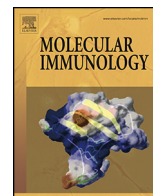




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Molecular Immunology

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IL-23, rather than IL-17, is crucial for the development of ovalbumin-induced allergic rhinitis

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ARTICLE INFO

Article history:

Received 25 April 2015

Received in revised form 28 June 2015

Accepted 8 July 2015

Available online xxx

Keywords:

Allergic rhinitis

IL-23

IL-17

Eosinophil

ABSTRACT

Interleukin-23 (IL-23) and IL-17 are involved in the pathogenesis of allergic rhinitis (AR). However, the roles of IL-23 and IL-17 in ovalbumin (OVA)-induced AR remain unclear. Therefore in this study we aim to investigate the precise roles of IL-23 and IL-17 in a mouse model of OVA-induced AR. We found that during OVA-induced AR, eosinophil and goblet cells in the nose were significantly decreased in IL-23-deficient, but not in IL-17-deficient mice. However, there was no difference in the serum IgE and IgG1 levels between IL-23-deficient or IL-17-deficient and wild-type mice. Moreover, IL-4 levels in lymph node cell culture supernatants were significantly decreased in IL-23-deficient, but not IL-17-deficient, compared with wild-type mice. Furthermore, OVA-induced AR developed similarly in wild-type mice transferred with either IL-23-deficient BM cells or wild-type BM cells. These findings suggest that IL-23, but not IL-17 is crucial for the development of OVA-induced AR, and IL-23 neutralization may be a potential approach for treatment of OVA-induced AR in humans.

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1. Introduction

The prevalence of allergic diseases, including asthma, rhinitis, eczema, and food allergies, has risen sharply (Holgate, 1999). Allergic rhinitis, which is a condition similar to common cold, could affect mental and learning capacities up to 30% (Global Allergy and Asthma European Network addresses the allergy and asthma epidemic) (Bousquet et al., 2009). AR is an inflammatory disease of the nasal mucosa induced by an immunoglobulin E (Helbling et al., 2014)-mediated reaction in allergen-sensitized individuals. It has been reported that numerous loci and candidate genes show an association with AR (Gaddam et al., 2012; Genuneit et al., 2009; Ibrahim et al., 2012; Tomazic et al., 2014). In spite of that, the molecular mechanisms underlying the development of AR have not been completely determined.

Interleukin-IL-23 (IL-23), a heterodimeric cytokine consisting of a p19 subunit specific for IL-23 and a p40 subunit shared with IL-12, has been shown to play a significant role in the maintenance (Langrish et al., 2005) and acquisition of pathogenic function of Th17 cells (McGeachy et al., 2007). Whereas interleukin-17 (IL-

17; also known as IL-17A) is a member of IL-17 cytokine family produced predominantly by a subpopulation of CD4⁺ T cells, Th17 cells (Harrington et al., 2005). Indeed, IL-23-IL-17 link has been found to be associated with the development of various inflammatory diseases, such as psoriasis (Di Cesare et al., 2009), arthritis (Cornelissen et al., 2009) and inflammatory bowel disease (IBD) (Sarra et al., 2010). To date, studies in patients with AR indicate that IL-23 and IL-17 are also likely to be involved in the pathogenesis of this condition (Nieminen et al., 2010). However, the role of IL-23 and Th17 in the regulation of allergic airway inflammation remains largely unknown. Therefore, in this study, we aimed to determine the roles of IL-23 and IL-17 in the development of OVA-induced AR, using AR model by treating wild-type, IL-23-deficient and IL-17-deficient mice with OVA.

2. Materials and methods

2.1. Mice

Female CD45.1-C57BL/6J, CD45.2-C57BL/6J, CD45.1-C57BL/6N wild-type mice, IL-23^{-/-}-CD45.1-C57BL/6N, C57BL/6J-wild-type and IL-17^{-/-} mice (6–8-week-old) were purchased from Shanghai SLAC Laboratory Animal Co., Ltd. (Shanghai, China) and were housed in the Animal Resource Facility. All procedures and experiments involving animals in this study were performed in

