

Accepted Manuscript

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PII: S0091-6749(16)31023-5

DOI: [10.1016/j.jaci.2016.08.022](https://doi.org/10.1016/j.jaci.2016.08.022)

Reference: YMAI 12356

To appear in: *Journal of Allergy and Clinical Immunology*

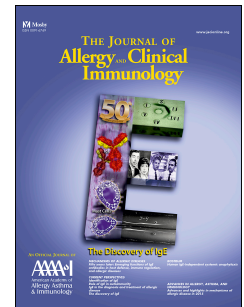
Received Date: 19 December 2015

Revised Date: 14 August 2016

Accepted Date: 23 August 2016

Please cite this article as: Matsuki A, Takatori H, Makita S, Yokota M, Tamachi T, Suto A, Suzuki K, Hirose K, Nakajima H, T-bet inhibits innate lymphoid cell-mediated eosinophilic airway inflammation by suppressing IL-9 production, *Journal of Allergy and Clinical Immunology* (2016), doi: 10.1016/j.jaci.2016.08.022.

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Full-length article

T-bet inhibits innate lymphoid cell-mediated eosinophilic airway inflammation by suppressing IL-9 production

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Word count: 4264. Word count in Abstract: 250

Number of Figures: 7

Running head: Role of T-bet in ILC2-mediated airway inflammation

The authors have no conflicting financial interests.

Abstract

Background: Innate lymphoid cells (ILCs) are emerging subsets of immune cells that produce large amounts of cytokines upon cytokine and/or alarmin stimulation. Recent studies have shown that T-bet plays pivotal roles in the development of ILC3s and ILC1s; however, the roles of T-bet in lung ILC2s remain unknown.

Objective: To determine the role of T-bet in ILC2-mediated airway inflammation.

Methods: The expression of T-bet in lung ILCs (defined as Thy1.2⁺ Lin⁻ cells) was examined. The roles of T-bet in the development of lung ILC2s and airway inflammation induced by IL-33 administration were examined by using T-bet-deficient (T-bet^{-/-}) mice. Gene expression profiles of T-bet^{-/-} lung ILCs were analyzed by RNA sequencing.

Results: T-bet was expressed in lung ILC2s (defined as Thy1.2⁺ Lin⁻ cells expressing ST2 or CD25) and IFN- γ enhanced its expression. While the development of lung ILC2s at steady-state conditions was normal in T-bet^{-/-} mice, IL-33-induced accumulation of lung ILC2s and eosinophilic airway inflammation were exacerbated in T-bet^{-/-} mice. The exacerbated accumulation of ILC2s and eosinophilic airway inflammation by the absence of T-bet was evident even in a RAG2^{-/-} background, suggesting that T-bet expressed in non-T/non-B population is involved in the suppression of IL-33-induced eosinophilic airway inflammation. Transcriptome analysis revealed that IL-9 expression in IL-33-stimulated lung ILCs was up-regulated in T-bet^{-/-} mice compared with that in wild-type mice. Importantly, neutralization of IL-9 markedly attenuated IL-33-induced accumulation of lung ILC2s and eosinophilic inflammation in T-bet^{-/-} mice.

Conclusion: T-bet suppresses IL-9 production from lung ILC2s and thereby inhibits IL-33-induced eosinophilic airway inflammation.

Key messages

T-bet expressed in lung ILC2s is involved in the counter-regulatory mechanisms of ILC2-mediated immune responses in the airways.

Capsule summary

T-bet suppresses IL-9 production from lung ILC2s and thereby inhibits IL-33-induced accumulation of lung ILC2s and ILC2-mediated eosinophilic airway inflammation.

Key words: T-bet, innate lymphoid cells, IL-9, eosinophils

Abbreviations used in this paper:

AhR; aryl hydrocarbon receptor

BALF; bronchoalveolar lavage fluid

ILCs; Innate lymphoid cells

KLRG1; killer cell lectin like receptor G1

Lin; lineage markers

MIG; MSCV-IRES-GFP

NCR; Natural cytotoxicity receptor

T-bet; T-box expressed in T-cells

Tregs; regulatory T cells

WT; wild-type

Introduction

Innate lymphoid cells (ILCs) are emerging subsets of immune cells that exist in mucosal and lymphoid tissues.^{1, 2, 3, 4} ILCs do not express antigen receptors or cell-lineage markers but do produce large amounts of cytokines upon cytokine and/or alarmin stimulation.¹ ILCs have been classified into several subsets by the expression pattern of surface molecules, cytokines, and transcription factors analogous to helper T cells. Type 1 ILCs (ILC1s) consist of conventional NK cells and ILCs that express T-box expressed in T-cells (T-bet) and IFN- γ .² Type 2 ILCs (ILC2s), which express ST2 and CD25 and produce large amounts of IL-5 and IL-13 in response to epithelial cell-derived cytokines such as IL-25 and IL-33, have been shown to be required for anti-helminth responses and the development of allergic diseases such as asthma and chronic rhinosinusitis.³ Recent studies have shown that ROR α , GATA3, and T cell factor 1 (Tcf1) are required for the development and the function of ILC2s.^{5, 6, 7, 8, 9} Type 3 ILCs (ILC3s) are comprised of three subsets: lymphoid tissue inducer (LTi) cells, natural cytotoxicity receptor (NCR)⁺ ILC3, and NCR⁻ ILC3, and are defined by the expression of ROR γ t and the production of IL-17A and/or IL-22.^{10, 11, 12} In addition to ROR γ t, GATA3 has been reported to be involved in the development of ILC3s.¹³

T-bet is well known as the master regulator of Th1 cells.^{14, 15} In addition, T-bet has been shown to be important for the development and/or function of cytotoxic CD8⁺ T cells, NKT cells, DCs, and ILC1s.¹⁵ Moreover, it has recently been shown that T-bet is highly expressed in NCR⁺ ILC3s in the gut of mice^{16, 17, 18} and that T-bet^{-/-} mice show a marked reduction of ROR γ t expression and a decrease in IL-22 production in ILC3s,¹⁶ suggesting that T-bet plays a pivotal role in gut NCR⁺ ILC3s. However, the roles of T-bet in the development of ILC2s remain to be determined.

Allergic airway inflammation is an immunohistopathological feature of asthma.¹⁹ Not only Th2 cells induced by allergens but also ILC2s activated by epithelial cell-derived cytokines such as IL-25 and IL-33 are involved in the induction of allergic airway inflammation.^{20, 21} Regarding the role of T-bet in allergic airway inflammation, it has been

98 shown that T-bet^{-/-} mice on a C57BL/6 background develop eosinophilic airway inflammation
99 spontaneously.²² By using ovalbumin-induced asthma models on a BALB/c background, we
100 have shown that T-bet expressed in CD4⁺ T cells suppresses both Th2 cell-induced
101 eosinophilic airway inflammation and Th17 cell-induced neutrophilic airway inflammation.²³
102 However, the roles of T-bet in ILC2-mediated airway inflammation remain unknown.

103 In this study, we examined the role of T-bet in the development of lung ILC2s and
104 eosinophilic airway inflammation induced by the administration of IL-33. While the
105 development of lung ILC2s at steady-state conditions was normal in T-bet^{-/-} mice,
106 IL-33-induced eosinophilic airway inflammation and accumulation of lung ILC2s was
107 exacerbated in T-bet^{-/-} mice even in the absence of T cells. Importantly, IL-9 production was
108 enhanced in T-bet^{-/-} ILC2s and the neutralization of IL-9 markedly attenuated IL-33-induced
109 eosinophilic airway inflammation in T-bet^{-/-} mice, suggesting that T-bet inhibits the expression
110 of IL-9 in lung ILC2s and thus suppresses IL-33-induced eosinophilic airway inflammation.

Methods

Mice

T-bet^{-/-} mice²² on a BALB/c background (Jackson Laboratory, Bar Harbor, Me) were crossed with RAG2^{-/-} mice to obtain T-bet^{-/-} RAG2^{-/-} mice. All mice were housed in microisolator cages under pathogen-free conditions. All experiments were performed according to the guidelines of Chiba University.

Reagents

Antibodies and cytokines used in this study are shown in Online Repository Methods.

Induction of airway inflammation

IL-33 (500 ng), IL-25 (1 µg), or papain (25 µg) was administered to mice (age 8-12 weeks) intranasally on days 0, 3, and 6 and airway inflammation was evaluated at 12 hs after the last administration. Where indicated, a neutralizing anti-IL-9 antibody (25 µg) or anti-IFN-γ antibody (500 µg) was administered to mice at 30 min before each of IL-33 administration.

Histological analysis

Lung sections (3 µm thick) were stained with H&E or PAS according to the standard protocols. Histological score on H&E-stained sections and goblet cell score on PAS stained sections were determined in a blinded manner as described previously.²⁴

Flow cytometric analysis

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

Intracellular cytokine staining

Cells were stimulated with PMA (20 ng/ml; Calbiochem, San Diego, CA) and ionomycin (1 μ g/ml; Calbiochem) for 4 hs, with monensin (2 μ M; Sigma-Aldrich) added for final 3 hs. Intracellular staining of IL-4, IL-5, IL-9, IL-13, and IL-17A was performed using antibodies to IL-4 (11B11; BD Biosciences), IL-5 (TRFK5; BioLegend), IL-9 (RM9A4; BioLegend), IL-13 (eBio13A; eBioscience), and IL-17A (TC11-18H10.1; BioLegend).

Isolation and culture of ILCs

Lineage-negative cells were roughly purified from single-cell suspensions of lung cells by negative sorting with a Lineage cell depletion kit (Miltenyi Biotec, Auburn, CA) according to the manufacturer's instruction. The resultant cells were stained with FITC-labeled antibodies to surface lineage markers (CD3 ϵ , CD4, CD8 α , CD19, CD5, B220, CD11b, CD11c, CD49b, Fc ϵ RI, TER119, TCR β , TCR $\gamma\delta$, and GR-1), anti-ST2-APC, and anti-Thy1.2-PerCP. Thy1.2⁺ Lin⁻ cells and ST2⁺ Thy1.2⁺ Lin⁻ cells were purified by a cell sorter SH800 (SONY, Tokyo, Japan). These cells (1.0 $\times$ 10⁶ cells/ml) were cultured in MEM α medium (Thermo Fisher Scientific, Waltham, MA) supplemented with 20% heat-inactivated FCS and 2-ME (50 μ M) (complete medium) containing IL-2 (10 ng/ml) and IL-7 (10 ng/ml). Where indicated, cells were cultured in the presence of IL-33 (10 ng/ml), IFN- γ (10 ng/ml), or anti-IL-9 antibody (50 μ g/ml).

ELISA

The levels of IL-4, IL-5 and IL-13 were measured by DuoSet ELISA kits (R&D Systems, Minneapolis, MN).

qPCR analysis

qPCR was performed as described previously.²⁵ The expression levels of each gene were normalized to the levels of β -actin. The sequences of PCR primers are shown in Online Repository Methods.

Retroviral transduction of T-bet

Retroviruses of MSCV-IRES-GFP (MIG) and MIG-T-bet (a kind gift from Dr. Steven L. Reiner) were produced as described previously.²⁵ For retroviral transduction, isolated lung Thy1.2⁺ Lin⁻ cells were cultured overnight in the presence of IL-2 (10 ng/ml), IL-7 (10 ng/ml), and IL-33 (10 ng/ml), then infected with retroviruses in the presence of IL-2, IL-7, and IL-33, and cultured for 4 days.

