



# T-bet and STAT6 Coordinately Suppress the Development of IL-9–Mediated Atopic Dermatitis–Like Skin Inflammation in Mice

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T-bet and signal transducer and activator of transcription (STAT) 6 are critical factors for helper T-cell differentiation in humans and mice. Additionally, polymorphisms in *TBX21* (T-bet) and *STAT6* are associated with the susceptibility of allergic diseases. However, precise mechanisms of the reciprocal regulation between T-bet and STAT6 in allergy remain unclear. To determine the reciprocal regulation in vivo, we investigated the phenotype of T-bet/STAT6 double-deficient (T-bet<sup>−/−</sup> STAT6<sup>−/−</sup>) mice. Unexpectedly, T-bet<sup>−/−</sup> STAT6<sup>−/−</sup> mice but not T-bet<sup>−/−</sup> mice or STAT6<sup>−/−</sup> mice spontaneously developed severe dermatitis. Not only eosinophils and mast cells but also CD4<sup>+</sup> T cells infiltrated into the skin of T-bet<sup>−/−</sup> STAT6<sup>−/−</sup> mice. Adoptive transfer of CD4<sup>+</sup> T cells of T-bet<sup>−/−</sup> STAT6<sup>−/−</sup> mice into severe combined immunodeficient mice induced the accumulation of eosinophils and mast cells in the skin, whereas depletion of CD4<sup>+</sup> T cells ameliorated the dermatitis in T-bet<sup>−/−</sup> STAT6<sup>−/−</sup> mice. Comprehensive transcriptome analyses revealed that IL-9 expression was enhanced in T-bet<sup>−/−</sup> STAT6<sup>−/−</sup> CD4<sup>+</sup> T cells. Indeed, IL-9 neutralization ameliorated the dermatitis in T-bet<sup>−/−</sup> STAT6<sup>−/−</sup> mice. T-bet<sup>−/−</sup> STAT6<sup>−/−</sup> CD4<sup>+</sup> T cells expressed functional thymic stromal lymphopoietin receptors and produced large amounts of IL-9 on thymic stromal lymphopoietin stimulation. These results indicate that T-bet and STAT6 coordinately suppress atopic dermatitis–like skin inflammation, possibly by inhibiting thymic stromal lymphopoietin–dependent IL-9 production in CD4<sup>+</sup> T cells.

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## INTRODUCTION

The classic view of T helper type (Th) 2 cell–mediated diseases such as asthma, allergic rhinitis, and atopic dermatitis is based on the fact that the pathology of these diseases is characterized by infiltration of inflammatory cells including eosinophils, mast cells, and activated CD4<sup>+</sup> T cells producing Th2 cytokines such as IL-4, IL-5, and IL-13 (Nakayama et al., 2017; Zhu and Paul, 2010). In accordance with these findings, it is generally believed that signal transducer and

activator of transcription (STAT) 6, which is an essential signaling molecule for IL-4 and IL-13, is vital for the induction of Th2 cell–mediated allergic diseases, whereas T-bet, a master regulator of Th1 cell development, is required for the downregulation of Th2 cell–mediated diseases (Nakayama et al., 2017; Zhu and Paul, 2010).

Regarding the role of T-bet beyond the suppression of Th2 cell–mediated immune responses, we have shown previously that T-bet expressed in CD4<sup>+</sup> T cells is crucial for the inhibition of Th17 cell–mediated neutrophilic inflammation in the airways (Fujiwara et al., 2007). In addition, we have reported recently that T-bet expressed in innate lymphoid cells (ILCs) inhibits IL-33–induced, type 2 ILC (ILC2)–mediated eosinophilic airway inflammation (Matsuki et al., 2017), suggesting that T-bet suppresses innate-type as well as acquired-type allergic airway inflammation in vivo. Moreover, it has been reported that gene variants in *TBX21* and *STAT6* are associated with asthma and allergy in humans (Vercelli, 2008). However, the precise roles of T-bet and STAT6 in the development of allergic diseases remain unclear.

IL-9, a pleiotropic cytokine, promotes the development of allergic diseases (Noelle and Nowak, 2010; Stassen et al., 2012). Concerning the source of IL-9, multiple Th cell subsets, including Th2 cells, Th9 cells, Th17 cells, and regulatory T cells, and other subsets of immune cells, such as ILC2s, mast cells, and NK T cells, have been shown to produce IL-9 (Noelle and Nowak, 2010; Schmitt et al., 2014). With regard

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Abbreviations: BM, bone marrow; ILC, innate lymphoid cell; ILC2, type 2 innate lymphoid cell; LN, lymph node; RNA-seq, RNA sequencing; SCID, severe combined immunodeficient; STAT, signal transducer and activator of transcription; Th, T helper type; TSLP, thymic stromal lymphopoietin; WT, wild-type

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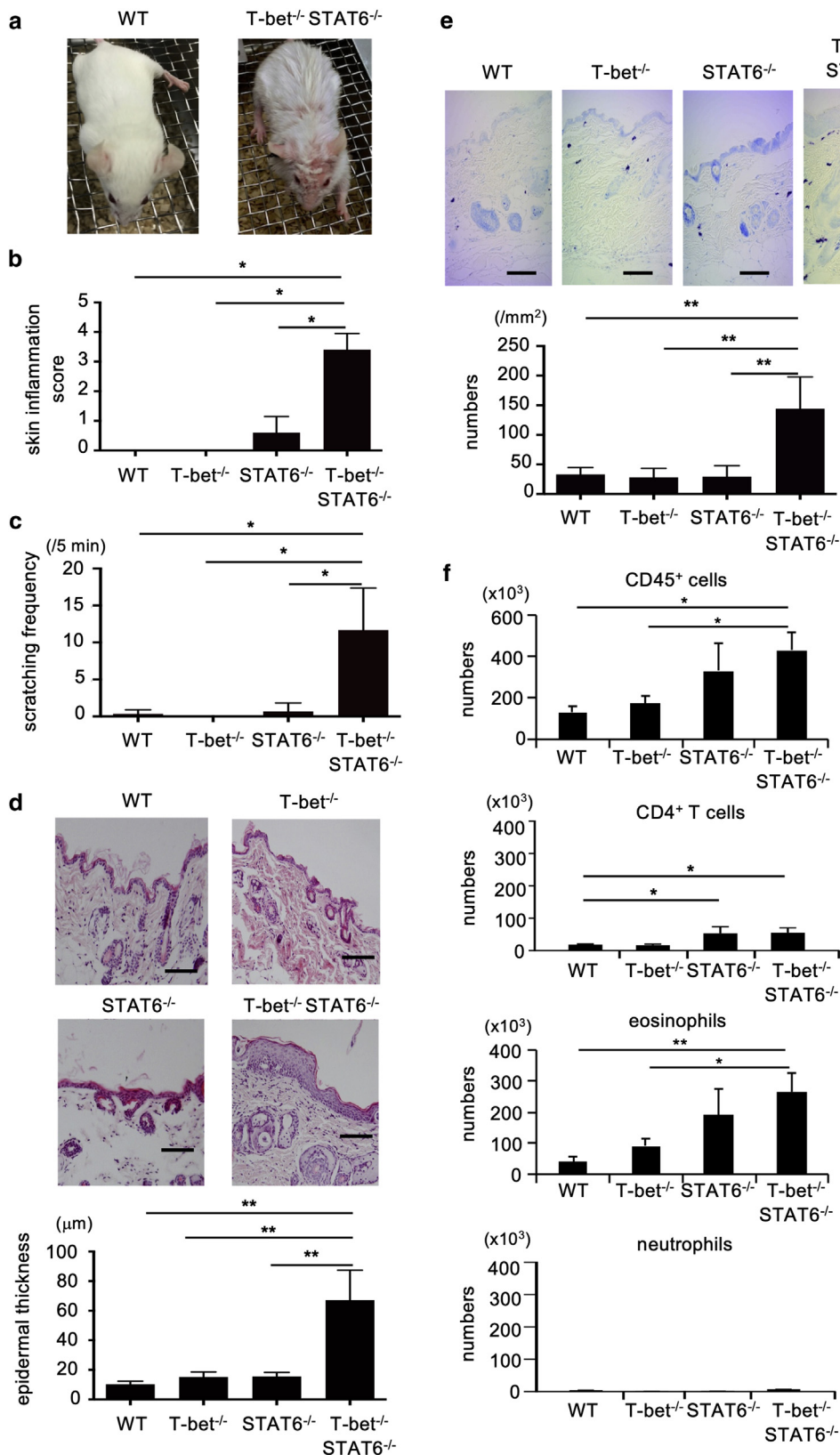
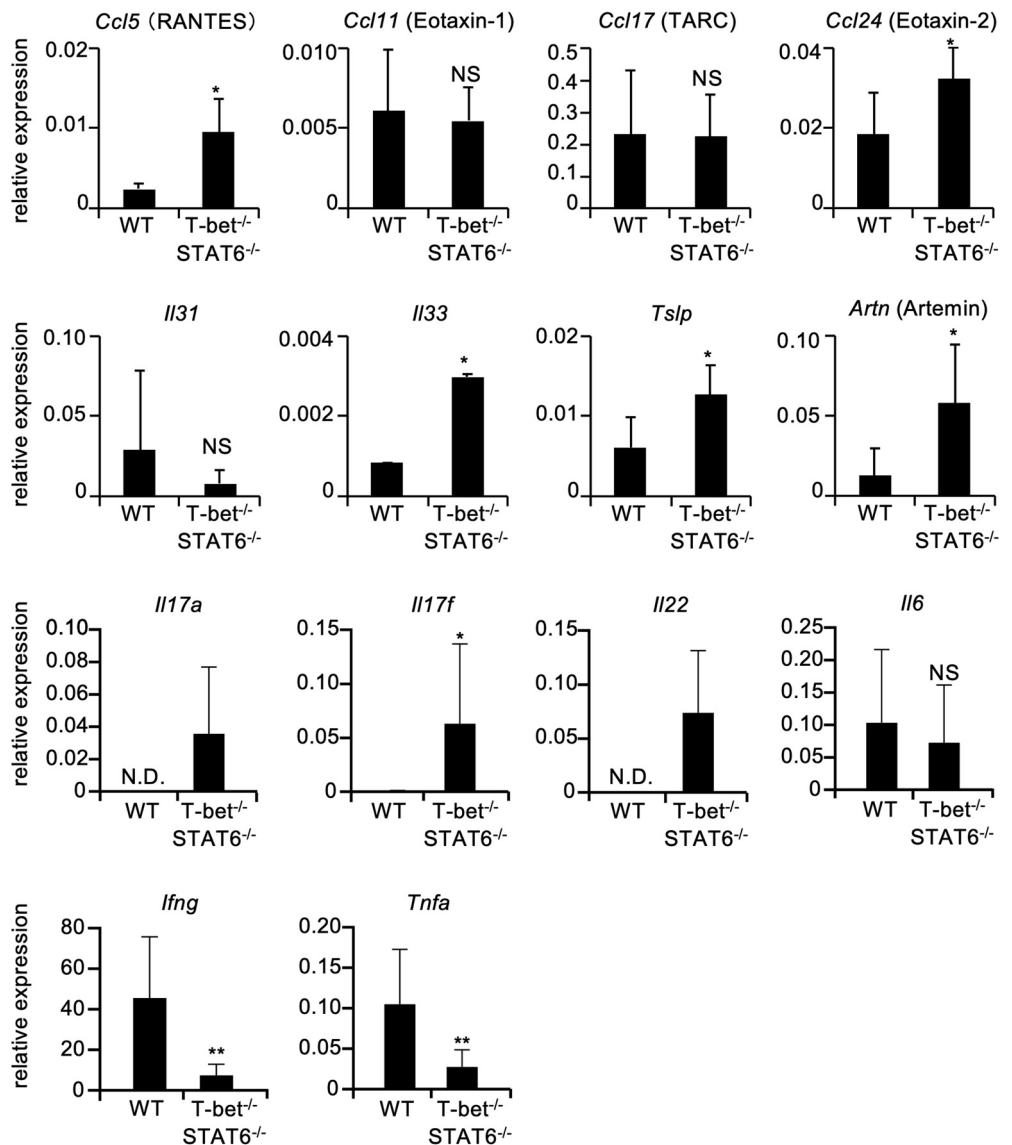


**Figure 1. T-bet<sup>-/-</sup> STAT6<sup>-/-</sup> mice spontaneously develop atopic dermatitis-like skin inflammation.** (a) Representative photographs of mice (13-week-old). (b) Means ± SD of skin inflammation score at 13 weeks old (n = 5, each). (c) Means ± SD of scratching frequency per 5 minutes (n = 3, each). (d) Representative photomicrographs of H&E staining and means ± SD of epidermal thickness at 10 random points at 13 weeks old (n = 3, each). (e) Representative photomicrographs of toluidine blue staining and means ± SD of mast cell numbers in five random microscope fields (×100). (f) Means ± SD of numbers of CD45<sup>+</sup> cells, CD4<sup>+</sup> T cells, Siglec-F<sup>+</sup> CD11c<sup>+</sup> eosinophils, and Ly6G<sup>+</sup> CD11b<sup>+</sup> neutrophils in dorsal skin (per 900 mm<sup>2</sup>) at 13 weeks old (n = 3, each). All results are representative of three independent experiments. Bar = 50 μm. \*P < 0.05, \*\*P < 0.005. STAT, signal transducer and activator of transcription; WT, wild-type.

to the development of IL-9-producing CD4<sup>+</sup> T cells, various cytokine signaling pathways, such as IL-2/STAT5, IL-4/STAT6, type I IFN/STAT1, and TGF-β/SMADs, have been reported to promote IL-9 production in CD4<sup>+</sup> T cells (Kaplan et al.,

2015). In contrast, we found that T-bet expressed in ILCs suppresses IL-9 production of lung ILC2s during IL-33-induced airway inflammation (Matsuki et al., 2017). However, little is known about the regulatory mechanisms of

**Figure 2. Cytokine and chemokine expression in the skin of T-bet<sup>-/-</sup> STAT6<sup>-/-</sup> mice.** Dorsal skin was resected from T-bet<sup>-/-</sup> STAT6<sup>-/-</sup> mice or WT mice (12-week-old), and mRNA levels of indicated molecules were analyzed by qPCR. Data are means  $\pm$  SD of three independent experiments. \* $P < 0.05$ . N.D., not detected; NS, not significant; STAT, signal transducer and activator of transcription; WT, wild-type.



