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## IL-23 signaling enhances Th2 polarization and regulates allergic airway inflammation

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### Abstract

IL-23/IL-17 axis is an important regulator in various inflammatory diseases. However, the role of IL-23 in allergic airway inflammation is not well understood. In this study, we show that in an allergen-induced asthma model, mice with transgenic overexpression of IL-23R exhibited increased airway infiltration of eosinophils and Th2 cytokine production, whereas those deficient in IL-23 displayed reduced airway inflammation. *In vitro*, IL-23-IL-23R signaling promoted GATA-3 expression and enhanced Th2 cytokine expression. Conversely, in the absence of this signal, Th2 cell differentiation was partially inhibited. Therefore, IL-23 signaling may regulate allergic asthma through modulation of Th2 cell differentiation.

### Keywords

cytokines; helper T cells; T cell differentiation; allergic airway inflammation

### Introduction

Allergic asthma is a common health problem in developed countries. It is characterized by chronic airway inflammation associated with a T helper (Th) 2 immune response to environment allergens. Upon allergen challenge, naïve CD4<sup>+</sup> T cells differentiate to a prevailing Th2 effector phenotype which predominantly secret interleukin-4 (IL-4), IL-5, and IL-13. Furthermore, these cytokines induce the recruitment of eosinophils, mast cells and lymphocytes, hyperplasia of smooth muscle and goblet cells, and airway hyperresponsiveness, which are often associated with increased serum IgE concentration 1–3.

Interleukin-23 (IL-23) is a member of the IL-12 family of heterodimeric cytokines. It is composed of a unique p19 subunit and a common p40 subunit sharing with IL-12. IL-23 binds to a heterodimeric receptor composed of the IL-12 receptor  $\beta$ 1 (IL-12R $\beta$ 1) and IL-23

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receptor (IL-23R) and this subsequently activates Jak2, Tyk2, and signal transducers and activators of transcription (STATs) 1, 3, 4, and 5. As IL-12, IL-23 is primarily secreted by activated DCs, monocytes and macrophages. It has been shown that IL-23 is important in some inflammatory diseases including experimental autoimmune encephalitis (EAE), collagen-induced arthritis (CIA), and intestinal inflammation. Moreover, polymorphisms in the gene encoding the IL-23R are important susceptibility factor for these disorders.

The proinflammatory features of IL-23 have been linked with T helper 17 (Th17) cell responses, through expansion and/or maintenance of the Th17 cells. Th17 cells are a recently described T helper subset characterized by production of IL-17 (IL-17A), IL-17F and IL-22. They have been associated with the induction of autoimmune tissue inflammation. Moreover, it has been shown that IL-17 is expressed in the airway of patients with asthma. Recently, we showed IL-17 positively while IL-17F negatively regulates allergic airway inflammation. A recent report revealed dual effects of IL-17 on allergic asthma. It is required for induction but negatively regulates established asthma. Although many studies have been focused on the IL-23-Th17 axis, the effect of IL-23 is evidenced in some diseases to be independent of IL-17 production. For instance, anti-IL-17 treatment had little impact on the T cell-mediated colitis, although the colitis was dependent on IL-23. Furthermore, development of IL-23-dependent colitis did not require IL-17 secretion by T cells. In this context, IL-23 targets not only Th17 cells but also other cell types to modulate inflammatory response. Recently, Wakashin et al. found IL-23 mediated enhancement of antigen-induced Th2 cytokine production and eosinophil recruitment in the airways, which remains in IL-17-deficient mice, suggesting IL-23 may regulate allergic airway inflammation through an IL-17-independent pathway. Whether IL-23 directly regulates Th2 cell responses is unclear.

Here, we found IL-23 deficiency alleviate airway inflammation by decreasing eosinophil recruitment and Th2 cytokine production, whereas T cell-specific IL-23R transgenic (Tg) overexpression exaggerated Th2 response and enhanced airway inflammation. Notably, allergen-specific Th17 responses were not altered in IL-23-deficient or IL-23 Tg mice compared with their controls. On the other hand, IL-23-IL-23R signaling promoted GATA3 expression *in vitro*, but inhibited T-bet expression and enhanced Th2 differentiation. Our results thus indicate an essential role of IL-23-IL-23R signaling in allergic asthma through regulating Th2 differentiation.

## Materials and methods

### Mice

Generation of IL-23R transgenic mice. IL-23R cDNA was amplified from a Th17 cell cDNA pool and inserted into pCD2 containing a human CD2 mini-locus. The transgene construct was isolated by digestion with Xho I and Xba I and microinjected into B6 mice at the Genetic Engineering Mouse Facility at University of Texas M. D. Anderson Cancer Center. Transgenic founders were maintained by breeding with B6 mice. IL-23 alpha subunit p19 target mutant mice were purchased from NIH Mutant Mouse Regional Resource Centers. Homozygous knockout (hereafter referred to as IL-23KO) and wild-type animals

on the 129×C57BL/6 mixed background were bred and used in experiments. C57BL/6 mice were purchased from the Jackson Laboratory. All animal experiments were performed using protocols approved by the Institutional Animal Care and Use Committee at the University of Texas M. D. Anderson Cancer Center.