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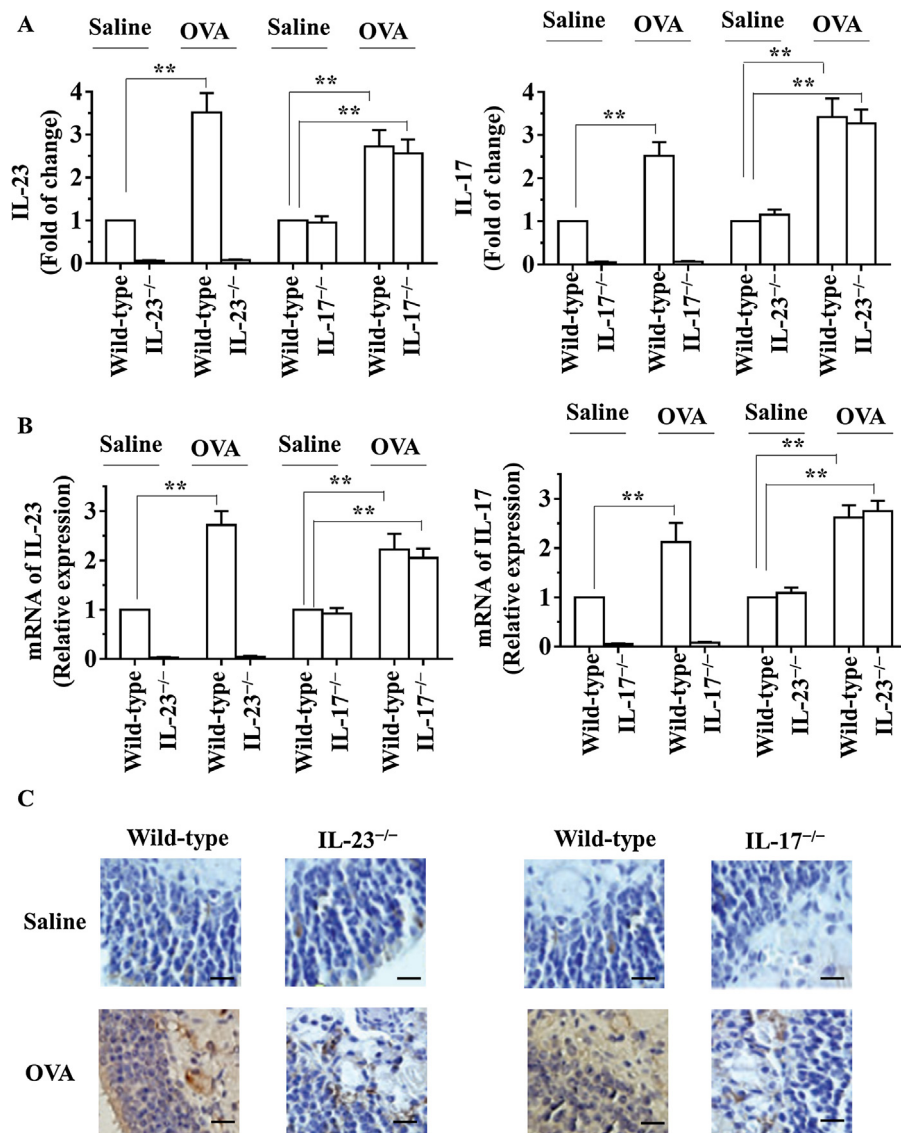


Fig. 1. Up-regulation of IL-23 and IL-17 in the mouse model of AR. (A and B) Blood and nasal mucosa were collected 24 h after the last challenge and analysed for serum IL-23 and IL-17 levels (A), relative mRNA level of IL-23 and IL-17 in nasal mucosa (B). (C) The sections were stained with anti-rabbit-IL-23 or anti-rabbit-IL-17 Abs. IL-23 and IL-17 were detected in the cytoplasm of epithelial cells (brown) from OVA-treated mice. Scale bars, 100 μ m. Data are presented as the mean $\pm$ SD from 6 mice in each group ($n=6$). ** $p < 0.01$, versus control. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

accordance with the National Institutes of Health Guide for Care and Use of Laboratory Animals. The protocol was approved by the Committee on the Ethics of Animal Experiments of Qingdao Municipal Hospital. All surgery was performed under sodium pentobarbital anesthesia, and all efforts were made to minimize suffering.

2.2. Mouse model of OVA-induced allergic rhinitis

The sensitization challenge for the murine model of AR was performed as previously described with slight modification (Saito et al., 2002). Under pathogen-free conditions, mice were sensitized using OVA (Sigma-Aldrich, St. Louis, MO, USA) as follows. Ovalbumin (40 μ g/kg) OVA diluted in sterile normal saline was administered along with aluminum hydroxide gel (alum adjuvant, 40 mg/kg) to un-anesthetized animals four times by intraperitoneal injection on days 1, 5, 14, and 21. This was followed by daily intranasal challenge with OVA diluted with sterile normal saline intranasally (20 μ L of 25 mg/ml OVA per mouse) from days 22 to 35. Twenty-four hours

after the last OVA challenge, blood and tissues were collected from each mouse.

2.3. Bone marrow cell transfer

CD45.1-C57BL/6J mice were irradiated with X-rays (8 Gy) and then injected intravenously with bone marrow cells (2×10^7 cells) from CD45.2-C57BL/6N-wild-type or -IL-23^{-/-} mice. One month later, the mice were used for experiments (CD45.2⁺ cells >95% in the spleen by FACS).

