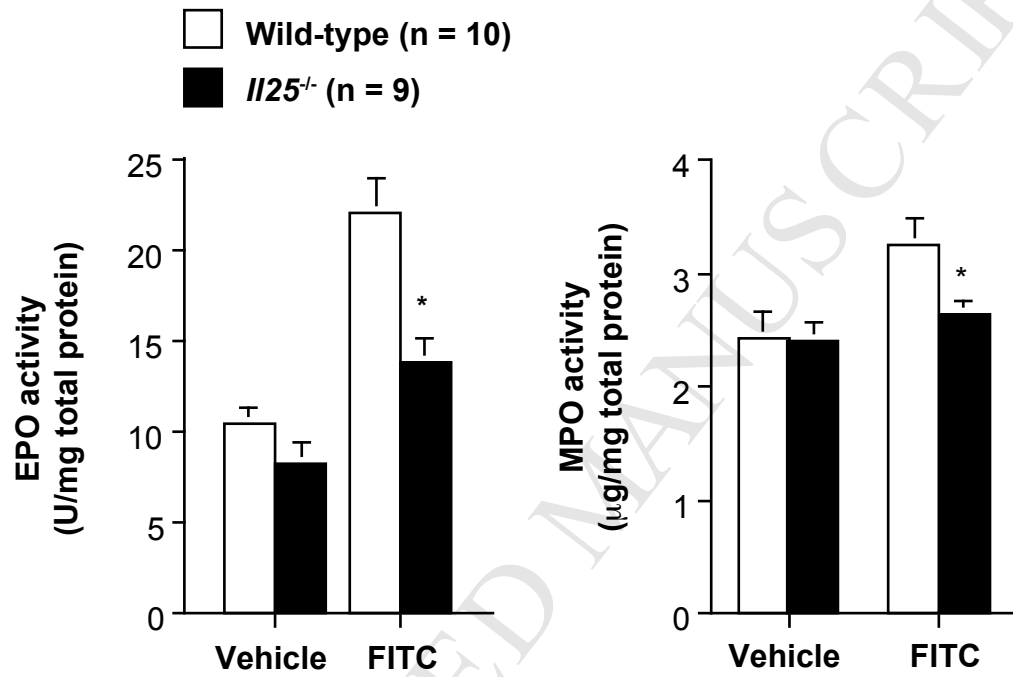
Twenty-four hours after epicutaneous challenge with FITC or vehicle alone, with and without intradermal injection of PBS or rmIL-1 $\beta$ , the ear thickness in naïve wild-type (WT) mice injected with FITC-responsive WT IL-17<sup>+</sup> Th17 cells (WT Th17 cells → WT mice; n=5) and naïve WT mice injected with FITC-responsive *Il1r1*<sup>-/-</sup> IL-17<sup>+</sup> Th17 cells (*Il1r1*<sup>-/-</sup> Th17 cells → WT mice; n=5) was examined. The

