Original article

Restoration of T-box–containing protein expressed in T cells expression in T cells protects against allergen-induced asthma

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Background: A T_H1-specific transcription factor, T-box–containing protein expressed in T cells (T-bet), controls the production of both T_H1 and T_H2 cytokines in T_H cell differentiation by means of distinct mechanisms. T-bet–deficient mice overproduce T_H2 cytokines and have spontaneous airway inflammation.
Objectives: We tested whether T-bet overexpression could protect against the development or progression of asthma.
Methods: We generated a T cell–specific and inducible line of T-bet–transgenic mice on a T-bet–deficient genetic background and used it to study the function of T-bet in an ovalbumin (OVA)–induced asthma model.
Results: Induction of T-bet in a T cell–specific manner in an OVA model of asthma concomitant with OVA injection prevented airway hyperresponsiveness, eosinophilic and lymphocytic inflammation, and IL-5 and IL-13 production in bronchoalveolar lavage fluid and also reduced serum IgE and T_H2 cytokine production by peripheral T cells. Even when T-bet expression was induced during later stages of asthma progression, T-bet overexpression still attenuated airway hyperresponsiveness and goblet cell hyperplasia, as well as T_H2 cytokine production.
Conclusions: Our results suggest that T-bet expression in T cells can prevent the initiation of airway inflammation and progression of chronic inflammation and might be extrapolated to human asthma. (J Allergy Clin Immunol ■■■■;■■■:■■■-■■■.)

Key words: *T-bet, T cell, transgenic mice, OVA, asthma, airway hyperresponsiveness*

$CD4^+$ T_H precursor cells differentiate into multiple effector lineages that include T_H1 and T_H2 cells,[1] as well as the more recently discovered T_H17 and regulatory T cells.[2-4] These T_H subsets are distinct in cytokine production profiles and immune functions but reciprocally influence each other. For example, the development and function of the T_H17 lineage is influenced by T_H1- and T_H2-secreted cytokines.[5-8] Asthma and atopic disease are typified by a T_H2-skewed cytokine profile with overproduction of IL-4, IL-5, and IL-13, which is responsible for IgE class switching, eosinophil recruitment, and histamine release from mast cells.[9]

T-cell lineage commitment is determined in early life through the interaction of environmental and genetic determinants.[10-14] One of the primary molecular components of this cellular programming is T-box–containing protein expressed in T cells (T-bet; Tbx21), a tyrosine phosphorylated T-box family transcription factor.[15-17] Members of the T-box gene family share a 200-amino-acid DNA-binding domain called T-box and play critical roles in diverse developmental processes.[17-20] T-bet is expressed in developing T_H1, but not T_H2, cells and is the factor that controls the T_H1-specific expression of the potent inflammatory cytokine IFN-γ. T-bet is a potent activator of IFN-γ transcription, and retroviral transduction of T-bet in T_H2 clones from asthmatic patients converts them into IFN-γ producers[17] while repressing T_H2 cytokine expression (IL-4, IL-5, IL-10, and IL-13) through mechanisms we and others have recently defined.[15,21]

We have demonstrated that mice harboring either homozygous or heterozygous targeted deletions of T-bet spontaneously show physiologic and histopathologic changes in the lung that mirror human asthma.[22] In response to immunologic sensitization but in the absence of challenge, T-bet knockout mice exhibit airway hyperresponsiveness (AHR) to methacholine. T-bet knockout mice also had eosinophilic and lymphocytic inflammation of the peribronchial and perivascular spaces reminiscent of acute asthma and accompanied by airway remodeling pathognomonic of chronic human asthma. Selective alterations in cytokine expression patterns are also observed in bronchoalveolar lavage fluid (BALF), with increased levels of TNF-α, IL-4, IL-5, and IL-13 that drive TGF-β production.[22,23] T-bet intimately interacts with profibrotic factors, such as TGF-β1 and IL-13, that mediate airway fibrosis. The disruption of T-bet leads to dramatic impairment in IFN-γ production in $CD4^+$, but not $CD8^+$, T cells and a

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Abbreviations used
AHR: Airway hyperresponsiveness
BALF: Bronchoalveolar lavage fluid
DTg/KO: Double-transgenic knockout
OVA: Ovalbumin
PAS: Periodic acid–Schiff
rtTA: Tetracycline-controlled transactivator
T-bet: T-box containing protein expressed in T cells
TRE: Tetracycline operon-responsive element
WT: Wild-type

concomitant increase in TGF-β, IL-4, and IL-13 production. These findings suggest that T-bet deficiency can mimic asthma in the mouse; importantly, additional evidence from human studies supports a role for T-bet in controlling IFN-γ expression and in asthma.[24-27] The expression of T-bet in human tissues mirrors that in mouse tissues because T-bet is present in $CD4^+$ cells in the peripheral blood and lung and is significantly reduced in the lungs of patients with allergic asthma compared with those of nonasthmatic patients.[28] Moreover, we and others have recently demonstrated that common T-bet polymorphisms are associated with asthma and related clinical phenotypes, including AHR and response to corticosteroid therapy.[29-34]

Mice constitutively overexpressing T-bet throughout development have an overabundance of T_H1 cells, whereas mice lacking T-bet have no T_H1 cells. Therefore neither T-bet–deficient nor constitutively overexpressing T-bet mutant mice accurately reflect the immune system of individuals who have had normal levels of T-bet throughout development and thus a normal repertoire of T_H1 and T_H2 cells. As a consequence, the effectiveness of T-bet overexpression as a therapeutic treatment for airway remodeling cannot be predicted with confidence from studying the response of these mice. Here we have used our recently generated, T cell–targeted, tetracycline-inducible T-bet–transgenic mice and bred them onto the T-bet knockout background, where a T_H2-skewed immune system was established during development (a state mimicking an atopic phenotype in early childhood).[35] Such mice lack expression of T-bet throughout development and thus will have spontaneous airway inflammation by 6 to 8 weeks of age. On induction with the tetracycline derivative doxycycline, the animal becomes a T-bet overexpressor, permitting the evaluation of T-bet overexpression in airway inflammation. Our results show that selectively inducing T-bet expression in T cells results in amelioration of allergic airway inflammation and suggest that small molecules that augment T-bet activity might be useful therapeutics in human asthma.

