Lack of WDFY4 Aggravates Ovalbumin-Induced Asthma via Enhanced Th2 Cell Differentiation

Yan Li  Anran Wang  Feng Long  Fei Gao  Shang Gao  Shijun Wei  Ai Liu  Xi Li  Wenjie Sun  Jiangxia Li  Qiji Liu

Key Laboratory for Experimental Teratology of the Ministry of Education, Department of Medical Genetics, School of Basic Medical Sciences, Cheeloo College of Medicine, Shandong University, Jinan, China

Keywords
WDFY4 · Th2 cell · Asthma

Abstract
Background: Asthma is a chronic inflammatory airway disease, and Th2 cells play an important role in asthma. WDFY4 (WDFY family member 4) is a susceptibility gene in several autoimmune diseases. Objective: In this study, the roles of WDFY4 in Th2 cell differentiation and Th2-dependent asthma were investigated. Methods: Naïve CD4+ T cells were isolated from wild-type and WDFY4-deficient mice and induced to differentiate in vitro. Subsequently, a mouse model of asthma was established by sensitization with ovalbumin. Results: Study results showed that WDFY4 deficiency could promote the differentiation of Th2 cells and the production of Th2 cytokines. WDFY4-deficient asthmatic mice showed higher levels of Th2 cytokines in the lungs and bronchoalveolar lavage fluid than wild-type mice. Moreover, infiltration of inflammatory cells, hyperplasia of goblet cells, production of mucus, and deposition of collagen were enhanced in WDFY4-deficient asthmatic mice. Conclusions: Our study demonstrates the pivotal role of WDFY4 in the pathogenesis of asthma and in Th2 cell differentiation.

Introduction
Asthma is a chronic disease characterized by airway inflammation, airway hyperresponsiveness and airway remodeling [1]. It affects more than 300 million people worldwide [2]. A variety of immune cells and their corresponding cytokines are involved in the development of asthma. The important role of Th2 cells in asthma has been confirmed by previous studies [3].

Inhaled allergens can induce the differentiation of Th2 cells, which leads to the production of allergen-specific IgE by B cells, activation of mast cells, and the recruitment of eosinophils to the lungs; all these processes eventually cause persistent airway inflammation along with symptoms of asthma [4, 5]. Th2 cells are involved in the development of asthma mainly via the secretion of Th2 cytokines, such as IL-4, IL-5 and IL-13 [6]. IL-4 is involved in the synthesis of the immunoglobulin IgE, activation of eosinophils, production of mucus, and remodeling of the airways [7]. IL-5 is important for the differentiation, maturation and survival of eosinophils [6]. As a major regulator of asthma, IL-13 can be involved in the regulation of eosinophilic inflammation, mucus secretion and airway hyperresponsiveness [6].
WDFY4 (WDFY family member 4) is the fourth member of the WDFY family and is conserved among multiple species. Several studies have revealed associations between WDFY4 and autoimmune diseases, such as systemic lupus erythematosus (SLE), rheumatoid arthritis, juvenile idiopathic arthritis and clinically amyopathic dermatomyositis [8–11]. These studies imply an important role for WDFY4 in the immune system. Recently, two studies revealed the involvement of WDFY4 in cross-presentation of cell-associated antigens [12] and maintenance of B cells [13]. Global knockout mice showed that WDFY4 had no influence on the development of conventional type 1 dendritic cells (cDC1s). However, deficiency in WDFY4 could impede the cross-presentation of cell-associated antigens by cDC1s and result in an impaired antigen-specific immune response [12]. Moreover, WDFY4 could modulate B cell fate via noncanonical autophagy. Selective deficiency in WDFY4 in B cells could lead to impaired antibody responses; thus, SLE phenotypes induced by pristane were effectively alleviated in WDFY4 conditional knockout mice [13]. A new method based on two linked approaches to speculate on gene function and connect genes with biological processes was developed by Li et al. [14]. In this way, they found that WDFY4 is associated with antigen processing, T-cell activation and the immune response in humans, rats and mice. However, this work was a mathematical study rather than a biological study. The specific effects of WDFY4 on T cells remain unclear and need to be explored in further biological studies.

