

ORIGINAL ARTICLE

Der p2 recombinant bacille Calmette-Guerin priming of bone marrow-derived dendritic cells suppresses Der p2-induced T helper17 function in a mouse model of asthma

SHUO-YAO QU,^{1*} HAI-FENG OU-YANG,^{1*} YA-LONG HE,² ZHI-KUI LI,¹ JIE-RAN SHI,¹ LI-QIANG SONG¹ AND CHANG-GUI WU¹

¹Department of Pulmonary and Critical Care Medicine and ²Department of Neurosurgery, Xijing Hospital, Fourth Military Medical University, Xi'an, China

ABSTRACT

Background and objective: Previous studies have demonstrated that our recombinant bacille Calmette-Guerin (rBCG), which expresses Der p2 in house dust mite (Der p2 rBCG) suppresses asthmatic airway inflammation by regulating the phenotype and function of dendritic cells (DC) and reprogramming T helper (Th) 0 cell differentiation into different T cell (Th1/Th2/Treg) subtypes. However, the exact role of Der p2 rBCG in reprogramming Th17 differentiation and the relevant mechanisms are not known. The aim of this study was to examine whether Der p2 rBCG-mediated inhibition of allergic airway inflammation is mediated by regulating Th17 differentiation in a murine asthma model.

Methods: Primary mouse bone marrow-derived dendritic cells (BMDC) were infected with Der p2 rBCG and adoptively transferred to Der p2-intranasally sensitized mice. The role of Der p2 rBCG-BMDC on the regulation of airway inflammation and Th17 cell differentiation was assessed.

Results: Adoptive transfer of Der p2 rBCG-BMDC suppressed airway inflammation and mucin secretion. Der p2 rBCG-BMDC inhibited excessive Th17 immune responses but not BCG-BMDC. Furthermore, Der p2 rBCG decreased jagged-2 and increased delta-like-4 expressions on BMDC to a greater extent than BCG.

Conclusions: These findings suggest that DC plays a key role in Der p2 rBCG-induced immunoregulation. Der p2 rBCG also displayed a potent inhibitory effect on Th17 differentiation, and these findings increase our understanding of the cellular basis of Der p2 BCG-mediated inhibition of asthma.

Key words: airway inflammation, dendritic cell, Notch signaling, recombinant bacille Calmette-Guerin, T helper 17.

Correspondence: Chang-Gui Wu, Department of Pulmonary and Critical Care Medicine, Xijing Hospital, Fourth Military Medical University, Xi'an 710032, China. Email: changgui@fmmu.edu.cn

*These authors contributed equally to this study.

Received 17 April 2013; invited to revise 7 June 2013; revised 27 June 2013; accepted 22 July 2013 (Associate Editor: Darryl Knight).

SUMMARY AT A GLANCE

This study shows that in a mouse model of asthma, Der p2 recombinant BCG is more effective in suppressing asthmatic airway inflammation than BCG alone. This effect is related to Th17 regulation during differentiation, which is associated with the regulation of IL-6 secretion and Notch ligand expression by dendritic cells.

Abbreviations: BCG, bacille Calmette-Guerin; DC, dendritic cell; IL, interleukin; MLR, mixed lymphocyte reaction; PS, physiological saline; rBCG, recombinant BCG; ROR γ t, retinoic acid-related orphan receptor γ t; TGF- β , transforming growth factor- β ; Th, T helper; TLR, toll-like receptor.

INTRODUCTION

Excessive T helper (Th) 2 cells play a key role in orchestrating chronic airway inflammation in allergic asthma. However, Th2 cytokine inhibition has been unsuccessful in treating established asthma in patients.¹ Recently, knowledge of the pathogenesis of asthma has broadened to incorporate the contribution of Th17 cells. Th17 cells have been shown to be effective in upregulating neutrophilic and macrophage inflammation in the lung.² Recombinant bacille Calmette-Guerin (rBCG), which expresses the Der p2 of house dust mites (Der p2 rBCG), can suppress asthmatic airway inflammation by inducing more allergen-specific T regulatory cells, while native BCG cannot.³ Further studies have shown that these effects are involved in regulating the functions and phenotypes of dendritic cells (DC).⁴ There is a close relationship between mycobacterial infection and Th17 differentiation; extended freeze-dried BCG blocked transcriptional regulator signatures of Th17 (retinoic acid-related orphan receptor γ t (ROR γ t)) cells.⁵ Similarly, the frequency of Th17 cells in patients with active tuberculosis is significantly less than that in healthy donors.⁶

The Notch signalling pathway is an important pathway that regulates development with high conservation between species.⁷ Notch receptors and ligands are involved in the interaction between DC and T cells. The effects produced by the different Notch receptors and ligands are different. It has been reported that Delta-like proteins 1 and 4 can promote naive T cells to differentiate into Th1, while in the pro-Th2/Th17 environment, the level of the protein jagged-2 on the DC surface is elevated.⁸

Hence, we hypothesized that Der p2 rBCG plays an important role in regulating Th17 differentiation causing inhibition of allergic airway inflammation. In this study, we compared the immunoregulatory role of Der p2 rBCG with native BCG in Th17 differentiation by employing a mouse asthma model.