METHODS

Reagents

All cytokines and antibodies for ELISA, recombinant human IL-2, and anti-CD3 and anti-CD28 antibodies were purchased from BD PharMingen (San Diego, Calif). Doxycycline, ovalbumin (OVA), alum, pentobarbital, and methacholine were from Sigma-Aldrich (St Louis, Mo).

Generation of double-transgenic knockout mice

Double-transgenic knockout (DTg/KO) mice were constructed by means of the introduction into T-bet knockout mice[18] on a C57BL/6 genetic background of a tetracycline-inducible expression system (Invitrogen, Carlsbad, Calif). DTg/KO mice consist of both a tetracycline operon-responsive element (TRE) placed upstream of a cDNA encoding mouse T-bet (TRE-T-bet Tg) and a reverse tetracycline-controlled transcriptional activator linked to the human CD2 promoter (CD2-rtTA Tg).[36] Single-transgenic mice on the knockout background were bred together to generate double-transgenic progeny containing both transgenes. T-bet mRNA expression in these mice was restricted to the thymus, lymph nodes, and spleen. DTg/KO mice were fed with the tetracycline derivative doxycycline containing water freshly made up every other day. All mice were housed in specific pathogen-free conditions at Ewha Womans University, and all animal experiments were performed in accordance with Institutional Animal Care and Use Committee guidelines.

In vitro T-cell activation and ELISA

Single cell suspensions were prepared from draining lymph nodes and incubated with mouse CD4 or CD8 beads (Miltenyi Biotech, Auburn, Calif). Isolated $CD4^+$ or $CD8^+$ T cells were stimulated with anti-CD3 and anti-CD28 antibodies (BD PharMingen) in the presence or absence of doxycycline. Cell supernatants were collected and used for measuring cytokines by means of ELISA, according to the manufacturer's instructions (BD PharMingen and R&D Systems, Minneapolis, Minn).

OVA-induced murine asthma model and doxycycline administration

Mice were sensitized with OVA absorbed to alum on days 0 and 14 and challenged with 1% OVA by means of nebulization or intranasal administration, as indicated in the figures and figure legends. Mice were fed with 2% sucrose water as a control and doxycycline water (5 mg/mL in 2% sucrose) for the indicated time periods in the figures. Doxycycline water was replaced every 2 days.

Measurement of AHR

AHR was assessed as a change in airway resistance (in centimeters of H_2O pr milliliter per second) determined by using the FlexiVent system (SCIREQ, Montreal, Quebec, Canada) after delivery of aerosolized methacholine. Each mouse was anesthetized with pentobarbital sodium (70 mg/kg administered intraperitoneally), tracheostomized with an 18-gauge cannula, and mechanically ventilated with 10 mL/kg tidal volume at 160 breaths/min (FlexiVent, SCIREQ). A positive end-expiratory pressure of 3 cm H_2O was applied by submerging the expiratory line. Methacholine aerosol (3.1-100 mg/mL) was administrated for 10 seconds, after which airway resistance was recorded at 60 and 120 seconds. Lung resistance and compliance were measured with the FlexiVent system.

BALF analyses

Lungs were lavaged through the tracheal tube with 1 mL of HBSS (Gibco, Grand Island, NY). Total cell numbers were counted with a hemocytometer. Differential cell counts were performed in a blinded fashion by counting at least 200 cells on cytocentrifuged preparations (Model Cytospin 3; Shandon Ltd, Runcorn, United Kingdom) stained with Leukostat (Fisher Diagnostics, Fair Lawn, NJ). Lungs were harvested and homogenized in PBS containing 0.05% Triton X-100 and Complete Protease Inhibitor (Roche Applied Science, Mannheim, Germany). Cytokines in the lung homogenates were determined by using ELISA kits. Detection limits were 15 pg/mL for IL-5, 1.5 pg/mL for IL-13, and 50 pg/mL for IFN-γ.

Histologic analyses

After obtaining BALF, the lungs were fixed in formalin (Sigma-Aldrich) and embedded in paraffin. Tissue sections of 4 to 5 μm in thickness were stained with hematoxylin and eosin for routine histologic studies and periodic acid–Schiff (PAS) and Masson trichrome for identification and quantification of mucus-containing goblet cells and collagen deposition. The number of PAS^+ cells per millimeter of epithelial basement membrane was applied for quantitative analysis. For quantitative analysis of collagen content in the lung, Masson trichrome–stained lung parenchyma was photographed with an Olympus BX40 microscope in conjunction with an Olympus U-TV0.63XC digital camera (Olympus Corp, Melville, NY). Images were

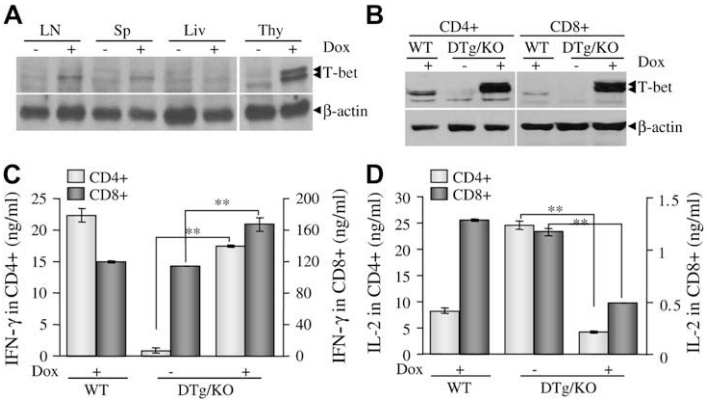


FIG 1. DTg/KO mice express T-bet in a T cell–specific and doxycycline *(Dox)*–inducible manner. **A**, DTg/KO mice on the C57BL6 genetic background were given control *(−)* or doxycycline *(+)* water for 4 weeks (n = 6). T-bet expression was analyzed in the lymph nodes *(LN)*, spleen *(Sp)*, liver *(Liv)*, and thymus *(Thy)* of DTg/KO mice. **B-D**, $CD4^+$ and $CD8^+$ T cells were isolated from C57BL6 WT and DTg/KO mice (n = 6) and stimulated with anti–T-cell receptor for 24 hours in the absence *(−)* or presence *(+)* of doxycycline. Whole cell extracts were resolved by means of SDS-PAGE and incubated with monoclonal T-bet antibody (Fig 1, *B*). Cell supernatants from the activated $CD4^+$ and $CD8^+$ T cells were collected for measuring the cytokines IFN-γ (Fig 1, *C*) and IL-2 (Fig 1, *D*). Data were expressed as means ± SD (n = 6). $^{**}P < .001$.