2.4. Measurement of OVA-specific IgE and total IgE

Serum levels of total IgE and OVA-specific IgG1 were measured by solid-phase enzyme-linked immunosorbent assay (ELISA) in accordance with the manufacturer's instructions. Bound immunoglobulin isotypes were detected with specific secondary antibody (biotin-conjugated rat anti-mouse IgE and IgG1 Abs were purchased from BD Pharmingen, San Jose, CA, USA).

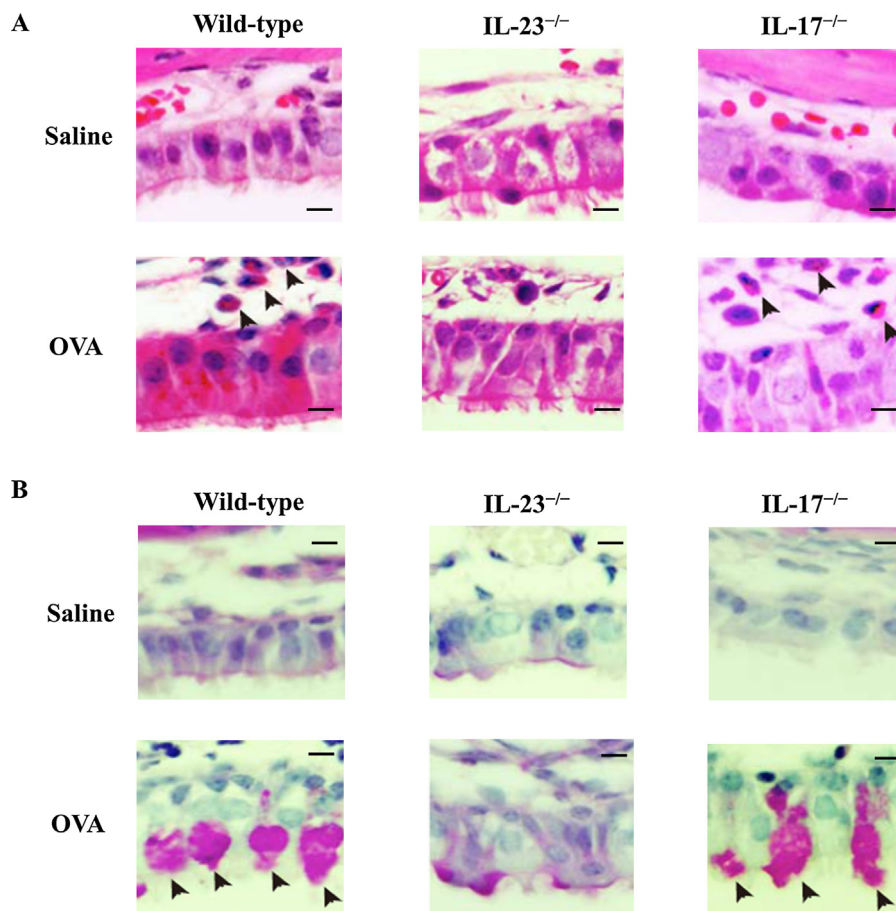


Fig. 2. Histological analysis for infiltration of leukocytes and hyperplasia of goblet cells. Histological analysis of nasal mucosa (maxillary turbinate) from wild-type, IL-23^{-/-} and IL-17^{-/-} mice 24 h after the last inhalation of OVA or normal saline. (A) H&E staining. Arrowheads indicate eosinophils. (B) Periodic acid-Schiff (PAS) staining. Arrowheads indicate PAS-positive goblet cells. Scale bars, 100 μ m.

2.5. Lymph node cell culture

At 24 h after the last intranasal treatment, cervical lymph nodes (LNs) were collected, and suspended in RPMI 1640 (Hyclone, GE Healthcare Life Sciences, Piscataway, NJ, USA) and 10% fetal bovine serum (FBS, Gibco, Grand Island, NY, USA). The cervical LN cells (5×10^5 cells/well in 96-well flat-bottom plates) were cultured in the presence or absence of 200 μ g/mL OVA at 37 °C for 48 h. Cell proliferation was assessed by pulsing with 1 μ Ci/mL [³H] thymidine (Amersham Biosciences, Little Chalfont, UK) at 37 °C for 6 h, harvesting the cells onto a glass-filter (Perkin Elmer, Waltham, MA) with a Micro 96 cell harvester (Skatron, Lier, Norway) and measuring the incorporated [³H] thymidine using a Micro Beta System (Amersham Biosciences).

2.6. Flow cytometry assay

Single-cell suspensions of superficial cervical LNs were prepared and cultured in RPMI-1640 with 10% FCS at a concentration of 5×10^6 cells/mL. For detection of IL-4⁺ and IFN- γ ⁺ cells, the cells were stimulated with 0.1 μ g/mL PMA (Sigma-Aldrich) and 1 μ g/mL ionomycin (Sigma-Aldrich) in the presence of 1 μ M Monensin (Sigma-Aldrich) at 37 °C for 4 h. After washing, the cells were incubated with PE-conjugated anti-rabbit IL-4 or brilliant Violet-conjugated anti-rabbit IFN- γ mAbs at 4 °C for 30 min. Isotype-matched antibodies were used as negative controls. All antibodies were purchased from BD Pharmingen. Samples were

assessed flow cytometry (FACSCalibur; Becton Dickinson, San Jose, CA) and data were analysed using the CellQuest software (BD Biosciences).

