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

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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

Cell Research (2010) 20:62–71. doi: 10.1038/cr.2009.128; published online 24 November 2009

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⁺ T cells differentiate into a prevailing Th2 effector phenotype, and these cells predominantly secrete 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].

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 [4]. IL-

23 binds to a heterodimeric receptor composed of the IL-12 receptor β 1 (IL-12R β 1) and IL-23 receptor (IL-23R) and this subsequently activates Jak2, Tyk2, and signal transducers and activators of transcription (STATs) 1, 3, 4, and 5 [5]. Like IL-12, IL-23 is primarily secreted by activated DCs, monocytes, and macrophages [6]. It has been shown that IL-23 is important in some inflammatory diseases including experimental autoimmune encephalitis, collagen-induced arthritis, and intestinal inflammation [7–9]. Moreover, polymorphisms in the gene encoding the IL-23R are an important susceptibility factor for these disorders [10–12].

The proinflammatory features of IL-23 have been linked with Th17 cell responses, through expansion and/or maintenance of the Th17 cells [11]. Th17 cells are a recently described Th subset characterized by the production of IL-17 (IL-17A), IL-17F, and IL-22 [13, 14], and have been associated with the induction of autoimmune tissue inflammation [15–17]. Moreover, it has been shown that IL-17 is expressed in the airway of patients with asthma [18–20]. Recently, we showed that IL-17 regulates allergic airway inflammation positively, while IL-17F regulates it negatively [21]. A recent report revealed dual effects of IL-17 on allergic asthma [22]. It is required for asthma induction, but negatively regulates established asthma. Although many studies have been

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Received 24 July 2009; revised 8 September 2009; accepted 17 September 2009; published online 24 November 2009

focused on the IL-23-Th17 axis, the effect of IL-23 is evidenced in some diseases to be independent of IL-17 production [23, 24]. For instance, anti-IL-17 treatment had little impact on the T-cell-mediated colitis, although the colitis was dependent on IL-23 [24]. Furthermore, development of IL-23-dependent colitis did not require IL-17 secretion by T cells [23]. In this context, IL-23 targets not only Th17 cells but also other cell types to modulate inflammatory responses [25]. Recently, Wakashin *et al.* [26] 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 that IL-23 may regulate allergic airway inflammation through an IL-17-independent pathway. Whether IL-23 directly regulates Th2 cell responses, however, is unclear.

Here, we found that IL-23 deficiency alleviated 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-23R Tg mice compared with their controls. On the other hand, IL-23-IL-23R signaling promoted GATA-3 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.

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 (chicken ovalbumin)-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 (Figure 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 associate with inflammatory diseases [10, 12], we further examined IL-23R mRNA expression in the lungs. After OVA challenge, expression of IL-23R mRNA was greatly increased (Figure 1B). These results suggest that 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-23 knock out (KO) 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 h after the last challenge. As shown in Figure 2A, antigen-induced inflammatory cell infiltration was greatly inhibited in the lungs from IL-23 KO mice compared with that from WT mice, consistent with a previous observation using IL-23-neutralizing antibody [26]. Cellular profiles in bronchoalveolar lavage fluid (BALF) were assessed by cytopsin with May-Gruenwald Giemsa stain. Eosinophils, macrophages, and neutrophils were significantly decreased in IL-23-deficient mice (Figure 2B). Consistently, RT-PCR analysis indicated that IL-23 deficiency led to dramatically decreased expression of eosinophil peroxidase (EPO) in IL-23 KO mice (Figure 2C).

IgE responses are a hallmark of allergic responses. In fact, in IL-23-deficient mice, OVA-specific IgE expres-

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, β -actin. Data shown represent mean $\pm$ SD from two independent experiments ($n = 4$ –6 per group). Student's *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 and E staining. Bar, 100 μ m. **(B)** Total cells of BALF from the asthmatic mice. Horizontal bars represent the means. Cellular profiles in BALF upon OVA challenge were assessed by cytospin 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 β -actin. Data shown are 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 two independent experiments with consistent results ($n = 4-6$). Student's *t*-test, * $P < 0.05$; ** $P < 0.005$.

sion is significantly lower than that in WT mice (Figure 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-23 KO mice was significantly lower in comparison with WT cells (Figure 2E). Splenocytes from IL-23 KO mice also exhibited greatly reduced Th2 cytokine production (Figure 2E), indicating that IL-23 may affect Th2 responses at the priming phase. However, OVA-specific Th17 responses were observed at similar low levels in both IL-23 KO and WT mice (Figure 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 Tg 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 Tg mice with IL-23R overexpression in T cells using the human CD2 mini locus promoter [27] (Figure 3A). One of the two lines (Line 2) was extensively analyzed. Next, we verified the function of IL-23R Tg during Th17 cell differentiation. Naïve $CD4^+$ T cells from IL-23R Tg mice or their littermate control were differentiated into Th17 cells

Figure 3 Transgenic expression of IL-23R enhanced Th17 differentiation but 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 two 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. Four days later, IFN- γ and IL-17-producing cells were analyzed by intra-cellular staining. Numbers within the quadrants indicate the percentage of positive cells. Data shown represent as least two independent experiments with consistent results.