acquired by using DP Controller and Manager software (Olympus Corp) and then analyzed with MetaMorph 4.6 software (Universal Imaging Corp, Downingtown, Pa). Detection thresholds were set for the red color based on an intensity-labeled point and a default color threshold. The degree of labeling in each section (as a percentage of relative red staining) was determined from the area within the color range divided by the area of interest. All sections were examined in a single-blind fashion.[37]

Statistical analysis

Statistical comparisons were performed with the unpaired Student *t* test. A *P* value of less than .05 was considered statistically significant. Data are presented as means ± SD, and *P* values for significance are described in the figure legends.

RESULTS

T-bet expression is induced in T cells in DTg/KO mice by doxycycline treatment

We generated a line of transgenic mice (TgTRE-T-bet) expressing a T-bet cDNA downstream of the TRE from the tetracycline operon. These mice have been crossed to CD2-rtTA mice (TgrtTA). In this experimental design the reverse tetracycline-controlled transactivator (rtTA) is constitutively expressed in CD2-expressing cells (ie, T cells). However, in the absence of doxycycline, rtTA is unable to bind to the TRE, and T-bet should not be expressed. In contrast, addition of doxycycline allows rtTA to initiate transcription at the TRE, and T-bet should be expressed. This line was bred onto the T-bet knockout strain (DTg/KO). DTg/KO mice contain 2 transgenes (TRE-T-bet and CD2-rtTA) and lack T-bet, as confirmed by genotyping (see Fig E1, *A*, in this article's Online Repository at www.jacionline.org). Wild-type (WT), knockout, or DTg/KO mice were given doxycycline-containing drinking water for 14 days, and T-bet expression in lymphoid organs was determined by means of Western blot analysis. Endogenous T-bet is expressed in the thymus but is barely detectable without stimulation. As expected, we did not detect T-bet expression in WT or knockout thymus, but T-bet was readily detected in DTg/KO thymus after doxycycline treatment, showing 2 bands that could be phosphorylated and unphosphorylated forms (see Fig E1, *B*).[15,19] DTg/KO mice given doxycycline water for 4 weeks displayed T-bet expression in the thymus, lymph nodes, and spleen but not in other organs, such as the liver, lungs, heart, or kidneys (Fig 1, *A*, and data not shown). To confirm the T cell–specific expression of T-bet in DTg/KO mice, we have isolated $CD4^+$ and $CD8^+$ T cells and $B220^+$ B cells and stimulated them with anti–T-cell receptor or phorbol 12-myristate 13-acetate/ionomycin for 24 hours. T-bet expression was induced by doxycycline treatment in the activated $CD4^+$ and $CD8^+$ T cells but not B cells (Fig 1, *B*, and see Fig E1, *C*). Doxycycline-induced T-bet expression in T-bet–deficient $CD4^+$ T cells increased IFN-γ production to levels comparable with those seen in WT $CD4^+$ T cells. And T-bet–restored $CD8^+$ cells even increased IFN-γ values, although IFN-γ production was normal in T-bet–deficient $CD8^+$ T cells (Fig 1, *C*). Furthermore, introduced T-bet suppressed IL-2 production in both $CD4^+$ and $CD8^+$ T cells, but no changes of T-bet expression and IL-2 levels were detected in DTg/KO B cells (Fig 1, *D*, and see Fig E1, *D*). Hence DTg/KO mice display convincing doxycycline-inducible and T cell–specific expression of T-bet.

T cell–specific expression of T-bet prevents the development of OVA-induced airway inflammation

To test the role of T-bet expression in the development of airway inflammation, we used an OVA-induced asthma model (see Fig E2, *A*, in this article's Online Repository at www.jacionline.org). As previously reported,[22,23] T-bet–deficient lungs revealed eosinophil and lymphocyte infiltration in the absence of antigen (see Fig E2, *B*). Airway remodeling and IL-13 production in the lung were exaggerated by OVA challenge and sensitization, and IL-5, IL-13, and IL-17 levels in lymphocytes of draining lymph nodes were significantly increased in T-bet knockout mice (see Fig E2, *C*, and E3 in this article's Online Repository at www.jacionline.org). OVA sensitization and nebulization, as presented in Fig E4, *A*, (available in this article's Online Repository at www.jacionline.org), also induced AHR to methacholine in DTg/KO mice fed with control water. However, the development of AHR was suppressed in DTg/KO mice fed