2.7. Measurement of cytokines

The levels of IL-23 and IL-17 in serum, IL-4, IL-5 and IL-13 in the LN cell culture supernatants were measured by the Cytometric Bead Array (CBA) Flex Set (BD Biosciences, San Diego, CA, USA) according to the manufacturer's instructions.

2.8. Real-time polymerase chain reaction

Nasal mucosa was dissected 2 h after the last OVA challenge and immediately frozen in liquid nitrogen for storage at –80 °C. Total RNA was isolated with Trizol reagent and reverse transcribed into first-strand cDNA using oligo (Mathias et al., 2009) primer. Real-time polymerase chain reaction (PCR) was performed in the Rotor-GeneTM 6200 HRM (Corbett Life Science, Sydney, Australia). The expression of IL-23 and IL-17 mRNA was determined using the Platinum SYBR Green qPCRSuperMixUDG (Invitrogen, Carlsbad, CA, USA). The primer sequences used were as follows. *GAPDH*, forward: cggatttggccgtattggg, reverse: ctcgctcctggaagatgg; *IL-17*, forward: ttcttgatgctgtgtgctgct, reverse: cgtggaacgggtgaggtag; *IL-23*, forward: ccagcagctctctcggaatc, reverse: tcatatgtcccgtgtgc. Comparative Ct method (delta delta Ct) method was used for relative gene expression analysis.

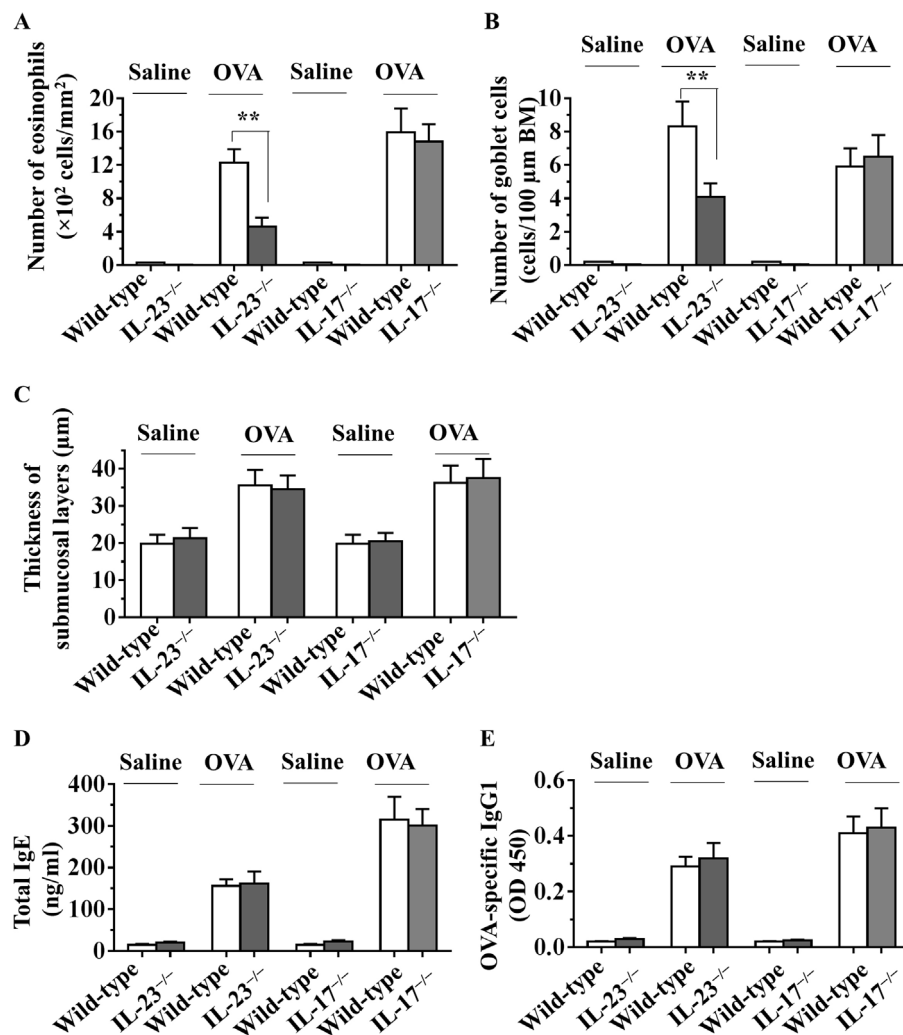


Fig. 3. IL-23, but not IL-17, is crucial for eosinophilia and goblet cell hyperplasia, but neither IL-23 nor IL-17 is essential for IgG1 or IgE production and affects the sub-mucosal thickness in OVA-induced AR. (A)–(C) The numbers of eosinophils (A) and goblet cells (B) in the nasal mucosa and the thickness of sub-mucosal layers (C). (D and E) The levels of total IgE (D) and OVA-specific IgG1 (E) in serum of wild-type and IL-23^{-/-} mice and in wild-type and IL-17^{-/-} mice 24 h after the last inhalation of OVA or normal saline. Data are presented as the mean $\pm$ SD ($n=6$). ** $p < 0.01$, versus control. BM = basement membrane.