RNA-Seq analysis

RNA-Seq analysis was performed as described previously.²⁵ Genes whose expression was enhanced by IL-33 stimulation and was higher in T-bet^{-/-} Thy1.2⁺ Lin⁻ cells than that in WT Thy1.2⁺ Lin⁻ cells were selected with weighted average difference (WAD) method.²⁶

Statistical analysis

Data are summarized as means $\pm$ SD. The statistical analysis of the results was performed by an unpaired *t* test. *P* values <0.05 were considered significant.

Results

IFN- γ induces T-bet expression in ST2⁺ Thy1.2⁺ Lin⁻ cells.

A recent study has shown that IFN- γ inhibits IL-33-induced proliferation and cytokine production in ILC2s.²⁷ More recently, it has been reported that IL-27 as well as IFN- γ suppresses the activation and function of ILC2s in a STAT1-dependent manner in IL-33-induced lung inflammation.^{28, 29} To examine the roles of T-bet, a representative IFN- γ -inducible gene in CD4⁺ T cells,³⁰ in IFN- γ -mediated inhibition of ILC2 function, we first examined the expression of T-bet in Thy1.2⁺ Lin⁻ cells isolated from lungs of wild-type (WT) mice. As shown in Fig. 1A, lung Thy1.2⁺ Lin⁻ cells modestly expressed T-bet in neutral conditions and the expression was significantly increased by IFN- γ stimulation to the levels similar to Th1 cells. In addition, the expression of IL-12R β 2, which is induced by T-bet in CD4⁺ T cells,³¹ was induced in Thy1.2⁺ Lin⁻ cells by IFN- γ (Fig. 1A). On the other hand, IFN- γ itself was not significantly induced by IFN- γ in Thy1.2⁺ Lin⁻ cells (Fig. 1A), consistent with poor IFN- γ -producing ability of lung ILC2s.³²

We next examine protein expression of T-bet in Thy1.2⁺ Lin⁻ cells in the lung by intracellular staining. As described elsewhere³³, lung Thy1.2⁺ Lin⁻ cells consist of two populations; ST2⁺ CD25⁺ cells and ST2⁻ CD25⁻ cells (Fig. 1B), which represent ILC2s and ILC1/3s, respectively. T-bet was expressed not only in ST2⁻ CD25⁻ population but also in ST2⁺ CD25⁺ population in steady-state conditions (Fig. 1B). The frequency of T-bet expressing cells in ST2⁺ Thy1.2⁺ Lin⁻ cells was increased by IFN- γ but decreased by IL-33 (Fig. 1C). IFN- γ partly antagonized the inhibitory effect of IL-33 on T-bet expression in ST2⁺ Thy1.2⁺ Lin⁻ cells (Fig. 1C). These results suggest that T-bet is expressed in lung ILC2s and IFN- γ enhances its expression.

T-bet is dispensable for the development of lung ILC2s.

Because T-bet has been shown to be indispensable for the development and function of a subset of NCR⁺ ILC3s,^{16, 17, 18} we next examined whether T-bet is essential for the

development of lung ILC2s. As shown in Fig. E1A, the numbers of Thy1.2⁺ Lin⁻ cells in the lung in T-bet^{-/-} mice were comparable to those in WT mice. The number of CD25⁺ Thy1.2⁺ Lin⁻ cells in the lung was also similar between T-bet^{-/-} mice and WT mice (Fig. E1B). Given that T-bet is required for the expression of IFN- γ in intestinal ILCs,³⁴ we next investigated cytokine production from lung Thy1.2⁺ Lin⁻ cells in T-bet^{-/-} mice. In steady-state conditions, the frequencies of IL-4-, IL-5-, IL-13-, or IL-17A-producing Thy1.2⁺ Lin⁻ cells were not significantly different between T-bet^{-/-} mice and WT mice (Fig. E1C). The expression levels of GATA3 and ROR γ t in lung Thy1.2⁺ Lin⁻ cells were also similar between T-bet^{-/-} mice and WT mice (Fig. E1D). These results indicate that T-bet is dispensable for the development and cytokine production of lung ILC2s in steady-state conditions.

IL-33-induced eosinophilic airway inflammation is exacerbated in T-bet^{-/-} mice.

It is well established that intranasal administration of IL-33 induces IL-5 and IL-13 production from ILC2s and causes eosinophilic airway inflammation.³² To determine the roles of T-bet in IL-33-induced airway inflammation, recombinant IL-33 was administered to WT mice and T-bet^{-/-} mice intranasally at day 0, 3, and 6 and the numbers of eosinophils in bronchoalveolar lavage fluid (BALF) were evaluated at 12 hs after the last administration. The proportion and absolute numbers of eosinophils (Siglec-F⁺ CD11c⁻ cells) in the BALF were significantly higher in IL-33-stimulated T-bet^{-/-} mice than those in IL-33-stimulated WT mice (Fig. 2A). Consistently, histological analyses showed that peribronchial inflammation with eosinophil infiltration (Fig. 2B) as well as goblet cell hyperplasia (Fig. 2C) was more obvious in IL-33-stimulated T-bet^{-/-} mice than that in IL-33-stimulated WT mice. In addition, the levels of IL-5 and IL-13 in the BALF were significantly higher in IL-33-stimulated T-bet^{-/-} mice (Fig. 2D). Eosinophilic airway inflammation induced by IL-25, another epithelial cell-derived cytokine that induces IL-5 and IL-13 production from ILC2s,³² was also significantly exacerbated in T-bet^{-/-} mice (Fig. E2). Furthermore, Papain-induced eosinophilic inflammation, which largely depends on ILC2s,³⁵ was significantly exacerbated in T-bet^{-/-}

mice (Fig. E2). These results suggest that T-bet plays an inhibitory role in ILC2-mediated eosinophilic airway inflammation.

IL-33-induced accumulation of lung ILC2s is exacerbated in T-bet^{-/-} mice.

To address the mechanism underlying the enhanced IL-33-induced eosinophilic inflammation in T-bet^{-/-} mice, we examined the numbers of ILC2s in the lung. As shown in Fig. 3A, the number of lung Thy1.2⁺ Lin⁻ cells was significantly increased in IL-33-stimulated T-bet^{-/-} mice as compared with that in IL-33-stimulated WT mice. In addition, while the frequency of CD25⁺ cells in lung Thy1.2⁺ Lin⁻ cells (Fig. 3B, left panel) as well as the frequencies of CD25⁺ Thy1.2⁺ Lin⁻ cells expressing ST2 and killer cell lectin like receptor G1 (KLRG1) (Fig. 3C, left panels), markers of ILC2s,^{7, 32, 36} in IL-33-stimulated T-bet^{-/-} mice was comparable to that in IL-33-stimulated WT mice, the absolute numbers of CD25⁺ Thy1.2⁺ Lin⁻ cells, ST2⁺ CD25⁺ Thy1.2⁺ Lin⁻ cells, and KLRG1⁺ CD25⁺ Thy1.2⁺ Lin⁻ cells were increased in IL-33-stimulated T-bet^{-/-} mice (Fig. 3B and 3C, right panels). Consistently, the frequency of IL-5- or IL-13-producing cells in lung Thy1.2⁺ Lin⁻ cells was similar between IL-33-stimulated T-bet^{-/-} mice and WT mice (Fig. 3D). Even in IL-33-stimulated T-bet^{-/-} mice, IL-4 production was negligible in lung Thy1.2⁺ Lin⁻ cells, consistent with poor ability of IL-4 production in ILC2s.³² Taken together, these results suggest that the accumulation of lung ILC2s is significantly enhanced but the ability of IL-5 and IL-13 production of ILC2s is not affected by the absence of T-bet. Meanwhile, the frequency of IL-17A-producing Thy1.2⁺ Lin⁻ cells was increased in T-bet^{-/-} mice (Fig. 3D), consistent with a previous report showing the increase of IL-17A-secreting ILCs in colon by the absence of T-bet.³⁷ As expected, the frequency of CD4⁺ T cells was similar between IL-33-stimulated T-bet^{-/-} mice and WT mice (Fig. E3A) and the production of IL-4, IL-5, and IL-13 from lung CD4⁺ T cells was negligible in IL-33-stimulated T-bet^{-/-} mice and WT mice (Fig. E3B).

T-bet expressed in non-T/non-B cells is involved in the suppression of IL-33-induced eosinophilic airway inflammation.

It has been shown that ILC2s and Th2 cells form a positive feedback loop to mount type 2 immune responses.³⁸ In addition, a recent study has shown that regulatory T cells (Tregs) inhibit ILC2-mediated allergic airway inflammation.³⁹ To exclude the possibility that the lack of T-bet in adaptive immune cells including CD4⁺ T cells is primarily involved in the exacerbation of IL-33-induced airway inflammation in T-bet^{-/-} mice, we compared IL-33-induced airway inflammation between RAG2^{-/-} mice and T-bet^{-/-} RAG2^{-/-} mice. Consistent with the comparison between WT mice and T-bet^{-/-} mice (Fig. 2), IL-33-induced eosinophil infiltration into the BALF was significantly enhanced in T-bet^{-/-} RAG2^{-/-} mice as compared with that in RAG2^{-/-} mice (Fig. 4A). Moreover, the number of lung Thy1.2⁺ Lin⁻ cells was significantly higher in IL-33-stimulated T-bet^{-/-} RAG2^{-/-} mice than that in IL-33-stimulated RAG2^{-/-} mice (Fig. 4B). Interestingly, the frequency of CD25⁺ cells in lung Thy1.2⁺ Lin⁻ cells and the expression levels of KLRG1 on CD25⁺ Thy1.2⁺ Lin⁻ cells were significantly up-regulated in IL-33-stimulated T-bet^{-/-} RAG2^{-/-} mice as compared with those in IL-33-stimulated RAG2^{-/-} mice (Fig. 4C and 4D). Furthermore, the production of IL-5, IL-13, and IL-17A in lung Thy1.2⁺ Lin⁻ cells was significantly enhanced in IL-33-stimulated T-bet^{-/-} RAG2^{-/-} mice (Fig. 4E). Taken together with the finding that T-bet is expressed in ILCs (Fig. 1), these results suggest that T-bet expressed in ILCs suppresses IL-33-induced accumulation of lung ILC2s and subsequent eosinophilic airway inflammation and that adaptive immune cells are involved in the suppression of cytokine production from lung ILCs in T-bet^{-/-} mice.

T-bet suppresses IL-9 production from lung Thy1.2⁺ Lin⁻ cells.

To determine the regulatory mechanism by which T-bet expressed in ILCs suppresses IL-33-induced airway inflammation, we searched for genes whose expression is up-regulated by the deficiency of T-bet in IL-33-stimulated lung Thy1.2⁺ Lin⁻ cells. RNA-Seq analysis identified IL-9 as one of the genes up-regulated in IL-33-stimulated T-bet^{-/-} Thy1.2⁺ Lin⁻ cells as compared with IL-33-stimulated WT Thy1.2⁺ Lin⁻ cells (Fig. 5A and Online Repository Table 1). qPCR analyses confirmed that mRNA levels of IL-9 but not of IL-4, IL-5, or IL-13 were higher in IL-33-stimulated T-bet^{-/-} Thy1.2⁺ Lin⁻ cells than those in IL-33-stimulated WT

Thy1.2⁺ Lin⁻ cells (Fig. 5B). Moreover, flow cytometric analysis revealed that IL-9 production by IL-33-stimulated CD25⁺ Thy1.2⁺ Lin⁻ cells was enhanced in T-bet^{-/-} mice than that in WT mice, whereas the production of IL-5 as well as the expression of CD25 and ST2 in IL-33-stimulated Thy1.2⁺ Lin⁻ cells was similar between T-bet^{-/-} mice and WT mice (Fig. 5C).