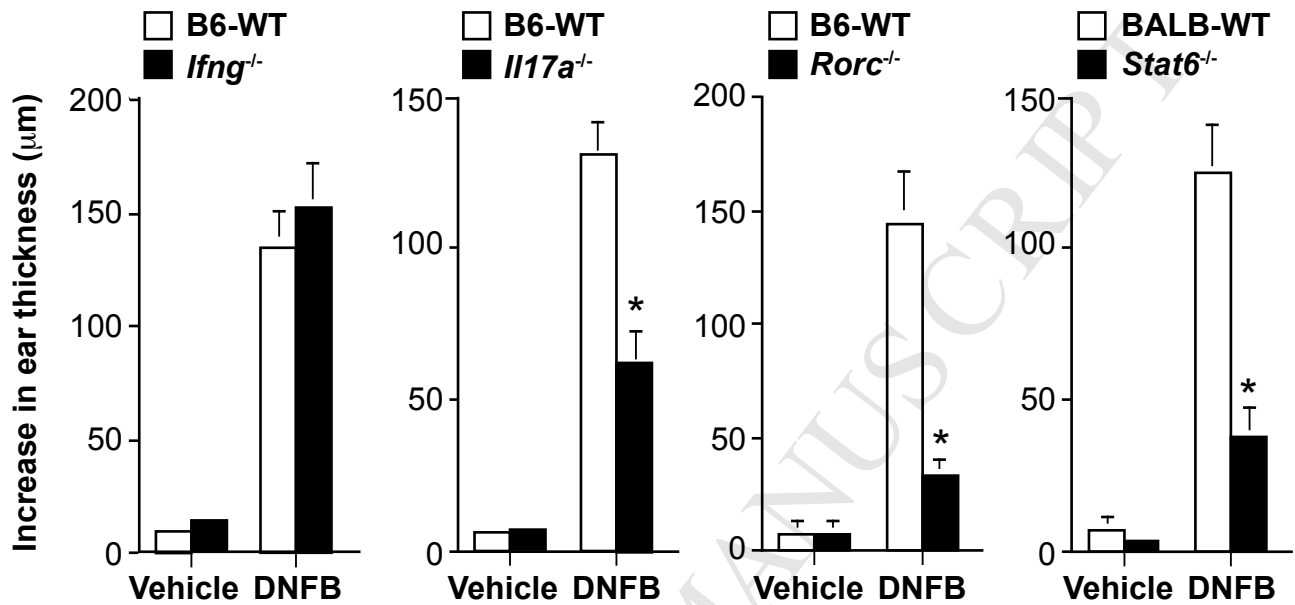
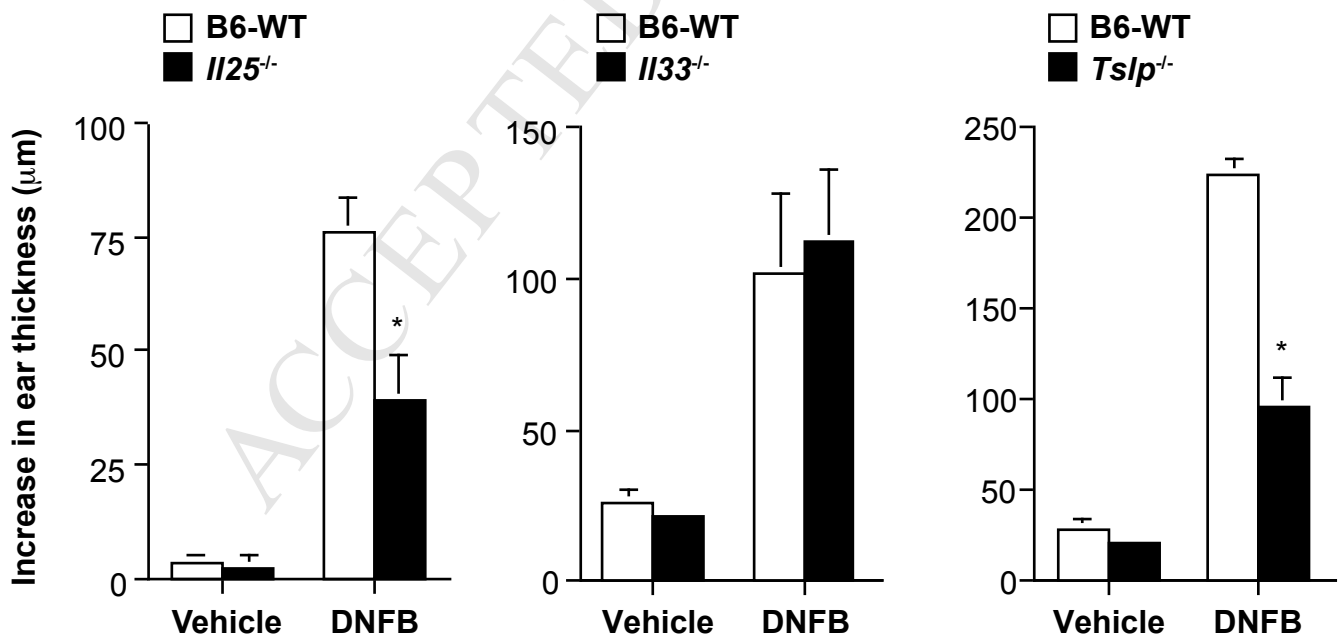
data show the mean + SEM. \* $p < 0.05$  vs. WT Th17 cells → WT mice.