### Induction of asthma

Mice were i.p. immunized twice at a 2-week intervals with 0.2 ml saline containing 100 µg chicken ovalbumin (OVA) in aluminum hydroxide (alum) and sensitized at day 14 and rechallenged intranasally three more times at days 25, 26, and 27 with 100 µg OVA. 24 hours after the last challenge, mice were killed and bronchoalveolar lavage fluid (BALF) and lungs were collected. BAL fluid was analyzed for cellular composition using May-Gruenwald Giemsa staining. The left Lung was homogenized in TRIzol for RNA extraction and the right lung was for histology. Splenocytes and lung-draining mediastinal lymph node cells from the asthma mice were further cultured with OVA for 3 days, and supernatants were analyzed for cytokine expression by ELISA.

### Lung histology

The right lung was removed and fixed in 4% buffered paraformaldehyde. Paraffin-embedded sections were made and stained with H&E. Peribronchial and perivascular inflammation was assessed using light microscopy under × 50 magnifications.

### T cell differentiation

CD4<sup>+</sup>CD25<sup>-</sup>CD62L<sup>high</sup>CD44<sup>low</sup> naïve T cells were FACS-sorted from lymph node cells and splenocytes of the indicated mice. For Th17 differentiation, Naïve T cells were stimulated with anti-CD3 (2 µg/ml) and anti-CD28 (2 µg/ml) in the presence of TGF-β (5 ng/ml, Peprotech), IL-6 (20 ng/ml, Peprotech), anti-IFN-γ (5 µg/ml; XMG 1.2) and anti-IL-4 (5 µg/ml; 11B11) with or without recombinant mouse IL-23 (20 ng/ml, R&D). For Th1 differentiation, Naïve T cells were stimulated with anti-CD3 (2 µg/ml) and anti-CD28 (2 µg/ml) in the presence of IL-12 (10 ng/ml) and anti-IL-4 (5 µg/ml; 11B11) with or without IL-23 (20 ng/ml). For intracellular cytokine analysis, 4 days later, cells were washed and restimulated with PMA (50 ng/ml), ionomycin (500 ng/ml), and GolgiPlug (BD Biosciences) for 5 hours.

For Th2 differentiation, Naïve T cells were activated with 2 µg/ml of plate-bound anti-CD3 and irradiated T cell-depleted splenic antigen-presenting cells or 2 µg/ml of plate-bound anti-CD28 in the presence of human IL-2 (50 U/ml), IL-4 (10 ng/ml) and anti-IFN-γ (10 µg/ml; XMG 1.2) with or without IL-23 (20 ng/ml). 4 days later, cells were washed and restimulated with 2 µg/ml of plate-bound anti-CD3 for 24 hours, and culture supernatants were analyzed for cytokine expression by ELISA. For intracellular cytokine analysis, cells were washed and restimulated with 2 µg/ml of plate-bound anti-CD3 for overnight, and with GolgiStop (BD Biosciences) for the last 5 hours. The cells were permeabilized and stained with a Cytotfix/Cytoperm kit (BD Biosciences).

### Quantitative real-time PCR

Total RNA was extracted using TRIzol reagent and cDNA was synthesized using oligo-dT and SuperScript reverse transcriptase II (Invitrogen). Gene expression was examined with a Bio-Rad iCycler Optical System using an iQ™ SYBR green real-time PCR kit (Bio-Rad Laboratories, Inc.). The data were normalized to a reference *Actb*. The primer pairs were as following (5'-3'): IL-17F: forward, 5'-tgcagaaggctgggaactgtcc and reverse, 5'-tgacctgggcattgatgcagc. GATA3: forward, 5'-aggacatctctgcgcgaactgt and reverse, 5'-catcttccggtttcgggtctgg. Other primers were described previous 14, 28, 29.

### Statistical analysis

Results were expressed as mean  $\pm$  SD. Differences between groups were calculated for statistical significance using the unpaired Student's *t* test.  $P < 0.05$  was considered as significant.

## Results

### IL-23 and IL-23R mRNA were both induced in the lung upon allergen challenge

To test the role of IL-23-IL-23R signaling in allergen-induced airway inflammation, we first assessed IL-23 and IL-23R expression in OVA immunized mice. We found that IL-23 p19 and IL-12/IL-23p40 mRNA were highly induced in the lungs from OVA challenged mice compared with those from non-immunized mice (Fig. 1A), consistent with a recent report by Wakashin et al. 26. Notably, IL-12p35 mRNA was also upregulated in OVA challenged mice. Since IL-23R polymorphisms associated with inflammatory diseases 10, 12. We further examined the IL-23R mRNA expression in the lungs. After OVA challenge, expression of IL-23R mRNA was greatly increased (Fig. 1B). These results suggest IL-23-IL-23R signaling may be involved in allergen-induced airway inflammation.

### IL-23 deficiency alleviates allergic airway inflammation

Since IL-23 was highly induced in asthmatic mice, we then tested the role of IL-23 in allergen-induced airway inflammation. IL-23KO and wild-type (WT) mice were sensitized i.p. with OVA followed by intranasal administration of OVA, and lung histology analysis was performed at 24 hours after the last challenge. As shown in Fig. 2A, antigen-induced inflammatory cell infiltration was greatly inhibited in the lungs from IL-23KO mice compared with that from WT mice, consistent with a previous observation using IL-23 neutralizing antibody 26. Cellular profiles in BALF upon OVA-challenge were assessed by cytospin with May-Gruenwald Giemsa stain. Eosinophils, macrophages and neutrophils were significantly decreased in IL-23 deficient mice (Fig. 2B). Consistently, RT-PCR analysis indicated that IL-23 deficiency led to dramatically decreased expression of eosinophil peroxidase (EPO) in IL-23KO mice (Fig. 2C).