To elucidate the molecular mechanisms of T-bet-mediated suppression of IL-9 production in ILC2s, we investigated the expression of transcription factors that are involved in IL-9 production in CD4⁺ T cells such as GATA3, IRF4, BATF, PU.1, and aryl hydrocarbon receptor (AhR).^{40, 41, 42, 43} As shown in Fig. E4, the expression levels of GATA3, IRF4, BATF, and AhR were not significantly different between IL-33-stimulated WT and T-bet^{-/-} Thy1.2⁺ Lin⁻ cells, while PU.1 was not detectable in both IL-33-stimulated WT and T-bet^{-/-} Thy1.2⁺ Lin⁻ cells, suggesting that T-bet down-regulates IL-9 production in ILC2s in GATA3-, IRF4-, BATF-, AhR-, or PU.1-independent mechanisms. On the other hand, the expression of IL-9 receptor (IL-9R) tended to be up-regulated in IL-33-stimulated T-bet^{-/-} Thy1.2⁺ Lin⁻ cells (Fig. E4). In addition, the number of surviving ST2⁺ Thy1.2⁺ Lin⁻ cells in culture was increased in T-bet^{-/-} mice as compared with that in WT mice and was more strongly reduced by the neutralization of IL-9 in T-bet^{-/-} mice (Fig. 5D).

We further examined the effect of enforced T-bet expression on IL-9 production in ILCs. Lung Thy1.2⁺ Lin⁻ cells isolated from WT mice were infected with retrovirus of MSCV-IRES-GFP (MIG)-T-bet or MIG (as a control) and cultured for an additional 4 days in the presence of IL-2, IL-7, and IL-33. As shown in Fig. 5E, IL-9 production was markedly reduced by the enforced expression of T-bet. On the other hand, the expression of ST2 and KLRG1 as well as the production of IL-5 and IL-13 was not significantly affected by the enforced expression of T-bet (Fig. 5E), although the enforced expression of T-bet reproducibly resulted in the reduction in the numbers of surviving infected Thy1.2⁺ Lin⁻ cells (data not shown). Taken together, these results suggest that T-bet preferentially down-regulates IL-9 production in ILC2s and may suppress an autocrine and/or paracrine loop of IL-9-IL-9R signaling in ILC2s.

To determine the role of IFN- γ -T-bet axis in the regulation of IL-33-induced IL-9 production from ILC2s in vivo, we next examined the effect of in vivo administration of anti-IFN- γ antibody on IL-9 production from CD25⁺ Thy1.2⁺ Lin⁻ cells in IL-33-stimulated WT mice and T-bet^{-/-} mice. Without anti-IFN- γ antibody administration, IL-9 production from CD25⁺ Thy1.2⁺ Lin⁻ cells was significantly exacerbated in IL-33-stimulated T-bet^{-/-} mice as compared with that in IL-33-stimulated WT mice (Fig. 6A), consistent with in vitro data shown in Fig. 5. Importantly, anti-IFN- γ antibody significantly enhanced IL-9 production from CD25⁺ Thy1.2⁺ Lin⁻ cells in IL-33-stimulated WT mice but not in IL-33-stimulated T-bet^{-/-} mice (Fig. 6A). These results suggest that endogenously produced IFN- γ suppresses IL-33-induced IL-9 production from ILC2s in a T-bet-dependent manner. Meanwhile, the expression of T-bet in CD25⁺ Thy1.2⁺ Lin⁻ cells was undetectable in IL-33-treated mice (Fig. 6B), consistent with the inhibitory effect of IL-33 on T-bet expression in ILC2s (Fig. 1C).

Neutralization of IL-9 cancels the enhanced IL-33-induced airway inflammation in T-bet^{-/-} mice.

It has been shown that the main producers of IL-9 in the lung during papain-induced airway inflammation⁴⁴ and infection with *Nippostrongylus brasiliensis*⁴⁵ were ILC2s. With respect to the function, IL-9 has been reported to protect ILC2s from apoptosis.⁴⁵ To determine whether the excessive production of IL-9 is involved in the enhanced IL-33-induced airway inflammation in T-bet^{-/-} mice, we finally examined the effect of neutralizing anti-IL-9 antibody on IL-33-induced airway inflammation in T-bet^{-/-} mice and WT mice. Whereas anti-IL-9 antibody did not significantly inhibit eosinophilic airway inflammation in IL-33-stimulated WT mice, anti-IL-9 antibody significantly suppressed eosinophilic airway inflammation in IL-33-stimulated T-bet^{-/-} mice (Fig. 7A). Anti-IL-9 antibody also suppressed the accumulation of CD25⁺ Thy1.2⁺ Lin⁻ cells in IL-33-stimulated T-bet^{-/-} mice but not in IL-33-stimulated WT mice (Fig. 7B). These results suggest that the excessive production of IL-9 from lung ILC2s is involved in the exacerbation of IL-33-induced, ILC2-mediated eosinophilic airway inflammation in T-bet^{-/-} mice.

Discussion

In this study, we show that ILC2-mediated eosinophilic airway inflammation is exacerbated by the absence of T-bet in mice. Regarding the involvement of T-bet in the pathogenesis of asthma, various studies have shown that single nucleotide polymorphisms (SNPs) or variants in *TBX21* gene, encoding T-bet, are associated with the development of asthma in humans.^{46, 47, 48, 49} With respect to T-bet-mediated regulation of asthma, we have previously shown that T-bet expressed in CD4⁺ T cells is crucial for the inhibition of Th2 cell-mediated eosinophilic airway inflammation in ovalbumin-induced asthma models.²³ In this study, we show that IL-33-induced eosinophilic airway inflammation, which is known to be dependent on ILC2s but not on T cells,⁵⁰ is exacerbated in T-bet^{-/-} mice (Fig. 2). These results suggest that T-bet is pleiotropically involved in the suppression of eosinophilic airway inflammation.

We show that IL-33-induced accumulation of ST2⁺ CD25⁺ Thy1.2⁺ Lin⁻ cells, which represent ILC2s,³³ in the lung is exacerbated in T-bet^{-/-} mice (Fig. 3). It is well established that T-bet is the most critical transcriptional factor for Th1 cell differentiation.¹⁵ On the other hand, it has been shown that T-bet, together with runt-related transcription factor 3 (Runx3), is required for silencing of *Il4* gene in Th1 cells.⁵¹ Moreover, it has been reported that T-bet interacts with GATA3 and dissociates GATA3 from the promoters of *Il5* and *Il13*.²² These findings suggest that T-bet directly suppresses Th2 cytokine production in CD4⁺ T cells. By contrast, we found that IL-33-induced production of IL-5 and IL-13 in Thy1.2⁺ Lin⁻ cells was not affected by the absence of T-bet (Fig. 3D). In addition, we showed that the enforced expression of T-bet did not suppress IL-5 and IL-13 production in Thy1.2⁺ Lin⁻ cells (Fig. 5E), suggesting that T-bet does not directly regulate the production of IL-5 and IL-13 in IL-33-stimulated ILC2s. On the other hand, we found that IL-33-induced accumulation of lung ST2⁺ CD25⁺ Thy1.2⁺ Lin⁻ cells was enhanced in T-bet^{-/-} mice (Fig. 3). The enhanced accumulation of lung ST2⁺ CD25⁺ Thy1.2⁺ Lin⁻ cells by the absence of T-bet was observed even in a RAG2^{-/-} background (Fig. 4). Taken together, these results suggest that T-bet

expressed in non-T/non-B population plays an inhibitory role in IL-33-induced accumulation of lung ILC2s.

Regarding the mechanism underlying the enhanced IL-33-induced accumulation of lung ILC2s and eosinophilic airway inflammation in T-bet^{-/-} mice, we found that IL-9 production from IL-33-stimulated CD25⁺ Thy1.2⁺ Lin⁻ cells was up-regulated by the absence of T-bet (Fig. 5B). We also found that the enforced expression of T-bet in lung Thy1.2⁺ Lin⁻ cells inhibited IL-9 production (Fig. 5E), suggesting that T-bet intrinsically inhibits IL-9 production in IL-33-stimulated ILC2s. In vivo, we showed that IL-9 neutralization by anti-IL-9 antibody attenuated IL-33-induced accumulation of lung CD25⁺ Thy1.2⁺ Lin⁻ cells and eosinophilic airway inflammation in T-bet^{-/-} mice (Fig. 7). Taken together, these results suggest that the enhanced IL-9 production of T-bet^{-/-} ILC2s seems responsible for the enhanced IL-33-induced eosinophilic airway inflammation in T-bet^{-/-} mice.

It has recently been shown that ILC2s represent a potentially important source of IL-9 during papain-induced lung inflammation and helminth lung infection by using IL-9 reporter mice and fate-mapping studies.^{44, 45} Importantly, one of the functions of IL-9 is to act on ILC2s in an autocrine manner to enhance their survival in a helminth infection model,⁴⁵ which is consistent with the expression of IL-9R on Thy1.2⁺ Lin⁻ cells (Fig. E4) and the reduction of surviving ST2⁺ Thy1.2⁺ Lin⁻ cells by IL-9 neutralization (Fig. 5D). Moreover, it has been shown that IL-9 expression during helminth infection precedes the expression of IL-5 and IL-13 and that the production of IL-5 and IL-13 is abrogated in IL-9R^{-/-} mice,⁴⁵ suggesting that IL-9-IL-9R signaling acts in the upstream of IL-5 and IL-13. Interestingly, it has been reported that the expression of IL-9 is more transient than that of IL-5 or IL-13 in ILC2s,⁴⁴ suggesting that IL-9 production is more tightly regulated than that of IL-13 or IL-5 in ILC2s. Importantly, we showed here that the expression of IL-9 but not of IL-13 or IL-5 in Thy1.2⁺ Lin⁻ cells was significantly enhanced by the absence of T-bet (Fig. 5B). In addition, we showed that IL-33-induced IL-9 production from CD25⁺ Thy1.2⁺ Lin⁻ cells was significantly enhanced by the neutralization of IFN- γ in WT mice but not in T-bet^{-/-} mice (Fig. 6). Taken

together, these results suggest that T-bet might be involved in the tight regulation of IL-9 expression in ILC2s as a downstream target of IFN- γ .

It has been reported that various transcription factors such as STAT5, STAT6, GATA3, IRF4, BATF, and PU.1 are involved in the differentiation of Th9 cells.^{41, 42, 43, 44, 52} More recently, analogous to the findings in Th9 cells, IRF4 has been shown to be essential for IL-9 production from ILC2s in response to IL-33 and thymic stromal lymphopoietin (TSLP).⁵³ On the other hand, we found that the expression of IRF4 was not significantly affected by the absence of T-bet in Thy1.2⁺ Lin⁻ cells (Fig. E4), suggesting that IRF4 might not be involved in T-bet-mediated suppression of IL-9 production in ILC2s. Our results also suggest that GATA3, BATF, AhR, and PU.1 are not responsible for the enhanced IL-9 production in T-bet^{-/-} ILC2s (Fig. E4). Further studies including comprehensive analyses of DNA binding of T-bet in ILC2s are needed to reveal the mechanisms of T-bet-mediated suppression of IL-9 expression in ILC2s.