2.9. Histological analysis of nasal mucosa

Heads of mice were excised and fixed in 10% neutral buffered formalin for 24 h at room temperature. After fixation, the heads were decalcified in 8.8% formic acid for 6 days and then embedded in paraffin. Samples were cut into 4 μ m cross sections and sections were stained with hematoxylin and eosin, or with periodic acid-Schiff (PAS). The number of eosinophils in the nasal submucosa extending from the maxillary turbinate to the lateral wall was counted under a microscope ($\times 400$). PAS-positive cells were counted to a depth of 100 μ m below the basement membrane, and the data show the mean of the results from 5 random areas. For IL-23 and IL-17 staining, rabbit anti-IL23 (abcam, 115759) and anti-IL-17 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) were used as primary antibodies, and horseradish peroxidase (HRP)-conjugated anti-rabbit IgG (Zhongshanjinqiao, Beijing, China) was used as secondary antibody. For neutrophils staining, rat neutrophil antibody NIMP-R14 (abcam, 2557) was used as primary antibody, HRP-conjugated anti-rat IgG (mouse adsorbed) (abcam, 7097) was used as secondary antibody. As a negative control, the primary antibodies were substituted by isotype- and concentration-matched control antibodies. The positive cells

were visualized by further incubation with 3,3'-diaminobenzidine which rendered the positive cells brown.

2.10. Statistical analysis

Data are presented as mean $\pm$ SD unless otherwise indicated. The statistical significance of the difference between the values of control and treatment groups was determined by either Student's t test using Prism version 5 (GraphPad Software, Inc.). Values of $p < 0.05$ were considered statistically significant.

3. Results

3.1. IL-23, rather than IL-17, is crucial for development of OVA-induced AR in mice

To evaluate the roles of IL-23 and IL-17 in the development of OVA-induced AR, mice were repeatedly treated with OVA intranasally. As shown in Fig. 1A, the concentration of serum IL-23 and IL-17 was significantly increased in OVA-treated C57BL/6 wild-type mice, but not IL-23^{-/-} or IL-17^{-/-} mice, compared to normal saline control group. The concentration of IL-23 and IL-17 were