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 cells in IL-23R Tg T cells (Figure 3B). Furthermore, under the Th1 condition, addition of IL-23 greatly inhibited the generation of IFN- γ -producing cells in IL-23R Tg T cells (Figure 3C). Thus, Tg overexpression of IL-23R enhances Th17 but inhibits Th1 differentiation.

IL-23R Tg-enhanced allergic airway inflammation

To examine whether IL-23 directly acts on CD4⁺ T cells in regulation of airway inflammation, we induced asthma in IL-23R Tg and littermate WT mice. Histology analysis revealed that Tg overexpression of IL-23R resulted in increased inflammatory cell infiltration in the lung (Figure 4A). Consistent with the histological analy-

sis, in BALF, the total cell numbers, eosinophils, macrophages, and neutrophils were significantly increased in IL-23R Tg mice compared with their littermate controls (Figure 4B). Dramatically elevated expression of EPO in the lung of IL-23R Tg mice indicated enhanced eosinophil function (Figure 4C).

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

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 and E staining. Bar, 100 μ m. **(B)** Total cells in BALF. Horizontal bars represent the means. Cellular profiles in BALF upon OVA challenge were assessed by cytopspin. **(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 β -actin. Data shown are 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 two independent experiments with similar results ($n = 4-6$). Student's *t*-test, * $P < 0.05$; ** $P < 0.005$.

IL-23 regulates Th2 differentiation *in vitro*

To further clarify 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^{high}CD44^{low} naïve CD4⁺ T cells from WT mice were differentiated into Th2 cells with splenic APCs from IL-23 KO or WT mice, and cytokine expression patterns were assessed. CD4⁺ T cells activated with IL-23-deficient APCs exhibited reduced IL-4-producing cell numbers, and visually no IL-17-producing cells were observed (Figure 5A). As revealed by ELISA, IL-23-deficient APCs led to significantly reduced amounts of IL-4 and IL-5 expression by effector T cells (Figure 5B). Consistently, GATA-3 mRNA expression was significantly decreased in T cells treated with IL-23

KO APCs compared with those treated with WT APCs (Figure 5C). Similar results were observed when naïve T cells were activated with plate-bound anti-CD3 and anti-CD28 in the presence of WT or IL-23 KO APC culture supernatants rather than the APCs (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⁺ 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 (Figure 6A, 6B). In WT T cells, only IL-13 was significantly enhanced by IL-23 treatment (Figure 6A, 6B). Consistently,

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, and anti-IFN- γ . **(A)** Five 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, GATA-3, and Foxp3 mRNA expression was analyzed by quantitative real-time RT-PCR. **(B, C)** The data are expressed as the mean $\pm$ SD of triplicate samples. Student's *t*-test, **P* < 0.05; ***P* < 0.005; *P*-values were calculated from two to three independent experiments with consistent results.

in response to IL-23 stimulation, IL-23R Tg but not WT T cells highly expressed GATA-3 mRNA (Figure 6C). Taken together, the IL-23-IL-23R signaling can promote Th2 differentiation through direct action on CD4⁺ 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 Tg 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, Tg overexpression of IL-23R enhanced the symptoms of allergic airway inflammation. Moreover, our *in vitro* data on 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 [28]. During antigen challenge, we detected profoundly reduced levels of inflammatory cells

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, and anti-IFN- γ with or without IL-23. **(A)** Five 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, GATA-3, and Foxp3 mRNA expression was analyzed by quantitative real-time RT-PCR. **(B-C)** The data are expressed as the mean $\pm$ SD of triplicate samples. Student's *t*-test, **P* < 0.05; *P*-values were calculated from two to three independent experiments with consistent results.

infiltrating into the lung tissue in IL-23 KO mice, including both eosinophils and neutrophils. These results are consistent with a recent report that neutralization of IL-23 attenuated antigen-induced eosinophil and neutrophil recruitment [26]. 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 was dramatically reduced in the lung-draining mediastinal lymph node cell and splenocyte cultures from IL-23 KO mice. 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-23 KO WT or IL-23R Tg mice, we observed that neither IL-17 nor IL-17F showed significantly altered expression in the lymph node cell and in splenocyte cultures compared with WT control. 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 showing that development of IL-23-dependent colitis did not require IL-17 secretion by T cells [23]. However, we found that in lungs, IL-23 deficiency led to reduced expression, while IL-23R Tg enhanced the expression of IL-17 mRNA (data not shown), suggesting that a non-Th17 source of IL-17 (maybe also IL-17F) may be involved in local inflammatory responses in agreement with a previous report that in lungs, the major source of IL-17 and IL-17F is $\gamma\delta$ -T cells but not Th17 cells during tuberculosis infection [29].