IgE responses are a hallmark of allergic responses. In fact, in IL-23 deficient mice, OVA-specific IgE expression is significantly lower than that in WT mice (Fig. 2D). Since Th2 cytokines regulate IgE class switching, airway inflammation and recruitment of eosinophils. We then asked whether IL-23 deficiency has an impact on type 2 cytokine expression. Upon *ex vivo* OVA restimulation, the expression of IL-4, IL-5 and IL-13 in lung-draining

mediastinal lymph node cells from OVA-challenged IL-23KO mice was significantly lower in comparison with WT cells (Fig. 2E). Splenocytes from IL-23KO mice also exhibited greatly reduced Th2 cytokine production (Fig. 2E), indicating IL-23 may affect Th2 responses at the priming phase. However, OVA-specific Th17 responses were only observed at similar low levels in both IL-23KO and WT mice (Fig. 2F). Taken together, IL-23 is a crucial factor regulating the antigen-induced airway inflammation, eosinophil and neutrophil recruitment and Th2 cytokine production, possibly in a Th17-independent manner.

### Generation of T cell-specific IL-23R transgenic mice

Since IL-23 influences airway inflammation in a Th17-independent manner, we then asked whether IL-23 functions through T cells in regulation of airway inflammation. First, we generated two lines of IL-23R transgenic mice with IL-23R overexpression in T cells using human CD2 mini locus 27 (Fig. 3A). One of the two lines (Line 2) was extensively analyzed. Next, we verified the function of IL-23R transgene (Tg) during Th17 cell differentiation. Naïve CD4<sup>+</sup> T cells from IL-23R Tg mice or their littermate control were differentiated to Th17 cells in the presence or absence of recombinant mouse IL-23. Under the Th17 condition, addition of IL-23 significantly increased the frequency IL-17-producing T cells in IL-23R Tg T cells (Figure. 3B). Furthermore, under the Th1 condition, addition of IL-23 greatly inhibited IFN- $\gamma$ -producing T cells in IL-23R Tg T cells (Figure. 3C). Thus, transgenic overexpression of IL-23R enhances Th17 but inhibits Th1 differentiation.

### IL-23R Tg enhanced allergic airway inflammation

To examine whether IL-23 directly act on CD4<sup>+</sup> T cells in regulation of airway inflammation, we induced asthma in IL-23R Tg and littermate WT mice. Histology analysis revealed transgenic overexpression of IL-23R resulted in increased inflammatory cell infiltration in the lung (Fig. 4A). Consistent with the histological analysis, in BALF, the total cell numbers, eosinophils, macrophages and neutrophils were significantly increased in IL-23R Tg mice compared with their littermate controls (Fig. 4B). Dramatically elevated expression of EPO in the lung of IL-23R Tg mice indicated enhanced eosinophil function (Fig. 4C).

In contrast to IL-23 deficiency, transgenic overexpression of IL-23R led to significantly elevated expression of OVA-specific IgE in sera (Fig. 4D) and Th2 cytokines in lung-draining mediastinal lymph node cells and splenocytes in response to *ex vivo* recall with OVA (Fig. 4E). In addition, OVA-specific Th17 responses were not significantly changed in the transgenic mice (Fig. 4F). These results further suggested IL-23-IL-23R signaling regulates allergic airway inflammation, potentially through targeting T cells and modulating Th2 responses.

### IL-23 regulates Th2 differentiation *in vitro*

To further confirm whether IL-23-IL-23R signaling can influence Th2 differentiation directly rather than through the Th17 pathway, we examined the impact of IL-23 signaling during *in vitro* Th2 differentiation.

FACS-sorted CD62L<sup>high</sup>CD44<sup>low</sup> naïve CD4<sup>+</sup> T cells from IL-23KO mice or WT mice were differentiated to Th2 cells with splenic APCs from IL-23KO or WT mice and cytokine expression were assessed. CD4<sup>+</sup> T cells activated with IL-23-deficient APCs exhibited reduced IL-4 producing cell numbers and visually no IL-17 producing cells were observed (Fig. 5A). As revealed by ELISA, IL-23-deficient APCs led to significantly reduced amounts of IL-4 and IL-5, IL-13 expression by effector T cells (Fig. 5B). Consistently, GATA-3 mRNA expression was significantly decreased in T cells treated with IL-23KO APCs compared with those treated with WT APCs (Fig. 5C). When naïve T cells were activated with plate-bound anti-CD3 and anti-CD28 in the presence of WT or IL-23KO APC culture supernatants rather than the APCs led to the same results (data not shown). Therefore, lack of IL-23 partially inhibited Th2 differentiation.