Here, we demonstrated that lack of WDFY4 could enhance the differentiation of Th2 cells and the production of Th2 cytokines. As a result, WDFY4-deficient mice showed more severe airway inflammation and airway remodeling than wild-type mice. Our findings contribute to the understanding of the involvement of WDFY4 in Th2 cell differentiation and establish a link between WDFY4 and asthma.

Materials and Methods

Mice

The genetic background of the mice was C57BL/6. WDFY4-deficient mice were constructed based on the LoxpP-Cre system as described previously [13]. Lck-Cre+/− transgenic mice were crossed with Wdfy4floxp/floxp mice to generate conditional knockout (referred to as CKO) mice with selective deficiency in WDFY4 in T cells and Wdfy4floxp/floxp mice (referred to as WT mice) in the same litter were used for comparison. All mice were maintained under specific-pathogen-free conditions. All experiments involving mice were approved by the Animal Experiment Ethics Review Committee of Shandong University School of Medicine.

Isolation of Splenocytes and Naïve CD4+ T Cells

Spleens were processed through nylon mesh cell strainers (BD Biosciences, USA) to obtain single-cell suspension. Naïve CD4+ T cells were purified from splenocytes using the naïve CD4+ T-Cell Isolation Kit (Miltenyi Biotec, Germany) according to the manufacturer’s protocol.

Differentiation in vitro

Splenocytes and naïve CD4+ T cells were cultured with plate-bound anti-CD3 (10 μg/mL) and soluble anti-CD28 (6 μg/mL) to differentiate the cells into Th1, Th2, and Th17 cells. For Th2 polarization, naïve CD4+ T cells were cultured with plate-bound anti-CD3, soluble anti-CD28, IL-4 (10 ng/mL), and anti-IFN-γ (10 μg/mL). Cells were cultured for 44 h and then treated with Cell Stimulation Cocktail. After 4 h, the cells were collected for flow cytometry. Anti-CD3 and anti-CD28 were purchased from BioLegend (USA). Murine IL-4 was obtained from PeproTech (USA). Anti-IFN-γ was obtained from BD Biosciences. Cell Stimulation Cocktail was from eBioscience (USA).

Flow Cytometry

Cultured cells were stained with anti-CD4 and then fixed with paraformaldehyde. Triton X-100 was used for permeabilization, and then the cells were stained with anti-IFN-γ, anti-IL-4 and anti-IL-17a. Data were analyzed by using FlowJo (TreeStar, USA). Anti-CD4, anti-IFN-γ and anti-IL-17a were purchased from BD Biosciences. Anti-IL-4 was purchased from BioLegend (USA). Murine IL-4 was obtained from PeproTech (USA), respectively.

RNA Extraction and Quantitative RT-PCR

RNA extraction, reverse transcription and quantitative RT-PCR were performed as described previously [15]. Relative expression of the target gene was normalized to the level of Gapdh, calculated by the 2−ΔΔCt method. The sequences of the primers (5′–3′) were as follows: Gata3 TCCGGTCTCATTACGGAAAC/CAGCAGAGAGGATGA; c-Maf GGAGACCGACCGCATGTCGTTT/CCCTCGACTCCTACATCTTCTGA; IL-4 GGGCGAGTGCTGTGCTTGCGGTTACAGAGG; and Col1a1 CAGTACCC; IL-13 TGAGCAACATCACAAGAGCC/GGCCCTGGGGTTACAGAGG; and Colla1 AGGGCGAGTGCTGTGCTTGCGTTT/CCCTCGACTCCTACATCTTCTGA.

Western Blot Analysis

Western blotting was performed to detect the expression of GATA3 in Th2 cells. The details were described previously [15]. Antibodies against mouse GATA3 and GAPDH were purchased from Proteintech (USA).