IL-9 production from CD4<sup>+</sup> T cells. We examine here the reciprocal regulation of T-bet and STAT6 in vivo by comparing T-bet<sup>-/-</sup> mice, STAT6<sup>-/-</sup> mice, and T-bet<sup>-/-</sup> STAT6<sup>-/-</sup> mice. Our findings highlight cooperative roles of T-bet and STAT6 in the suppression of IL-9-producing CD4<sup>+</sup> T cells that cause atopic dermatitis-like skin inflammation.

## RESULTS

### T-bet/STAT6 double-deficient mice develop atopic dermatitis-like skin inflammation spontaneously

To investigate the reciprocal regulation of T-bet and STAT6 in vivo, we generated T-bet and STAT6 double-deficient (T-bet<sup>-/-</sup> STAT6<sup>-/-</sup>) mice and examined the phenotype of the mice. Unexpectedly, we found that T-bet<sup>-/-</sup> STAT6<sup>-/-</sup> mice but not wild-type (WT) mice, T-bet<sup>-/-</sup> mice, or STAT6<sup>-/-</sup> mice spontaneously developed inflammatory skin diseases (Figure 1a and b). The dermatitis started being apparent at the age of 8 weeks, and both male and female T-bet<sup>-/-</sup> STAT6<sup>-/-</sup> mice developed dermatitis by the age of 12 weeks (Supplementary Figure S1). In addition, T-bet<sup>-/-</sup> STAT6<sup>-/-</sup>

mice but not WT mice, T-bet<sup>-/-</sup> mice, or STAT6<sup>-/-</sup> mice scratched the skin frequently (Figure 1c). Histopathological analyses of the dorsal skin revealed that T-bet<sup>-/-</sup> STAT6<sup>-/-</sup> mice exhibited extensive thickening of the epidermis (acanthosis), which was associated with hyperkeratosis and infiltration of inflammatory cells, including mast cells (Figure 1d and e). Analyses of cells harvested from the skin showed that the numbers of skin-infiltrating CD45<sup>+</sup> inflammatory cells, Siglec-F<sup>+</sup> CD11c<sup>+</sup> eosinophils, and CD4<sup>+</sup> T cells were significantly higher in T-bet<sup>-/-</sup> STAT6<sup>-/-</sup> mice than those in WT mice or T-bet<sup>-/-</sup> mice (Figure 1f). In contrast, the numbers of Ly6G<sup>+</sup> CD11b<sup>+</sup> neutrophils were very low and compatible among these mice (Figure 1f).

qPCR analysis of the dorsal skin demonstrated that mRNA levels of Th2-related cytokines and chemokines, including *Il33*, *Tslp*, *Ccl5* (encoding RANTES), and *Ccl24* (encoding eotaxin-2), were enhanced in T-bet<sup>-/-</sup> STAT6<sup>-/-</sup> mice as compared with those in WT mice (Figure 2). In addition, mRNA expression of *Artn* (encoding artemin), which is shown to be responsible for epidermal hyperinnervation and

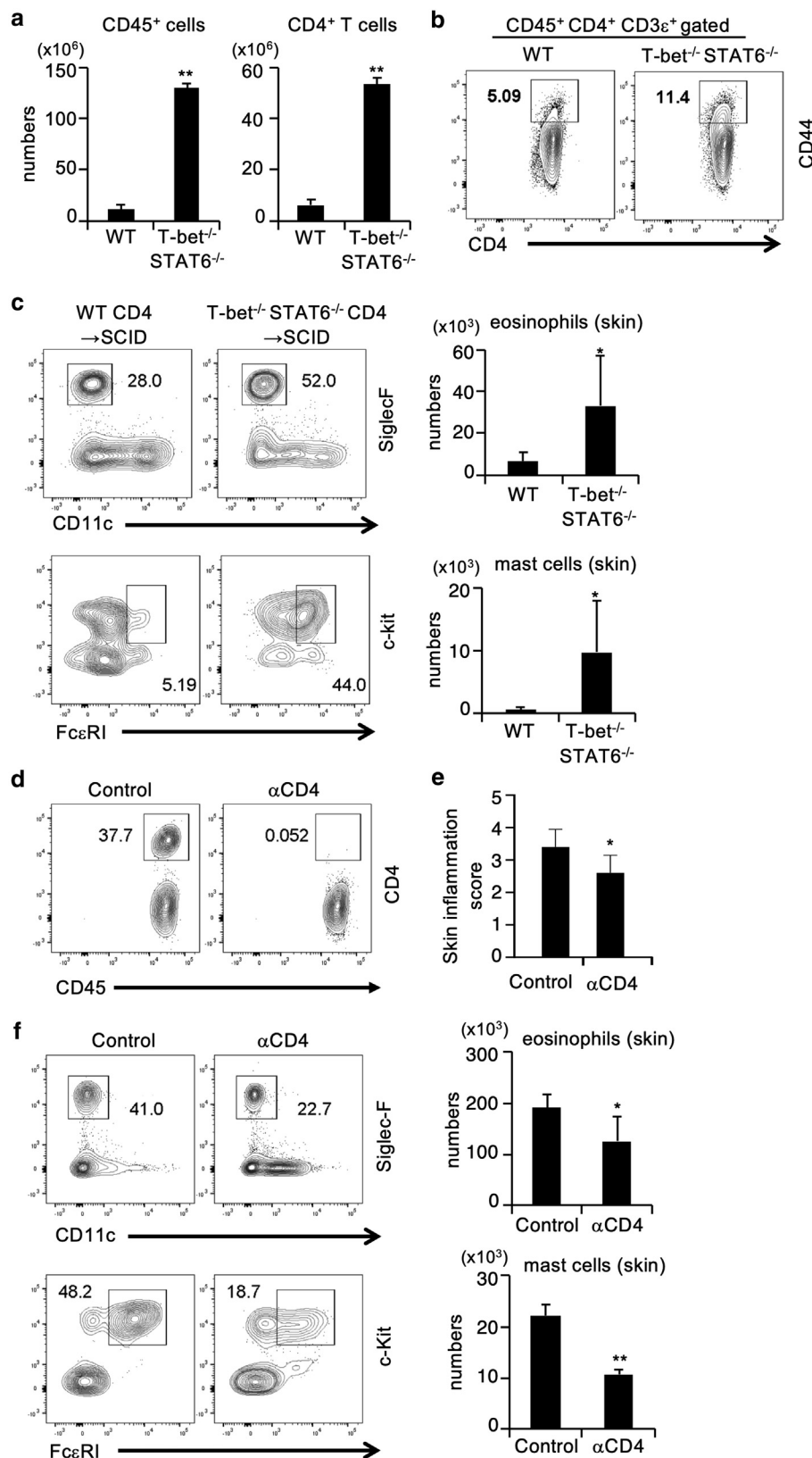


**Figure 3. T-bet<sup>-/-</sup> STAT6<sup>-/-</sup> CD4<sup>+</sup> T cells induce the accumulation of eosinophils and mast cells in the skin.**

Cells of skin-draining LNs were harvested from the mice (12-week-old). (a) Means ± SD of absolute numbers of CD45<sup>+</sup> cells and CD4<sup>+</sup> CD3ε<sup>+</sup> cells. (b) Representative CD4 versus CD44 staining of CD4<sup>+</sup> CD3ε<sup>+</sup> T cells (n = 3, each). (c) CD4<sup>+</sup> T cells isolated from WT mice or T-bet<sup>-/-</sup> STAT6<sup>-/-</sup> mice (8- to 12-week-old) were transferred to SCID mice. Eight weeks later, cells from the dorsal skin were analyzed by FACS.

Representative CD11c versus Siglec-F and FcεRI versus c-kit staining of CD45<sup>+</sup> CD3<sup>-</sup> cells and means ± SD of the numbers of Siglec-F<sup>+</sup> CD11c<sup>-</sup> eosinophils and c-kit<sup>+</sup> FcεRI<sup>+</sup> mast cells (n = 3, each). (d–f) Mice were treated with anti-CD4 antibody as described in the Materials and Methods.

(d) Representative CD45 versus CD4 staining of CD45<sup>+</sup> CD3<sup>-</sup> cells in the skin (n = 3, each). (e) Means ± SD of skin inflammation score at 13 weeks (n = 3, each). (f) Representative CD11c versus Siglec-F and FcεRI versus c-kit staining of CD45<sup>+</sup> CD3<sup>-</sup> cells in the skin and means ± SD of the numbers of eosinophils and mast cells (n = 3, each). Data are representative of two independent experiments. \*P < 0.05, \*\*P < 0.005. LN, lymph node; SCID, severe combined immunodeficient; STAT, signal transducer and activator of transcription; WT, wild-type.

hypersensitivity in atopic dermatitis (Hidaka et al., 2017), was upregulated in the skin in T-bet<sup>-/-</sup> STAT6<sup>-/-</sup> mice (Figure 2) consistent with the scratching behavior. Moreover, mRNA expression of Th17 cell-related cytokines, such as

*Il17a*, *Il17f*, and *Il22*, but not of the Th1 cell-related cytokine *Ifng* was upregulated in the skin of T-bet<sup>-/-</sup> STAT6<sup>-/-</sup> mice (Figure 2). In contrast, no significant difference was observed in the expression of *Il31*, *Ccl17* (encoding TARC), *Ccl11*

(encoding eotaxin-1), or *Il6* mRNA (Figure 2). These results suggest that T-bet and STAT6 coordinately suppress the development of inflammatory skin diseases and that the inflammatory skin diseases developed in T-bet<sup>-/-</sup> STAT6<sup>-/-</sup> mice resemble atopic dermatitis rather than psoriasis, although some characteristics of atopic dermatitis including, TARC elevation, are lacking.

#### CD4<sup>+</sup> T cells cause atopic dermatitis-like skin inflammation in T-bet<sup>-/-</sup> STAT6<sup>-/-</sup> mice

Because ILC2s in the skin of T-bet<sup>-/-</sup> STAT6<sup>-/-</sup> mice do not produce IL-5 (Makita et al., 2018) and thus seem not to be responsible for eosinophilic skin inflammation, we evaluate the roles of CD4<sup>+</sup> T cells in atopic dermatitis-like skin inflammation. As shown in Figure 3a, CD4<sup>+</sup> T cells were increased in the skin-draining lymph nodes (LNs) in T-bet<sup>-/-</sup> STAT6<sup>-/-</sup> mice compared with those in WT mice. FACS analyses revealed that CD44<sup>+</sup> (memory) CD4<sup>+</sup> T cells were markedly increased in the skin-draining LNs in T-bet<sup>-/-</sup> STAT6<sup>-/-</sup> mice (Figure 3b). In contrast, the numbers of CD4<sup>+</sup> T cells were not significantly increased in mesenteric LNs or mediastinal LNs in T-bet<sup>-/-</sup> STAT6<sup>-/-</sup> mice (Supplementary Figure S2), suggesting that the skin inflammation in T-bet<sup>-/-</sup> STAT6<sup>-/-</sup> mice seems to be a local manifestation rather than one of the systemic manifestations.

To determine whether CD4<sup>+</sup> T cells are involved in the development of dermatitis, we first performed adoptive transfer experiments of CD4<sup>+</sup> T cells. In this experiment, CD4<sup>+</sup> T cells isolated from spleen and skin-draining LNs of either T-bet<sup>-/-</sup> STAT6<sup>-/-</sup> mice (8- to 12-week-old) that had developed the skin inflammation or control WT mice (8- to 12-week-old) were intravenously transferred to severe combined immunodeficient (SCID) mice. Because some of the SCID mice receiving CD4<sup>+</sup> T-cell transfer develop colitis, which makes it difficult to evaluate the skin inflammation score, we evaluated skin inflammation by measuring the number of eosinophils and mast cells in the skin rather than the skin inflammation score. The numbers of eosinophils and mast cells in the skin were significantly increased by the adoptive transfer of CD4<sup>+</sup> T cells of T-bet<sup>-/-</sup> STAT6<sup>-/-</sup> mice but not of WT mice at 8 weeks after the transfer (Figure 3c).

We further examined the roles of CD4<sup>+</sup> T cells in the development of atopic dermatitis-like skin inflammation by depletion experiments. In this experiment, anti-CD4 mAb or control IgG was injected intraperitoneally to T-bet<sup>-/-</sup> STAT6<sup>-/-</sup> mice from 3 weeks to 13 weeks of age. We found that the depletion of CD4<sup>+</sup> T cells (Figure 3d) modestly but significantly reduced the skin score (Figure 3e) and the numbers of eosinophils and mast cells in the skin (Figure 3f) in T-bet<sup>-/-</sup> STAT6<sup>-/-</sup> mice. These results suggest that CD4<sup>+</sup> T cells are involved in the development of atopic dermatitis-like skin inflammation in T-bet<sup>-/-</sup> STAT6<sup>-/-</sup> mice.

