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Letter to the editor

Analyses of dermal innate lymphoid cells in mice lacking T-bet and STAT6

Dear Editor,

Innate lymphoid cells (ILCs) are novel subsets of immune cells which lack antigen-specific receptors and cell-lineage markers.^{1–3} ILCs are tissue-resident cells, which are found not only in lymphoid tissues and skin, but also at the mucosal surface of lung and gut.² ILCs protect epithelial barriers in the front line by preventing infection and by promoting tissue repair to maintain tissue homeostasis.^{2,3} ILCs consist of type 1 ILCs (ILC1s), type 2 ILCs (ILC2s), and type 3 ILCs (ILC3s), which are classified based on the expression pattern of surface molecules, transcription factors, and cytokines, analogous to helper T cells like Th1 cells, Th2 cells, and Th17 cells.^{1–3} ILC1s express T-bet, and produce IFN- γ in response to IL-12, IL-15, and IL-18.^{1,3} ILC3s express ROR γ t, and are involved in immune responses against extracellular bacteria by producing IL-17A and IL-22 in response to IL-1 β and IL-23.^{1,3} On the other hand, ILC2s express GATA3, and produce Th2 cytokines such as IL-5, IL-9, and IL-13 in response to IL-33 and IL-25.^{1,3,4} It became clear that ILC2s are required for anti-helminth responses but the excessive activation of ILC2s results in the development of allergic inflammation.^{3,4} With respect to the regulatory mechanism of ILC2 activation in vivo, we have recently reported that T-bet suppresses IL-9 production from lung ILC2s and thereby inhibits IL-33-induced eosinophilic airway inflammation.⁴

Regarding the relationship between ILC2s and atopic dermatitis, Salimi *et al.* have reported that the number of ILC2s is increased in the skin of patients with atopic dermatitis as compared with that of healthy controls.⁵ In addition, ILC2s have been shown to be critical for the development of MC903 (vitamin D3 analog; calcipotriol)-induced atopic dermatitis-like skin inflammation in mice.⁵ Furthermore, transgenic mice overexpressing IL-33 in the skin have been shown to spontaneously develop an atopic dermatitis-like skin disease, along with the activation of ILC2s.⁶ However, it is poorly understood how the accumulation of ILCs in the skin is regulated and whether ILCs are involved in the development of eosinophilic skin inflammation.

In this regard, we have recently found that T-bet- and STAT6-double-deficient (T-bet^{-/-} STAT6^{-/-}) mice spontaneously develop an atopic dermatitis-like skin disease (Makita *et al.*, submitted). The skin disease started being apparent at 8-week-old and almost all T-bet^{-/-} STAT6^{-/-} mice developed the skin disease by 12-week-old. In T-bet^{-/-} STAT6^{-/-} mice, the numbers of eosinophils, mast cells, and CD4⁺ T cells in the skin are increased, and the depletion of CD4⁺ T cells by anti-CD4 antibody decreases the number of

eosinophils in the skin and attenuates the atopic dermatitis-like skin disease, suggesting that CD4⁺ T cells are responsible at least in part for the development of the atopic dermatitis-like skin disease in T-bet^{-/-} STAT6^{-/-} mice (Makita *et al.*, submitted). However, importantly, even when CD4⁺ T cells were depleted, a considerable extent of eosinophilic skin inflammation was still observed in T-bet^{-/-} STAT6^{-/-} mice, suggesting that in addition to CD4⁺ T cells, other cell types are involved in the development of eosinophilic skin inflammation in T-bet^{-/-} STAT6^{-/-} mice.