To rule out the possibility of IL-23 acting on APCs, we utilized an APC-free system. Naïve CD4<sup>+</sup> T cells from IL-23R Tg mice or their littermate controls were activated with plate-bound anti-CD3 and anti-CD28 in the presence or absence of recombinant mouse IL-23. Addition of IL-23 increased IL-4-producing cell numbers and protein expression of IL-4 and IL-13 in IL-23R Tg cells (Fig. 6A-B). In WT T cells, only IL-13 was significantly enhanced by IL-23 treatment (Fig. 6A-B). Consistently, in response to IL-23 stimulation, IL-23R Tg but not WT T cells highly expressed GATA-3 mRNA (Fig. 6C). Taken together, the IL-23-IL-23R signaling can promote Th2 differentiation through direct action with CD4<sup>+</sup> T cells.

## Discussion

IL-23, a member of the IL-12 family, participates in a variety of inflammatory disorders 7–9. Here we show that IL-23-specific p19 mRNA and IL-23R mRNA were both induced in the lungs upon allergen challenge, suggesting the involvement of IL-23-IL23R signaling in regulating allergic airway inflammation. By using IL-23-deficient and IL-23R transgenic mice, we have demonstrated that IL-23-IL-23R signaling plays a critical role in allergic asthma. In the absence of IL-23, the development of airway inflammation was inhibited in a mouse model of allergic asthma. Conversely, transgenic overexpression of IL-23R enhanced the symptoms of allergic airway inflammation. Moreover, our *in vitro* data on the Th2 differentiation revealed that IL-23-IL-23R signaling can modulate pathogenic type 2 responses independently of its role in promoting Th17 cell development.

A significant hallmark of asthma is accumulation of eosinophils, neutrophils, lymphocytes and macrophages in the lung, and this cellular influx is directly proportional to disease severity 30. During antigen challenge, we detected profoundly reduced levels of inflammatory cells into the lung tissue in IL-23KO mice, including both eosinophils and neutrophils. These results are consistent with a recent report that neutralization of IL-23 attenuated antigen-induced eosinophils and neutrophils recruitment 26. The eosinophilia is associated with type 2 immune responses characterized by production of Th2 cytokines IL-4, IL-5 and IL-13. As expected, the production of the Th2 cytokines were dramatically reduced in the lung-draining mediastinal IL-23KO lymph node cell and splenocyte cultures. Furthermore, we found that enforced expression of IL-23R in T cells enhanced the type 2 immune responses in allergic airway inflammation. These results collectively suggest that



IL-23-IL-23R signaling can promote the allergic airway inflammation by regulating the type 2 immune responses.

IL-23 was generally implicated in stabilizing the phenotype of Th17 cells and maintaining IL-17 production by Th17 cells 11, and many proinflammatory functions of IL-23 seem linked with the Th17 subset. However, in antigen challenged IL-23KO WT or IL-23R Tg versus their WT control mice, we observed that neither IL-17 nor IL-17F had significantly altered in the lymph node cell and splenocyte cultures (data not shown). Thus, the role of IL-23-IL-23R signaling in allergen-induced airway inflammation may be independent of the IL-23/Th17 pathway. This idea is supported by a recent study that development of IL23-dependent colitis did not require IL-17 secretion by T cells 23. However, we found in lung, IL-23 deficiency led to reduced while IL-23R Tg enhanced the expression of IL-17 mRNA (data not shown), suggesting a non-Th17 cellular source of IL-17 (maybe also IL-17F) may be involved in local inflammatory responses in agree with a previous report that in lung, the major source of IL-17 and IL-17F is  $\gamma\delta$  T but not Th17 cells during tuberculosis infection 31.

As well as Th17 cells, IL-23 can target multiple cell types, such as NK cells, DC and macrophages to modulate inflammatory responses 25. To test the influence of IL-23 on T cells, we generated IL-23R Tg mice and tested T cell differentiation using *in vitro* culture systems. We found transgenic overexpression of IL-23R significantly enhanced the frequencies of IL-17-producing T cells under the Th17 condition, while reduced IFN- $\gamma$ -producing T cell numbers under the Th1 condition. At this point, whether IL-23 signaling enhances Th2 differentiation indirectly through inhibition of Th1 pathway remains unclear. Next, we tested the impact of IL-23 in Th2 cell differentiation. IL-23-deficient APCs partially inhibited the Th2 differentiation and resulted in greatly reduced GATA-3 mRNA expression. This effect was also observed when using IL-23-deficient or sufficient APC culture media instead of APC themselves (data not shown). Furthermore, using an APC-free system, we found transgenic overexpression of IL-23R led to up-regulated Th2 cytokines and dramatically enhanced GATA3 expression, suggesting IL-23-IL-23R signaling can directly target Th2 cells independent of APCs. Previous studies showed IL-23R when binding IL-23 activates STAT1, 3, 4 and 5 5. Whether IL-23 signaling enhances Th2 differentiation through activation of STAT5 and in turn promotes Th2 differentiation is not clear.

In conclusion, our study highlights the importance of IL-23-IL-23R signaling in the development of allergic airway inflammation and suggests that IL-23 can act on Th2 cells. Thus, our findings have important implications for therapeutically targeting IL-23 and its receptor in allergic asthma.