#### IL-9 is partly responsible for the development of atopic dermatitis-like skin inflammation in T-bet<sup>-/-</sup> STAT6<sup>-/-</sup> mice

To address the mechanisms by which T-bet and STAT6 expressed in CD4<sup>+</sup> T cells coordinately suppress the development of atopic dermatitis-like skin inflammation, we searched for genes whose expression was upregulated in memory CD4<sup>+</sup> T cells in the skin-draining LNs in T-bet<sup>-/-</sup>

STAT6<sup>-/-</sup> mice by RNA sequencing (RNA-seq) analyses. A substantial number of genes were upregulated in T-bet<sup>-/-</sup> STAT6<sup>-/-</sup> CD4<sup>+</sup> T cells compared with WT CD4<sup>+</sup> T cells (cluster I–IV) (Figure 4a). Some of the genes were upregulated in T-bet<sup>-/-</sup> STAT6<sup>-/-</sup> CD4<sup>+</sup> T cells even compared with T-bet<sup>-/-</sup> CD4<sup>+</sup> T cells or STAT6<sup>-/-</sup> CD4<sup>+</sup> T cells (cluster I) (Figure 4b). Consistent with a previous report showing enhanced Th17 cell differentiation in T-bet<sup>-/-</sup> STAT6<sup>-/-</sup> mice (Das et al., 2009), cluster I contained several Th17 cell-related genes, such as *Il17a*, *Il17f*, *Il22*, and *Rorc*; however, the most significantly induced gene was *Il9* (Figure 4c). Recent studies shed light on the importance of IL-9 in the context of atopic dermatitis (Angkasekwinai, 2019; Esaki et al., 2016; Ma et al., 2014). Therefore, we focused on the excessive IL-9 production in T-bet<sup>-/-</sup> STAT6<sup>-/-</sup> CD4<sup>+</sup> T cells. qPCR analysis of isolated memory CD4<sup>+</sup> T cells from the skin-draining LNs confirmed elevated expression of *Il9* mRNA in T-bet<sup>-/-</sup> STAT6<sup>-/-</sup> mice (Figure 4d). Indeed, the frequency of IL-9-producing CD4<sup>+</sup> T cells in the dorsal skin was significantly higher in T-bet<sup>-/-</sup> STAT6<sup>-/-</sup> mice than in WT mice (Figure 4e).

Because T-bet and STAT6 are expressed not only in T cells but also in other cell populations, including nonhematopoietic cells, we next examined whether the increased IL-9-producing CD4<sup>+</sup> T cells in T-bet<sup>-/-</sup> STAT6<sup>-/-</sup> mice was cell-intrinsic or tissue environment-dependent. We first employed usual bone marrow (BM) chimera experiments in which BM cells from either T-bet<sup>-/-</sup> STAT6<sup>-/-</sup> mice or WT mice were transferred to irradiated WT recipient mice. IL-9 production of isolated CD4<sup>+</sup> T cells from these mice was examined seven weeks after the cell transfer. We found that CD4<sup>+</sup> T cells derived from T-bet<sup>-/-</sup> STAT6<sup>-/-</sup> BM cells produced higher amounts of IL-9 than those derived from WT BM cells (Figure 4f). We also performed other BM chimera experiments in which BM cells from WT mice were transferred to either irradiated T-bet<sup>-/-</sup> STAT6<sup>-/-</sup> or WT recipient mice. As shown in Figure 4g, CD4<sup>+</sup> T cells from these mice produced similar amounts of IL-9. We finally performed mixed BM chimera experiments in which BM cells from T-bet<sup>-/-</sup> STAT6<sup>-/-</sup> mice (Thy1.2) and WT mice (Thy1.1) were cotransferred to irradiated WT recipient mice (Thy1.1). We found that T-bet<sup>-/-</sup> STAT6<sup>-/-</sup> CD4<sup>+</sup> T cells isolated from the recipient mice produced higher levels of IL-9 than WT CD4<sup>+</sup> T cells isolated from the same mice (Figure 4h). These results suggest that T-bet and STAT6 expressed in hematopoietic cells are primarily involved in suppressing the development of IL-9-producing CD4<sup>+</sup> T cells.

To determine whether the excessive production of IL-9 is involved in the development of atopic dermatitis-like skin inflammation in T-bet<sup>-/-</sup> STAT6<sup>-/-</sup> mice, we next examined the effect of neutralizing anti-IL-9 antibody on the development of atopic dermatitis-like skin inflammation. As shown in Figure 5a and b, anti-IL-9 antibody significantly ameliorated skin disease in T-bet<sup>-/-</sup> STAT6<sup>-/-</sup> mice. Consistently, the number of skin-infiltrating eosinophils was significantly reduced by anti-IL-9 antibody in T-bet<sup>-/-</sup> STAT6<sup>-/-</sup> mice (Figure 5c). In contrast, the numbers of mast cells and CD4<sup>+</sup> T cells were not significantly reduced by anti-IL-9 antibody (Figure 5c). These results indicate that the enhanced production of IL-9 leads to eosinophilic inflammation in the skin in T-bet<sup>-/-</sup> STAT6<sup>-/-</sup> mice.

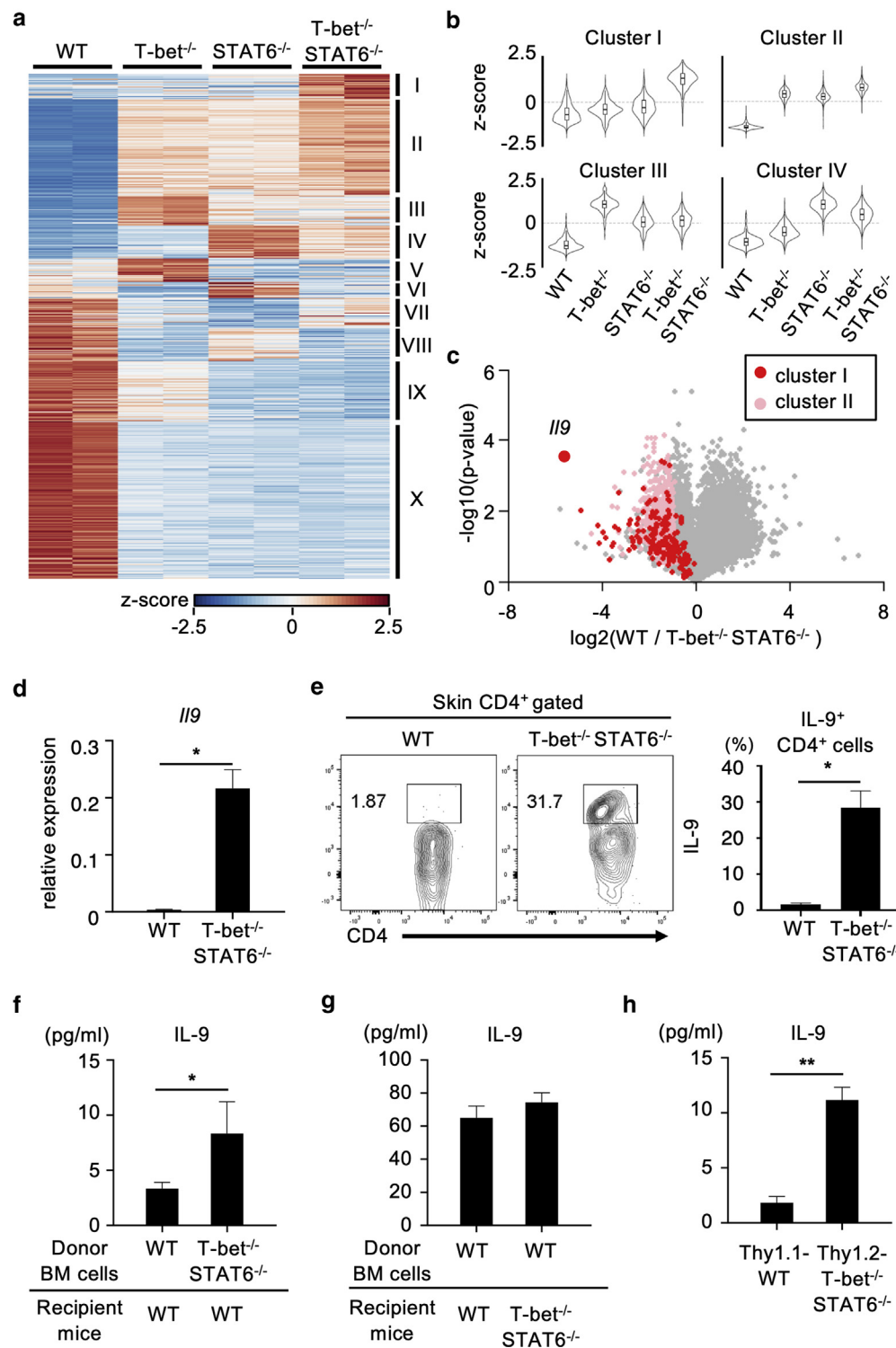


**Figure 4. IL-9-producing CD4<sup>+</sup> T cells are increased in the skin in T-bet<sup>-/-</sup> STAT6<sup>-/-</sup> mice.** (a–c) Total RNA of CD44<sup>+</sup> CD4<sup>+</sup> T cells in skin-draining LNs was isolated from WT mice, T-bet<sup>-/-</sup> mice, STAT6<sup>-/-</sup> mice, or T-bet<sup>-/-</sup> STAT6<sup>-/-</sup> mice and subjected to RNA-seq analysis. Shown are (a) K-means++ clustering, (b) z-score distribution of the indicated clusters, and (c) a volcano plot comparing WT CD4<sup>+</sup> T cells and T-bet<sup>-/-</sup> STAT6<sup>-/-</sup> CD4<sup>+</sup> T cells. (d) CD44<sup>+</sup> CD4<sup>+</sup> T cells from skin-draining LNs were stimulated with PMA and ionomycin for 2 hours and mRNA levels of *Il9* were analyzed by qPCR analysis (n = 3, each). (e) CD44<sup>+</sup> CD4<sup>+</sup> T cells from the skin were stimulated with PMA and ionomycin for 4.5 hours with monensin added for the last 30 minutes, and intracellular IL-9 staining was performed. Representative CD4<sup>+</sup> CD3ε<sup>+</sup> cells and means ± SD of the frequency of IL-9<sup>+</sup> cells (n = 3, each). (f, g) BM cells (5 × 10<sup>6</sup> cells) from indicated mice were injected into irradiated recipient mice. (h) A mixture of BM cells from Thy1.1<sup>+</sup> WT mice (2.5 × 10<sup>6</sup> cells) and Thy1.2<sup>+</sup> T-bet<sup>-/-</sup> STAT6<sup>-/-</sup> mice (2.5 × 10<sup>6</sup> cells) were injected into irradiated Thy1.1<sup>+</sup> WT mice. Four to seven weeks later, CD4<sup>+</sup> T cells were isolated from the skin-draining LNs of the chimeric mice (n = 3 mice per group). Thy1.1<sup>+</sup> WT CD4<sup>+</sup> T cells and Thy1.2<sup>+</sup> T-bet<sup>-/-</sup> STAT6<sup>-/-</sup> CD4<sup>+</sup> T cells were purified from the same mice as described in the Materials and Methods. Cells were then stimulated with anti-CD3/CD28 mAbs + TSLP for 24 hours, and IL-9 levels in culture supernatants were measured by ELISA. Means ± SD, n = 3, each. Results are representative of three independent experiments. \*P < 0.05, \*\*P < 0.005. BM, bone marrow; LN, lymph node; PMA, phorbol 12-myristate 13-acetate; RNA-seq, RNA sequencing; STAT, signal transducer and activator of transcription; TSLP, thymic stromal lymphopoietin; WT, wild-type.

#### IL-9-producing CD4<sup>+</sup> T cells do not develop in T-bet<sup>-/-</sup> STAT6<sup>-/-</sup> mice under conventional Th9 cell-inducing conditions

To address the mechanism underlying the enhanced IL-9 production by the absence of T-bet and STAT6 in CD4<sup>+</sup> T cells, we evaluated in vitro differentiation of IL-9-producing CD4<sup>+</sup> T cells. In this experiment, naïve CD4<sup>+</sup> T cells isolated from WT mice or T-bet<sup>-/-</sup> STAT6<sup>-/-</sup> mice were stimulated with anti-CD3/CD28 mAb under

conventional Th9 cell-inducing conditions (in the presence of IL-4 plus TGF-β) for 4 days, and IL-9 production was examined by intracellular staining. Consistent with a previous report showing that STAT6 is required for Th9 cell differentiation under Th9 cell-inducing conditions (Goswami et al., 2012), IL-9-producing CD4<sup>+</sup> T cells developed under Th9 cell-inducing conditions in WT mice but not in T-bet<sup>-/-</sup> STAT6<sup>-/-</sup> mice (Supplementary Figure S3).