Our findings suggest that T-bet expressed in lung ILC2s plays an inhibitory role in ILC2s-mediated immune responses. Recently, several groups have investigated the counter-regulatory mechanisms of ILC2s and have shown that IFN- β , IFN- γ , and IL-27 regulate the activation and function of ILC2s during lung inflammation in a STAT1-dependent manner.^{27, 28, 29, 54} Importantly, Kudo et al. have shown that IFN- γ produced by activated NKT cells is important for the suppression of IL-5 and IL-13 production from lung ILC2s.⁵⁴ In this regard, we found that IFN- γ induced the expression of T-bet in lung ST2⁺ Thy1.2⁺ Lin⁻ cells (Fig. 1). Moreover, we found that the enforced expression of T-bet suppressed IL-33-induced IL-9 but not IL-5 or IL-13 production in lung Thy1.2⁺ Lin⁻ cells (Fig. 5D), consistent with a previous finding that IFN- γ inhibits IL-33-induced IL-5 production in a manner dependent on STAT1 but not on T-bet.²⁸ Therefore, it is likely that IFN- γ suppresses the function of ILC2s via both T-bet-dependent and -independent mechanisms.

In conclusion, our findings indicate that T-bet intrinsically suppresses the expression of IL-9 in lung ILC2s during IL-33-induced lung inflammation and thereby suppresses the

433 accumulation of lung ILC2s and subsequent eosinophilic airway inflammation. Although
434 further studies are needed, our results should add a new insight into the counter-regulatory
435 mechanisms for ILC2s in asthma and suggest that the induction of T-bet expression in ILC2s
436 could be a therapeutic strategy in the treatment of asthma.

Acknowledgements

We thank Drs. Ken Nonaka and Osamu Ohara (Kazusa DNA Research Institute, Japan) for RNA-Seq analysis and Dr. Steven L. Reiner (University of Pennsylvania) for the MIG-T-bet. We also thank Ms. Juri Iwata, Kazumi Nemoto, and Yumiko Hanabuchi (Chiba University, Chiba, Japan) for technical support. This work was supported in part by Grants-in-Aids for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology (MEXT, WG24390207, G26461486), and LGS (Leading Graduate School at Chiba University) Program, MEXT, Japan, and the Takeda Science Foundation, Japan.

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Figure Legends**Figure 1. IFN- γ induces T-bet expression in lung ILC2s.**

(A) Thy1.2⁺ Lin⁻ cells were isolated from the lung of WT mice and cultured with IL-2 and IL-7 in the presence or absence of IFN- γ for 6 days. mRNA levels of T-bet, IL-12R β 2, and IFN- γ were analyzed by qPCR analysis. Naïve CD4⁺ T cells, Th0 cells, and Th1 cells were used as controls. Data are means $\pm$ SD of three independent experiments. **p<0.01. (B) The expression of T-bet and GATA3 in lung CD25⁺ ST2⁺ Thy1.2⁺ Lin⁻ cells or CD25⁻ ST2⁻ Thy1.2⁺ Lin⁻ cells was examined by intracellular staining in WT mice. T-bet^{-/-} mice were used as controls. Data are representative of three independent experiments. (C) ST2⁺ Thy1.2⁺ Lin⁻ cells were isolated from the lung of WT mice and cultured with IL-2 and IL-7 in the presence or absence of IFN- γ and/or IL-33 for 6 days. Representative data of T-bet staining and means $\pm$ SD of the frequency of T-bet-expressing cells are shown. *p<0.05. Data are representative of three independent experiments.

Figure 2. IL-33-induced eosinophilic airway inflammation is exacerbated in T-bet^{-/-} mice.

(A) Representative CD11c vs. Siglec-F staining, and means $\pm$ SD of the frequency and absolute numbers of Siglec-F⁺ CD11c⁻ cells (eosinophils) in the BALF are shown (n=5, each). *p<0.05. (B) Representative photomicrographs (H&E staining) and means $\pm$ SD of histological score of the lung are shown. *p<0.05. n=4, each. Scale bars, 100 μ m. (C) Representative photomicrographs (PAS staining) and means $\pm$ SD of goblet cell score are shown. *p<0.05. n=4, each. Scale bars, 50 μ m. (D) Means $\pm$ SD of the levels of IL-4, IL-5, and IL-13 in the BALF are shown (n=3, each). *p<0.05. NS=not significant. ND=not detectable.

Figure 3. IL-33-induced accumulation of lung ILC2s is exacerbated in T-bet^{-/-} mice.

(A) Representative Lin vs. Thy1.2 staining of isolated lung cells, and means $\pm$ SD of the frequency and absolute numbers of Thy1.2⁺ Lin⁻ cells in the lung are shown (n=5, each).

* $p < 0.05$. **(B)** Means $\pm$ SD of the frequency of CD25⁺ cells in Thy1.2⁺ Lin⁻ cells and absolute numbers of CD25⁺ Thy1.2⁺ Lin⁻ cells in the lung are shown ($n=3$, each). * $p < 0.05$. NS=not significant. **(C)** Means $\pm$ SD of the frequency of ST2⁺ cells and KLRG1⁺ cells in CD25⁺ Thy1.2⁺ Lin⁻ cells and absolute numbers of ST2⁺ CD25⁺ Thy1.2⁺ Lin⁻ cells and KLRG1⁺ CD25⁺ Thy1.2⁺ Lin⁻ cells are shown ($n=3$, each). **(D)** Isolated lung cells were stimulated with PMA and ionomycin for 4 hs, and subjected to intracellular staining for IL-4, IL-5, IL-13, and IL-17A. Shown are representative Thy1.2 vs. IL-4, IL-5, IL-13, or IL-17A staining of Thy1.2⁺ Lin⁻ cells, and means $\pm$ SD of the frequency of the indicated cells ($n=3$, each). * $p < 0.05$. Data are representative of four independent experiments.

Figure 4. T-bet expressed in non-T/non-B cells is involved in the suppression of IL-33-induced eosinophilic airway inflammation.

(A) Representative CD11c vs. Siglec-F staining of BALF cells and means $\pm$ SD of the absolute numbers of Siglec-F⁺ CD11c⁻ cells in the BALF are shown ($n=3$, each). **(B)** Representative Lin vs. Thy1.2 staining of isolated lung cells and means $\pm$ SD of the absolute numbers of Thy1.2⁺ Lin⁻ in the lung are shown ($n=3$, each). * $p < 0.05$. **(C)** Representative histogram of CD25 staining gating on Thy1.2⁺ Lin⁻ cells and means $\pm$ SD of the frequency of CD25⁺ cells are shown ($n=3$, each). * $p < 0.05$. **(D)** Representative histogram of ST2 or KLRG1 staining gating on CD25⁺ Thy1.2⁺ Lin⁻ cells and means $\pm$ SD of the frequency of ST2⁺ or KLRG1⁺ cells are shown ($n=3$, each). ** $p < 0.01$. NS=not significant. **(E)** Isolated lung cells were stimulated with PMA and ionomycin for 4 hs, and subjected to intracellular staining for IL-4, IL-5, IL-13, and IL-17A. Shown are representative CD25 vs. IL-4, IL-5, IL-13, or IL-17A staining of Thy1.2⁺ Lin⁻ cells and means $\pm$ SD of the frequency of cytokine-producing cells ($n=3$, each). * $p < 0.05$. Data are representative of three independent experiments.

Figure 5. T-bet suppresses IL-9 production in lung ILC2s.

(A) Isolated lung Thy1.2⁺ Lin⁻ cells from WT mice and T-bet^{-/-} mice were cultured with IL-2 and IL-7 in the presence or absence of IL-33 for 6 days, and RNA-Seq analysis was

performed. Shown is a heatmap of top 10 genes whose expression is up-regulated in IL-33-stimulated T-bet^{-/-} Thy1.2⁺ Lin⁻ cells. **(B)** Isolated lung Thy1.2⁺ Lin⁻ cells from IL-33-stimulated WT mice and T-bet^{-/-} mice were cultured with IL-2, IL-7, and IL-33 for 6 days, and mRNA levels for indicated cytokines were analyzed by qPCR. Data are means $\pm$ SD of 3 independent experiments. * p <0.05. **(C)** Isolated lung Thy1.2⁺ Lin⁻ cells from IL-33-stimulated WT mice and T-bet^{-/-} mice were cultured with IL-2, IL-7, and IL-33 for 6 days and then subjected to flow-cytometric analysis. Representative CD25 vs. ST2 staining (upper panels) and IL-5 vs. IL-9 staining gating on CD25⁺ population (lower panels) are shown. Data are representative of three independent experiments. **(D)** Isolated lung ST2⁺ Thy1.2⁺ Lin⁻ cells from WT mice and T-bet^{-/-} mice were cultured with IL-2 and IL-7 in the presence or absence of anti-IL-9 antibody for 6 days, and the number of surviving cells was analyzed. Data are means $\pm$ SD of surviving cells. * p <0.05, ** p <0.01. **(E)** Isolated lung Thy1.2⁺ Lin⁻ cells from IL-33-stimulated WT mice were stimulated with IL-2, IL-7, and IL-33 for 24 hs. These cells were infected with retroviruses of MSCV-IRES-GFP (MIG) (as a control) or MIG-T-bet, cultured for 4 days in the presence of IL-2, IL-7, and IL-33, and subjected to flow cytometric analyses. Shown are representative FACS profiles of ST2 vs. KLRG1, IL-5 vs. IL-13, and IL-5 vs. IL-9 gating on GFP⁺ CD25⁺ cells. Data are representative of three independent experiments.

Figure 6. Anti-IFN- γ antibody enhances IL-33-induced IL-9 production from ILC2s in WT mice but not in T-bet^{-/-} mice.

Representative CD25 vs. IL-9 staining (A) and CD25 vs. T-bet staining (B) of Thy1.2⁺ Lin⁻ cells in the lung and means $\pm$ SD of the absolute numbers of IL-9-producing CD25⁺ Thy1.2⁺ Lin⁻ cells in the lung are shown (n=3, each). * p <0.05. ** p <0.01. NS=not significant. Results are representative of two independent experiments.

Figure 7. Anti-IL-9 antibody cancels the enhanced IL-33-induced airway inflammation in T-bet^{-/-} mice.

688 (A) Representative CD11c vs. Siglec-F staining of BALF cells and means $\pm$ SD of the
689 absolute numbers of Siglec-F⁺ CD11c⁻ cells in the BALF are shown (n=3, each). *p<0.05.
690 **p<0.01. NS=not significant. (B) Representative Lin vs. Thy1.2 staining of isolated lung
691 cells and means $\pm$ SD of the absolute numbers of Thy1.2⁺ Lin⁻ cells and CD25⁺ Thy1.2⁺ Lin⁻
692 cells in the lung are shown (n=3, each). Results are representative of two independent
693 experiments.