## Acknowledgments

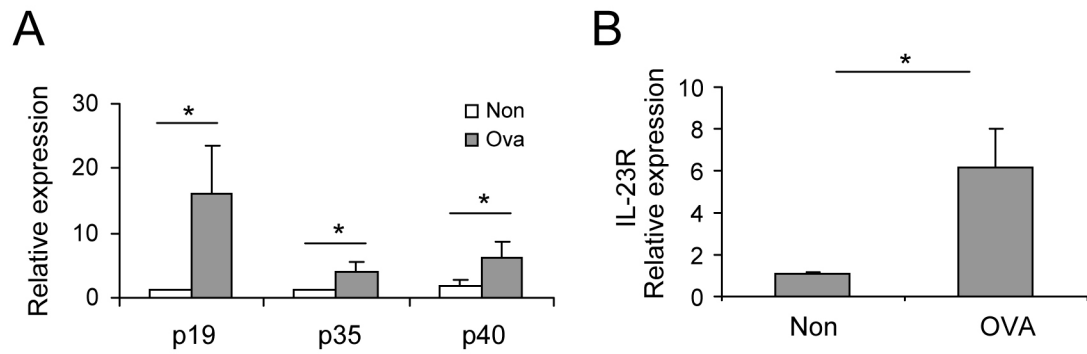
We thank Dr. Jan Parker-Thornburg for her help in generation of IL-23R transgenic animals and the Dong lab members for their help and discussion. The work is supported by research grants from NIH, and MD Anderson Center for Targeted Therapy (to CD). XOY receives a Beginning Grant-in-Aid from American Heart Association, JP receives a fellowship from Ministry of Education of China, and CD is a Trust Fellow of the MD Anderson Cancer Center and a Leukemia and Lymphoma Society Scholar.

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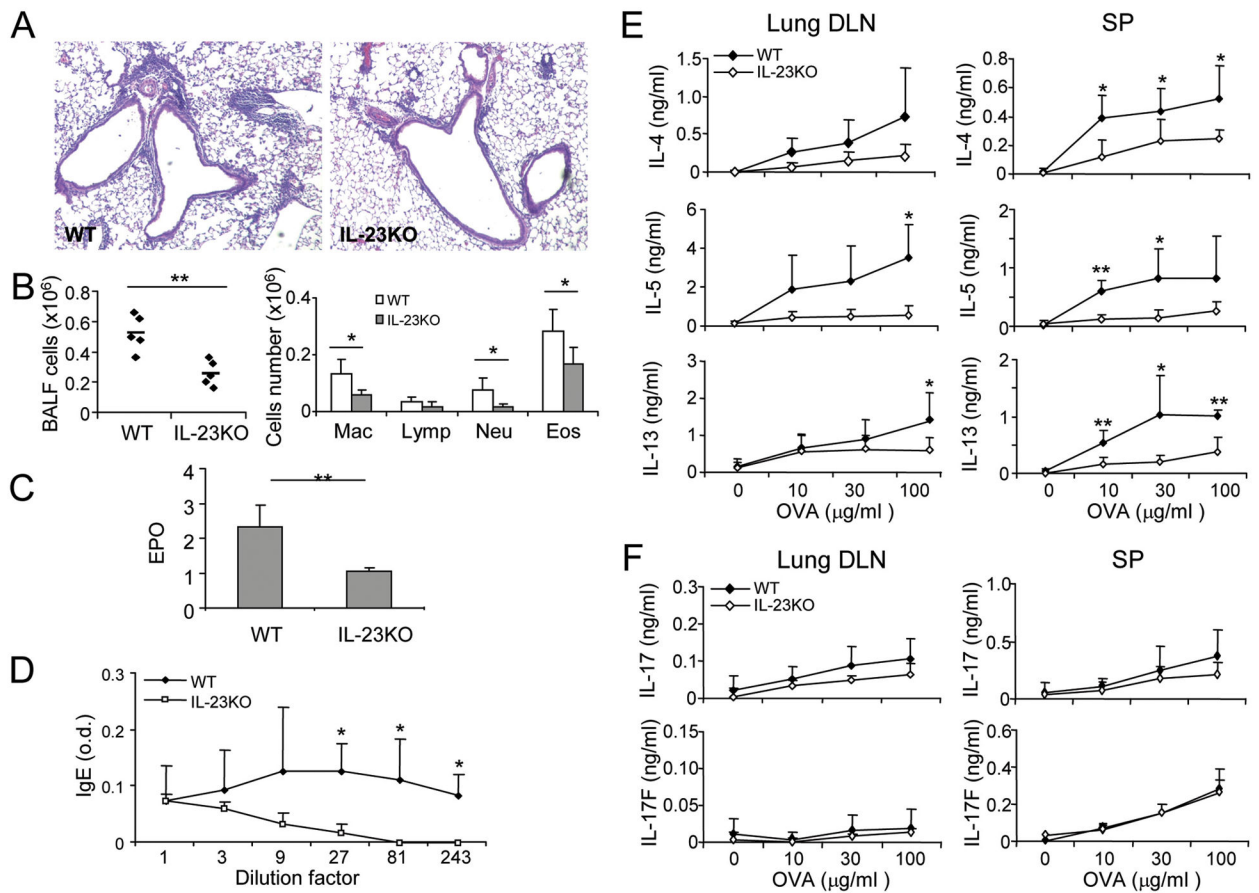
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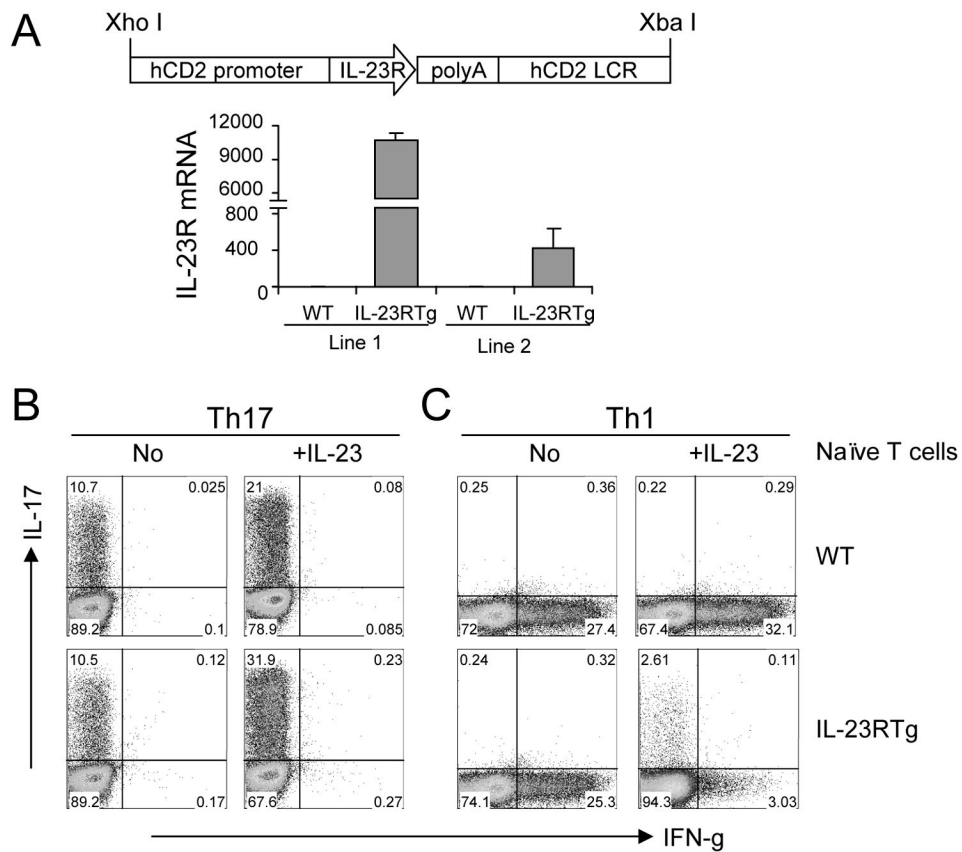