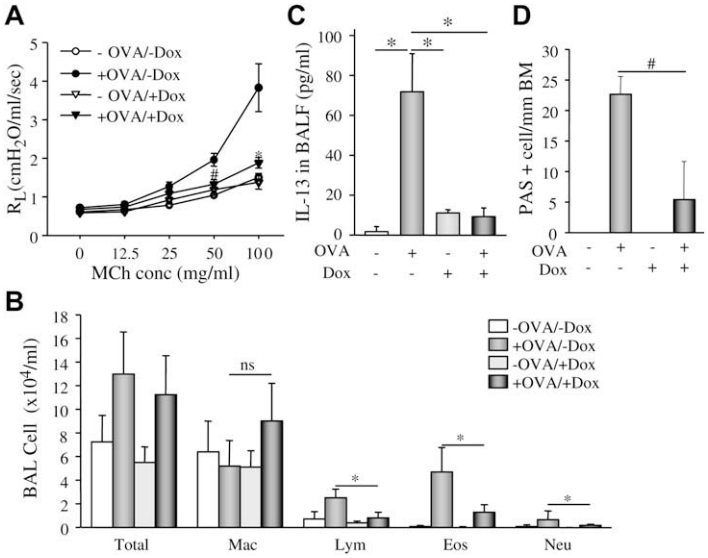


FIG 2. T-bet overexpression attenuates allergic airway inflammation. DTg/KO mice were sensitized and challenged with OVA concomitant with doxycycline *(Dox)* administration (n = 6 per group). **A**, AHR was measured by using airway resistance *(R_L)* to methacholine. **B**, Immune cells in BALF were counted. **C**, IL-13 expression in BALF was measured by means of ELISA. **D**, Lung tissues were stained with PAS, and the number of PAS^+ cells per millimeter of epithelial basement membrane was counted. Data are presented as means ± SD for 6 mice in each group. *ns*, Not significant; *Mac*, macrophages; *Lym*, lymphocytes; *Eos*, eosinophils; *Neu*, neutrophils. #$P < .05$, *$P < .01$.

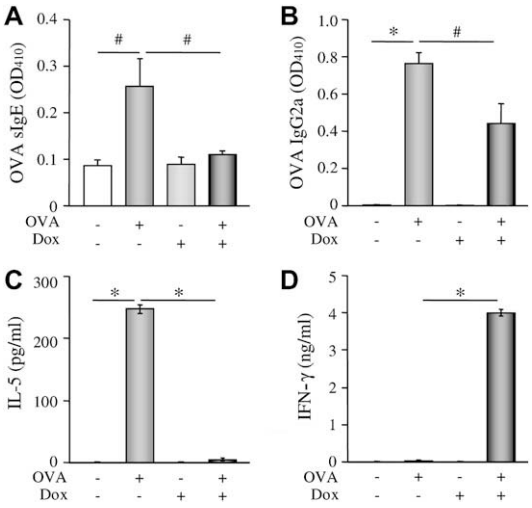


FIG 3. OVA-specific sIgE, IgG2a, and cytokines were modulated by T-bet. DTg/KO mice were sensitized and challenged with OVA concomitant with doxycycline *(Dox)* administration (n = 6 per group). OVA-specific sIgE **(A)** and IgG2a **(B)** levels were measured in the serum by means of ELISA. **C**, Lymphocytes were isolated from draining lymph nodes and stimulated with anti-CD3 (2 μg/mL) overnight. Supernatants were collected for measuring the cytokines IL-5 (Fig 3, *C*) and IFN-γ **(D)**. Data are presented as means ± SD for 6 mice. #$P < .05$, *$P < .01$.

with doxycycline water coincident with OVA sensitization (Fig 2, *A*). In addition, increased eosinophilic and lymphocytic inflammation and IL-13 production in BALF caused by OVA sensitization and challenge were substantially reduced in DTg/KO mice fed with doxycycline water (Fig 2, *B* and *C*). PAS staining of respiratory epithelial goblet cells revealed the significant reduction of PAS^+ goblet cells through the induction of T-bet (Fig 2, *D*, and see Fig E4, *B*).

Induction of T-bet expression also suppressed production of IgE and T_H2 cytokines

We next performed an ELISA to determine the level of OVA-specific circulating IgE (sIgE), which is a key mediator and diagnostic marker of allergic asthma. OVA-specific sIgE and IgG2a antibodies were increased in the absence of T-bet but reduced by restoration of T-bet expression (Fig 3, *A* and *B*). In addition, cytokine production by T cells in the draining lymph node was assayed by means of ELISA. Consistent with previous reports of T-bet overexpression *in vitro*,[17] lymphocytes from DTg/KO mice fed with doxycycline had diminished production of the T_H2 cytokine IL-5 and, concomitantly, increased levels of IFN-γ (Fig 3, *C* and *D*). Although IL-4 production was barely detected in the lung and lymph nodes by means of ELISA, IL-4 mRNA levels in the lung determined by means of real-time PCR were reduced by the induction of T-bet (data not shown).

Induction of T-bet expression in chronic airway inflammation also attenuates AHR and eosinophilia

When we induced T cell–specific T-bet expression at the same time as OVA sensitization, the development of allergic airway inflammation was significantly reduced *in vivo* (Fig 2, *A*). To more faithfully mimic the situation in human asthmatic patients, we next asked whether induction of T-bet expression once airway inflammation has already begun to develop might dampen the progression of acute and chronic airway inflammation. DTg/KO mice were sensitized with OVA and fed with doxycycline water for 10 days before death. T-bet induction after OVA sensitization in an acute asthma model also decreased AHR but did not reduce eosinophilic inflammation (see Fig E5 in this article's Online Repository at www.jacionline.org). Furthermore, DTg/KO mice were sensitized and challenged with OVA for 8 weeks and fed with doxycycline water for the entire period (Dox group) or for

2 weeks before death (DoxL group; Fig 4, *A*). We evaluated whether T-bet expression in the DoxL group during chronic airway inflammation attenuated asthmatic symptoms compared with those seen in knockout and DTg/KO mice given doxycycline for the entire period. The expression levels of T-bet were assessed in the thymus and revealed comparable amounts of T-bet between the Dox and DoxL groups (Fig 4, *B*). Notably, induction of T-bet expression in the DoxL group reduced airway resistance to methacholine comparably with the Dox group (Fig 4, *C*). Consistent with the decreased AHR observed, the number of eosinophils in BALF was markedly reduced in the Dox group and less but still significantly reduced in DoxL mice. Numbers of lymphocytes were decreased by T-bet overexpression; however, no significant changes were detected in macrophages (Fig 4, *D*).