Fig. 1

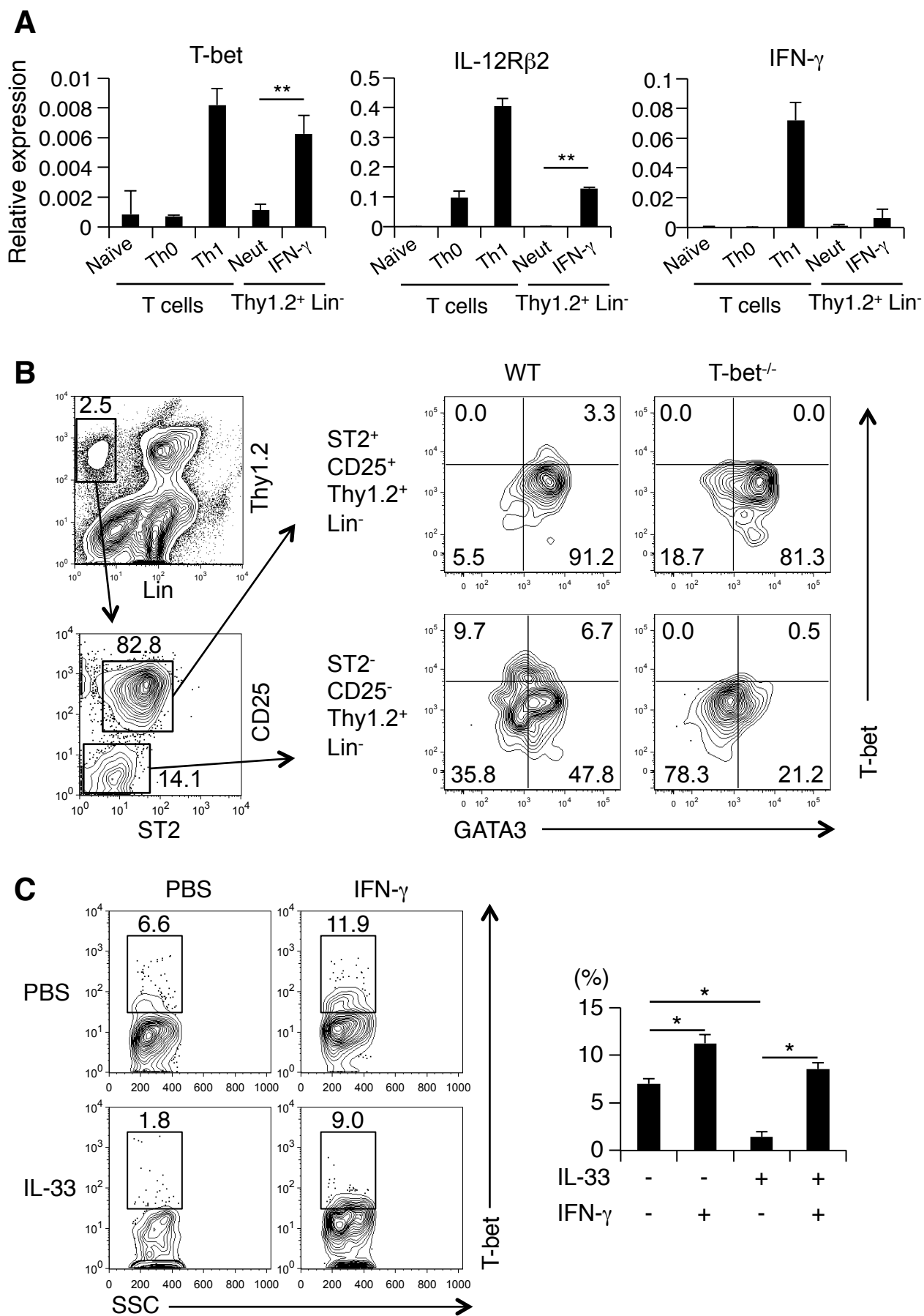


Fig. 2

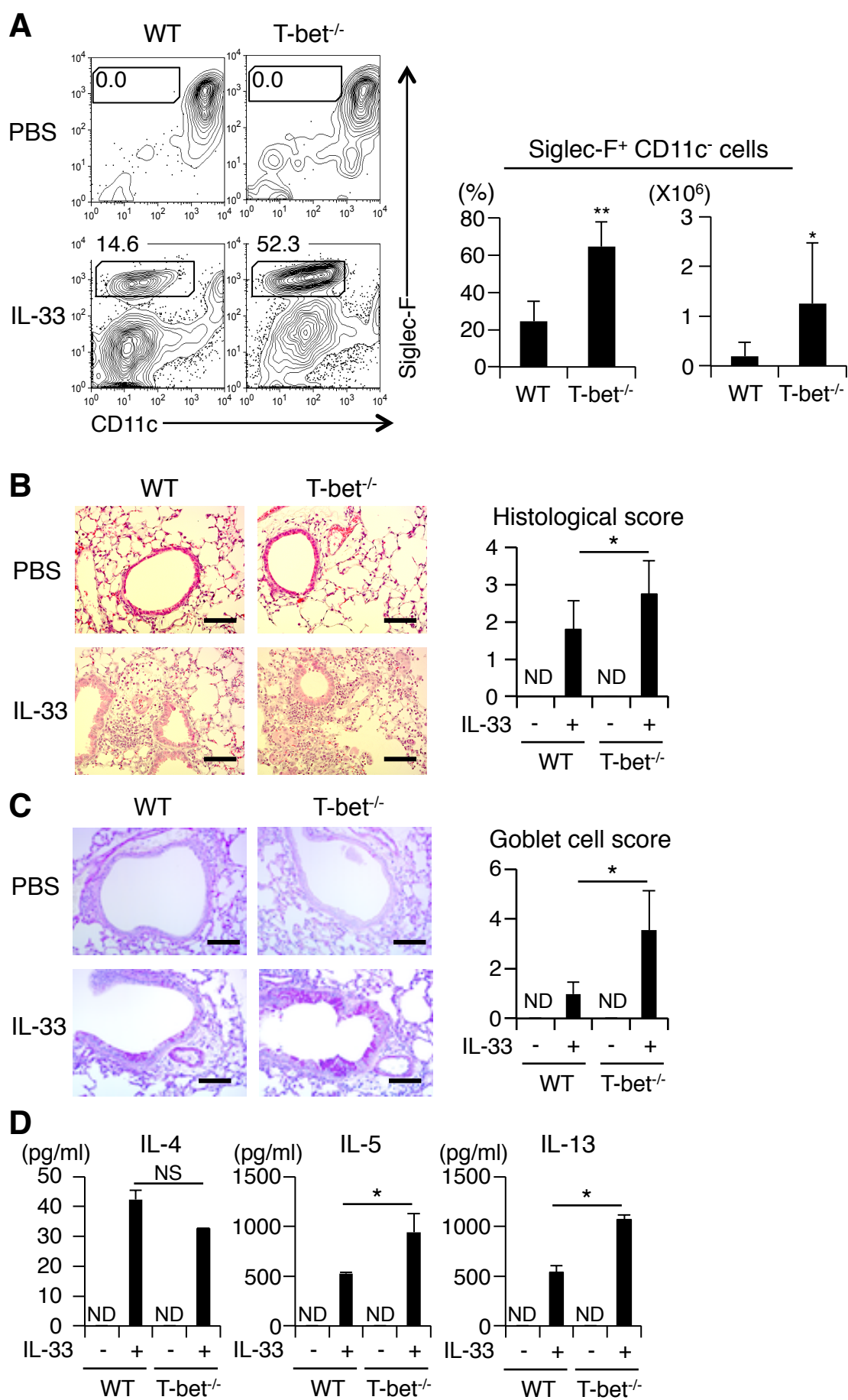


Fig. 3

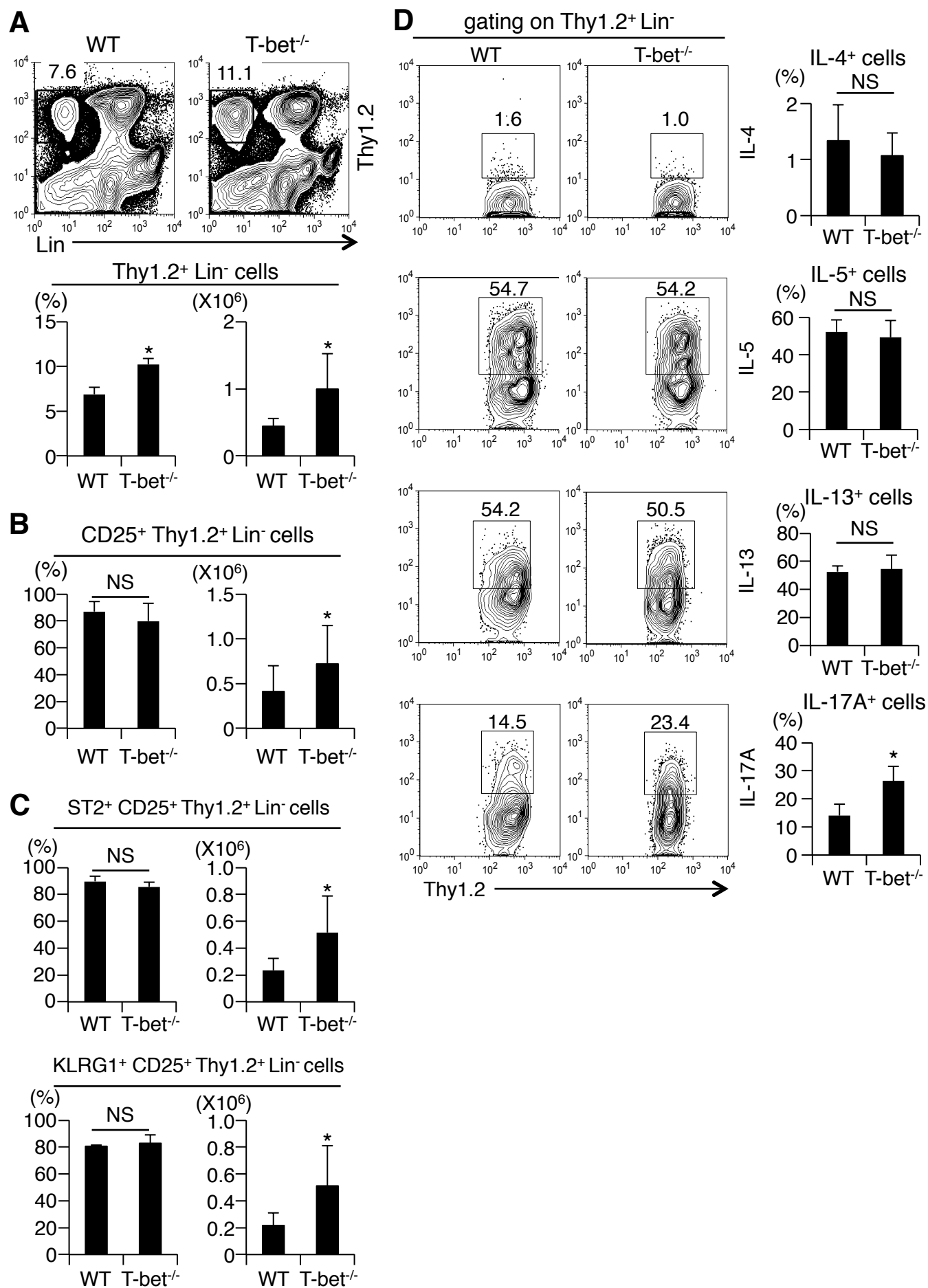


Fig. 4

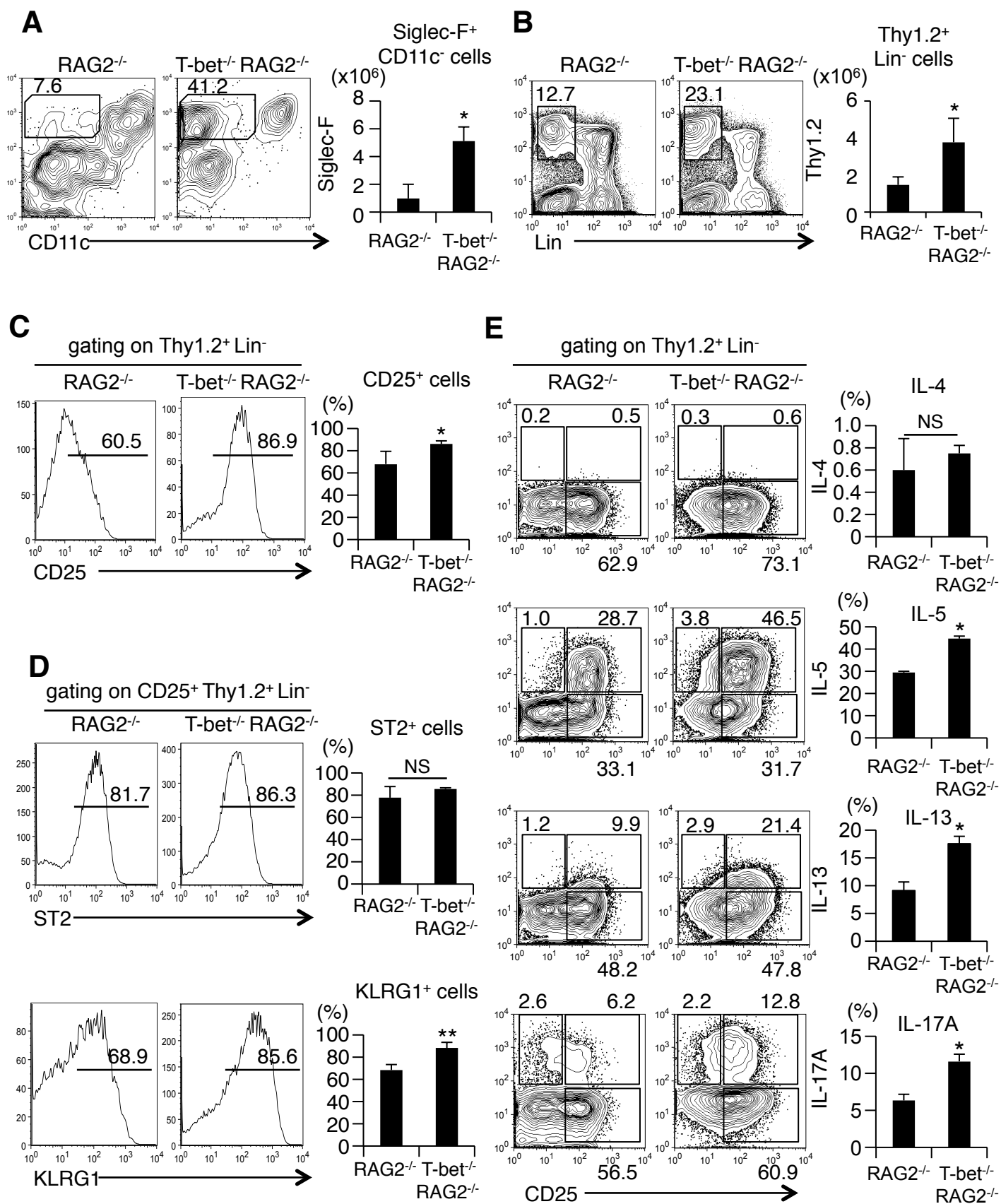


Fig. 5

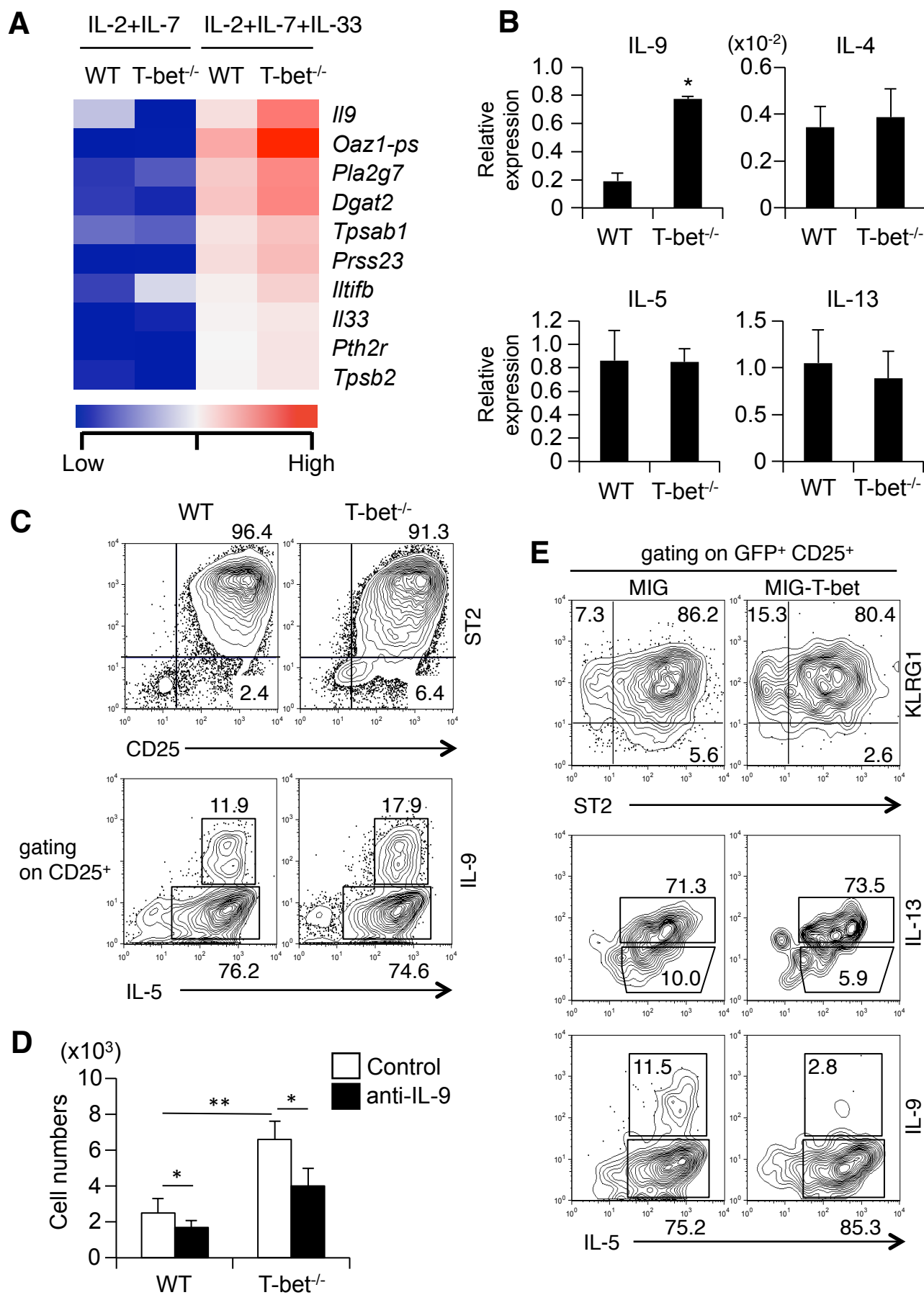


Fig. 6

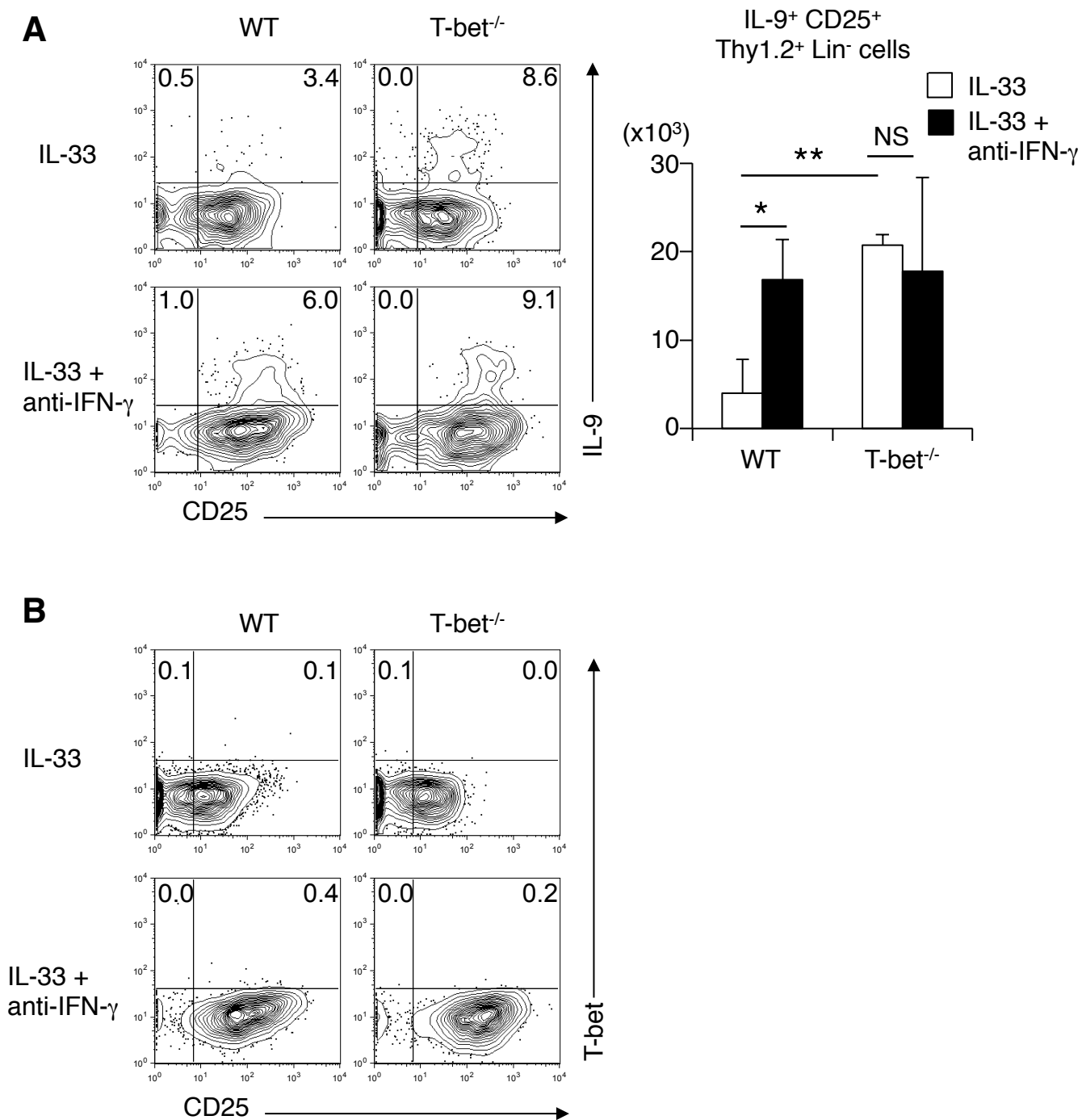


Fig. 7

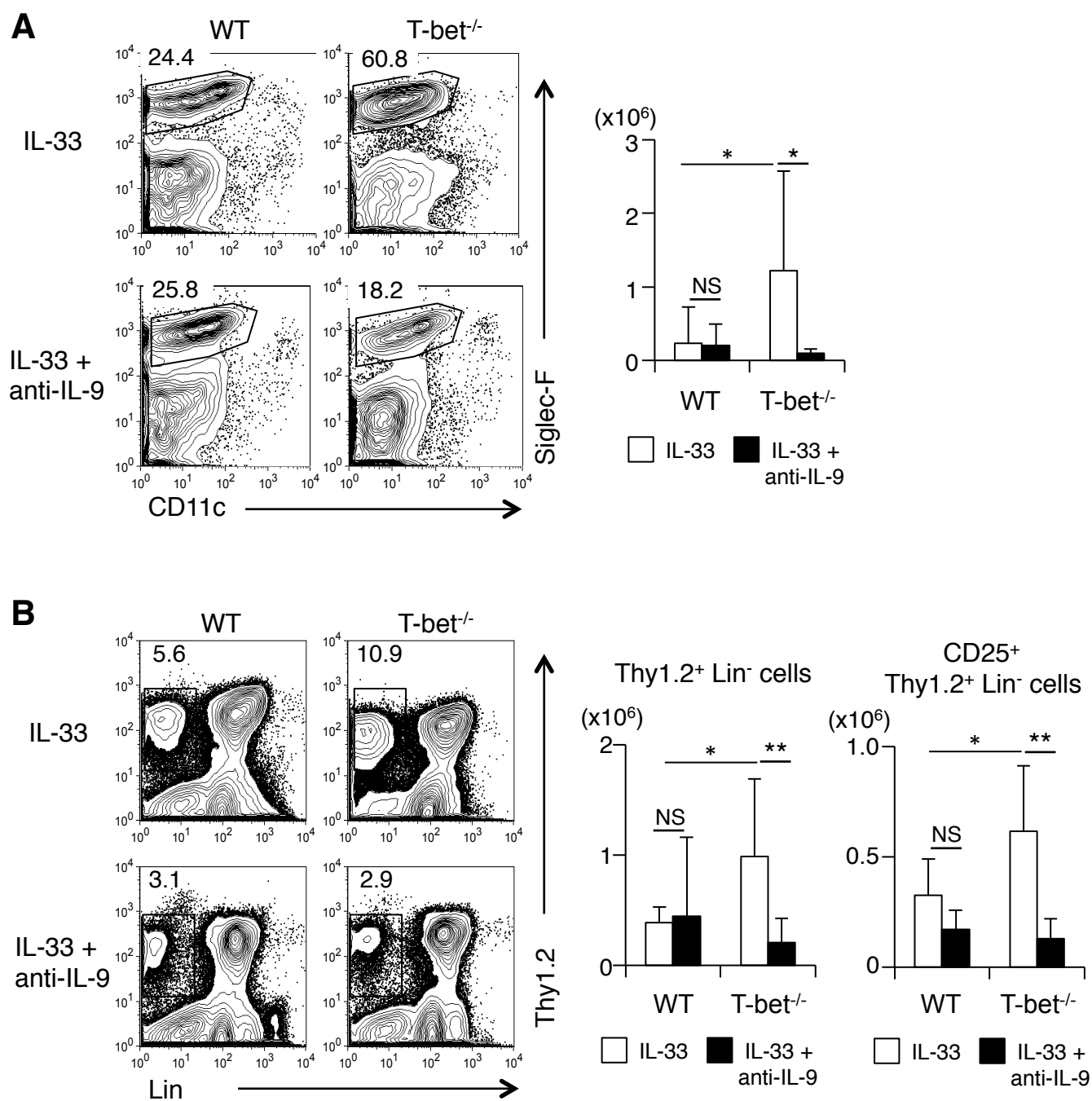


Fig. E1

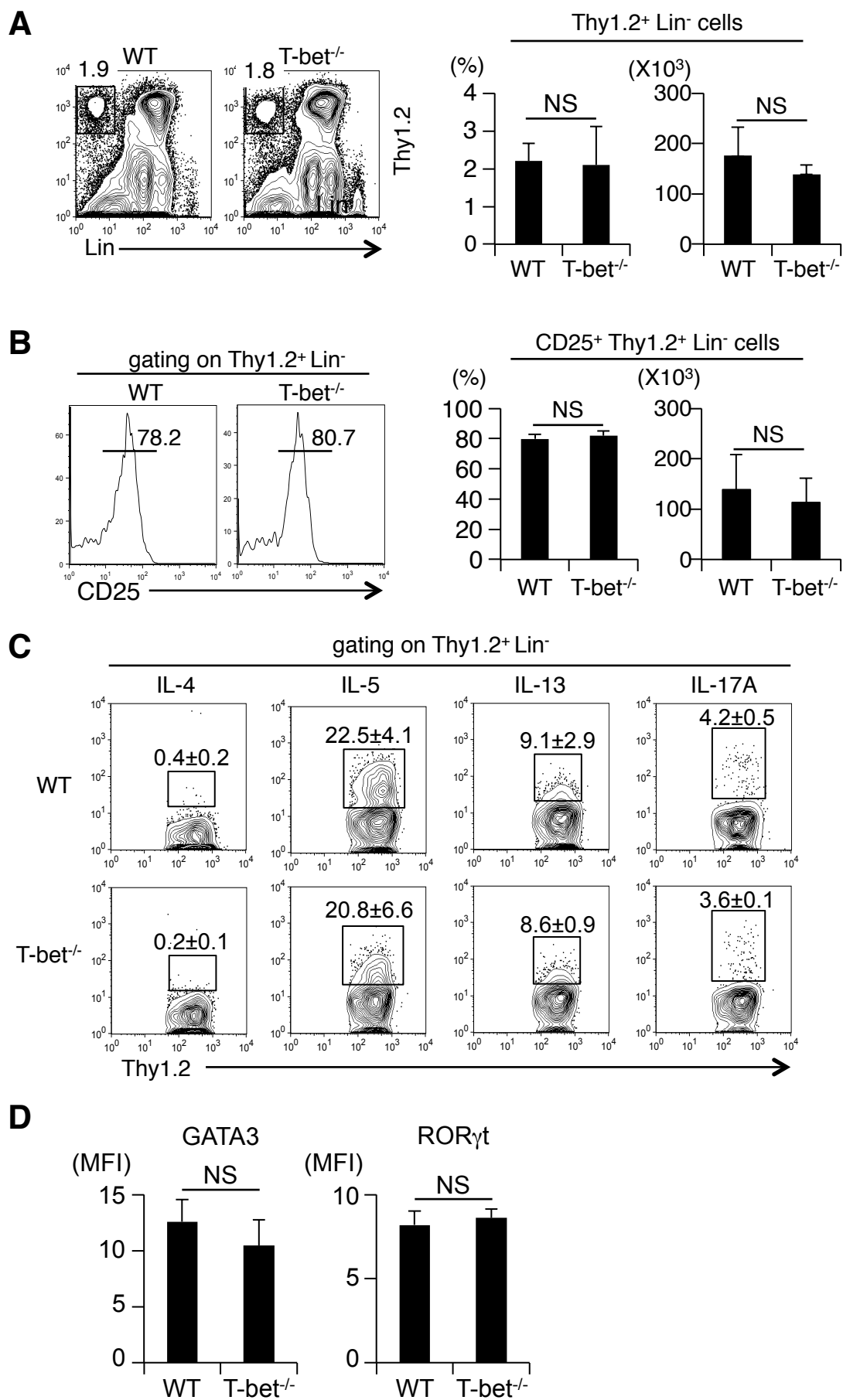


Fig. E2

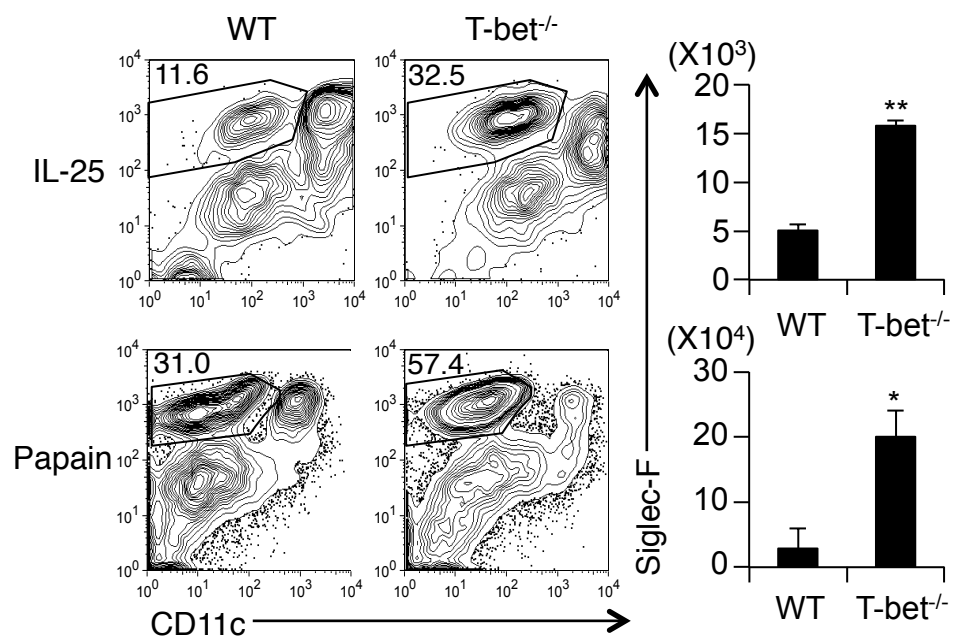


Fig. E3

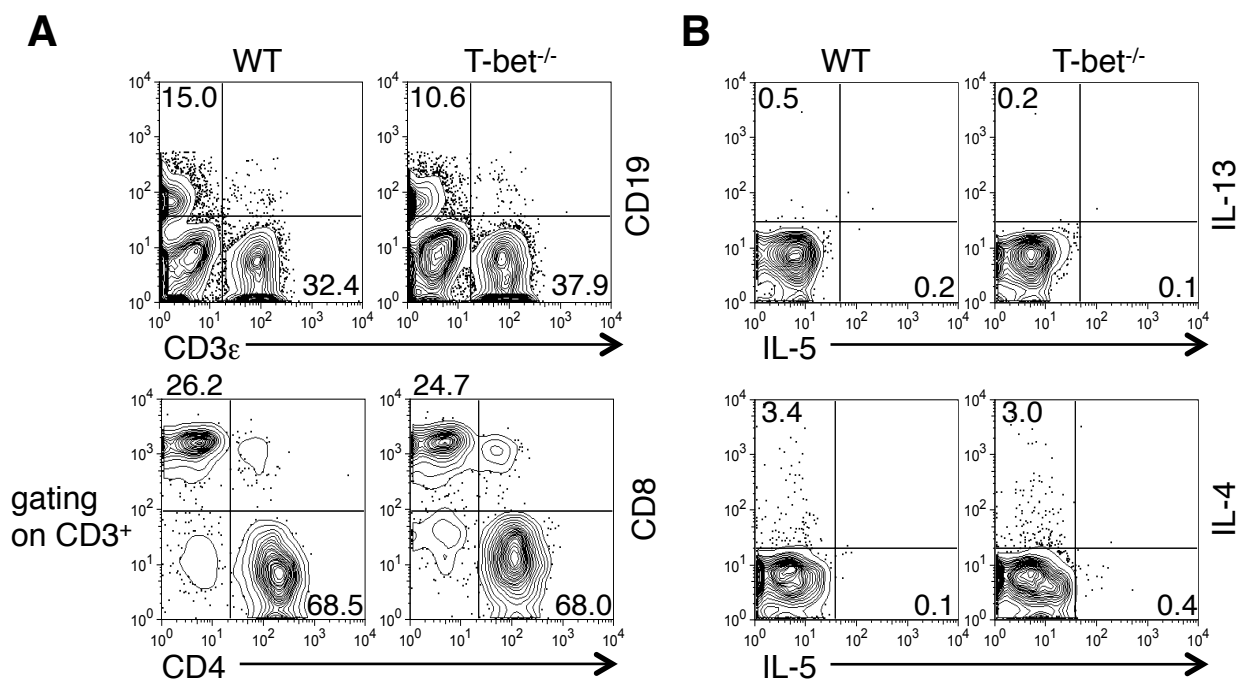
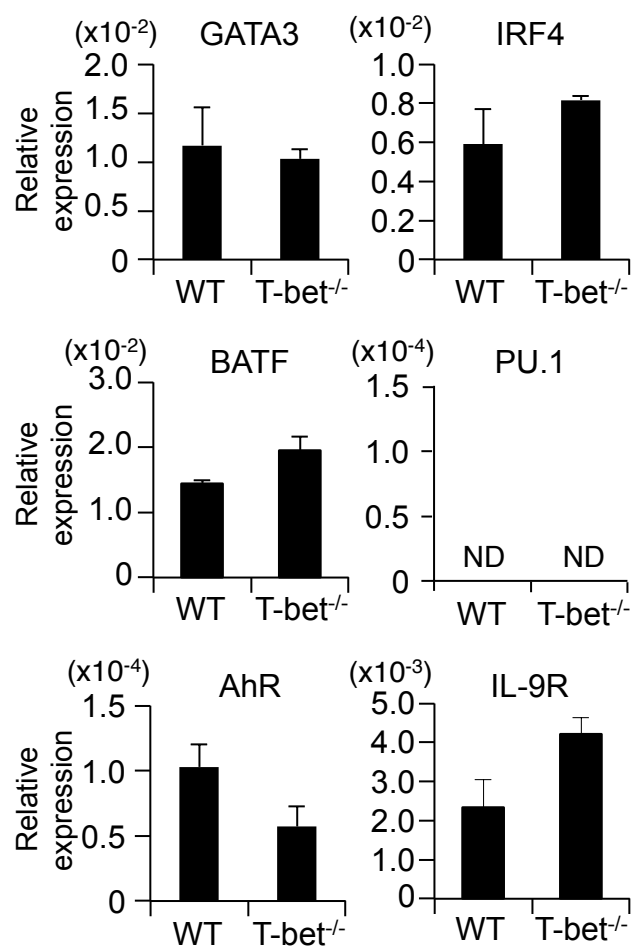


Fig. E4



Online Repository**T-bet inhibits innate lymphoid cell-mediated eosinophilic airway inflammation by suppressing IL-9 production**

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Online Repository Methods

Reagents

Antibodies to murine CD3 ϵ (145-2C11), CD4 (RM4-5), CD8 α (53-6.7), CD19 (6D5), CD5 (53-7.3), B220 (RA3-6B2), CD11b (M1/70), CD11c (N418), CD49b (Dx5), Fc ϵ RI (MAR-1), TER119, TCR β (H57-597), TCR $\gamma\delta$ (UC7-13D5), GR-1 (RB6-8C5), Thy1.2 (30-H12), CD25 (PC61), IL-33R α (ST2) (DIH9), and KLRG1 (2F1/KLRG1) were purchased from BioLegend (San Diego, CA). Anti-Siglec-F antibody (E50-2440) was purchased from BD Biosciences (San Jose, CA). Anti-IL-9 antibody (MM9C1) and anti-IFN- γ antibody (XMG1.2) were purchased from Bio X Cell (West Lebanon, NH). Anti-GATA3 antibody (TWAJ), anti-T-bet antibody (eBio4B10), and anti-ROR γ t antibody (B2D) were purchased from eBioscience (San Diego, CA). Recombinant murine IL-2, IL-7, IL-25, and IL-33 were purchased from BioLegend. Recombinant murine IFN- γ was purchased from PeproTech (Rocky Hill, NJ). Papain was purchased from Sigma-Aldrich (St. Louis, MO).