ELISA

The concentration of IL-4 in the medium supernatant and bronchoalveolar lavage fluid (BALF) was detected by using the Mouse IL-4 Precoated ELISA Kit (Dakewe, China) according to the manufacturer’s protocol.
Fig. 1. a–c. Splenocytes from WT mice and CKO mice were treated with anti-CD3 (10 μg/mL) and anti-CD28 (6 μg/mL) for 48 h. Intracellular cytokine staining was performed to assess the proportions of Th cells by flow cytometry. The numbers in the FACS pseudocolour images indicate the percentages of Th cells in the CD4+ T-cell population. d–f. Statistical results for a–c. Data are shown as the mean ± SEM (n = 4:4). *p < 0.05, Mann-Whitney U test.

g–i. Naïve CD4+ T cells were purified and treated with anti-CD3 (10 μg/mL) and anti-CD28 (6 μg/mL) for 48 h. Intracellular cytokine staining was performed to assess the proportions of Th cells by flow cytometry. In the FACS pseudocolour images, the numbers adjacent to the outlined areas indicate the percentages of CD4+ T cells or Th cells. j–l. Statistical results for g–i. Data are shown as the mean ± SEM (n = 2:2). Every mouse was evaluated in 2–3 replicates. *p < 0.05, Mann-Whitney U test. Only statistically significant results are marked.
Histopathological Analysis

The lungs of asthmatic mice were fixed with paraformaldehyde and dehydrated with an alcohol series. Subsequently, the samples were embedded in paraffin and cut into 5-μm sections. Then, the sections were stained with hematoxylin and eosin (HE), periodic acid-Schiff or Sirius red. Images were captured with a microscope (Olympus, Japan).

Statistical Analysis

All data were recorded as the mean ± SEM. GraphPad Prism (GraphPad Software, USA) was used to generate graphs. Data were analyzed by using Student’s t test or the Mann-Whitney U test, and p < 0.05 was considered statistically significant. All experiments were repeated independently at least three times.

Results

Lack of WDFY4 Promotes Th2 Cell Differentiation

To explore the influences of WDFY4 on T cells, WDFY4-deficient mice were constructed based on the LoxP-Cre system as described previously [13]. Mice with selective deficiency in WDFY4 in T cells were constructed by crossing Lck-Cre+/− mice and Wdfy4loxp/loxp mice. Conditional knockout mice were referred to as CKO mice, and Wdfy4loxp/loxp mice (referred to as WT mice) were used as controls.

Splenocytes from WT mice and CKO mice were treated with plate-bound anti-CD3 and soluble anti-CD28 for activation and cultured in vitro for 48 h. Then flow cytometry was performed to detect the proportions of Th cells. As shown in Figure 1, cells treated with anti-CD3 and anti-CD28 showed higher percentages of Th cells than cells treated with PBS. After activation, more Th1 cells and Th2 cells and fewer Th17 cells were observed in WDFY4-deficient T cells (Fig. 1a–f).

To further confirm the above results, naïve CD4+ T cells were isolated from WT mice and CKO mice using magnetic beads and activated in vitro. Consistently, deficiency in WDFY4 promoted the differentiation of naive CD4+ T cells into Th2 cells (Fig. 1h, k). However, there were no significant differences in the proportions of Th1 cells or Th17 cells between WT T cells and WDFY4-deficient T cells (Fig. 1g, i, j, l). Overall, lack of WDFY4 promotes the differentiation of Th2 cells.

WDFY4-Deficient Th2 Cells Express Relatively High Levels of Th2 Cytokines

Naïve CD4+ T cells from WT mice and CKO mice were purified and cultured under Th2 polarization conditions to clarify the involvement of WDFY4 in Th2 cell differentiation. Quantitative RT-PCR results showed upregulated expression of Gata3 and c-Maf, important transcription factors for Th2 cell differentiation, in WDFY4-deficient Th2 cells compared with WT Th2 cells (Fig. 2a). GATA3 is defined as the specific transcription factor for Th2 cell differentiation. Western blotting was performed to detect the expression of GATA3, and higher levels of
GATA3 were observed in WDFY4-deficient Th2 cells (Fig. 2b). Th2 cells participate in immune responses mainly by secreting cytokines, such as IL-4, IL-5 and IL-13. We found that deficiency in WDFY4 could promote the production of Th2 cytokines (Fig. 2c). The medium supernatant of cultured Th2 cells was collected, and the concentration of IL-4 in the medium supernatant was measured by ELISA. The result showed higher level of IL-4 in the medium supernatant of WDFY4-deficient Th2 cells than that of WT Th2 cells (Fig. 2d). The above data indicate the important roles of WDFY4 in the differentiation of Th2 cells and production of Th2 cytokines.