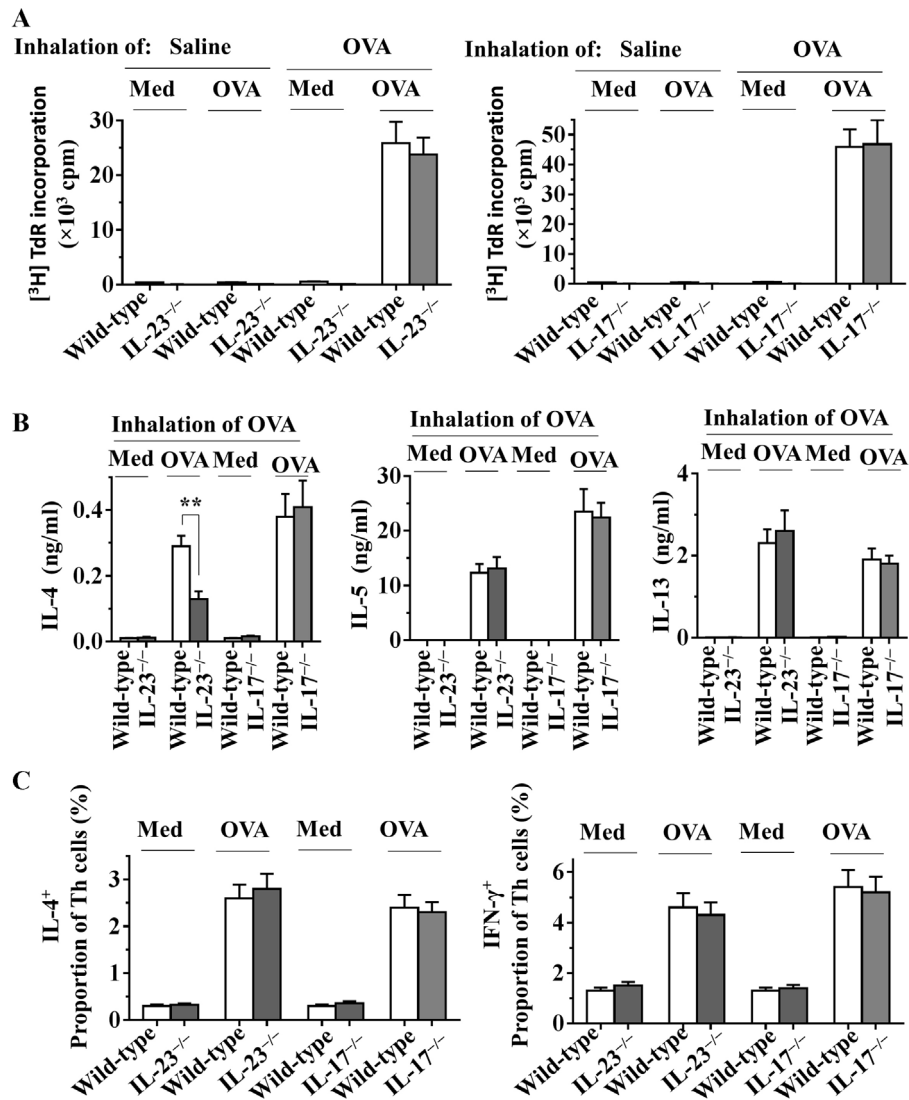


Fig. 4. IL-23, but not IL-17, is required for IL-4 production, but not Th2 cell differentiation, during OVA-induced AR in mice. (A) and (B) OVA-specific LN cell proliferative responses (A) and cytokine production (B) of wild-type and IL-23^{-/-} mice and of wild-type and IL-17^{-/-} mice 24 h after the last inhalation of OVA or normal saline. (C) Quantitative analysis for proportions of IL-4⁺ and IFN- γ ⁺ T cells in the supernatants of LN cells. Data are presented as the mean $\pm$ SD ($n=6$). ** $p<0.01$, versus control. Med = medium alone.

significantly enhanced in OVA-treated IL-17^{-/-} mice and IL-23^{-/-} mice, respectively. In addition, the mRNA levels of IL-23 and IL-17 in the nasal mucosa was significantly increased in OVA-treated mice, but not IL-23^{-/-} or IL-17^{-/-} mice, compared to the control group (Fig. 1B). The mRNA levels of IL-23 and IL-17 were significantly increased in OVA-treated IL-17^{-/-} mice and IL-23^{-/-} mice, respectively. Moreover, histological analysis found infiltration of leukocytes and hyperplasia of goblet cells in the nasal mucosa of OVA treatment mice, but not IL-23^{-/-} or IL-17^{-/-} mice and control group mice (Fig. 1C). These findings suggested that IL-23 and IL-17 may somehow contribute to the development of OVA-induced AR. We further determined the roles of IL-23 and IL-17 in the development of OVA-induced AR. Histological analysis results showed the infiltration of leukocytes and hyperplasia of goblet cells in the nasal mucosa of wild-type mice 24 h after the last inhalation of OVA, but not normal saline (Fig. 2A and B). In that setting, inflammation of the nasal mucosa in IL-17^{-/-} mice was similar to that in wild-type mice (Fig. 2A), but markedly reduced in IL-23^{-/-} mice compared with wild-type mice (Fig. 2B). In addition, the numbers of eosinophils and goblet cells in the nasal mucosa have no significant difference between wild-type mice and IL-17^{-/-} mice (Fig. 3A–C). On the

other hand, the numbers of eosinophils and goblet cells in the nasal mucosa were significantly reduced in IL-23^{-/-} mice compared with wild-type mice 24 h after the last inhalation of OVA (Figs. 3A and B), although the thickness of the submucosal layers was comparable between these groups (Fig. 3C). These observations suggested that IL-23, but not IL-17, was crucial for eosinophilia and goblet cell hyperplasia. However, neither IL-23 nor IL-17 affected the submucosal thickness in OVA-induced AR. Furthermore, the serum levels of total IgE and OVA-specific IgG1 in IL-17^{-/-} mice and IL-23^{-/-} mice were elevated similarly to those in wild-type mice 24 h after the last OVA inhalation (Fig. 3D and E), indicating that neither IL-23 nor IL-17 was essential for IgE or IgG1 production in OVA-induced AR.