The sequences of PCR primers:

IFN- γ , forward, 5'-TCAAGTGGCATAGATGTGGAAGAA-3',
reverse, 5'-TGGCTCTGCAGGATTTTCATG-3';
IL-4, forward, 5'-GGCATTGTTGAACGAGGTCACA-3',
reverse, 5'-GACGTTTGGCACATCCATCTC-3';
IL-5, forward, 5'-ACGGAGGACGAGGCACTTC-3',
reverse, 5'-TCCATTGCCCACTCTGTACTCA-3';
IL-9, forward, 5'-CGTCCCCAGGAGACTCTTCA-3',
reverse, 5'-CGAAAAGCCATGCAACCAG-3';
IL-13, forward, 5'-GGTCCTGTAGATGGCATTGCA-3',
reverse, 5'-GGAGCTGAGCAACATCACACA-3';

- 48 IL-9R, forward, 5'-GGCAGCAGCGACTATTGCAT-3',
49 reverse, 5'-ACACAGGAAGGGCCACAGG-3';
50 IL-12R β 2, forward, 5'-TTCATAGTCCGTGTTACTGCC-3',
51 reverse, 5'-TCATCTTCCCCTGAGTGTA-3';
52 T-bet, forward, 5'-CAACAACCCCTTTGCCAAAG-3',
53 reverse, 5'-TCCCCCAAGCAGTTGACAGT-3';
54 IRF4, forward, 5'-TCCGACAGTGGTTGATCGAC-3',
55 reverse, 5'-CCTCACGATTGTAGTCCTGCTT-3';
56 GATA3, forward, 5'-AGAACCGGCCCTTATCAA-3',
57 reverse, 5'-AGTTCGCGCAGGATGTCC-3';
58 BATF, forward, 5'-CTGGCAAACAGGACTCATCTG-3',
59 reverse, 5'-GGGTGTCGGCTTTCTGTGTC-3';
60 AhR, forward, 5'-TTCTATGCTTCCTCCACTATCCA-3',
61 reverse, 5'-GGCTTCGTCCACTCCTTGT-3';
62 PU.1, forward, 5'-AGAGCTATACCAACGTCCAATGC-3',
63 reverse, 5'-TTCTCAAACCTCGTTGTTGTGGAC-3' ;
64 β -actin, forward, 5'-TGTTACCAACTGGGACGACA-3',
65 reverse, 5'-CCATCACAATGCCTGTGGTA-3'.

Online Repository Table

Online Repository Table 1. Top 10 genes whose expression is up-regulated in

IL-33-stimulated T-bet^{-/-} Thy1.2⁺ Lin⁻ cells

WAD ranking	Absolute FC	Gene symbol (name)	Function
1	4.25	<i>Il9</i> (interleukin 9)	cytokine
2	2.49	<i>Oaz1-ps</i> (ornithine decarboxylase antizyme 1)	enzyme
3	2.22	<i>Pla2g7</i> (phospholipase A2, group VII)	enzyme