Induction of T-bet reduces asthma-associated cytokines

Measurement of cytokines from lung homogenates revealed that IFN-γ was significantly increased in both the Dox and DoxL groups (Fig 5, *A*), whereas both IL-5 and IL-13 cytokines were reduced in both groups (Fig 5, *B* and *C*). There was no significant difference between the Dox and DoxL groups. Measurement of cytokine production in draining lymph nodes revealed increased IFN-γ levels in both Dox groups, although statistically less in the DoxL group (Fig 5, *D*). Levels of the T_H2 cytokines IL-5 and IL-13 were decreased by the induction of T-bet in both groups and were not significantly different (Fig 5, *E* and *F*). IL-17 levels were recently reported to be increased in patients with asthma and modulated by T-bet (see Fig E3, *D*).[38,39] We found that restoration of T-bet expression consistently decreased IL-17 production (Fig 5, *G*).

The duration of T-bet expression correlates with asthmatic phenotype

OVA sensitization and challenge induce intense inflammation characterized by an eosinophilic and mononuclear cell infiltrate along with significant mucus-like protein secretion. As shown in Fig 6, *A*, although eosinophilic inflammation was widespread, with dense cellular infiltration seen in T-bet knockout mice, it was significantly decreased in mice expressing T-bet. However, the amelioration of the airway inflammation was less marked in the DoxL group. Quantitation of PAS^+ cells revealed a statistical difference between the Dox and DoxL groups (Fig 6, *B*). In addition, the peribronchial collagen deposition around the airways observed in OVA-injected mice was significantly decreased by T-bet induction (Fig 6, *C*). Taken together, these data suggest that induction of T-bet in the setting of a chronic asthma model can suppress the progression of chronic allergic airway inflammation.

DISCUSSION

Allergic asthma is recognized as a T_H2-mediated immune system disease. Whether the induction of allergen-specific T_H1 cells can attenuate allergic asthma remains controversial. Several studies have shown that administration of T_H1 cytokines, such as IFN-γ and IL-12, at the time of sensitization can inhibit the induction of AHR and T_H2-driven inflammation.[40-42] However, contradictory results involving adoptive transfer of allergen-specific T_H1 cells have been obtained.[43-45] The relative importance of a T_H1 immune response in allergic diseases might differ between the

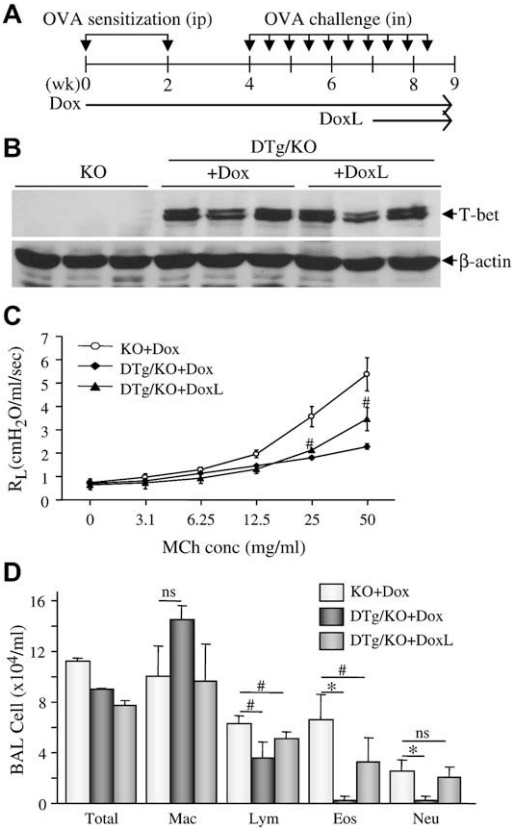


FIG 4. Chronic airway inflammation was attenuated by the induction of T-bet. **A**, Scheme of the chronic asthma murine model. **B**, Expression levels of T-bet were analyzed by means of immunoblotting with thymic extracts of 3 mice per group. **C**, AHR was measured based on airway resistance *(R_L)* to methacholine *(MCh)* in 3 groups of doxycycline-fed mice (n = 6 per group). #*P* < .05. **D**, Cytospin preparations of immune cells in the BALF were counted. *Dox*, Doxycycline; *KO*, knockout; *ns*, not significant; *Mac*, macrophages; *Lym*, lymphocytes; *Eos*, eosinophils; *Neu*, neutrophils. #*P* < .05, **P* < .01. Data are presented as means ± SD for 6 mice.

allergen sensitization and challenge phases. Meyts et al[46] reported that responses to the neutralization of IL-12 vary at different time points of administration during the asthmatic process. Neutralization of IL-12 during OVA sensitization aggravated AHR and eosinophilic inflammation, whereas neutralization of IL-12 during the OVA challenge phase attenuated AHR, eosinophilic inflammation, and expression of T_H2 cytokines, such as IL-5 and IL-13, through an IFN-γ–dependent pathway.[46] In addition, other groups[47] reported aggravation of airway inflammation by means of adoptive transfer of OVA-specific T_H1 cells just before OVA challenge. Further complicating the picture, lung IFN-γ might have pleiotropic actions in murine asthma models. It might enhance expression of IL-5 and IL-13 while simultaneously suppressing the development of AHR.[48]

Increasing evidence from both murine and human studies highlights T-bet as a central molecule in the pathobiology of asthma. We have demonstrated that common T-bet polymorphisms are associated with asthma and related clinical phenotypes, including AHR and response to corticosteroid therapy. A nonsynonymous polymorphism that replaces histidine for glutamine at amino acid residue 33 (H33Q) is present at 2.7% in asthmatic populations and is the only common coding polymorphism