3.2. IL-23, but not IL-17, is required for IL-4 production, but not Th2 cell differentiation, during OVA-induced AR in mice

To determine the effect of IL-23 and IL-17 on Th2 responses in OVA-induced allergic rhinitis, 24 h after the last OVA or normal saline inhalation, harvested cervical LN cells were cultured in the presence or absence of OVA. As shown in Fig. 4A and B,

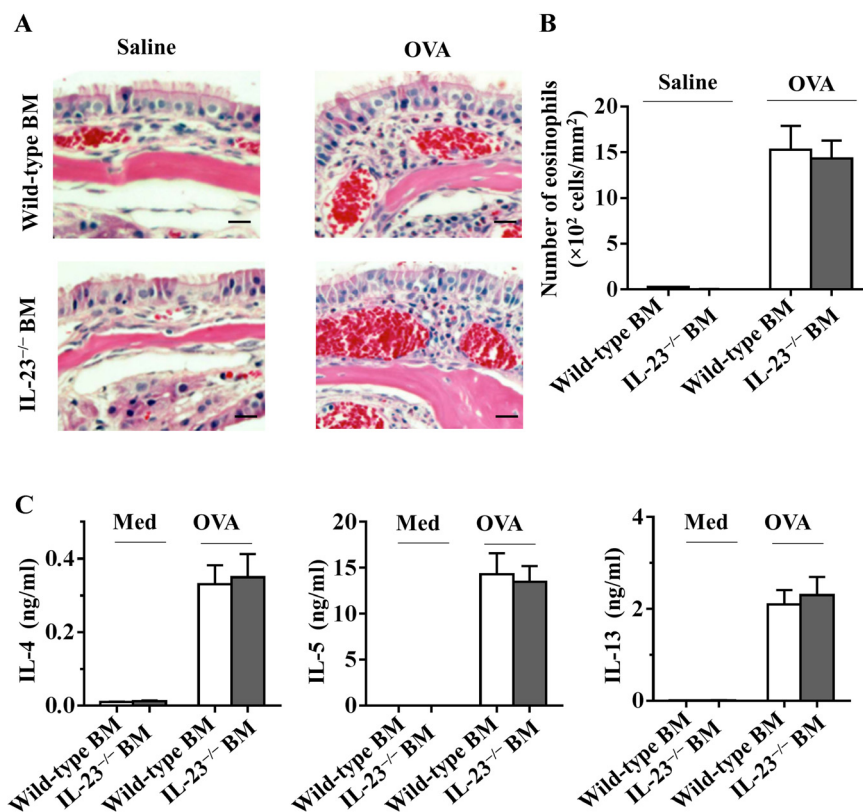


Fig. 5. IL-23 produced by immune cells derived from bone marrow stem cells is not crucial for development of OVA-induced AR. (A) H&E staining of nasal mucosa. (B and C) The number of eosinophils in the nasal mucosa (B) cytokine levels (C) in the culture supernatants of OVA-stimulated LN cells from wild-type mice injected with wild-type bone marrow (BM) cells or IL-23^{-/-} BM cells, 24 h after the last inhalation of OVA or normal saline. Data are presented as the mean $\pm$ SD ($n=6$). Scale bars, 100 μ m.

OVA-specific LN cell proliferation was observed in mice treated intranasally with OVA, but not normal saline. LN cells from IL-23^{-/-} mice (Fig. 4A) and IL-17^{-/-} mice (Fig. 4B) showed no difference from the wild-type cells. In addition, although the IL-5 and IL-13 levels in the supernatants of LNs cells cultured with OVA were equivalent between wild-type and IL-23^{-/-} mice, the level of IL-4 was significantly reduced in the IL-23^{-/-} mice (Fig. 4B). Moreover, the levels of IL-4, IL-5 and IL-13 in the supernatants of LNs cells cultured with OVA displayed no significant difference between wild-type and IL-17^{-/-} mice (Fig. 4B). Nevertheless, flow cytometry assay showed that the proportions of IL-4⁺ and IFN- γ ⁺ CD4⁺ T cells among the cervical LN cells cultured in the presence or absence of OVA did not differ among these mice (Fig. 4C). These observations suggested that neither IL-23 nor IL-17 was essential for Th2 cell differentiation, whereas IL-23, but not IL-17, was important for IL-4 production by Th2 cells in OVA-induced AR.

3.3. IL-23 produced by immune cells derived from bone marrow stem cells is not crucial for development of OVA-induced AR

To further investigate the role of IL-23 in OVA-induced AR, bone marrow (BM) cell transfer analysis was performed to elucidate the contribution of immune-cell-produced IL-23 to OVA-induced AR. The number of eosinophils in the nasal mucosa of CD45.1-C57BL/6 wild-type mice have no difference, regardless of whether they had been injected with CD45.2-C57BL/6N wild-type BM cells or IL-23^{-/-} BM cells (Fig. 5A and B). Consistent with these results, the levels of IL-4, IL-5 and IL-13 in the culture supernatants of cervical LN cells in the presence or absence of OVA also have no significant difference between both groups (Fig. 5C). These observations

suggested that immune-cell-derived IL-23 was not essential for development of OVA-induced AR.