**Figure 1. Induction of IL-23 and IL-23R in the lung upon allergen challenge**  
 mRNA expression of IL-23p19, IL-12p35, IL-12/IL-23p40 (A) and IL-23R (B) was determined by real-time PCR in whole lung tissue from OVA-challenged B6 mice. Non-challenged B6 mice were used as control. mRNA expression was normalized to a housekeeping gene  $\beta$ -actin. Data shown represent mean  $\pm$  SD from two independent experiments (n = 4–6 per group). Student t test, \*, p < 0.05.



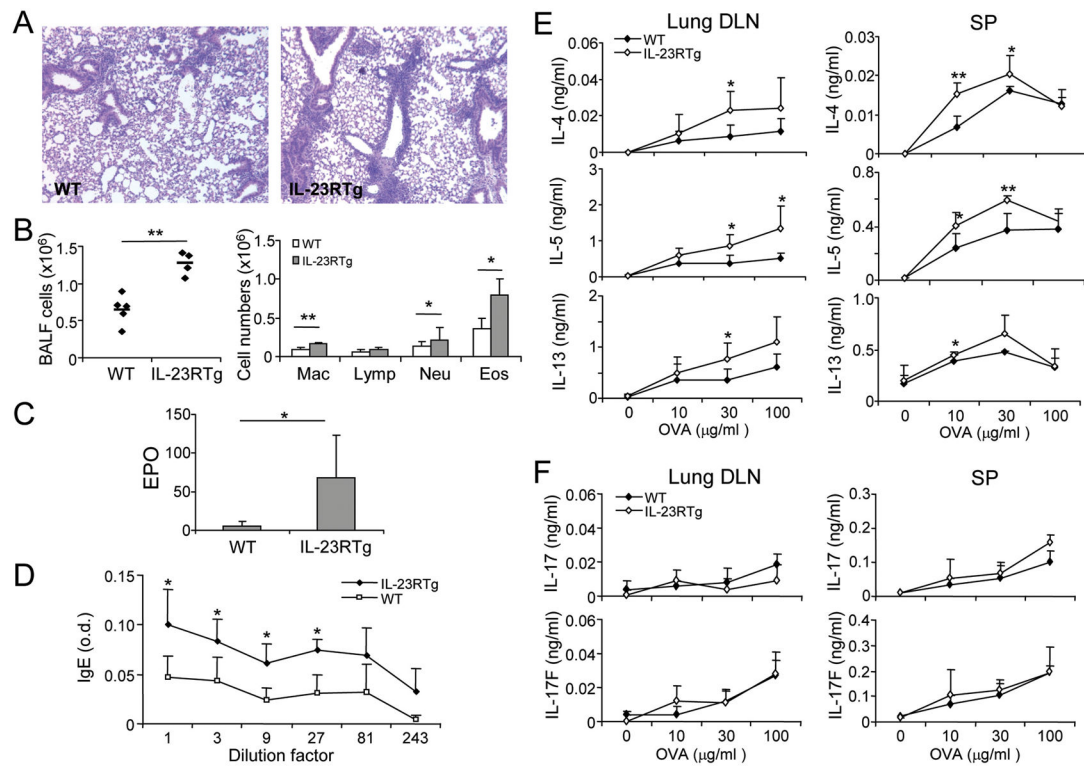
**Figure 2. IL-23 deficiency led to reduced allergic airway inflammation and Th2 cytokine expression**