Deficiency in WDFY4 Promotes the Production of Th2 Cytokines in Asthmatic Mice

To determine whether WDFY4 is involved in asthma by influencing the differentiation of Th2 cells, an OVA-induced asthmatic mouse model was established (Fig. 3a). After challenge, the mice treated with OVA showed head and face itching, fidgeting, and crouching. The expression of Gata3 and c-Maf in the lungs was detected by quantitative RT-PCR, and upregulated expression of Gata3 and c-Maf was observed in WDFY4-deficient asthmatic mice (Fig. 3b). Moreover, the BALF was collected, and the level of IL-4 was higher in the BALF of WDFY4-deficient asthmatic mice than that of WT asthmatic mice (Fig. 3c). These data reveal that deficiency in WDFY4 promotes the production of Th2 cytokines in asthmatic mice.

Aggravated Airway Inflammation Was Observed in WDFY4-Deficient Asthmatic Mice

Airway inflammation is the pathological basis of bronchial asthma [16]. Hematoxylin and eosin (HE) staining was performed to evaluate airway inflammation in asthmatic mice. As shown in Figure 4a, infiltration of inflammatory cells was observed in asthmatic mice, and inflammatory cell infiltration was increased in WDFY4-deficient asthmatic mice compared with WT asthmatic mice (Fig. 4a). Goblet cell hyperplasia and increased mucus production are the characteristic features of allergic inflammation [6]. Mucus overproduction and goblet cell hyperplasia were observed in asthmatic mice, and WDFY4-deficient asthmatic mice exhibited more severe phenotypes (Fig. 4b). These results suggest that deficiency in WDFY4 can aggravate airway inflammation in asthmatic mice.

Deficiency in WDFY4 Promotes Airway Remodeling in Asthmatic Mice

Airway remodeling is one of the common features of chronic asthma, and collagen deposition is an important feature of airway remodeling. Sirius red staining was performed, and we found that the collagen deposition in WDFY4-deficient asthmatic mice was more serious than that in WT asthmatic mice (Fig. 4c). Consistently, quantitative RT-PCR result showed upregulated expression of Coll1a1 in WDFY4-deficient asthmatic mice (Fig. 4d). In general, deficiency in WDFY4 promotes airway remodeling in asthmatic mice.
Discussion

The first report on *WDFY4* was a genome-wide association study that identified variants in *WDFY4* associated with SLE in Asian populations [8]. Subsequently, the association between *WDFY4* and SLE has been confirmed in several populations [17–19]. Zhao et al. [20] revealed the mechanism by which *WDFY4* leads to SLE susceptibility. They found that the functional variant rs877819 of *WDFY4* could specifically bind to the transcription factor YY1 and that the affinity between rare gene A and YY1 was significantly lower than

---

**Fig. 4.** a Lung sections were stained with HE; magnification, 40×. Bar = 200 μm. b Lung sections were stained with PAS; magnification, 100×. Bar = 100 μm. c Lung sections were stained with Sirius red; magnification, 100×. Bar = 100 μm. d The mRNA level of *Col1a1* in the lungs of asthmatic mice was detected by quantitative RT-PCR. Data are shown as the mean ± SEM. 4–8 mice were sacrificed for histopathological analysis, and 4 mice were sacrificed for quantitative RT-PCR. For quantitative RT-PCR, every mouse was evaluated in 4 replicates. HE, hematoxylin and eosin; PAS, periodic acid-Schiff.
that of allele G, thus downregulating the transcriptional activity of rare gene A. In addition to SLE, other autoimmune diseases, such as rheumatoid arthritis, juvenile idiopathic arthritis and clinically amyopathic dermatomyositis, have been reported to be associated with WDFY4 [9–11]. As a susceptibility gene in various autoimmune diseases, WDFY4 must be important for immune cells and immune responses. Although Li et al. [14] found that WDFY4 is associated with T-cell activation and the immune response in humans, rats and mice, the detailed effects of WDFY4 on T cells remain unclear. Th2 cells are involved in humoral immunity, allergic reactions and chronic inflammation. In this paper, we generated CKO mice with selective deficiency in WDFY4 in T cells. We found that compared to WT cells, WDFY4-deficient T cells were more likely to differentiate into Th2 cells after activation or under Th2 polarization conditions.