To determine whether ILCs are involved in the development of atopic dermatitis-like skin disease in T-bet^{-/-} STAT6^{-/-} mice, we first compared the numbers of ILCs in the skin in T-bet^{-/-} STAT6^{-/-} mice with that in wild type (WT) mice, T-bet^{-/-} mice, and STAT6^{-/-} mice. As shown in Figure 1A, the frequency and the absolute number of skin CD45.2⁺ Thy1.2⁺ CD2⁻ CD3e⁻ CD11b⁻ cells (dermal ILCs)⁷ were significantly higher in T-bet^{-/-} STAT6^{-/-} mice than those in WT mice, T-bet^{-/-} mice, or STAT6^{-/-} mice. While STAT6^{-/-} mice do not develop the atopic dermatitis-like skin disease spontaneously (Makita *et al.*, submitted), unexpectedly, the absolute number of dermal ILCs was modestly but significantly increased in STAT6^{-/-} mice as compared with that in WT mice (Fig. 1A). Further analyses revealed that the frequencies of dermal ILCs expressing CD103 or ICOS, surface markers of ILCs, were significantly higher in STAT6^{-/-} mice and T-bet^{-/-} STAT6^{-/-} mice than those in WT mice (Fig. 1B). These findings suggest that the numbers of ILCs were increased in the skin in mice lacking STAT6.

Given that the atopic dermatitis-like skin disease in T-bet^{-/-} STAT6^{-/-} mice is accompanied by massive infiltration of eosinophils (Makita *et al.*, submitted), we next examined whether the dermal ILCs are the major cellular source of IL-5. Inconsistent with a previous report showing that STAT6 is not essential for IL-5 production in lung ILC2s,⁸ we found that the frequency of IL-5-producing cells in dermal ILCs was significantly reduced in STAT6^{-/-} mice and T-bet^{-/-} STAT6^{-/-} mice as compared with that in WT mice (Fig. 2), indicating that IL-5 production in dermal ILCs depends on STAT6-mediated signaling.

In this study, we show that although dermal ILCs are increased in T-bet^{-/-} STAT6^{-/-} mice and STAT6^{-/-} mice (Fig. 1), the frequency of IL-5-producing ILCs was rather reduced in mice lacking STAT6 as compared with that in mice sufficient for STAT6 (Fig. 2). These findings suggest that STAT6 inhibits the accumulation of dermal ILCs that seem to be distinct from ILC2s. In addition, our findings suggest that the accumulation of ILCs may not be responsible for the development of eosinophilic skin inflammation in T-bet^{-/-} STAT6^{-/-} mice. However, we could not exclude the possibility that ILCs are involved in the eosinophilic skin inflammation in T-bet^{-/-}

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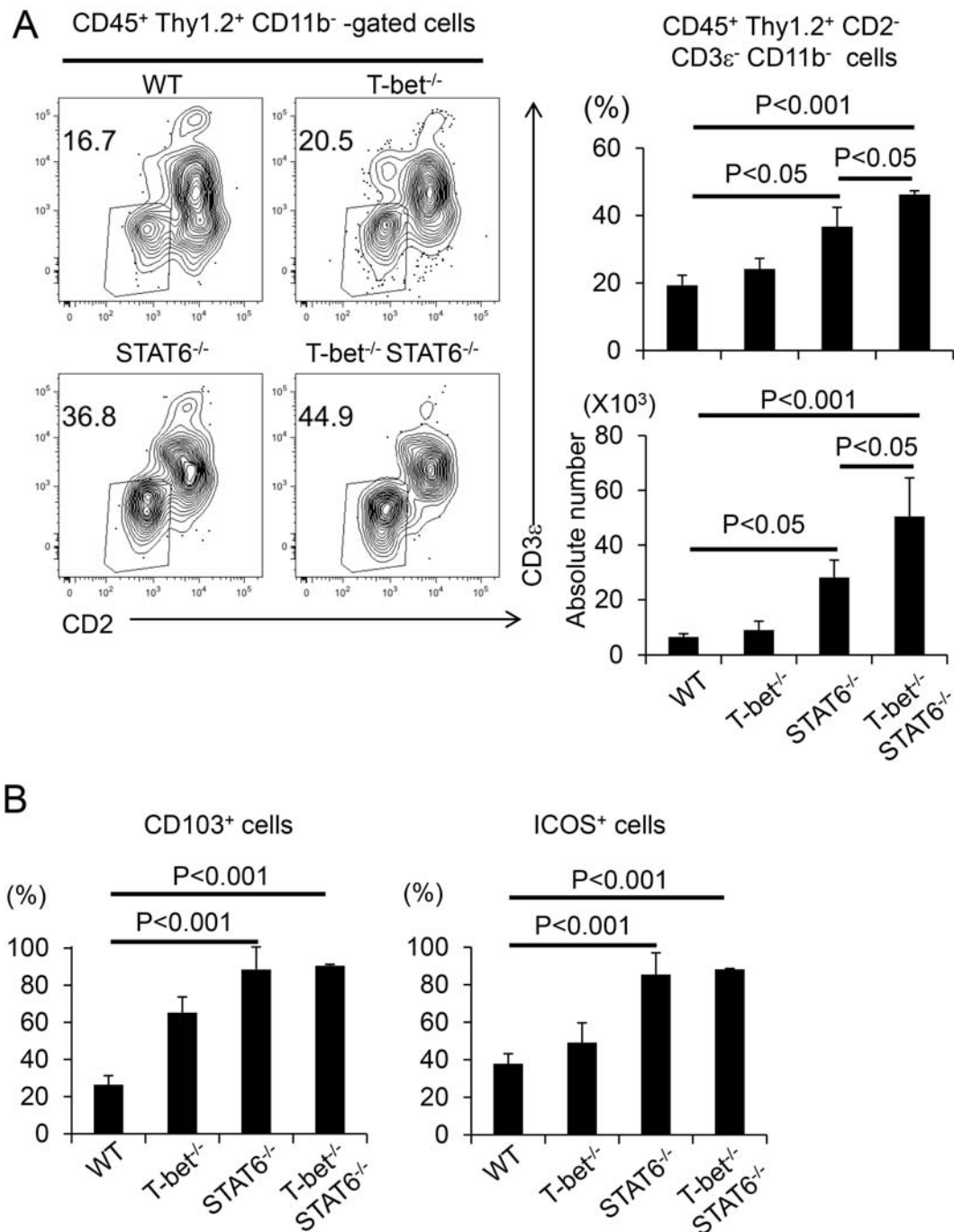