IL-23 KO and WT mice were subjected to OVA-sensitizing induced asthma. (A) Inflammatory infiltrates in lung were assessed by H&E staining. (B) Total cells of BALF from the asthmatic mice. Horizontal bars represent the means. Cellular profiles in BALF upon OVA-challenge were assessed by cytopsin with May-Gruenwald Giemsa staining. (C) EPO expression in lung. Whole lung mRNA was prepared and subjected to quantitative real time RT-PCR. Gene expression was normalized to expression of  $\beta$ -actin. Data shown are a representative of two independent experiments (n=4–6). (D) OVA-specific IgE expression in sera was measured by ELISA. (E) Expression of type 2 cytokines in lung lymph node cells and splenocytes after *ex vivo* OVA restimulation was assessed by ELISA. (F) OVA-specific Th17 cytokine expression was determined by ELISA. Data shown represent at least 2 independent experiments with consistent results (n=4–6). Student t test, \*, p < 0.05; \*\*, p < 0.005.



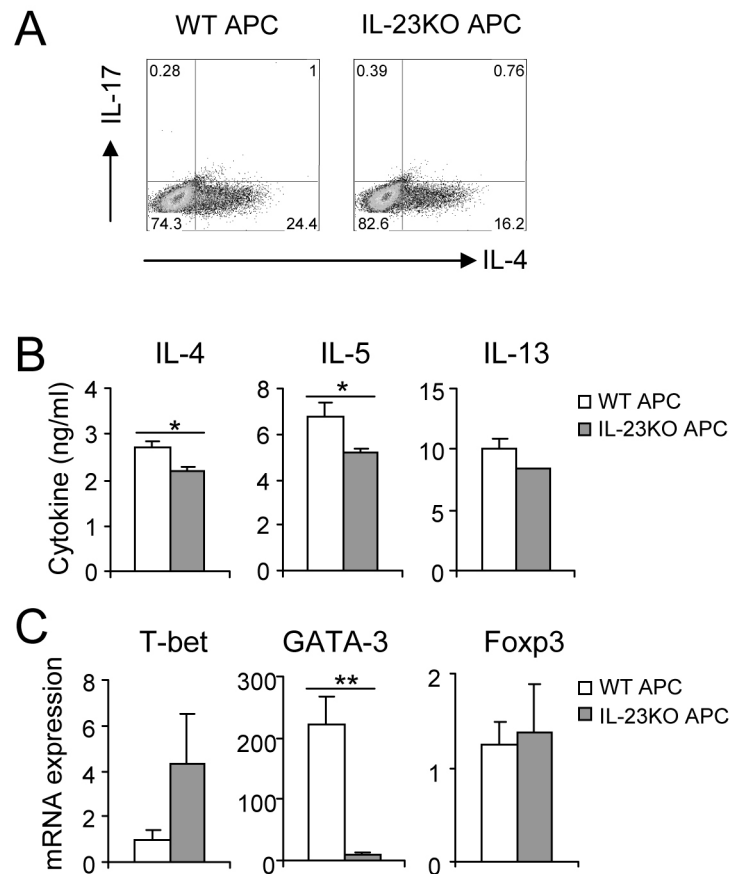
**Figure 3. Transgenic expression of IL-23R enhanced Th17 while inhibited Th1 differentiation *in vitro***

(A) Generation of IL-23R Tg mice. IL-23R was driven by an hCD2 promoter under the control of hCD2 LCR. IL-23R mRNA expression was tested in 2 lines. (B-C) Naïve T cells were FACS-sorted from IL-23R Tg or B6 mice and activated under Th17 and Th1 conditions with or without recombinant mouse IL-23. 4 days later, IFN- $\gamma$  and IL-17 producing cells were analyzed by intracellular staining. Numbers within the quadrants indicate the percentage of positive cells. Data shown represent as least 2 independent experiments with consistent results.