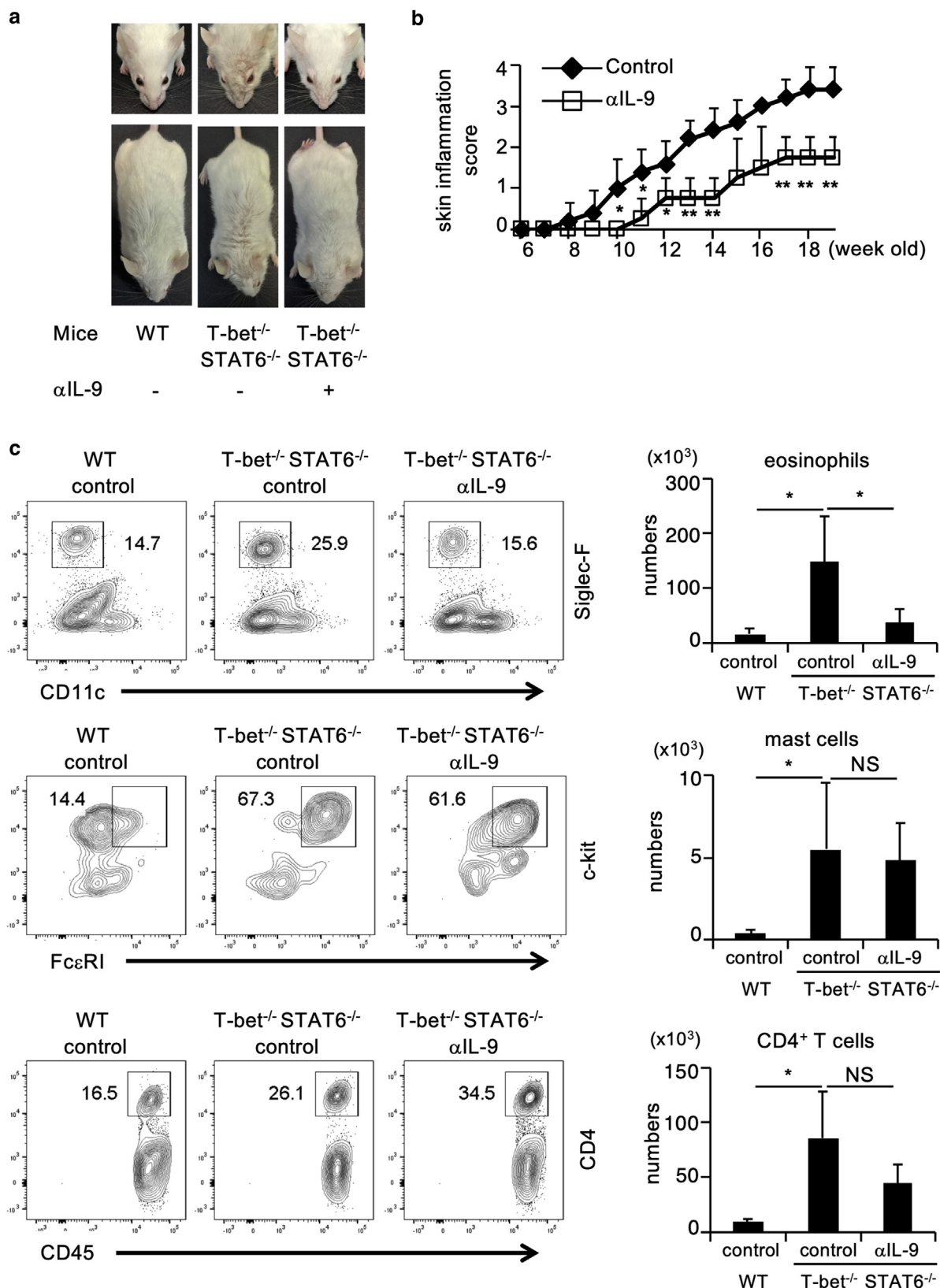


**Figure 5. Neutralization of IL-9 attenuates atopic dermatitis-like skin inflammation in T-bet<sup>-/-</sup> STAT6<sup>-/-</sup> mice.** (a) Shown are representative photographs of WT mice and T-bet<sup>-/-</sup> STAT6<sup>-/-</sup> mice (19-week-old) with or without anti-IL-9 antibody treatment (twice per week, from 6 to 19 weeks). (b) Severity of skin disease was assessed by the skin inflammation score. Means ± SD (n = 5, each). (c) Representative CD11c versus Siglec-F and FcεRI versus c-kit staining of CD45<sup>+</sup> CD3<sup>-</sup> cells; CD45 versus CD4 staining of CD45<sup>+</sup> CD3<sup>+</sup> cells in the skin; and means ± SD of the numbers of Siglec-F<sup>+</sup> CD11c<sup>-</sup> eosinophils, c-kit<sup>+</sup> FcεRI<sup>+</sup> mast cells, and CD45<sup>+</sup> CD4<sup>+</sup> T cells in the skin (n = 3, each). Results are representative of two independent experiments. \*P < 0.05, \*\*P < 0.005. NS, not significant; STAT, signal transducer and activator of transcription; WT, wild-type.

To further address the mechanisms underlying the enhanced IL-9 production in T-bet<sup>-/-</sup> STAT6<sup>-/-</sup> CD4<sup>+</sup> T cells, we explored mRNA expression of conventional Th9 cell-related molecules in memory CD4<sup>+</sup> T cells in the skin-draining LNs of T-bet<sup>-/-</sup> STAT6<sup>-/-</sup> mice. We found that the levels of *Smad2*, *Maf*, and *Rorc*, which are involved in the development of Th9 cells and Th17 cells (Wang et al., 2013), were significantly increased in T-bet<sup>-/-</sup> STAT6<sup>-/-</sup> CD4<sup>+</sup> T cells (Supplementary Figure S4). In contrast, the levels of *Gata3*, *Irf4*, *Batf*, *Spi1* (encoding PU.1), *Smad3*, and *Gcn5*, which are crucial for the development of IL-4/STAT6- and TGF- $\beta$ -dependent conventional Th9 cells (Kaplan et al., 2015), were not enhanced in T-bet<sup>-/-</sup> STAT6<sup>-/-</sup> CD4<sup>+</sup> T cells (Supplementary Figure S4). The expression of *Il2rg* was reduced in T-bet<sup>-/-</sup> STAT6<sup>-/-</sup> CD4<sup>+</sup> T cells (Supplementary Figure S4). Taken together, these findings suggest that IL-9-producing CD4<sup>+</sup> T cells in T-bet<sup>-/-</sup> STAT6<sup>-/-</sup> mice are likely to be distinct from conventional Th9 cells developed in the presence of IL-4 plus TGF- $\beta$ .

#### Thymic stromal lymphopoietin-induced IL-9 production is enhanced in T-bet<sup>-/-</sup> STAT6<sup>-/-</sup> CD4<sup>+</sup> T cells

To address the mechanism underlying the enhanced IL-9 production in T-bet<sup>-/-</sup> STAT6<sup>-/-</sup> CD4<sup>+</sup> T cells, we performed Gene Ontology analyses of the upregulated genes in T-bet<sup>-/-</sup> STAT6<sup>-/-</sup> CD4<sup>+</sup> T cells. We found that “cytokine-cytokine receptor interaction” was the most significantly enriched Gene Ontology term in the upregulated genes in T-bet<sup>-/-</sup> STAT6<sup>-/-</sup> CD4<sup>+</sup> T cells (Supplementary Figure S5a). Because it has been reported that not only STAT6 but also STAT5 induces the development of IL-9-producing CD4<sup>+</sup> T cells as a downstream signaling molecule of IL-2 or thymic stromal lymphopoietin (TSLP) (Kaplan et al., 2015; Yao et al., 2013), we next performed gene set enrichment analyses of the upregulated genes in T-bet<sup>-/-</sup> STAT6<sup>-/-</sup> CD4<sup>+</sup> T cells. Among IL-4, IL-2, and TSLP signaling, TSLP signaling was significantly enriched in the upregulated genes in T-bet<sup>-/-</sup> STAT6<sup>-/-</sup> CD4<sup>+</sup> T cells (Supplementary Figure S5b), which is consistent with the increased mRNA expression of *Tslp* in the skin in T-bet<sup>-/-</sup> STAT6<sup>-/-</sup> mice (Figure 2).

We then examined the expression of functional TSLP receptor, which consists of IL-7 receptor  $\alpha$  chain and TSLP receptor, in CD4<sup>+</sup> T cells in skin-draining LNs in T-bet<sup>-/-</sup> STAT6<sup>-/-</sup> mice. We found that the levels of *Il7ra* and *Crlf2* (encoding TSLP receptor) were significantly increased in T-bet<sup>-/-</sup> STAT6<sup>-/-</sup> CD4<sup>+</sup> T cells compared with those in WT CD4<sup>+</sup> T cells (Figure 6a). FACS analyses revealed that the frequency of CD4<sup>+</sup> T cells expressing IL-7 receptor  $\alpha$  chain and TSLP receptor was higher in T-bet<sup>-/-</sup> STAT6<sup>-/-</sup> mice than in WT mice (Figure 6b). Consistently, TSLP-induced STAT5 phosphorylation was enhanced in T-bet<sup>-/-</sup> STAT6<sup>-/-</sup> CD4<sup>+</sup> T cells (Figure 6c), suggesting that TSLP-STAT5 signaling was enhanced in T-bet<sup>-/-</sup> STAT6<sup>-/-</sup> CD4<sup>+</sup> T cells.

We finally investigated whether TSLP induces IL-9 production in T-bet<sup>-/-</sup> STAT6<sup>-/-</sup> CD4<sup>+</sup> T cells. CD4<sup>+</sup> T cells isolated from skin-draining LNs of WT or T-bet<sup>-/-</sup> STAT6<sup>-/-</sup> mice were stimulated with anti-CD3/CD28 mAb in the presence or absence of TSLP or IL-2. In the presence of TSLP, *Il9* mRNA was markedly induced in T-bet<sup>-/-</sup> STAT6<sup>-/-</sup> CD4<sup>+</sup> T cells (Figure 6d). In contrast, IL-2 did not induce *Il9* mRNA

expression in T-bet<sup>-/-</sup> STAT6<sup>-/-</sup> CD4<sup>+</sup> T cells (Figure 6d), consistent with the reduced expression of *Il2rg*. TSLP markedly promoted anti-CD3/CD28 mAb-induced IL-9 production from T-bet<sup>-/-</sup> STAT6<sup>-/-</sup> CD4<sup>+</sup> T cells at protein levels (Figure 6e). Even when naïve CD4<sup>+</sup> T cells were stimulated with anti-CD3/CD28 mAb plus TSLP, IL-9 production was significantly increased in T-bet<sup>-/-</sup> STAT6<sup>-/-</sup> CD4<sup>+</sup> T cells (Figure 6f). These results suggest that T-bet and STAT6 cooperatively suppress the expression of functional TSLP receptors in CD4<sup>+</sup> T cells and suppress TSLP-mediated IL-9 production.

#### DISCUSSION

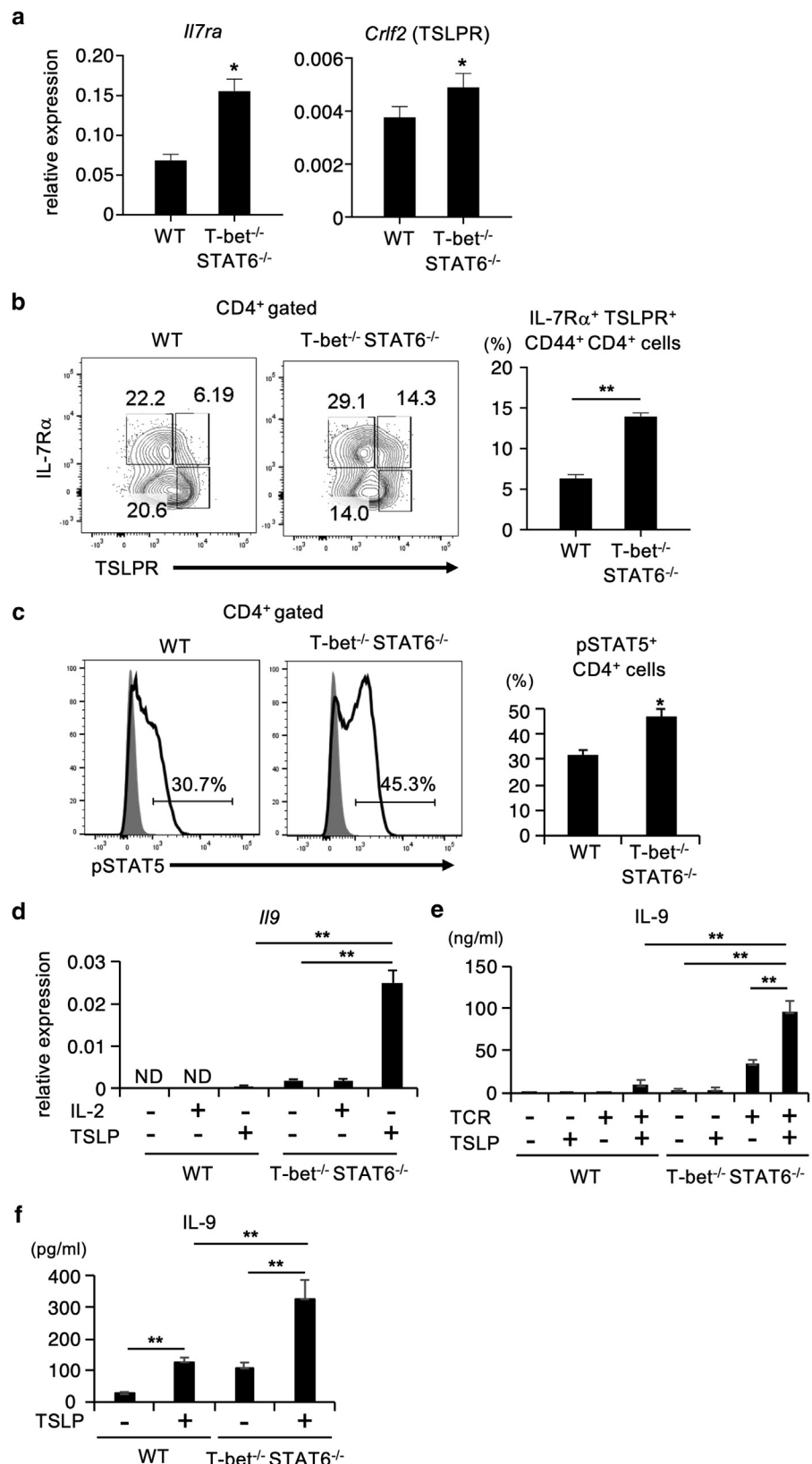
In this study, we showed that T-bet<sup>-/-</sup> STAT6<sup>-/-</sup> mice but not T-bet<sup>-/-</sup> mice or STAT6<sup>-/-</sup> mice developed atopic dermatitis-like skin inflammation (Figure 1). Regarding the mechanism underlying the skin inflammation in T-bet<sup>-/-</sup> STAT6<sup>-/-</sup> mice, we found that the depletion of CD4<sup>+</sup> T cells attenuated the skin inflammation in T-bet<sup>-/-</sup> STAT6<sup>-/-</sup> mice, whereas the adoptive transfer of T-bet<sup>-/-</sup> STAT6<sup>-/-</sup> CD4<sup>+</sup> T cells into SCID mice resulted in the accumulation of eosinophils and mast cells in the skin (Figure 3). Transcriptome analysis and intracellular staining revealed that the production of IL-9 was significantly enhanced in T-bet<sup>-/-</sup> STAT6<sup>-/-</sup> CD4<sup>+</sup> T cells (Figure 4). The neutralization of IL-9 improved the skin inflammation in T-bet<sup>-/-</sup> STAT6<sup>-/-</sup> mice (Figure 5). Finally, we showed that TSLP strongly induced IL-9 production in T-bet<sup>-/-</sup> STAT6<sup>-/-</sup> CD4<sup>+</sup> T cells, possibly because of the enhanced expression of TSLP receptors (Figure 6). These results suggest that T-bet and STAT6 coordinately suppress the development of atopic dermatitis-like skin inflammation through the inhibition of TSLP-induced IL-9 production in CD4<sup>+</sup> T cells.