Fig. 1. STAT6 inhibits the accumulation of innate lymphoid cells (ILCs) in the skin. Skin-infiltrating cells isolated from dorsal skin of indicated mice (12–13-week-old) were subjected to FACS analysis. **(A)** Representative CD2 vs. CD3 ϵ staining of CD45⁺ Thy1.2⁺ CD11b⁻ cells, means $\pm$ SD of the frequency of CD45⁺ Thy1.2⁺ CD11b⁻ CD2⁻ CD3 ϵ ⁻ cells, and absolute numbers of skin-infiltrating CD45⁺ Thy1.2⁺ CD11b⁻ CD2⁻ CD3 ϵ ⁻ cells are shown (n = 3, each). Data are representative of 3 independent experiments. **(B)** Means $\pm$ SD of the frequency of CD103⁺ cells or ICOS⁺ cells in CD45⁺ Thy1.2⁺ CD11b⁻ CD2⁻ CD3 ϵ ⁻ cells are shown (n = 3, each).

STAT6^{-/-} mice by producing some cytokines/chemokines other than IL-5 at this point, because it has been shown that dermal ILCs cannot be depleted by the administration of an antibody against CD25 or CD90 (Thy1).⁸ Given that STAT6 signaling is involved in gene expression in non-immune cells such as differentiating keratinocytes,⁹ it is also possible that STAT6 expressed in non-immune cells is involved in the skin disease of T-bet^{-/-} STAT6^{-/-} mice. Further studies including the analysis of bone marrow chimeric mice would be required to unveil the importance

of STAT6 signaling for the development and function of dermal ILCs in vivo.

In conclusion, our results indicate that STAT6 plays an important role in maintaining the accumulation and cytokine production of dermal ILCs in vivo. Further studies on the molecular mechanisms underlying our findings would lead to the development of novel strategy for the treatment of allergic skin diseases.

A detailed description of materials and methods in this study is provided in the [Supplementary Methods](#) section.