**Figure 4. Transgenic overexpression of IL-23R enhanced allergic airway inflammation and Th2 cytokine expression**

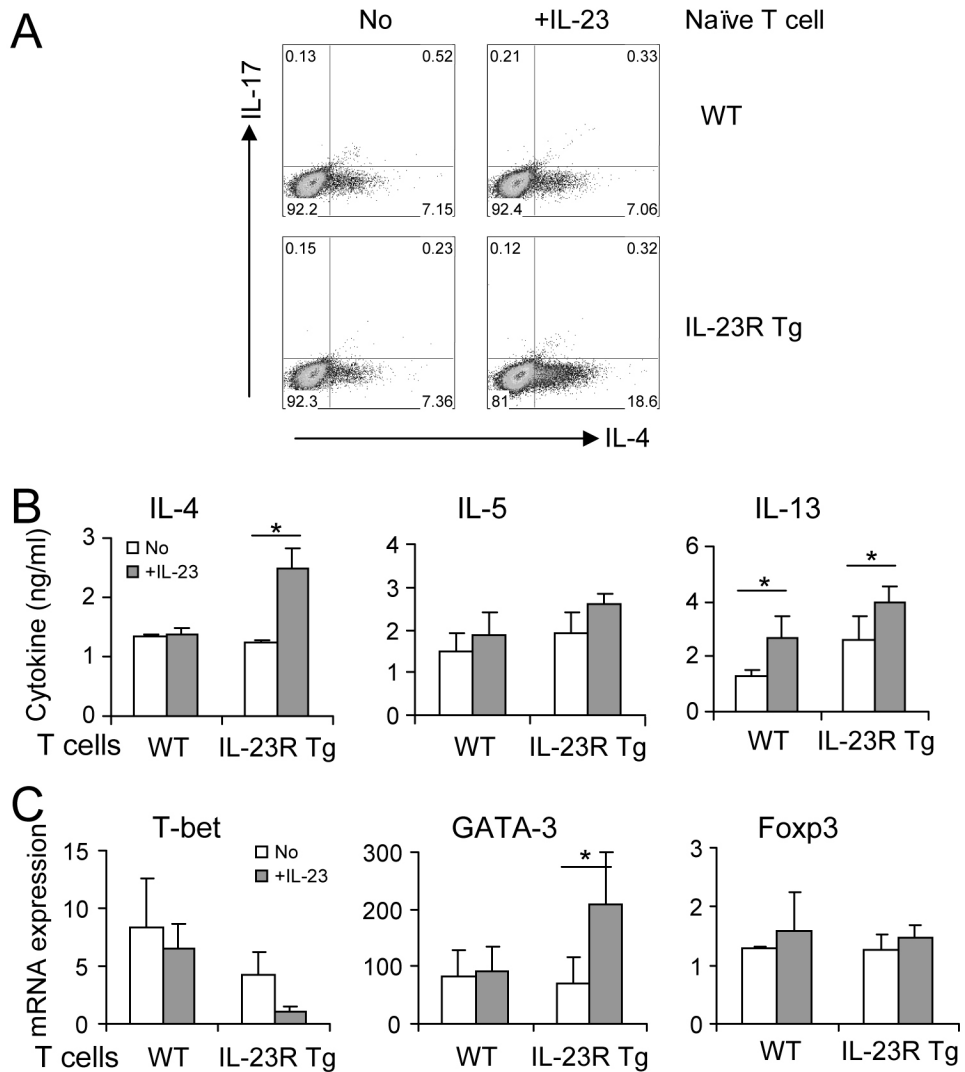
Asthma was induced in IL-23R Tg and B6 mice. (A) Inflammatory infiltrates in lung from the asthmatic mice were assessed by H&E staining. (B) Total cells in BALF. Horizontal bars represent the means. Cellular profiles in BALF upon OVA-challenge were assessed by cytopsin. (C) EPO expression in lung. Whole lung mRNA was prepared and subjected to quantitative real time RT-PCR. Gene expression was normalized relative to expression of  $\beta$ -actin. Data shown are a representative of two independent experiments (n=4–6). (D) OVA-specific IgE expression in sera was measured by ELISA. (E) Expression of type 2 cytokines in lung lymph node cells and splenocytes after *ex vivo* OVA restimulation was assessed by ELISA. (F) OVA-specific Th17 cytokine expression was determined by ELISA. Data shown represent 2 independent experiments with similar results (n=4–6). Student t test, \*,  $p < 0.05$ ; \*\*,  $p < 0.005$ .



**Figure 5. IL-23-deficiency inhibits Th2 differentiation *in vitro***

Naïve T cells were FACS-sorted and stimulated with anti-CD3 and irradiated splenic APC from IL-23 KO or WT mice in the presence of IL-2, IL-4, anti-IFN- $\gamma$ . (A) 5 days later, IL-4 and IL-17 producing cells were analyzed by intracellular staining. Numbers within the quadrants indicate the percentage of positive cells. (B) Cytokine production was measured by ELISA. (C) T-bet, GATA3 and Foxp3 mRNA expression was analyzed by quantitative realtime RT-PCR. (B-C) The data are expressed as the mean  $\pm$  SD of triplicate samples. Student t test, \*,  $p < 0.05$ ; \*\*,  $p < 0.005$ ;  $p$  values were calculated from 2–3 independent experiments with consistent results.





**Figure 6. IL-23-IL-23R signaling promotes Th2 differentiation *in vitro***  
 Naïve T cells were FACS-sorted from IL-23R Tg or B6 mice and stimulated with anti-CD3 and anti-CD28 in the presence of IL-2, IL-4, anti-IFN- $\gamma$ . (A) 5 days later, IL-4 and IL-17 producing cells were analyzed by intracellular staining. Numbers within the quadrants indicate the percentage of positive cells. (B) Cytokine production was measured by ELISA. (C) T-bet, GATA3 and Foxp3 mRNA expression was analyzed by quantitative realtime RT-PCR. (B–C) The data are expressed as the mean  $\pm$  SD of triplicate samples. Student t test, \*,  $p < 0.05$ ;  $p$  values were calculated from 2–3 independent experiments with consistent results.