We found that IL-9 production is increased in T-bet<sup>-/-</sup> STAT6<sup>-/-</sup> CD4<sup>+</sup> T cells (Figure 4) and that IL-9 is involved in the development of atopic dermatitis-like skin inflammation in T-bet<sup>-/-</sup> STAT6<sup>-/-</sup> mice (Figure 5). IL-9 is a pleiotropic cytokine that influences various functions of target cells, including T cells, B cells, ILC2s, mast cells, and eosinophils (Angkasekwinai, 2019; Kaplan et al., 2015; Noelle and Nowak, 2010; Stassen et al., 2012). It has been reported that Th9 cells may contribute to the pathogenesis of atopic dermatitis because IL-9 stimulates VEGF secretion in human keratinocytes, leading to the upregulation of angiogenesis and vascular permeability in the skin lesion (Ma et al., 2014). In addition, Schlapbach et al. (2014) have shown that human Th9 cells are predominantly found in the skin and capable of amplifying immune responses by enhancing cytokine production from Th1, Th2, Th9, and Th17 cells. Moreover, Th9 cells are involved in causing allergic contact dermatitis (Angkasekwinai, 2019). Therefore, the excessive differentiation and/or accumulation of IL-9-producing CD4<sup>+</sup> T cells in the skin could be involved in the pathogenesis of atopic dermatitis-like skin inflammation in T-bet<sup>-/-</sup> STAT6<sup>-/-</sup> mice.

It has been shown that the development of Th9 cells requires the coordinated activation of transcriptional factors such as IRF4 and BATF, which are downstream of IL-4/STAT6 signaling, and TGF- $\beta$ -induced SMAD2/3 as well as SMAD-independent induction of PU.1 (Chang et al., 2010; Kaplan, 2017). In addition, IL-2- or TSLP-STAT5 signaling is reported to be involved in IL-9 production from CD4<sup>+</sup> T cells

**Figure 6. TSLP-induced IL-9 production is enhanced in T-bet<sup>-/-</sup> STAT6<sup>-/-</sup> CD4<sup>+</sup> T cells.**

(a) mRNA expression of *Il7ra* and *Crlf2* (TSLPR) in CD44<sup>+</sup> CD4<sup>+</sup> T cells isolated from skin-draining LNs of WT mice or T-bet<sup>-/-</sup> STAT6<sup>-/-</sup> mice (n = 3, each). (b) Representative TSLPR versus IL-7R $\alpha$  staining of CD44<sup>+</sup> CD4<sup>+</sup> T cells in skin-draining LNs and means  $\pm$  SD of the frequency of IL-7R $\alpha$ <sup>+</sup> TSLPR<sup>+</sup> cells (n = 3, each). (c) Skin-draining LN cells were stimulated with or without TSLP (100 ng/ml) for 15 minutes. The levels of intracellular pSTAT5 in CD4<sup>+</sup> T cells were evaluated by FACS. Histograms indicate the levels of pSTAT5 in the absence (shaded) or presence (line) of TSLP. Means  $\pm$  SD of frequency of pSTAT5<sup>+</sup> cells (n = 3, each). (d) CD4<sup>+</sup> T cells from skin-draining LNs were stimulated with anti-CD3/CD28 mAbs in the presence or absence of IL-2 (100 ng/ml) or TSLP (100 ng/ml) for 18 hours, and mRNA levels of *Il9* were analyzed by qPCR. Means  $\pm$  SD of relative expression of *Il9* (n = 3, each). (e) CD4<sup>+</sup> T cells from skin-draining LNs were stimulated with or without anti-CD3/CD28 mAbs in the presence or absence of TSLP (100 ng/ml) for 3 days. Protein levels of IL-9 in the supernatants were measured by ELISA. Means  $\pm$  SD (n = 3, each). (f) Naïve CD4<sup>+</sup> T cells from skin-draining LNs were stimulated with anti-CD3/CD28 mAbs in the presence or absence of TSLP (100 ng/ml) for 5 days. IL-9 levels in culture supernatants were measured by ELISA. Means  $\pm$  SD, n = 3, each. Data are representative of two independent experiments. \**P* < 0.05, \*\**P* < 0.005. LN, lymph node; IL-17R $\alpha$ , IL-17 receptor  $\alpha$  chain; pSTAT, phosphorylated STAT; STAT, signal transducer and activator of transcription; TSLP, thymic stromal lymphopoietin; TSLPR, thymic stromal lymphopoietin receptor; WT, wild-type.



(Kaplan, 2017). Micossé et al. (2019) have reported that PPAR $\gamma$  contributes to the production of IL-9 in human Th2 cells. In this study, we found that IL-9-producing CD4<sup>+</sup> T cells were markedly increased in T-bet<sup>-/-</sup> STAT6<sup>-/-</sup> mice

in vivo (Figure 4), although in vitro stimulation under conventional Th9 conditions (IL-4 plus TGF- $\beta$ ) did not induce IL-9 production in naïve CD4<sup>+</sup> T cells in T-bet<sup>-/-</sup> STAT6<sup>-/-</sup> mice (Supplementary Figure S3). Consistently, the expression

of various transcription factors induced under conventional Th9 conditions such as PU.1 and IRF4 was not increased in T-bet<sup>-/-</sup> STAT6<sup>-/-</sup> CD4<sup>+</sup> T cells (Supplementary Figure S4). RNA-seq analyses revealed that the expression of PPAR $\gamma$  was not increased in T-bet<sup>-/-</sup> STAT6<sup>-/-</sup> CD4<sup>+</sup> T cells compared with WT CD4<sup>+</sup> T cells (cluster V in Figure 4a). These findings indicate that conventional Th9 cell-related transcriptional factors are not implicated in the increased IL-9 production in T-bet<sup>-/-</sup> STAT6<sup>-/-</sup> CD4<sup>+</sup> T cells.

Regarding the mechanisms underlying IL-9 production even in the absence of STAT6 activation, our findings suggest that the enhanced responsiveness to TSLP stimulation is involved in the enhanced IL-9 production in T-bet<sup>-/-</sup> STAT6<sup>-/-</sup> CD4<sup>+</sup> T cells. TSLP has been shown to be highly expressed in keratinocytes in patients with atopic dermatitis (Soumelis et al., 2002). In addition, forced expression of TSLP in keratinocytes induces atopic dermatitis-like skin inflammation (Soumelis et al., 2002), suggesting that TSLP expressed in the skin contributes to the pathogenesis of atopic dermatitis. TSLP is known to activate STAT5 to promote proliferation and cytokine production of Th2 cells (Pandey et al., 2000; Soumelis et al., 2002). Moreover, it has been shown that TSLP increases IL-9 production from human and murine Th9 cells and enhances Th9 cells' ability to promote allergic airway inflammation (Yao et al., 2013). In this regard, we found that TSLP signaling rather than IL-4 or IL-2 signaling is enriched in genes preferentially expressed in T-bet<sup>-/-</sup> STAT6<sup>-/-</sup> CD4<sup>+</sup> T cells (Supplementary Figure S5b). Indeed, TSLP induces IL-9 production more strongly in T-bet<sup>-/-</sup> STAT6<sup>-/-</sup> CD4<sup>+</sup> T cells than in WT CD4<sup>+</sup> T cells (Figure 6). We also found that TSLP-STAT5-mediated signaling is upregulated, possibly because of the increased expression of functional TSLP receptors in T-bet<sup>-/-</sup> STAT6<sup>-/-</sup> CD4<sup>+</sup> T cells (Figure 6). These results suggest that T-bet and STAT6 coordinately suppress the TSLP-STAT5-IL-9 axis in CD4<sup>+</sup> T cells. Further studies addressing the mechanisms underlying the suppression of the TSLP-STAT5-IL-9 axis by T-bet and STAT6 are required.

Our findings raise the possibility that IL-9-producing CD4<sup>+</sup> T cells in T-bet<sup>-/-</sup> STAT6<sup>-/-</sup> mice may share some characteristics with Th17 cells. qPCR analyses showed that the expression of *Il17a* and *Il17f*, as well as *Il9*, was increased in memory CD4<sup>+</sup> T cells in the skin-draining LNs in T-bet<sup>-/-</sup> STAT6<sup>-/-</sup> mice, consistent with previous findings that Th17 cell differentiation was enhanced, but Th1 and Th2 cell differentiation were impaired in T-bet<sup>-/-</sup> STAT6<sup>-/-</sup> mice (Das et al., 2009; Tamachi et al., 2009; Yang et al., 2008). Our attempts to detect both IL-9 and IL-17A in one cell by intracellular cytokine staining have failed likely because of technical reasons. Further analyses using highly sensitive single-cell RNA-seq analysis are required to address the relationship between IL-9-producing CD4<sup>+</sup> T cells and Th17 cells in T-bet<sup>-/-</sup> STAT6<sup>-/-</sup> mice.

We demonstrated that the numbers of CD4<sup>+</sup> T cells and eosinophils infiltrating into the skin were increased in STAT6<sup>-/-</sup> mice compared with those in WT mice (Figure 1f), suggesting that STAT6 suppresses skin infiltration of CD4<sup>+</sup> T cells and eosinophils. However, STAT6<sup>-/-</sup> mice did not develop atopic dermatitis-like skin diseases (Figure 1). The

mechanisms underlying the discrepancy remains unknown. Transcriptome analyses of CD4<sup>+</sup> T cells in the skin-draining LNs revealed that considerable numbers of genes, including *Il9* and Th17 cell-related genes such as *Il17a*, *Il17f*, and *Il22*, were increased in T-bet<sup>-/-</sup> STAT6<sup>-/-</sup> CD4<sup>+</sup> T cells compared with STAT6<sup>-/-</sup> CD4<sup>+</sup> T cells. A series of BM chimera experiments demonstrated that T-bet/STAT6 double deficiency in BM cells is involved in the enhanced IL-9 production in CD4<sup>+</sup> T cells (Figure 4f–h). These findings suggest that the enhanced production of IL-9 and Th17 cell-related cytokines by the absence of T-bet and STAT6 and the enhanced skin infiltration of CD4<sup>+</sup> T cells by the absence of STAT6 may synergistically cause atopic dermatitis-like skin diseases in T-bet<sup>-/-</sup> STAT6<sup>-/-</sup> mice. This notion is consistent with recent studies showing that Th17 cells contribute to the development of atopic dermatitis in some situations (Brunner et al., 2017; Esaki et al., 2016).

We found that the depletion of CD4<sup>+</sup> T cells by anti-CD4 antibody reproducibly reduced the severity of skin inflammation, but the effects are not strong. Because skin infiltration of eosinophils and mast cells has already been observed at 3 weeks of age in T-bet<sup>-/-</sup> STAT6<sup>-/-</sup> mice (unpublished data), it is plausible that the depletion of CD4<sup>+</sup> T cells starting from 3 weeks of age is not sufficient to eliminate the preexisting inflammation. It is also possible that other cell populations contribute to the development of skin inflammation. In this regard, we have previously reported that ILCs may not be responsible for the development of skin inflammation because ILCs in the skin of T-bet<sup>-/-</sup> STAT6<sup>-/-</sup> mice did not produce IL-5 (Makita et al., 2018). However, it remains possible that ILCs contribute to the development of skin inflammation by producing other cytokines such as IL-9. Further studies using cell type-specific IL-9-deficient mice are required to address this possibility.

In conclusion, our findings indicate that T-bet and STAT6 coordinately suppress the development of atopic dermatitis-like skin inflammation, possibly through the downregulation of the TSLP-IL-9 axis in CD4<sup>+</sup> T cells. Our findings could associate with the mechanisms underlying the development of conjunctivitis after administration of dupilumab, an inhibitor of IL-4/IL-13 signaling, to treat atopic dermatitis (Gooderham et al., 2018). Although further studies are needed, our results should add insights into the pathogenesis of atopic dermatitis and indicate that we should be careful when we apply STAT6 inhibition to the therapeutic strategy for atopic dermatitis.

## MATERIAL AND METHODS

### Mice

T-bet<sup>-/-</sup> mice (Finotto et al., 2002) on a BALB/c background were obtained from Jackson Laboratory (Bar Harbor, ME). BALB/c T-bet<sup>-/-</sup> mice were crossed with STAT6<sup>-/-</sup> mice (Takeda et al., 1996) to obtain T-bet<sup>-/-</sup> STAT6<sup>-/-</sup> mice. BALB/c-*scid/scid* mice (SCID mice) were purchased from CLEA (Tokyo, Japan). BALB/c Thy1.1<sup>+</sup> mice were kindly provided by Dr S. Hori (The University of Tokyo, Tokyo, Japan). All mice were housed in microisolator cages under specific pathogen-free conditions, and all experiments were performed according to the guidelines of Chiba University and the National Institutes of Health guide for the care and use of laboratory animals.