4	2.07	<i>Dgat2</i> (diacylglycerol O-acyltransferase 2)	enzyme
5	2.31	<i>Tpsab1</i> (tryptase alpha/beta 1)	enzyme
6	2.05	<i>Prss23</i> (protease, serine 23)	enzyme
7	3.33	<i>Iltifb</i> (interleukin 10-related T cell-derived inducible factor beta)	cytokine
8	2.23	<i>Il33</i> (interleukin 33)	cytokine
9	4.17	<i>Pth2r</i> (parathyroid hormone 2 receptor)	receptor
10	3.92	<i>Tpsb2</i> (tryptase beta 2)	enzyme

WAD: Weighted average difference, FC: fold change

Online Repository Figure Legends

Figure E1. T-bet is dispensable for the development of ILCs in the lung.

(A-D) Single-cell suspensions of lung cells were isolated from WT mice and T-bet^{-/-} mice at steady-state conditions and subjected to flow-cytometric analysis. **(A)** Representative lineage markers (Lin⁻ vs. Thy1.2 staining of isolated lung cells, and means $\pm$ SD of the frequency and absolute numbers of Thy1.2⁺ Lin⁻ cells in the lung are shown (n=3, each). NS=not significant. **(B)** Representative histograms of CD25 staining gating on Thy1.2⁺ Lin⁻ cells, and means $\pm$ SD of the frequency of CD25⁺ cells and absolute numbers of CD25⁺ Thy1.2⁺ Lin⁻ cells are shown (n=3, each). **(C)** Isolated lung cells were stimulated with PMA and ionomycin for 4 hs, and subjected to intracellular staining for IL-4, IL-5, IL-13, and IL-17A. Representative Thy1.2 vs. IL-4, IL-5, IL-13, or IL-17A staining of Thy1.2⁺ Lin⁻ cells, and means $\pm$ SD of the frequencies of cytokine-producing cells are shown (n=3, each). **(D)** Means $\pm$ SD of mean fluorescent intensity (MFI) for GATA3 and ROR γ t staining of lung Thy1.2⁺ Lin⁻ cells are shown (n=3, each). NS=not significant.