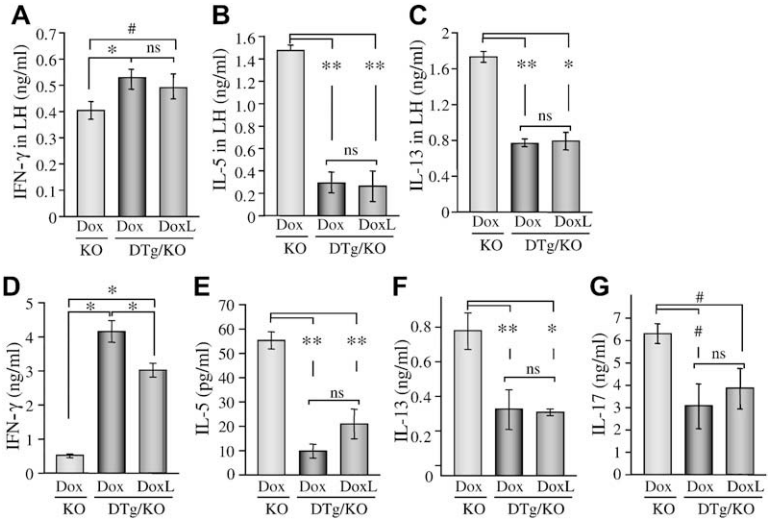


FIG 5. Restoration of T-bet expression modulates cytokine production. Lung homogenates were harvested from the Dox (knockout *[KO]*), Dox (DTg/KO), and DoxL (DTg/KO) groups (n = 6 per group), as depicted in Fig 4, *A*, and used for measuring the cytokines IFN-γ **(A)**, IL-5 **(B)**, and IL-13 **(C)**. Lymphocytes were isolated from draining lymph nodes and stimulated with anti-CD3 antibody overnight, and supernatants were harvested for ELISA of IFN-γ **(D)**, IL-5 **(E)**, IL-13 **(F)**, and IL-17 **(G)**. *Dox*, Doxycycline; *ns*, Not significant; *KO*, knockout. #$P < .05$, *$P < .01$, **$P < .001$. Data are presented as means ± SD for 6 mice.

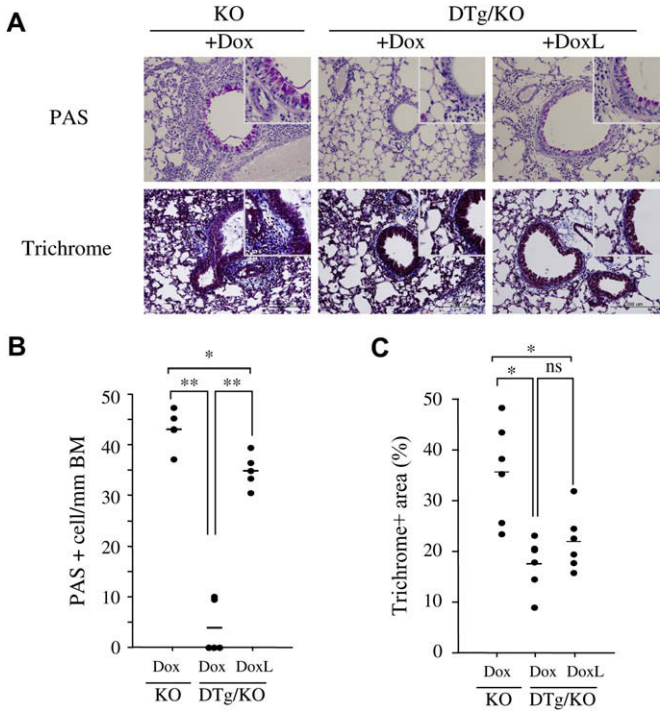


FIG 6. Goblet cell hyperplasia and collagen deposition were decreased by the induction of T-bet (n = 6 per group). Lung tissues were collected from the Dox (knockout *[KO]*), Dox (DTg/KO), and DoxL (DTg/KO) groups, as depicted in Fig 5, *A*, and cut at 5-μm thickness. **A**, Lung tissue sections were stained with PAS and Masson trichrome. **B**, Quantitative analysis of PAS^+ cells was performed (n = 5). **C**, Collagen contents in the lung (n = 6) were calculated by using image analysis. *$P < .01$, **$P < .001$. *Dox*, Doxycycline; *KO*, knockout; *ns*, not significant.

described in T-bet. Carriers of the T-bet H33Q polymorphism demonstrate enhanced responsiveness to corticosteroids in the treatment of asthma.[32] Other groups have published an association between aspirin-induced asthma and polymorphisms at the T-bet locus[29] and additional associations of TBX21 variants and asthma.[31,49] Together, these studies provide substantial evidence that T-bet polymorphisms affect asthma and its related phenotypes. Furthermore, a clinical association between T-bet expression and asthma was evidenced by the reduced levels of T-bet in T cells of asthmatic patients compared with those of control patients.[28]

Here we examined whether the induction of T-bet can block or prevent allergic asthma development induced by OVA sensitization and challenge. Our study shows that selective overexpression of the T-bet gene primarily in T cells can both prevent the development of airway inflammation and suppress an ongoing chronic inflammation. Timed induction of T-bet expression during both acute and chronic allergic airway inflammation attenuated AHR, eosinophilic inflammation, goblet cell hyperplasia of the respiratory epithelium, and collagen deposition in the lung. These effects were accompanied by reduced production of the T_H2 cytokines IL-5 and IL-13 and enhanced expression of IFN-γ in both the lung and the draining lymph node. Interestingly, production of the newly described cytokine IL-17, reported to be controlled by T-bet, was reduced as well. Moreover, levels of immunoglobulins classically associated with allergic asthma, in particular sIgE and IgG2a, were decreased by overexpression of T-bet in the asthma model. These results are consistent with earlier studies.[50] Our study suggests the possibility that manipulation of the T-bet gene or its products might be useful for the prevention or control of allergic asthma.

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Clinical implications: Small molecules that augment the expression or activity of T-bet might be beneficial therapeutic agents for asthma.