### Scoring of skin diseases

The severity of skin diseases was scored by a method based on the macroscopic diagnostic criteria described by Saunders et al. (2016) in a blinded manner. In brief, the signs of edema, erythema, scale, and erosion of skin lesion were graded 0–3 (0, none; 1, mild; 2, moderate; 3, severe), and total score on four regions (ear, face, head, and back) of each mouse was calculated by the sum of individual scores for each parameter.

### Measurement of scratching behavior

To measure the frequency of scratching, 13-week-old mice were put into an acrylic cage for approximately 5 minutes and allowed to adapt to the conditions. The behavior of mice was recorded for 5 minutes by a video camera, and scratching frequency was counted as described previously (Kuraishi et al., 1995).

### Isolation of inflammatory cells from the skin

Dorsal skin was cut into small pieces and incubated in RPMI 1640 medium containing Liberase TL (0.5 mg/ml, Roche, Basel, Switzerland), DNase (0.1 mg/ml, Wako, Tokyo, Japan), and Hepes (25 mM, Thermo Fisher Scientific, Waltham, MA) for 90 minutes at 37 °C. The samples were transferred to a Gentle MACS tube (Miltenyi Biotec, Bergisch Gladbach, Germany) with 10 ml of RPMI. The samples were dissociated by Gentle MACS (Miltenyi Biotec) with program C. Single-cell suspensions were prepared by passing through a 70-µm nylon cell strainer.

### Isolation of memory CD4<sup>+</sup> T cells from LNs

Memory CD4<sup>+</sup> T cells were purified from single-cell suspensions of skin-draining LNs by negative sorting with an EasySep Mouse Memory CD4<sup>+</sup> T Cell Isolation Kit (StemCell Technologies, Vancouver, British Columbia, Canada) according to the manufacturers' instructions.

### Flow cytometric analysis and intracellular staining

After dead cell staining with Zombie NIR (Biolegend, San Diego, CA) and Fc receptor blocking with anti-CD16/32 antibody (93; Biolegend), intracellular staining of CD4<sup>+</sup> T cells was performed as described previously (Kawashima et al., 2013) using anti-IL-9 antibody. After washing, cells were analyzed on a FACS Canto II (Becton Dickinson, San Jose, CA), and data were analyzed with FlowJo software (Becton Dickinson).

### Adoptive transfer of CD4<sup>+</sup> T cells

CD4<sup>+</sup> T cells were purified from LNs and spleen with a MojoSort Mouse CD4 T Cell Isolation Kit (Biolegend) and injected intravenously to SCID mice at 6 weeks of age ( $1 \times 10^6$  cells/mouse).

### In vivo depletion of CD4<sup>+</sup> T cells

Anti-CD4 mAb (GK1.5, 500 µg) or control IgG was injected intraperitoneally to T-bet<sup>-/-</sup> STAT6<sup>-/-</sup> mice twice per week from 3 weeks to 13 weeks of age. The mice were analyzed at 13 weeks of age.

### In vivo neutralization of IL-9

Anti-IL-9 mAb (MM9C1, 200 µg) or control IgG was injected intraperitoneally to T-bet<sup>-/-</sup> STAT6<sup>-/-</sup> mice twice per week from 6 weeks to 19 weeks of age. The mice were analyzed at 19 weeks of age.

### Bone marrow chimeras

BM chimera mice were prepared by intravenously injecting Thy1.2<sup>+</sup> WT BM cells ( $5 \times 10^6$  cells) or Thy1.2<sup>+</sup> T-bet<sup>-/-</sup> STAT6<sup>-/-</sup> BM cells ( $5 \times 10^6$  cells) into Thy1.1<sup>+</sup> WT mice at 5 hours after total body irradiation (4.5 Gy). In other experiments, Thy1.1<sup>+</sup> WT BM cells ( $5 \times$

$10^6$  cells) were injected intravenously into irradiated Thy1.2<sup>+</sup> WT mice or Thy1.2<sup>+</sup> T-bet<sup>-/-</sup> STAT6<sup>-/-</sup> mice. In mixed BM chimera experiments, mixtures of Thy1.1<sup>+</sup> WT BM cells ( $2.5 \times 10^6$  cells) and Thy1.2<sup>+</sup> T-bet<sup>-/-</sup> STAT6<sup>-/-</sup> BM cells ( $2.5 \times 10^6$  cells) were injected intravenously into irradiated Thy1.1<sup>+</sup> WT mice. Recipient mice were treated with antibiotics (sulfamethoxazole and trimethoprim suspension, Hi-Tech Pharmaceuticals, Norcross, GA) for two weeks after the injection. Four to seven weeks after the BM reconstitution, CD4<sup>+</sup> T cells were purified from skin-draining LNs of the chimeric mice. In mixed BM chimera experiments, after CD4<sup>+</sup> T-cell purification, Thy1.2<sup>+</sup> CD4<sup>+</sup> T cells were separated from Thy1.1<sup>+</sup> CD4<sup>+</sup> T cells using anti-Thy1.2 antibody and anti-Thy1.1 antibody with a cell sorter SH800 (SONY, Tokyo, Japan).

### RNA sequencing analysis

Total RNA was isolated by using a RNeasy Mini Kit (Qiagen, Hilden, Germany). RNA-seq was performed as described previously (Tanaka et al., 2014). In brief, RNA-seq libraries were prepared using a SureSelect Strand Specific RNA Library Preparation kit (Agilent, Santa Clara, CA). Sequencing was performed on an Illumina HiSeq1500 using a HiSeq Rapid SBS kit v2 (Illumina, San Diego, CA) in a 50-base single-end mode. mRNA profiles were calculated with a Cufflinks software and expressed as fragments per kilobase of exon model per million mapped fragments. K-means++ clustering was performed to z-transformed data using the R project (ver 3.6.2) and the LICORS package. Gene Ontology analysis was performed using Metascape (Zhou et al., 2019). Gene set enrichment analysis was performed using the GSEA software (4.0.3) (Subramanian et al., 2005). The gene sets used in this study were prepared from GSM2151577 and GSM2151575 for IL-4 signaling (Rochman et al., 2017), GSM2151585 and GSM2151571 for TSLP signaling (Rochman et al., 2017), and GSM1014577 and GSM1014578 for IL-2 signaling (Liao et al., 2014). RNA-seq data will be available from the National Center for Biotechnology Information's Gene Expression Omnibus on publication of the manuscript.

### Statistical analysis

Data are summarized as means  $\pm$  SD. The statistical analyses of the results were performed using Prism 5 for Mac OSX as follows: two-tailed Student's *t*-test for paired groups and two-way ANOVA followed by Tukey's post-hoc comparisons tests for multiple groups. *P*-values < 0.05 were considered significant.

### Data availability statement

The datasets generated for this study can be found in the National Center for Biotechnology Information's Gene Expression Omnibus (GSE156288).

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### CONFLICT OF INTEREST

The authors state no conflict of interest.

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## AUTHOR CONTRIBUTIONS

Conceptualization: SM, HT, HN; Formal Analysis: TT, KS, AS, AI, ST, KH; Investigation: SM, HT, AM, HK, DN; Resources: YO, RM; Writing - Original Draft Preparation: SM, HT, HN; Writing - Review and Editing: SM, HT, AI, ST, HN

## SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at [www.jidonline.org](http://www.jidonline.org), and at <https://doi.org/10.1016/j.jid.2020.08.029>.

## REFERENCES

- Angkasekwinai P. Th9 cells in allergic disease. *Curr Allergy Asthma Rep* 2019;19:29.
- Brunner PM, Guttman-Yassky E, Leung DY. The immunology of atopic dermatitis and its reversibility with broad-spectrum and targeted therapies. *J Allergy Clin Immunol* 2017;139:S65–76.
- Chang HC, Sehra S, Goswami R, Yao W, Yu Q, Stritesky GL, et al. The transcription factor PU.1 is required for the development of IL-9-producing T cells and allergic inflammation. *Nat Immunol* 2010;11:527–34.
- Das J, Ren G, Zhang L, Roberts AI, Zhao X, Bothwell AL, et al. Transforming growth factor beta is dispensable for the molecular orchestration of Th17 cell differentiation. *J Exp Med* 2009;206:2407–16.
- Esaki H, Brunner PM, Renert-Yuval Y, Czarnowicki T, Huynh T, Tran G, et al. Early-onset pediatric atopic dermatitis is  $T_H2$  but also  $T_H17$  polarized in skin. *J Allergy Clin Immunol* 2016;138:1639–51.
- Finotto S, Neurath MF, Glickman JN, Qin S, Lehr HA, Green FH, et al. Development of spontaneous airway changes consistent with human asthma in mice lacking T-bet. *Science* 2002;295:336–8.
- Fujiwara M, Hirose K, Kagami S, Takatori H, Wakashin H, Tamachi T, et al. T-bet inhibits both TH2 cell-mediated eosinophil recruitment and TH17 cell-mediated neutrophil recruitment into the airways. *J Allergy Clin Immunol* 2007;119:662–70.
- Gooderham MJ, Hong HC, Eshtiaghi P, Papp KA. Dupilumab: a review of its use in the treatment of atopic dermatitis. *J Am Acad Dermatol* 2018;78(Suppl. 1):S28–36.
- Goswami R, Jabeen R, Yagi R, Pham D, Zhu J, Goenka S, et al. STAT6-dependent regulation of Th9 development. *J Immunol* 2012;188:968–75.
- Hidaka T, Ogawa E, Kobayashi EH, Suzuki T, Funayama R, Nagashima T, et al. The aryl hydrocarbon receptor AhR links atopic dermatitis and air pollution via induction of the neurotrophic factor artemin. *Nat Immunol* 2017;18:64–73.
- Kaplan MH. The transcription factor network in Th9 cells. *Semin Immunopathol* 2017;39:11–20.
- Kaplan MH, Hufford MM, Olson MR. The development and in vivo function of T helper 9 cells. *Nat Rev Immunol* 2015;15:295–307.
- Kawashima H, Takatori H, Suzuki K, Iwata A, Yokota M, Suto A, et al. Tumor suppressor p53 inhibits systemic autoimmune diseases by inducing regulatory T cells. *J Immunol* 2013;191:3614–23.
- Kuraishi Y, Nagasawa T, Hayashi K, Satoh M. Scratching behavior induced by pruritogenic but not algesiogenic agents in mice. *Eur J Pharmacol* 1995;275:229–33.
- Liao W, Spolski R, Li P, Du N, West EE, Ren M, et al. Opposing actions of IL-2 and IL-21 on Th9 differentiation correlate with their differential regulation of BCL6 expression. *Proc Natl Acad Sci U S A* 2014;111:3508–13.
- Ma L, Xue HB, Guan XH, Shu CM, Zhang JH, Yu J. Possible pathogenic role of T helper type 9 cells and interleukin (IL)-9 in atopic dermatitis. *Clin Exp Immunol* 2014;175:25–31.
- Makita S, Takatori H, Tamachi T, Suto A, Suzuki K, Nakajima H. Analyses of dermal innate lymphoid cells in mice lacking T-bet and STAT6. *Allergol Int* 2018;67S:S51–3.
- Matsuki A, Takatori H, Makita S, Yokota M, Tamachi T, Suto A, et al. T-bet inhibits innate lymphoid cell-mediated eosinophilic airway inflammation by suppressing IL-9 production. *J Allergy Clin Immunol* 2017;139:1355–67.e6.
- Micossé C, von Meyenn L, Steck O, Kipfer E, Adam C, Simillion C, et al. Human “ $T_H9$ ” cells are a subpopulation of PPAR- $\gamma^+$   $T_H2$  cells. *Sci Immunol* 2019;4:eaat5943.
- Nakayama T, Hirahara K, Onodera A, Endo Y, Hosokawa H, Shinoda K, et al. Th2 cells in health and disease. *Annu Rev Immunol* 2017;35:53–84.
- Noelle RJ, Nowak EC. Cellular sources and immune functions of interleukin-9. *Nat Rev Immunol* 2010;10:683–7.
- Pandey A, Ozaki K, Baumann H, Levin SD, Puel A, Farr AG, et al. Cloning of a receptor subunit required for signaling by thymic stromal lymphopoietin. *Nat Immunol* 2000;1:59–64.
- Rochman Y, Dienger-Stambaugh K, Richgels PK, Lewkowich IP, Kartashov AV, Barski A, et al. TSLP signaling in CD4+ T cells programs a pathogenic T helper 2 cell state. *Sci Signal* 2018;11:eaam8858.
- Saunders SP, Moran T, Floudas A, Wurlod F, Kaszlikowska A, Salimi M, et al. Spontaneous atopic dermatitis is mediated by innate immunity, with the secondary lung inflammation of the atopic march requiring adaptive immunity. *J Allergy Clin Immunol* 2016;137:482–91.
- Schlapbach C, Gehad A, Yang C, Watanabe R, Guenova E, Teague JE, et al. Human TH9 cells are skin-tropic and have autocrine and paracrine proinflammatory capacity. *Sci Transl Med* 2014;6:219ra8.
- Schmitt E, Klein M, Bopp T. Th9 cells, new players in adaptive immunity. *Trends Immunol* 2014;35:61–8.
- Soumelis V, Reche PA, Kanzler H, Yuan W, Edward G, Homey B, et al. Human epithelial cells trigger dendritic cell mediated allergic inflammation by producing TSLP. *Nat Immunol* 2002;3:673–80.
- Stassen M, Schmitt E, Bopp T. From interleukin-9 to T helper 9 cells. *Ann N Y Acad Sci* 2012;1247:56–68.
- Subramanian A, Tamayo P, Mootha VK, Mukherjee S, Ebert BL, Gillette MA, et al. Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. *Proc Natl Acad Sci USA* 2005;102:15545–50.
- Takeda K, Tanaka T, Shi W, Matsumoto M, Minami M, Kashiwamura S, et al. Essential role of Stat6 in IL-4 signalling. *Nature* 1996;380:627–30.
- Tamachi T, Takatori H, Fujiwara M, Hirose K, Maezawa Y, Kagami S, et al. STAT6 inhibits T-bet-independent Th1 cell differentiation. *Biochem Biophys Res Commun* 2009;382:751–5.
- Tanaka S, Suto A, Iwamoto T, Kashiwakuma D, Kagami S, Suzuki K, et al. Sox5 and c-Maf cooperatively induce Th17 cell differentiation via ROR $\gamma$ t induction as downstream targets of Stat3. *J Exp Med* 2014;211:1857–74.
- Vercelli D. Discovering susceptibility genes for asthma and allergy. *Nat Rev Immunol* 2008;8:169–82.
- Wang A, Pan D, Lee YH, Martinez GJ, Feng XH, Dong C. Cutting edge: Smad2 and Smad4 regulate TGF- $\beta$ -mediated IL9 gene expression via EZH2 displacement. *J Immunol* 2013;191:4908–12.
- Yang Y, Xu J, Niu Y, Bromberg JS, Ding Y. T-bet and eomesodermin play critical roles in directing T cell differentiation to Th1 versus Th17. *J Immunol* 2008;181:8700–10.
- Yao W, Zhang Y, Jabeen R, Nguyen ET, Wilkes DS, Tepper RS, et al. Interleukin-9 is required for allergic airway inflammation mediated by the cytokine TSLP. *Immunity* 2013;38:360–72.
- Zhou Y, Zhou B, Pache L, Chang M, Khodabakhshi AH, Tanaseichuk O, et al. Metascape provides a biologist-oriented resource for the analysis of systems-level datasets. *Nat Commun* 2019;10:1523.
- Zhu J, Paul WE. Peripheral CD4+ T-cell differentiation regulated by networks of cytokines and transcription factors. *Immunol Rev* 2010;238:247–62.