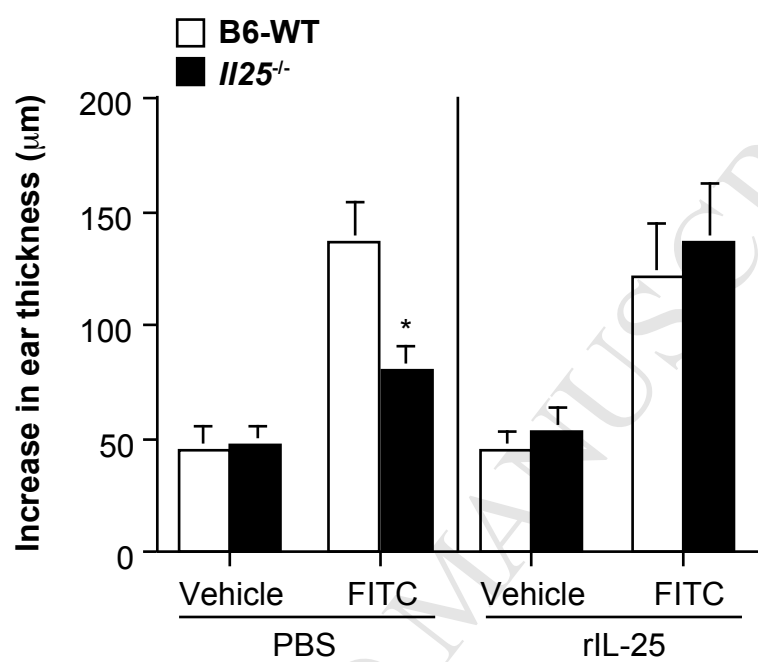
**Table E1. Sequences of primers**

| Gene           | Forward                         | Reverse                          |
|----------------|---------------------------------|----------------------------------|
| <i>Gapdh</i>   | 5'-ttcaccacatggagaaggc-3'       | 5' -agtgggagttgctgtgaagtcg-3'    |
| <i>Il1a</i>    | 5'-tcgggaggagacgactctaa-3'      | 5' -tggttctggcaactcctca-3'       |
| <i>Il1b</i>    | 5'-caaccaacaagtgatattctccatg-3' | 5' -gatccacactctccagctgca-3'     |
| <i>Il4</i>     | 5'-gggtctcaacccccagctagt-3'     | 5' -gccgatgatctctctcaagtgtat-3'  |
| <i>Il6</i>     | 5'-gaggataccactccaacagacc-3'    | 5' -aagtgcacatcatggtgtcataca-3'  |
| <i>Il13</i>    | 5'-cctggctctgcttgccctt-3'       | 5' -gggtctgtgtgatgttgctca-3'     |
| <i>Il21</i>    | 5'-ggaccctgtctgtctggtag-3'      | 5' -tgtggagctgtagaagttcagg-3'    |
| <i>Il23p19</i> | 5'-tggtgtgcttaggagtagca-3'      | 5' -catcctcttcttcttagtag-3'      |
| <i>Il33</i>    | 5'-tccaactccaagatttccccg-3'     | 5' -catgcagtagacatggcagaa-3'     |
| <i>Tnfa</i>    | 5'-gcctccctctcatcagttct-3'      | 5' -cacttggtggttgctacga-3'       |
| <i>Tslp</i>    | 5'-tatactctcaatcctatccctggc-3'  | 5' -catttctgagtaccgtcatttc-3'    |
| <i>Ccl11</i>   | 5'-gaatcaccaacaacagatgcac-3'    | 5' -atcctggaccacttcttctt-3'      |
| <i>Ccl17</i>   | 5'-aggaagttggtgagctggtataag-3'  | 5' -tcttcacatgttgctttggg-3'      |
| <i>Ccl20</i>   | 5'-gagctattgtgggttcacaagac-3'   | 5' -actcttaggctgaggaggttcac-3'   |
| <i>Ccl22</i>   | 5'-aggccctatggtgccaatgt-3'      | 5' -cggcaggatttgaggcca-3'        |
| <i>Cxcl10</i>  | 5'-tgggtctgagtgggactcaaggg-3'   | 5' -gctcatcattcttttcatcggtggc-3' |