Along with Th17 cells, IL-23 can target multiple cell types, such as NK cells, DCs, 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 that Tg overexpression of IL-23R significantly enhanced the frequencies of IL-17-producing T cells under the Th17 condition, while it reduced IFN- γ -producing T cell numbers under the Th1 condition. At this point, whether IL-23 signaling enhances Th2 differentiation indirectly through inhibition of the 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 IL-23-sufficient APC culture media instead of APC themselves (data not shown). Furthermore, using an APC-free system, we found that Tg overexpression of IL-23R led to upregulated Th2 cytokine levels and dramatically enhanced GATA-3 expression, suggesting that IL-23-IL-23R signaling can directly target Th2 cells independent of APCs. Previous studies showed that IL-23R when binding to IL-23 activates STAT1, 3, 4, and 5 [5]. Whether IL-23 signaling enhances Th2 differentiation through activation of STAT5, which 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.

Materials and methods

Mice

Generation of IL-23R Tg mice: IL-23R cDNA was amplified from a Th17 cell cDNA pool and inserted into pCD2 containing a human CD2 mini locus [27]. 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 MD Anderson Cancer Center. Transgenic founders were maintained by breeding with B6 mice. IL-23 α subunit p19 targeted mutant mice were purchased from NIH Mutant Mouse Regional Resource Centers. Homozygous knockout (hereafter referred to as IL-23 KO) and WT animals on the mixed 129 $\times$ C57BL/6 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 MD Anderson Cancer Center.

Induction of asthma

Mice were i.p. immunized twice at 2-week intervals with 0.2 ml saline containing 100 μ g chicken ovalbumin (OVA) in aluminum hydroxide (alum), sensitized at day 14, and rechallenged intranasally three more times at days 25, 26, and 27 with 100 μ g OVA. At 24 h after the last challenge, mice were killed and BALF and lungs were collected. BALF 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 collected 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 and E. Peribronchial and perivascular inflammation was assessed using light microscopy under $\times 50$ magnifications.

T-cell differentiation

CD4⁺CD25[−]CD62L^{high}CD44^{low} 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 μ g/ml) and anti-CD28 (2 μ g/ml) in the presence of TGF- β (5 ng/ml, Peprotech), IL-6 (20 ng/ml, Peprotech), anti-IFN- γ (5 μ g/ml; XMG 1.2), and anti-IL-4 (5 μ g/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 μ g/ml) and anti-CD28 (2 μ g/ml) in the presence of IL-12 (10 ng/ml) and anti-IL-4 (5 μ g/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 h.

For Th2 differentiation, naïve T cells were activated with 2 μ g/ml of plate-bound anti-CD3 and irradiated T-cell-depleted splenic antigen-presenting cells or 2 μ g/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 μ g/ml; XMG 1.2) with or without IL-23 (20 ng/ml). Four days later, cells were washed and restimulated with 2 μ g/ml of plate-bound anti-CD3 for 24 h, and culture supernatants were analyzed for cytokine expression by ELISA. For intracellular cytokine analysis, cells were washed and restimulated with 2 μ g/ml plate-bound anti-CD3 overnight, and with GolgiStop (BD Biosciences) for the last 5 h. The cells were permeabilized and stained with a Cytofix/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 following primer pairs were used: IL-17F forward, 5'-TGC AGA AGG CTG GGA ACT GTC C-3', and reverse, 5'-TGA CCC TGG GCA TTG ATG CAG C-3'. GATA-3 forward, 5'-AGG GAC ATC CTG CGC GAA CTG T-3', and reverse, 5'-CAT CTT CCG GTT TCG GGT CTG G-3'. Other primers were described previously [14, 30, 31].

Statistical analysis

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

Acknowledgements

We thank Dr Jan Parker-Thornburg (MD Anderson Cancer Center, USA) for her help in the generation of IL-23R transgenic animals and the Dong lab members for their help and discussion. The work was supported by research grants from the NIH, and the MD Anderson Center for Targeted Therapy (to CD) and from the American Heart Association (to XOY). JP receives a fellowship from the 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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