## MATERIALS AND METHODS

### Reagents

Antibodies to murine CD3 $\epsilon$  (2c11), CD4 (RM4-5), CD11b (M1/70), CD28 (37.51), CD45 (30F-11), c-kit (2B8), Fc $\epsilon$ RI (MAR-1), IL-7 receptor  $\alpha$  chain (SB/199), thymic stromal lymphopoietin receptor (22H9), Thy1.2 (30-H12), and IL-9 (RM9A4) were purchased from Biolegend (San Diego, CA). Antibodies to murine Siglec-F (E50-2440), Ly6G (RB6-8C5), CD44 (1M7), CD11c (HL3), Thy1.1 (OX-7), and phosphorylated signal transducer and activator of transcription 5 (pY694) were purchased from BD Biosciences (San Jose, CA). Anti-CD4 antibody (GK1.5) and anti-IL-9 antibody (MM9C1) were purchased from Bio X Cell (West Lebanon, NH). Murine thymic stromal lymphopoietin and IL-2 were purchased from R&D Systems (Minneapolis, MN) and Wako (Osaka, Japan), respectively.

### Histologic analysis

The dorsal skin was resected, fixed in 10% phosphate-buffered formalin, embedded in paraffin, sectioned, stained with either H&E or toluidine blue, and examined by light microscopy.

### IL-9 quantification

IL-9 in culture supernatants was measured by using an ELISA MAX Set (Biolegend) according to the manufacture's protocol.

### qPCR analysis

Total RNA was extracted with an Isogen II reagent (Nippon Gene, Tokyo, Japan), and reverse transcription was carried out using an iScript cDNA Synthesis Kit (BioRad, Hercules, CA). qPCR was performed as described previously (Kawashima et al., 2013). The expression level of each gene was normalized to the level of  $\beta$ -actin. The sequences of primers are as follows.

*Actb*: 5'-tggtaccaactgggacgaca-3' and 5'-ccatcaaatgcctgtggta-3'

*Artn*: 5'-ccctagctgtttagccctg-3' and 5'-agggttcttcgctgcaca-3'

*Batf*: 5'-gttctgtttctccagggtcc-3' and 5'-gaagaatcgcatcgctgc-3'

*Bcl6*: 5'-cctgtgaaatctgtggcactcg-3' and 5'-cgcagttggctttgtgacg-3'

*Ccl5*: 5'-cagcagcaagtgtccaatctt-3' and 5'-ttctgaaccacttcttctcg-3'

*Ccl11*: 5'-atgcagagctccacagcgct-3' and 5'-ttatggttttgagttggag-3'

*Ccl17*: 5'-caggaagttggtgagctggtata-3' and 5'-ttgttgcctgtagtgcata-3'

*Ccl24*: 5'-ctgtgcctgacctcagaac-3' and 5'-ctaaacctcggtctattgc-3'

*Gata3*: 5'-agggacatcctgcgcgaactgt-3' and 5'-catctccggttcgggtctgg-3'

*Gcn5*: 5'-aaggccaatgaaacctgcaag-3' and 5'-ctcagctacggcacaactc-3'

*Il1ng*: 5'-ggatgattcatgagttatgc-3' and 5'-cctttccgcttctgagg-3'

*Il2ra*: 5'-aaccatagtagccagttgtcgg-3' and 5'-tcctaagcaacgcatatagacca-3'

*Il2rb*: 5'-tggagcctgtccctctacg-3' and 5'-tccacatgcaagagacattgg-3'

*Il2rg*: 5'-ctcaggcaaccaacctcac-3' and 5'-gctggacacaaatgtctgtag-3'

*Il6*: 5'-gaggataccactcccaacagacc-3' and 5'-aagtgcatactgttgcataca-3'

*Il7ra*: 5'-acaactgtgaagaagccaaaaacga-3' and 5'-tcaacgcctttcacctcatg-3'

*Il9*: 5'-cttgtgtctctccgtcccaac-3' and 5'-actatcctttcaccgtagga-3'

*Il17a*: 5'-gagtccaggagagcttcatc-3' and 5'-cagtggttggacacgctgag-3'

*Il17f*: 5'-tgctactgttgatgtgggac-3' and 5'-aatgccctgggtttgttgaa-3'

*Il22*: 5'-tttctgaccaaactcagca-3' and 5'-tctggatgttctggtcgtca-3'

*Il31*: 5'-tcagcagacgaatcaatacagc-3' and 5'-tcgctcaacactttgactttct-3'

*Il33*: 5'-tctctggacctgccagggtgt-3' and 5'-cctgttgatggtcacgacgcg-3'

*Irf1*: 5'-aggcatcctgttgatgtcc-3' and 5'-aattccaacaaatccagg-3'

*Irf4*: 5'-gccaacaagctagaaag-3' and 5'-tctctgagggtctggaaact-3'

*Maf*: 5'-aaatacagagaagctggtgagcaa-3' and 5'-cgggagaggaagggtgtc-3'

*Rorc*: 5'-ccgctgagaggggttcac-3' and 5'-tgcaggagtaggccacattaca-3'

*Spi1*: 5'-gggcatccagaagggcaa-3' and 5'-ggtaggtgagcttcttctga-3'

*Smad2*: 5'-atgtcgtccatcttgccattc-3' and 5'-aacgcctctgtttcttagctt-3'

*Smad3*: 5'-cacgcagaacgtgaacacc-3' and 5'-ggcagtagataacgtgagga-3'

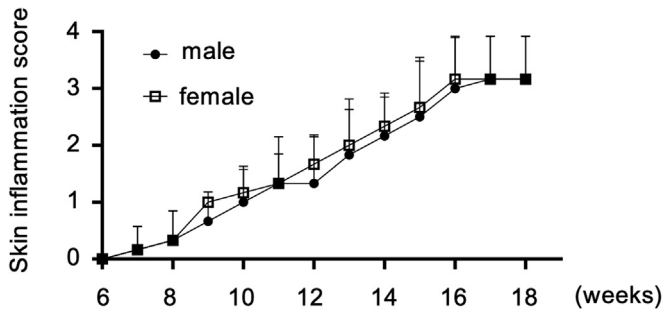
*Tnfa*: 5'-gcctcttctcattctgcttg-3' and 5'-ctgatgagggaggccatt-3'

*Tslp*: 5'-acggatgggctaacttaca-3' and 5'-agtcctcgattgtcgaact-3'

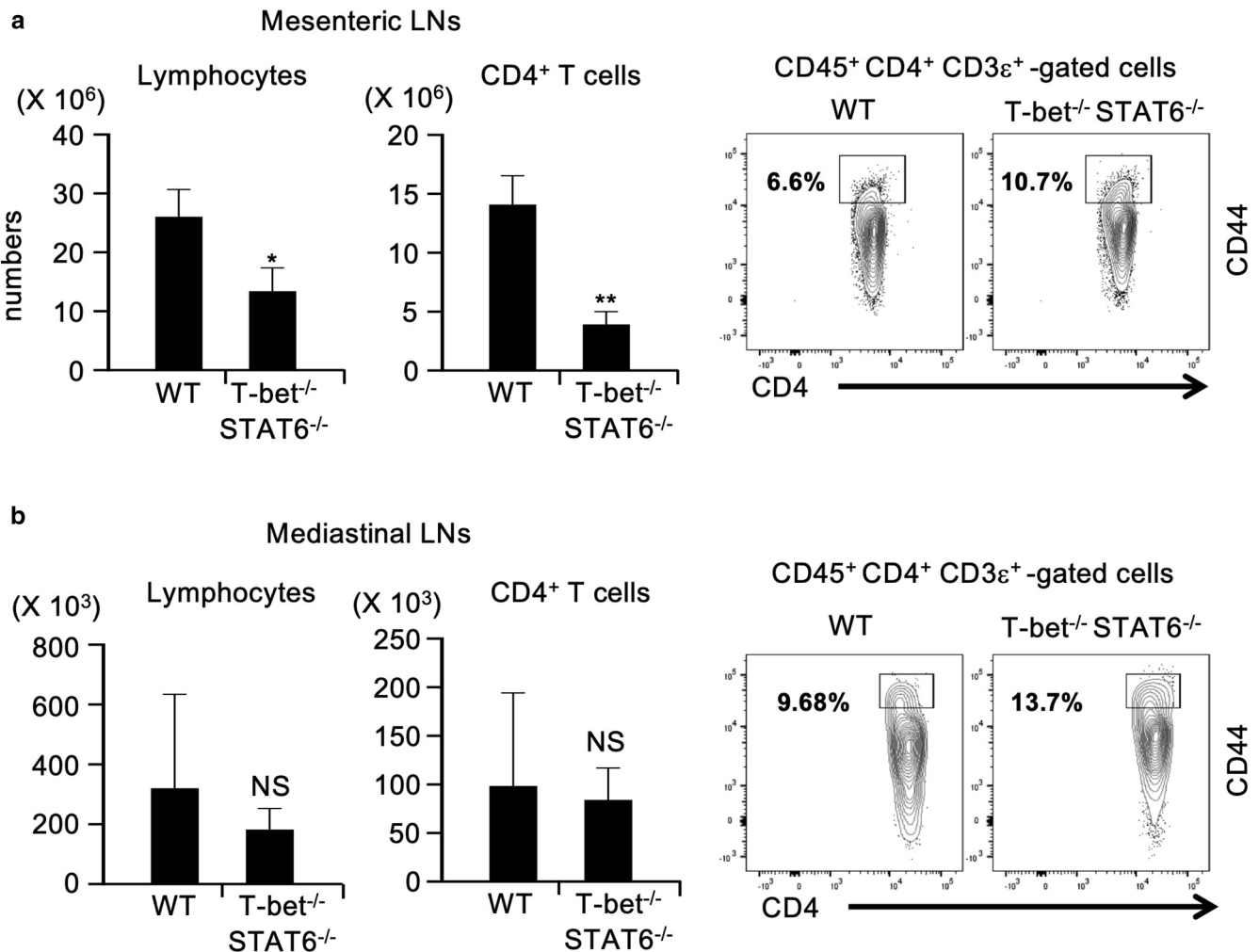
*Tslpr*: 5'-tcacggggtgatgtcacagt-3' and 5'-agtgccataacggaactccag-3'

## SUPPLEMENTARY REFERENCE

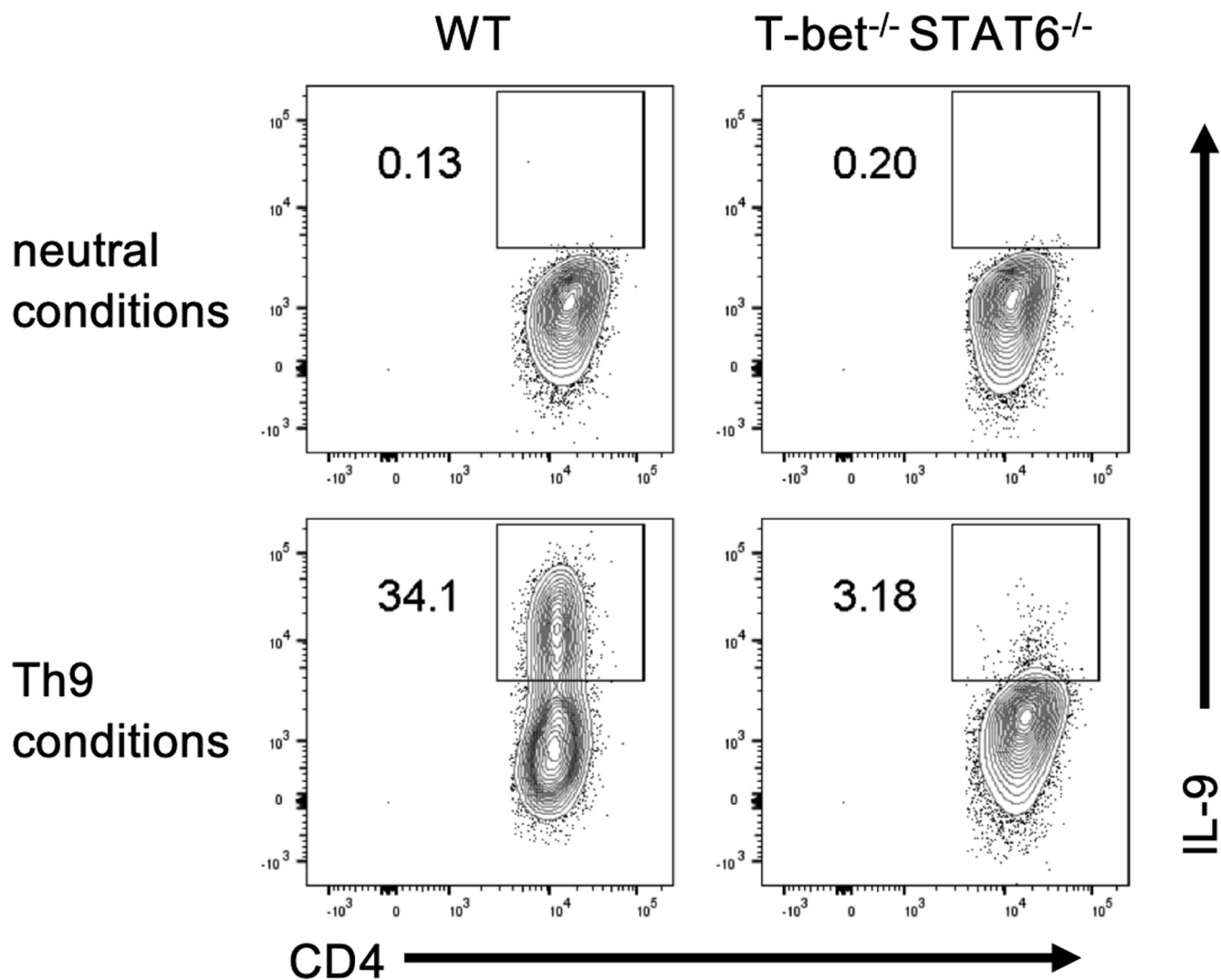
Kawashima H, Takatori H, Suzuki K, Iwata A, Yokota M, Suto A, et al. Tumor suppressor p53 inhibits systemic autoimmune diseases by inducing regulatory T cells. *J Immunol* 2013;191:3614–23.