4. Discussion

In allergic rhinitis patients, serum IL-17 responses have been shown to correlate with the symptoms severity scores, medication use, and peripheral eosinophil count (Ciprandi et al., 2008, 2009). The development and maintenance of Th17 cells is dependent on IL-23. IL-23 is a heterodimeric cytokine composed of IL-12p40 and p19 subunits (Korn et al., 2009), which is secreted by activated antigen-presenting cells (APC) such as dendritic cells and macrophages (Kastelein et al., 2007; Wilson et al., 2007). IL-23 has also been shown to enhance the expression of IL-17, by either *in vitro* generated human Th17 or *ex vivo* isolated memory Th17 cells, whereas IL-23 also exerts an inhibitory effect on the IL-12-induced IFN- γ responses in Th1 cells (Annunziato et al., 2007). Additionally, accumulating evidences suggest that both IL-23 and IL-17 are significantly upregulated in many tumors, such as hepatocellular carcinoma, non-small cell lung cancer, and colorectal cancer (Helbling et al., 2014; Xu et al., 2014; Zarogoulidis et al., 2014). Further, it has been reported that IL-23 signaling promotes tumor growth and progression, and development of a tumoral IL-17 response (Grivnenkov et al., 2012). However, the potential pathogenic mechanisms associated with these mediators in allergic rhinitis are largely unknown. In the current study, we found that IL-23, but not IL-17, was crucial for the development of OVA-induced AR.

Previous study has shown that IL-23 and IL-17 were up-regulated in OVA-induced AR mouse model, and anti-IL-23 and anti-IL-17 Ab treatment had different effects. Anti-IL-23 Ab

treatment significantly alleviated nasal symptoms, accompanied by reduced Th2 responses, serum OVA-specific IgE, as well as nasal neutrophil, eosinophil and mast cell infiltration (Wang et al., 2013). In this study, we found that the mRNA levels of IL-23 and IL-17 in the nasal mucosa were significantly increased in OVA-treated mice. Moreover, we observed the infiltration of leukocytes and hyperplasia of goblet cells in the nasal mucosa of OVA treatment mice. These findings suggest that IL-23 and IL-17 may contribute to the development of OVA-induced AR. Although IL-23 has been shown to be essential for Th17 response (Langrish et al., 2005; McGeachy et al., 2007), the different effects between anti-IL-23 and anti-IL-17 Ab indicate that IL-23 and IL-17 may play a pathogenic role in an established AR mouse model. Further studies are needed to elucidate the potential mechanisms associated with IL-23 and IL-17 in OVA-induced AR.

To date, the contribution of IL-23 and IL-17 to asthma is controversial, with different studies proposing either a pathogenic (Haworth et al., 2008; Li et al., 2011; Wakashin et al., 2008) or a protective role (Schnyder-Candrian et al., 2006). Wang et al. (2013) reported that IL-17 has a pathogenic role in this well-established mouse model of AR, and its effects are limited to the Th2 responses, nasal neutrophil and eosinophil infiltration. A previous study demonstrated that the failure of anti-IL-17Ab to inhibit OVA-specific Ig-E and mast cell infiltration may contribute to its failure to alleviate nasal symptoms, because IgE levels are related to mast cell migration and activation (Mathias et al., 2009). The limited effects of anti-IL-17 Ab indicated that IL-17 may not be a crucial pathogenic mediator in mouse model of AR. However, further studies with higher doses of anti-IL-17 Ab, and/or different routes of administration (e.g. giving intraperitoneally), as well as investigations in IL-17-deficient mice need to be done to confirm this. In the present study, we found that neither IL-23 nor IL-17 was essential for differentiation of OVA-specific Th2 cells, whereas IL-23 was required for enhancement of IL-4 secretion by Th2 cells, during OVA-induced AR. In contrast to ragweed-induced AR (Haenuki et al., 2012), we found that IL-23^{-/-} mice, as well as IL-17^{-/-} mice, showed serum IgG1 and IgE production levels similar to those in wild-type mice during OVA-induced AR. It is well documented that allergic inflammation in the upper respiratory tract (i.e., AR) is suspected to influence induction of allergic inflammation in the lower respiratory tract (i.e., asthma) (Shaaban et al., 2008). Others showed that IL-17 was involved in tissue remodeling during OVA-induced airway inflammation (Gregory et al., 2013). However, we found that neither IL-23 nor IL-17 was necessary for sub-mucosal thickening during chronic AR induced by OVA. Furthermore, IL-23 was reportedly produced/expressed by various types of immune cells (such as macrophages, dendritic cells and mast cells) as well as non-immune cells (such as epithelial cells, endothelial cells and fibroblasts) (Sherlock et al., 2015; Wang et al., 2013). Our findings suggest that immune-cell-derived IL-23 is not essential for development of OVA-induced AR.

In conclusion, our current study demonstrates a pathogenic role of IL-23 and IL-17 in an established mouse model of AR. IL-23, rather than IL-17, plays important roles in AR induced by OVA. Moreover, our present findings raise the possibility that IL-23 blockade may be a novel therapeutic method for AR patients.

Conflict of interest statement

The authors declare no conflict of interest.

Acknowledgements

None.

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