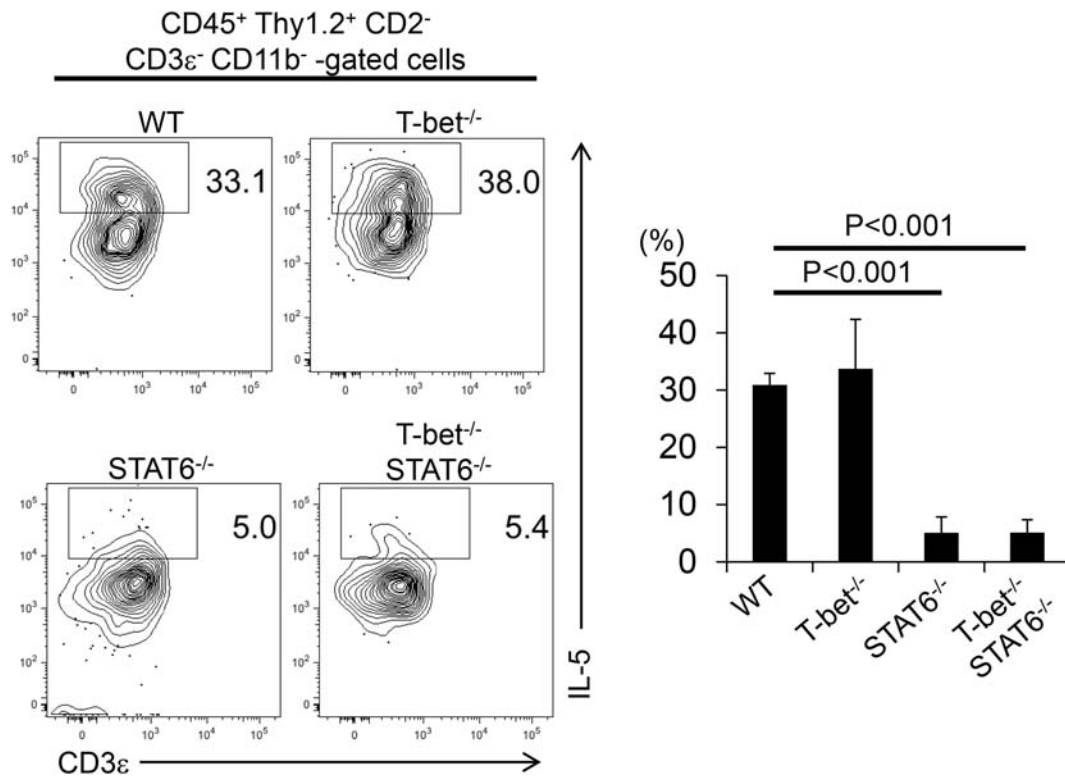


Fig. 2. STAT6-mediated signaling is crucial for IL-5 production in dermal ILCs. Skin-infiltrating cells isolated from dorsal skin of indicated mice (12–13-week-old) were stimulated with PMA and ionomycin for 4 h and the production of IL-5 was evaluated by intracellular staining. Representative IL-5 staining of CD45⁺ Thy1.2⁺ CD11b⁻ CD2⁻ CD3ε⁻ cells and means ± SD of the frequency of IL-5⁺ CD45⁺ Thy1.2⁺ CD11b⁻ CD2⁻ CD3ε⁻ cells are shown (n = 3, each).

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Appendix A. Supplementary data

Supplementary data related to this article can be found at <https://doi.org/10.1016/j.alit.2018.05.004>.

Conflict of interest

The authors have no conflict of interest to declare.

Sohei Makita ^{a,1}, Hiroaki Takatori ^{a,b,*1}, Tomohiro Tamachi ^a, Akira Suto ^a, Kotaro Suzuki ^a, Hiroshi Nakajima ^{a,**}

^a Department of Allergy and Clinical Immunology, Graduate School of Medicine, Chiba University, Chiba, Japan

^b Department of Rheumatology, Hamamatsu Medical Center, Hamamatsu, Japan

*, ** Corresponding authors. Department of Allergy and Clinical Immunology, Graduate School of Medicine, Chiba University, 1-8-1 Inohana, Chiba City, Chiba 260-8670, Japan.

E-mail addresses: takatori@faculty.chiba-u.jp (H. Takatori), nakajimh@faculty.chiba-u.jp (H. Nakajima).