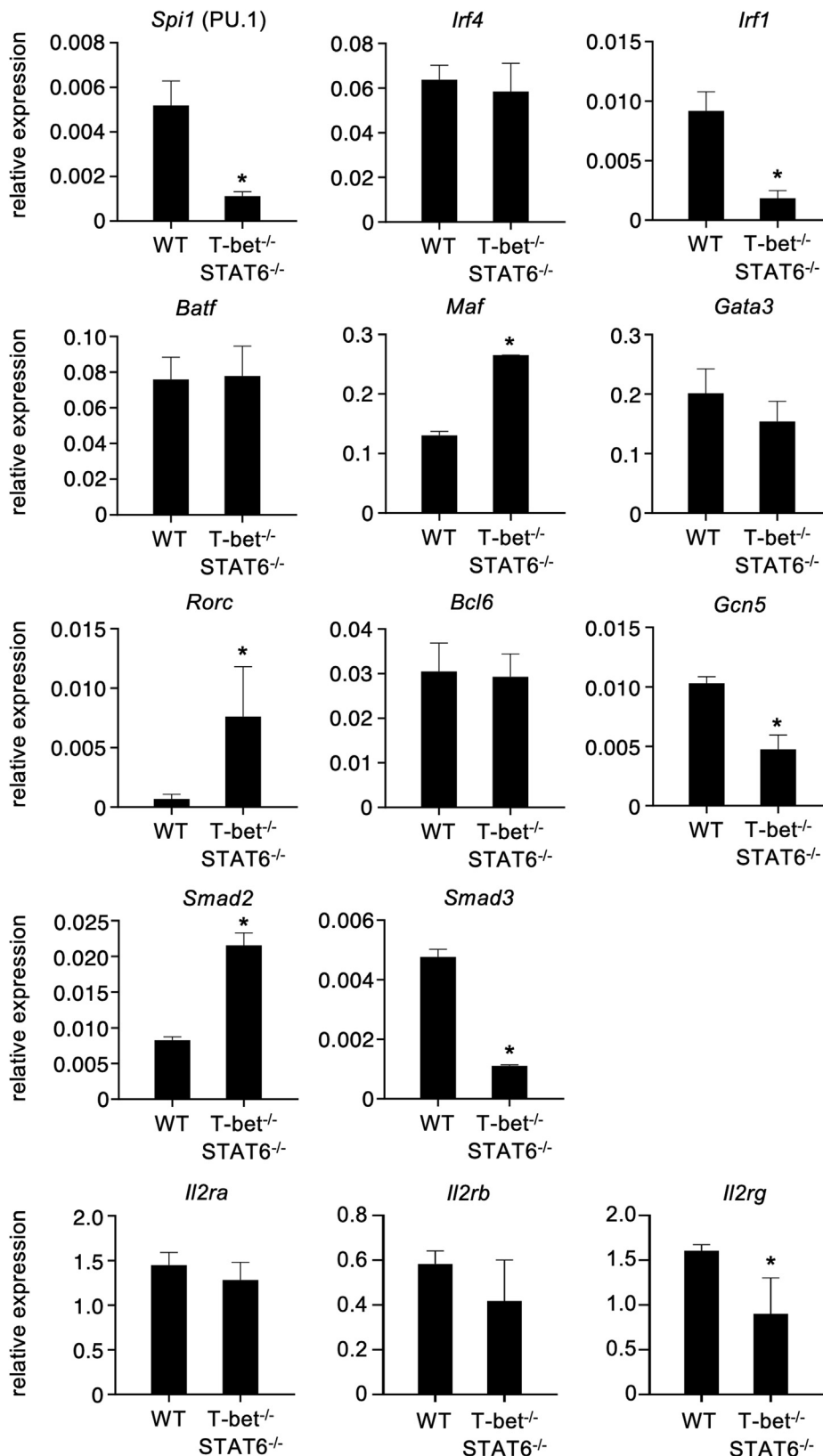
**Supplementary Figure S1. Skin inflammation score of male and female T-bet<sup>-/-</sup> STAT6<sup>-/-</sup> mice.** The severity of the skin disease in male and female T-bet<sup>-/-</sup> STAT6<sup>-/-</sup> mice was assessed by the skin inflammation score. Means  $\pm$  SD (n = 6, each). STAT, signal transducer and activator of transcription.



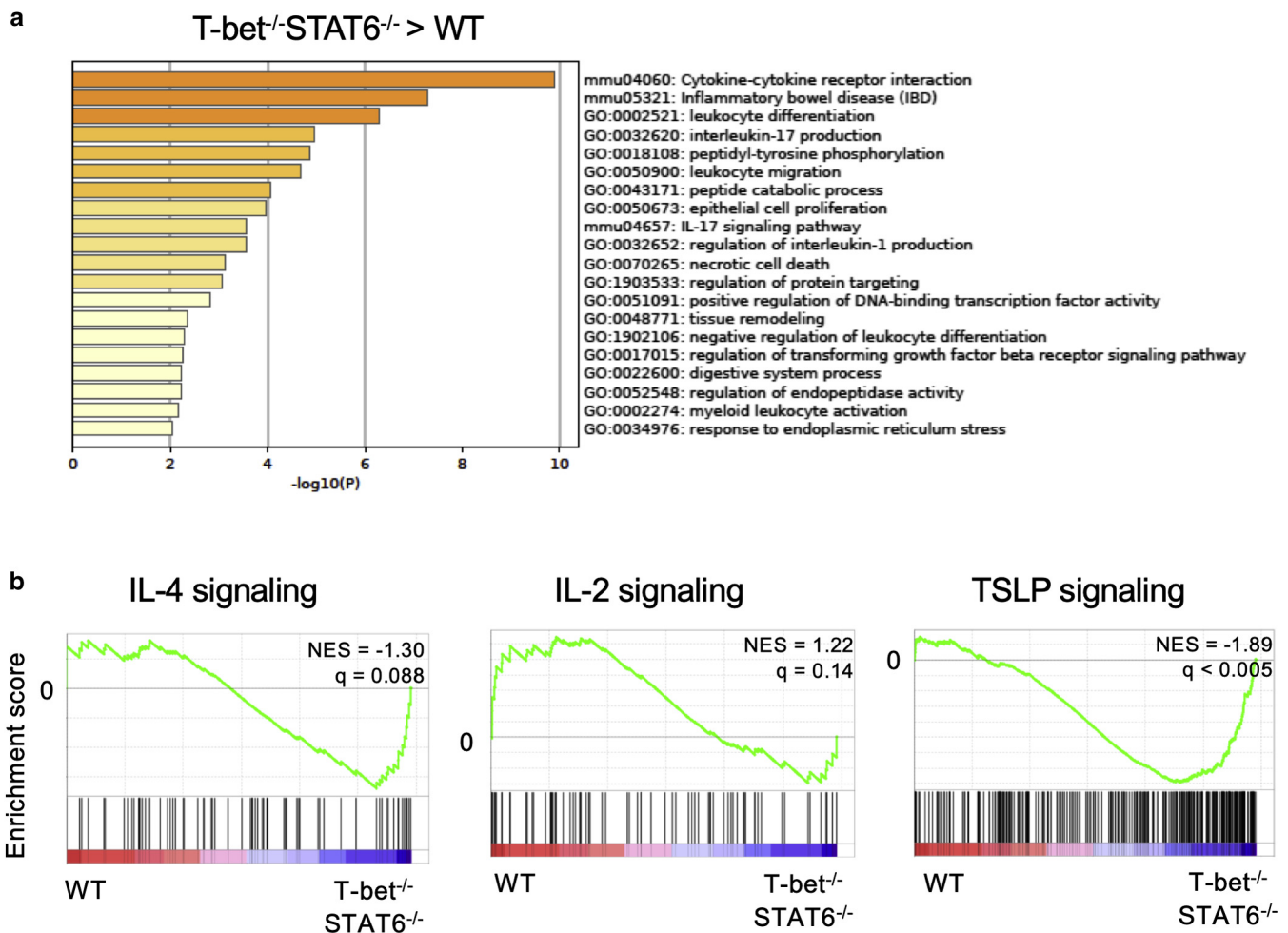
**Supplementary Figure S2. The numbers of CD4<sup>+</sup> CD3 $\epsilon$ <sup>+</sup> cells in mesenteric LNs and mediastinal LNs in T-bet<sup>-/-</sup> STAT6<sup>-/-</sup> mice.** Cells of (a) mesenteric LNs or (b) mediastinal LNs were harvested from WT and T-bet<sup>-/-</sup> STAT6<sup>-/-</sup> mice (12-week-old). Means  $\pm$  SD of absolute numbers of lymphocytes and CD4<sup>+</sup> CD3 $\epsilon$ <sup>+</sup> cells in LNs and representative CD4 versus CD44 staining of CD4<sup>+</sup> CD3 $\epsilon$ <sup>+</sup> T cells are shown (n = 3, each). LN, lymph node; NS, not significant; STAT, signal transducer and activator of transcription; WT, wild-type.



**Supplementary Figure S3. Development of conventional Th9 cells is impaired in T-bet<sup>-/-</sup> STAT6<sup>-/-</sup> mice.** Naïve CD4<sup>+</sup> T cells isolated from spleen of WT mice or T-bet<sup>-/-</sup> STAT6<sup>-/-</sup> mice were stimulated with anti-CD3/CD28 mAbs under neutral conditions or Th9-inducing conditions (IL-4, TGF-β, and anti-IFN-γ antibody) for 4 days. Cells were stimulated with PMA/ionomycin for 4 hours, and the expression of IL-9 in CD4<sup>+</sup> T cells was assessed by intracellular staining. Representative CD4 versus IL-9 staining in CD4<sup>+</sup> T cells are shown. Data are representative of two independent experiments. PMA, phorbol 12-myristate 13-acetate; STAT, signal transducer and activator of transcription; Th, T helper type; WT, wild-type.



**Supplementary Figure S4. The expression levels of Th9 cell-related molecules in T-bet<sup>-/-</sup> STAT6<sup>-/-</sup> CD4<sup>+</sup> T cells.** CD4<sup>+</sup> CD4<sup>+</sup> T cells were isolated from skin-draining LNs of WT mice or T-bet<sup>-/-</sup> STAT6<sup>-/-</sup> mice and stimulated with PMA/ionomycin for 2 hours. mRNA levels of indicated molecules were analyzed by qPCR analyses. Means  $\pm$  SD (n = 3, each). \*P < 0.05. LN, lymph node; PMA, phorbol 12-myristate 13-acetate; STAT, signal transducer and activator of transcription; Th, T helper type; WT, wild-type.



**Supplementary Figure S5. TSLP signaling-associated genes are enriched in T-bet<sup>-/-</sup> STAT6<sup>-/-</sup> memory CD4<sup>+</sup> T cells.** Total RNA of CD44<sup>+</sup> CD4<sup>+</sup> T cells in skin-draining LNs was isolated from WT mice or T-bet<sup>-/-</sup> STAT6<sup>-/-</sup> mice and subjected to RNA-seq analysis as described in Figure 4a–c. (a) GO enrichment analysis was performed for the upregulated genes in T-bet<sup>-/-</sup> STAT6<sup>-/-</sup> CD44<sup>+</sup> CD4<sup>+</sup> T cells by Metascape. (b) GSEA was performed for differentially expressed genes in CD44<sup>+</sup> CD4<sup>+</sup> T cells between WT mice and T-bet<sup>-/-</sup> STAT6<sup>-/-</sup> mice using gene sets involved in IL-4 signaling, IL-2 signaling, and TSLP signaling. The significance threshold was set at FDR < 0.05. FDR, false discovery rate; GSEA, gene set enrichment analysis; GO, Gene Ontology; NES, normalized enrichment score; RNA-seq, RNA sequencing; STAT, signal transducer and activator of transcription; TSLP, thymic stromal lymphopoietin; WT, wild-type.