**A****B**



**A****B**



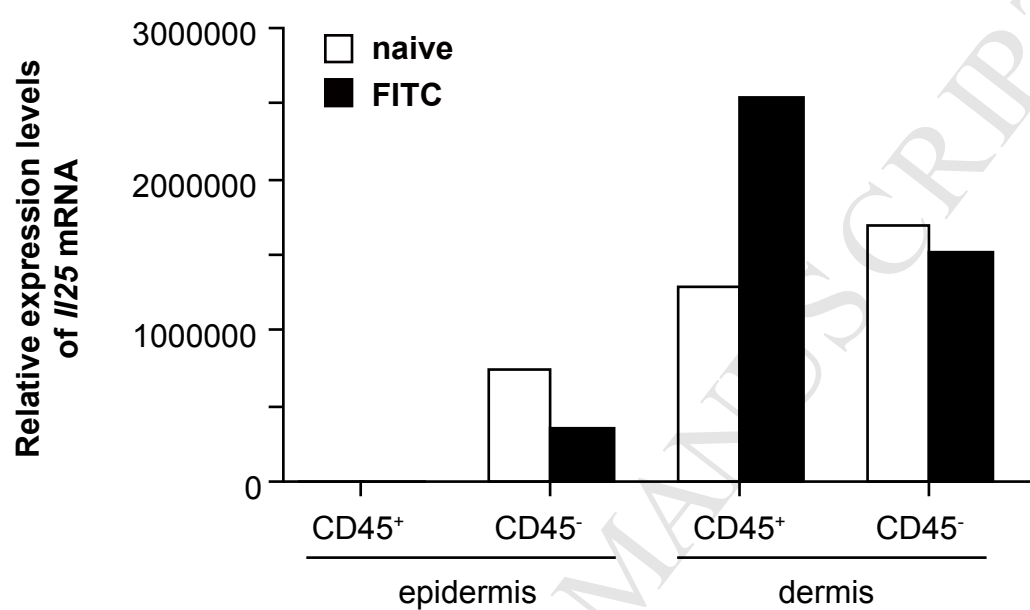
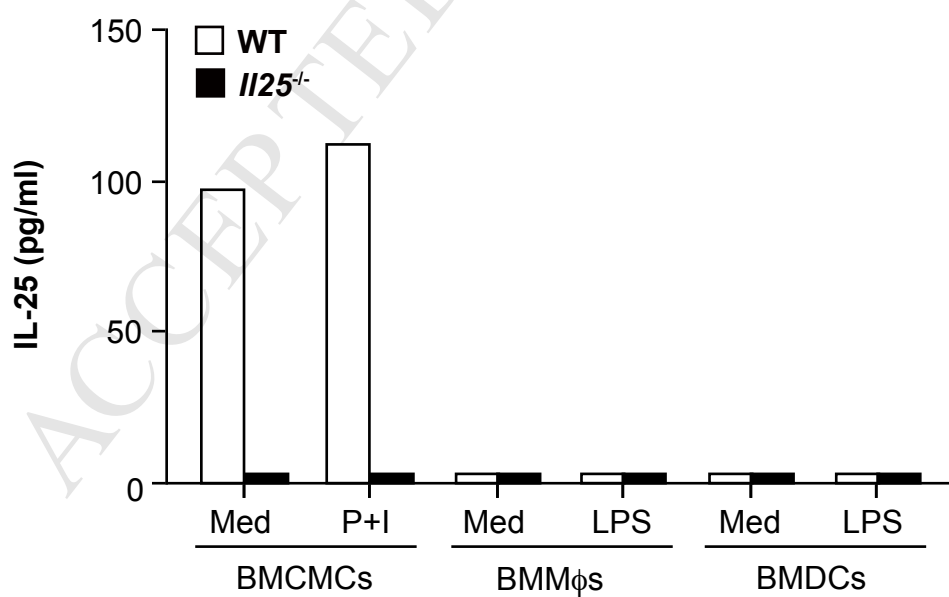
**A****B**

FIG E6. Suto et al.