References

- Artis D, Spits H. The biology of innate lymphoid cells. *Nature* 2015;**517**:293–301.
- Klose CS, Artis D. Innate lymphoid cells as regulators of immunity, inflammation and tissue homeostasis. *Nat Immunol* 2016;**17**:765–74.
- Morita H, Moro K, Koyasu S. Innate lymphoid cells in allergic and nonallergic inflammation. *J Allergy Clin Immunol* 2016;**138**:1253–64.
- 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.
- Salimi M, Barlow JL, Saunders SP, Xue L, Gutowska-Owsiak D, Wang X, et al. A role for IL-25 and IL-33-driven type-2 innate lymphoid cells in atopic dermatitis. *J Exp Med* 2013;**210**:2939–50.
- Imai Y, Yasuda K, Sakaguchi Y, Haneda T, Mizutani H, Yoshimoto T, et al. Skin-specific expression of IL-33 activates group 2 innate lymphoid cells and elicits atopic dermatitis-like inflammation in mice. *Proc Natl Acad Sci U S A* 2013;**110**:13921–6.
- Roediger B, Kyle R, Yip KH, Sumaria N, Guy TV, Kim BS, et al. Cutaneous immunosurveillance and regulation of inflammation by group 2 innate lymphoid cells. *Nat Immunol* 2013;**14**:564–73.
- Doherty TA, Khorram N, Chang JE, Kim HK, Rosenthal P, Croft M, et al. STAT6 regulates natural helper cell proliferation during lung inflammation initiated by *Alternaria*. *Am J Physiol Lung Cell Mol Physiol* 2012;**303**:L577–88.
- Omori-Miyake M, Yamashita M, Tsunemi Y, Kawashima M, Yagi J. In vitro assessment of IL-4- or IL-13-mediated changes in the structural components of keratinocytes in mice and humans. *J Invest Dermatol* 2014;**134**:1342–50.

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¹ The authors contributed equally to this work.

Supplementary Methods

Mice

T-bet^{-/-} mice^{10, 11} on a BALB/c background were obtained from Jackson Laboratory (Bar Harbor, ME). BALB/c T-bet^{-/-} mice were crossed with STAT6^{-/-} mice¹² to obtain T-bet^{-/-} STAT6^{-/-} mice. All mice were housed in microisolator cages under specific pathogen-free conditions. The Chiba University Animal Care and Use Committee approved animal procedures used in this study.

Reagents

Antibodies to murine CD3 ϵ (145-2C11), CD4 (RM4-5), CD2 (RM2.5), CD11b (M1/70), CD45 (30-F11), Thy1.2 (30-H12), CD103 (2E7), and IL-5 (TRFK5) were purchased from BioLegend (San Diego, CA). Antibody to murine ICOS (C398.4A) was purchased from eBioscience (San Diego, CA).

Isolation of inflammatory cells from the skin

Dorsal skin (3 x 3 cm) 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), and Hepes (25mM, Thermo Fisher Scientific, Waltham, MA) for 90 minutes at 37°C. Single cell suspensions were prepared by passing through a 70 μ m nylon cell strainer.

Flow cytometric analysis

After Fc receptors were blocked with anti-CD16/32 Ab (93; BioLegend), cells were stained and analyzed on a FACS Canto II (Becton Dickinson, San Jose, CA) using FlowJo software (Tree Star, Ashland, OR).

Intracellular cytokine staining

Isolated skin inflammatory cells were stimulated with PMA (20 ng/ml; Calbiochem, San Diego, CA) and ionomycin (1 μ g/ml; Calbiochem) for 4 hours, with monensin (2 μ M; Sigma-Aldrich) added for final 3 hours. Intracellular staining of IL-5 was performed by

using anti-IL-5 antibody (TRFK5) as described previously⁴.

Statistical analysis

Data are summarized as means $\pm$ SD. The statistical analysis of the results was performed by one-way ANOVA. P values <0.05 were considered significant.

References

10. Szabo SJ, Kim ST, Costa GL, Zhang X, Fathman CG, Glimcher LH. A novel transcription factor, T-bet, directs Th1 lineage commitment. *Cell*. 2000;**100**:655-69.
11. 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.
12. 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.