Transcription factors play an important role in regulating the differentiation of Th cells. GATA3 is defined as the main regulator of Th2 cell differentiation and is upregulated during Th2 cell differentiation [21]. Knockout or inhibition of GATA3 can not only suppress the differentiation of Th2 cells but also inhibit the production of Th2 cytokines and airway hyperresponsiveness [22–24]. c-Maf, another transcription factor involved in Th2 cell differentiation, is a potential target of GATA3 [25]. We found that WDFY4-deficient Th2 cells expressed higher levels of Gata3 and c-Maf than WT Th2 cells. It has been reported that GATA3 and c-Maf can promote the production of IL-4, IL-5 and IL-13 [24, 26, 27]. Consistently, higher levels of Th2 cytokines were observed in WDFY4-deficient Th2 cells and WDFY4-deficient asthmatic mice than in the corresponding WT controls.

Previous studies have shown that Th2 cells play an important role in asthma. Inhaled allergens can induce the differentiation of Th2 cells, which leads to the production of allergen-specific IgE by B cells, activation of mast cells, and recruitment of eosinophils to the lungs; all these processes eventually cause persistent airway inflammation along with symptoms of asthma [4, 5]. Airway inflammation is the pathological basis of asthma [16]. Inflammatory cell infiltration, goblet cell hyperplasia and increased mucus production are the characteristic features of airway inflammation in asthma. Histopathological analysis showed that the amounts of infiltrating inflammatory cells and goblet cells in WDFY4-deficient asthmatic mice were greater than those in WT asthmatic mice.

Unlike in most studies, a mouse model of chronic asthma, not acute asthma, was established in this study. In addition to airway inflammation, airway remodeling was observed in the mouse model of chronic asthma. Airway remodeling is defined as changes in the constituents, structure and amount of airway wall cells and extracellular components [28] and is induced by repeated injury/repair processes [29]. Collagen deposition is one of the characteristic features of airway remodeling. Prolonged and repeated allergen stimulation can lead to collagen deposition, which can result in structural alterations. As a consequence, airway contraction is restricted and airway hyperresponsiveness occurs [30]. Consistently, we observed more severe collagen deposition in WDFY4-deficient asthmatic mice than in WT asthmatic mice.

In this study, we demonstrate that lack of WDFY4 can promote the differentiation of Th2 cells and the production of Th2 cytokines, thus aggravating OVA-induced asthma. Our results establish a link between WDFY4 and asthma and provide a novel idea for the study of disease pathogenesis and treatment.

Statement of Ethics

The animal experiments conformed to internationally accepted standards and were approved by the Animal Experiment Ethics Review Committee of Shandong University School of Medicine (reference number: 201402030).

Conflict of Interest Statement

The authors have no conflicts of interest to declare.

Funding Sources

This work was supported by the National Natural Science Foundation of China (81873878, 32070586, 81021001, 81300835, and 81671114) and Shandong Natural Science Foundation (2018CXGC1211 and 2015GSF118050).

Author Contributions

Conception and design: Y. Li, X. Li, W. Sun, J. Li, and Q. Liu. Development of methodology: J. Li, X. Li, W. Sun, and Q. Liu. Acquisition of data: Y. Li, A. Wang, S. Gao, S. Wei, and A. Liu. Analysis and interpretation of data: Y. Li, A. Wang, F. Long, and F. Gao. Writing, review and/or revision of the manuscript: Y. Li and Q. Liu.
References