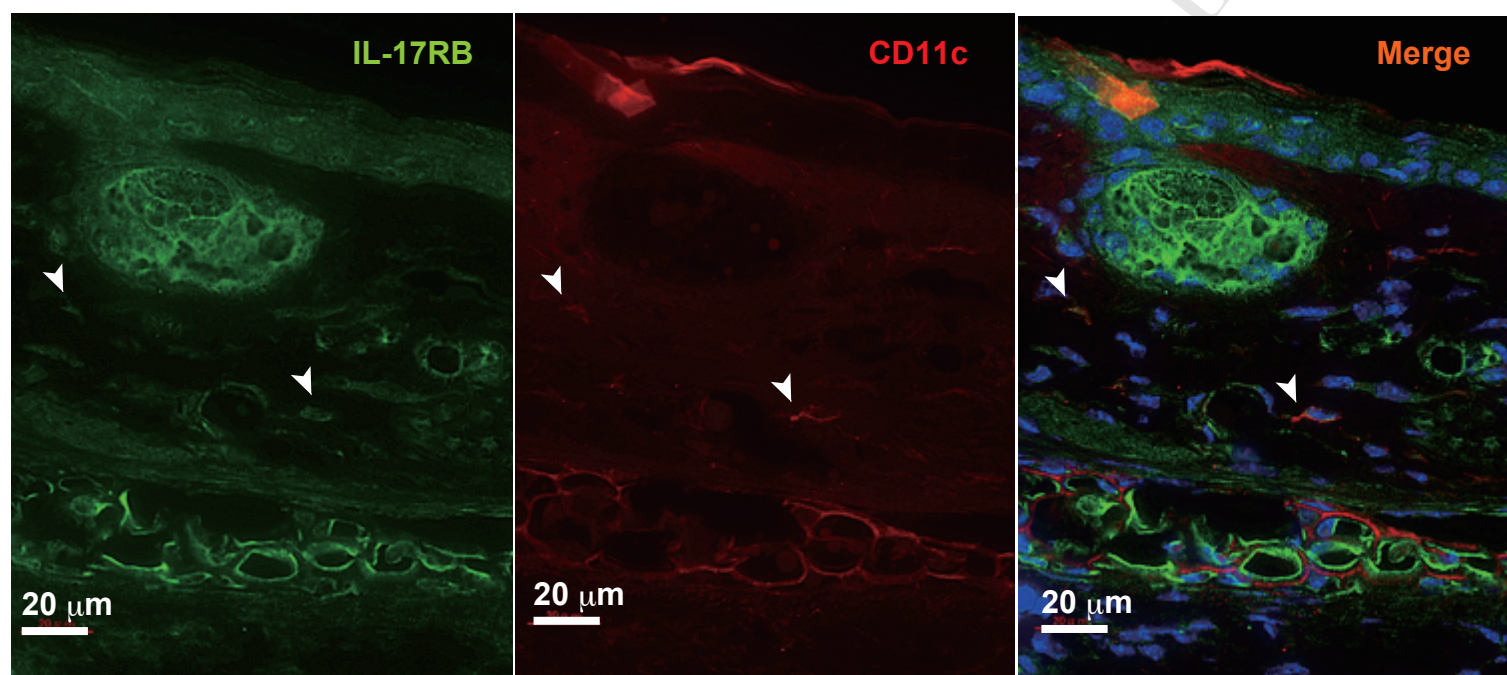


FIG E7. Suto et al.