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METHODS

Genotyping of DTg/KO mice by means of PCR

Tail genomic DNA was prepared from DTg/KO mice and used for genotyping by means of PCR. We have used primers as follows[E1]:

PCR primers of the TRE-T-bet transgene

TRE-T-bet forward 5′- GAAGGATCCAAGGAAGCGACCC-3′
TRE-T-bet reverse 5′-AGCAGAGCTCGTTTAGTGAACC-3′

PCR primers of the CD2-rtTA transgene

rtTA forward 5′-TAAAAGGGGAAAGCTGGCAAGATT-3′
rtTA reverse 5′-CCGCGGGGAGAAAGGACA-3′

PCR primers of the T-bet mutant of knockout mice

T-bet 596 forward 5′-TGGGCATACAGGAGGCAGGAACAAATA-3′
T-bet 314 reverse 5′-GACTGAAGCCCCGACCCCCACTCCTAAG-3′
pGKPR-1220 reverse 5′-GCGCGAAGGGGCCACCAAAGAACGGAG-3′.

OVA sensitization and challenge protocol for acute and chronic asthma models

Mice were sensitized by means of intraperitoneal injection with OVA absorbed to alum on days 0 and 14. Nonsensitized control mice received alum in saline. Sensitized mice were challenged with 1% OVA by means of nebulization or intranasal administration, as indicated in the figures and figure legends. Mice were fed with 2% sucrose water as a control and doxycycline water (5 mg/mL in 2% sucrose) for the time periods indicated in the figures.

Measurement of IL-13 in the BALF

Lungs were lavaged through the tracheal tube with 1 mL of HBSS (Gibco). Cytokines in BALF were determined by means of ELISA with a commercial ELISA kit (detection limits, −1.5 pg/mL for IL-13; R&D Systems).

In vitro T-cell activation and ELISA

Single cell suspensions were prepared from the lymphocytes of draining lymph nodes. Total lymphocytes were stimulated with anti-CD3 (BD PharMingen) overnight, and supernatants were collected for ELISA. Cell supernatants were collected and used for measuring the cytokines IL-2, IL-5, IL-13, and IL-17 by means of ELISA according to the manufacturer's instructions (BD PharMingen). All cytokines and antibodies for ELISA were from R&D Systems.

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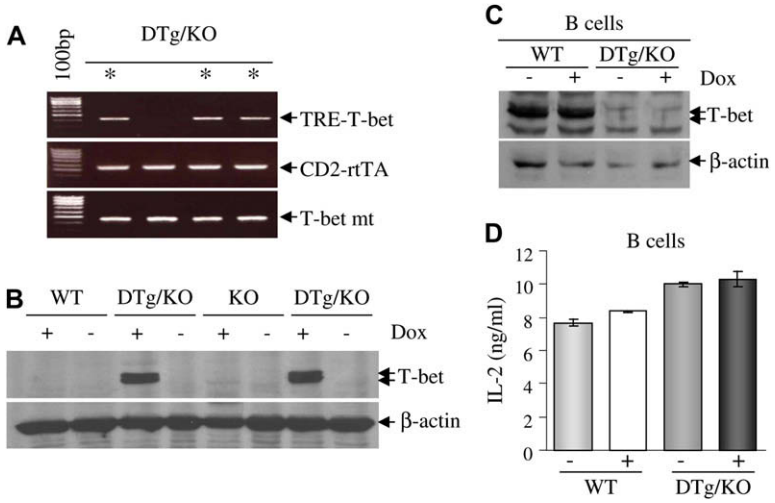


FIG E1. Generation of DTg/KO mice. **A**, The TRE-T-bet and CD2-rtTA transgenes and the mutant T-bet allele of DTg/KO mice were genotyped by means of PCR as indicated. *Asterisks* indicate DTg/KO mice. **B**, WT, T-bet knockout, and DTg/KO mice were given control *(−)* or doxycycline water *(+)* for 14 days (n = 5), and thymic whole-cell extracts were analyzed for T-bet protein expression by means of immunoblotting with the 4B10 T-bet mAb and anti-β-actin antibody. **C**, B220+ cells were isolated from WT and DTg/KO mice (n = 4) and stimulated with phorbol 12-myristate 13-acetate and ionomycin for 24 hours in the absence *(−)* and presence *(+)* of doxycycline. Cell extracts were harvested and resolved by means of SDS-PAGE, followed by immunoblotting of T-bet. **D**, Cell supernatants were assayed for measuring IL-2 cytokines by means of ELISA. Data are presented as means ± SDs for 4 mice. *Dox*, Doxycycline; *KO*, knockout.

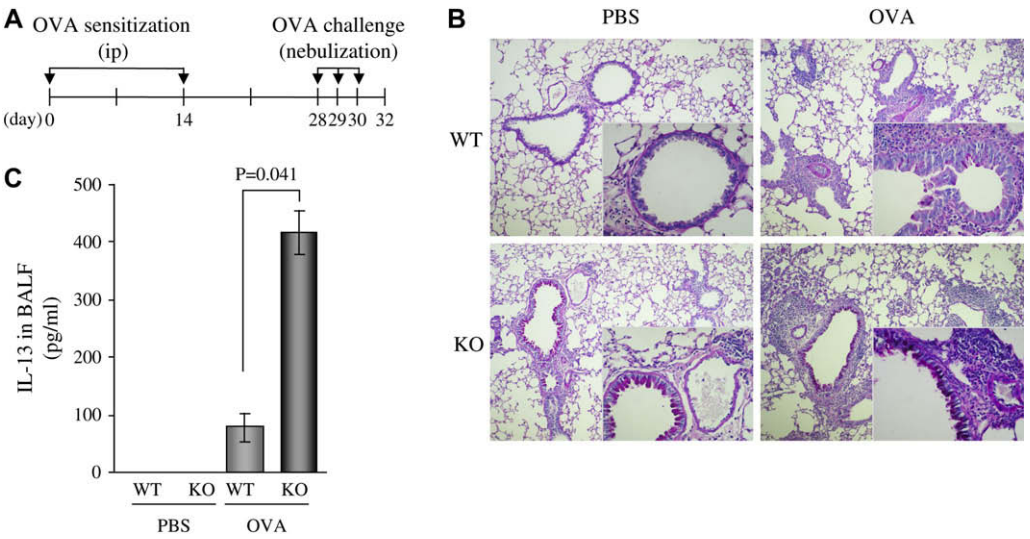


FIG E2. T-bet deficiency increased allergen-induced airway inflammation. **A**, Scheme of the OVA-induced asthma murine model. WT and T-bet knockout *(KO)* mice were sensitized and challenged with 1% OVA or PBS (n = 5). Mice were analyzed at day 32. **B**, Lung tissues were sectioned and stained with PAS. **C**, IL-13 production was measured in lung homogenates by means of ELISA. Data are presented as means ± SD (n = 5).