Figure E2. Eosinophilic inflammation is exacerbated in T-bet^{-/-} mice upon IL-25 or papain administration.

IL-25 (1 μ g) or papain (25 μ g) was administered to WT mice and T-bet^{-/-} mice intranasally at days 0, 3, and 6. Twelve hours after the last administration, cells harvested from the BALF were subjected to flow-cytometric analysis. Representative CD11c vs. Siglec-F staining of BALF cells and means $\pm$ SD of the absolute numbers of Siglec-F⁺ CD11c⁻ cells in the BALF are shown (n=3, each). Data are representative of two independent experiments.

Figure E3. The frequency and cytokine production of CD4⁺ T cells in**IL-33-stimulated T-bet^{-/-} mice.**

(A-B) IL-33 was administered to WT mice and T-bet^{-/-} mice intranasally as described in the Methods. **(A)** Twelve hours after the last administration, isolated lung cells were subjected to flow-cytometric analysis. Representative CD3ε vs. CD19 staining of single-cell suspensions of lung cells and CD4 vs. CD8 staining of CD3ε⁺ cells are shown. **(B)** Isolated lung cells were stimulated with PMA and ionomycin for 4 hs, and subjected to intracellular staining for IL-4, IL-5, and IL-13. Shown are representative IL-5 vs. IL-4 and IL-5 vs. IL-13 staining of CD4⁺ cells. Data are representative of 3 independent experiments.

Figure E4. The expression levels of Th9 cell-related genes in Thy1.2⁺ Lin⁻ cells.

Isolated lung Thy1.2⁺ Lin⁻ cells from IL-33-stimulated WT mice and T-bet^{-/-} mice were cultured with IL-2, IL-7, and IL-33 for 6 days, and mRNA levels for indicated molecules were analyzed by qPCR. Data are means ± SD of 3 independent experiments.

*p<0.05.