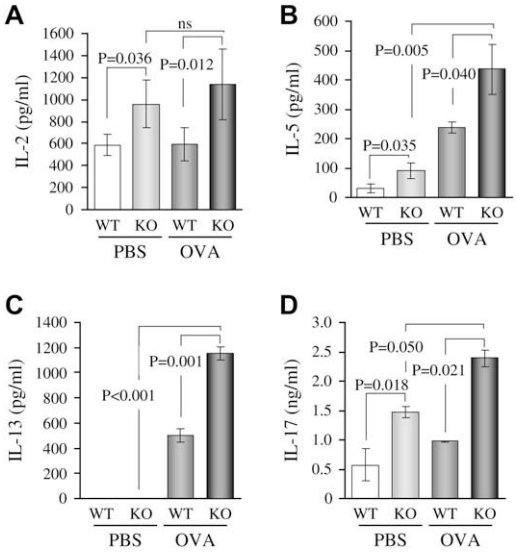


FIG E3. Asthma-related cytokines were increased in T-bet knockout *(KO)* mice. WT and T-bet knockout mice were sensitized and challenged with OVA, as described in Fig E2 (n = 5). Single cell suspensions of lymphocytes were obtained from draining lymph nodes and stimulated with anti-CD3 overnight. Cell supernatants were collected for measuring the cytokines IL-2 **(A)**, IL-5 **(B)**, IL-13 **(C)**, and IL-17 **(D)** by means of ELISA. Data are presented as means ± SD for 5 mice. *ns*, Not statistically significant.

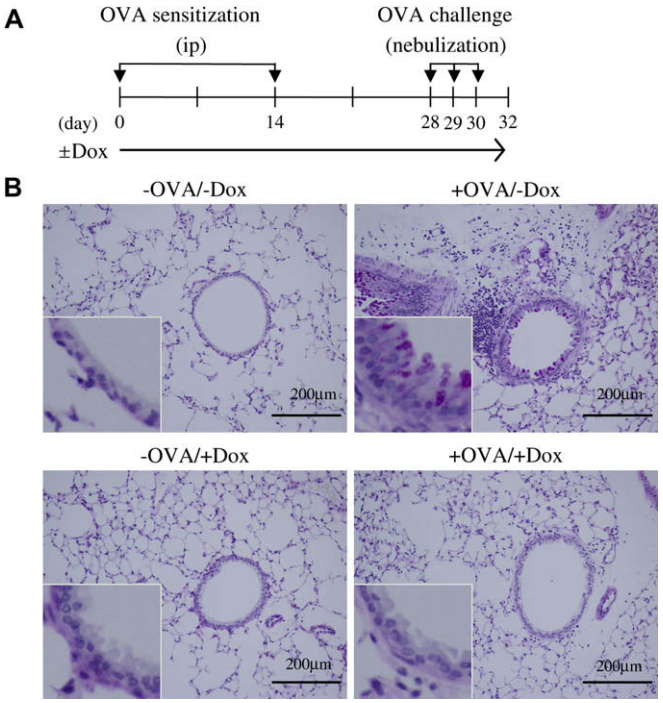


FIG E4. Allergen-induced eosinophilic inflammation was protected by T-bet expression. **A**, Scheme of the allergen-induced asthma model. DTg/KO mice were sensitized and challenged with OVA and killed at day 32. **B**, Representative photomicrographs of PAS staining of lung sections are shown (n = 6 per group). *ip*, Intraperitoneal; *Dox*, doxycycline.

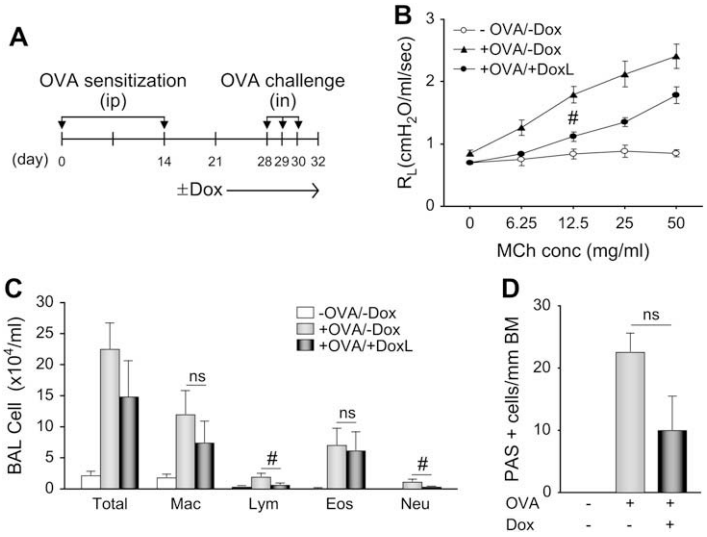


FIG E5. Progression of airway inflammation was attenuated by T-bet expression. **A**, Scheme of the allergen-induced asthma model. OVA-sensitized DTg/KO mice were fed with doxycycline water and challenged with OVA by means of intranasal administration (n = 6 per group). *ip*, Intraperitoneal; *in*, intranasal. **B**, AHR to methacholine *(MCh)* was measured in each group (n = 6). **C**, Immune cells in the BALF were counted (n = 6). *Mac*, macrophage; *Lym*, lymphocytes; *Eos*, eosinophils; *Neu*, neutrophils. **D**, Lung tissue sections were stained with PAS, and PAS^+ cells counted are shown (n = 6). Data are presented as means ± SDs for 6 mice. *ns*, Not significant. #$P < .05$.