METHODS

Animals

Healthy female C57BL/6 mice (6–8 weeks of age) were purchased from the Experimental Animal Center of Fourth Military Medical University (Xi'an, China). All research protocols were approved by the Animal Experiment Administration Committee of Fourth Military Medical University.

Der p2 rBCG

Der p2 rBCG was constructed as previously described.⁹ Der p2 rBCG and native BCG were cultured and harvested as previously described.⁴

Bone marrow-derived DC culture and stimulation

Mouse bone marrow was flushed from femurs and tibias, and a single-cell suspension was prepared, followed by erythrolysis in ammonium chloride-potassium buffer. Cells were cultured in Roswell Park Memorial Institute-1640 medium containing 20% foetal calf serum and 2 mmol/L L-glutamine (20 ng/mL murine granulocyte macrophage-colony stimulating factor, 10 ng/mL murine interleukin (IL)-4, PeproTech, Inc., Rocky Hill, NJ, USA), and the medium was refreshed on days 3 and 6.¹⁰ After 9 days, BMDC was harvested and stimulated with BCG or Der p2 rBCG (1×10^6 CFU/mL). Another group treated with physiological saline (PS) served as a control.

DC transfer and study design

Mice were randomly divided into five groups: a control group, asthma model group, Der p2 rBCG-BMDC transfer group, BCG-BMDC transfer group and PS-BMDC transfer group. Each group had six mice. In the control group, mice were treated, sensitized and challenged with PS. In the asthma model group, mice were sensitized with 100 µg of Der p2 (Indoor Biotechnologies, Inc., Charlottesville, VA, USA) absorbed to potassium alum (intraperitoneally) (A1577; Sigma-Aldrich, St. Louis, MO, USA) and challenged with Der p2 intranasally without any BMDC transfer. In other

groups, mice were first sensitized on days 0 and 7. BMDC that was pretreated with Der p2 rBCG, BCG or PS was irradiated (10 Gy) and injected (intranasally) (1×10^6 cells per mouse) into the sensitized mice on day 14. The mice were challenged with 100 µg of Der p2 intranasally on days 15, 16 and 17, and sacrificed 48 h after the last challenge.

Bronchoalveolar lavage fluid

Bronchoalveolar lavage fluid was collected as previously described.⁴ The cytokines concentrations of bronchoalveolar lavage fluid were assayed by enzyme-linked immunosorbent assay kits (Neobioscience, Beijing, China).

Lung histology

Lungs were fixed with 10% buffered formalin after bronchoalveolar lavage fluid collection. Lung samples were embedded in paraffin and then sectioned (4 µm). Sections were stained with haematoxylin and eosin as well as Alcian blue-periodic acid Schiff reagents. The histological mucus index was quantified by the formula as previously described.¹¹

Mixed lymphocyte reaction

Purified naive CD4⁺ T cells were isolated as previously described.⁴ Naive T cells were co-cultured with BMDC that were previously treated with Der p2 rBCG, BCG or PS (ratio, 1 : 5). Der p2 antigen was added at the beginning of the DC-T cell co-culture. After 72 h, the supernatants were collected for enzyme-linked immunosorbent assays, while the cells were collected for fluorescence-activated cell sorting analysis.

Fluorescence-activated cell sorting analysis of Th17 cells

Cell suspensions of lung tissues were prepared.⁴ Whole cells in mixed lymphocyte reaction were collected and resuspended in fluorescence-activated cell sorting buffer. Cells were labelled with PE-anti-mouse CD4 antibody (GK1.5, BD PharMingen, San Diego, CA, USA) and FITC-anti-mouse IL-17 (TC11-18H10.1, Biolegend, San Diego, CA, USA).

Real-time polymerase chain reaction analysis of ROR-γt messenger RNA

RNA was extracted with TRIZOL reagent (Invitrogen, Carlsbad, CA, USA), and complementary DNA was generated using reverse transcriptase (Fermentas, Vilnius, Lithuania) according to the manufacturer's instructions. Real-time polymerase chain reaction was performed using a kit (SYBR Premix EX Taq, Takara, Dalian, China) and ABI PRISM 7500 real-time polymerase chain reaction equipment. Sequences of primers used are listed in Table S1.

Analysing toll-like receptor function of DC phenotype regulation

BMDC was treated with toll-like receptor (TLR)-2/TLR-4 (OxPAPC) (30 µg/mL, Invivogen, San Diego,

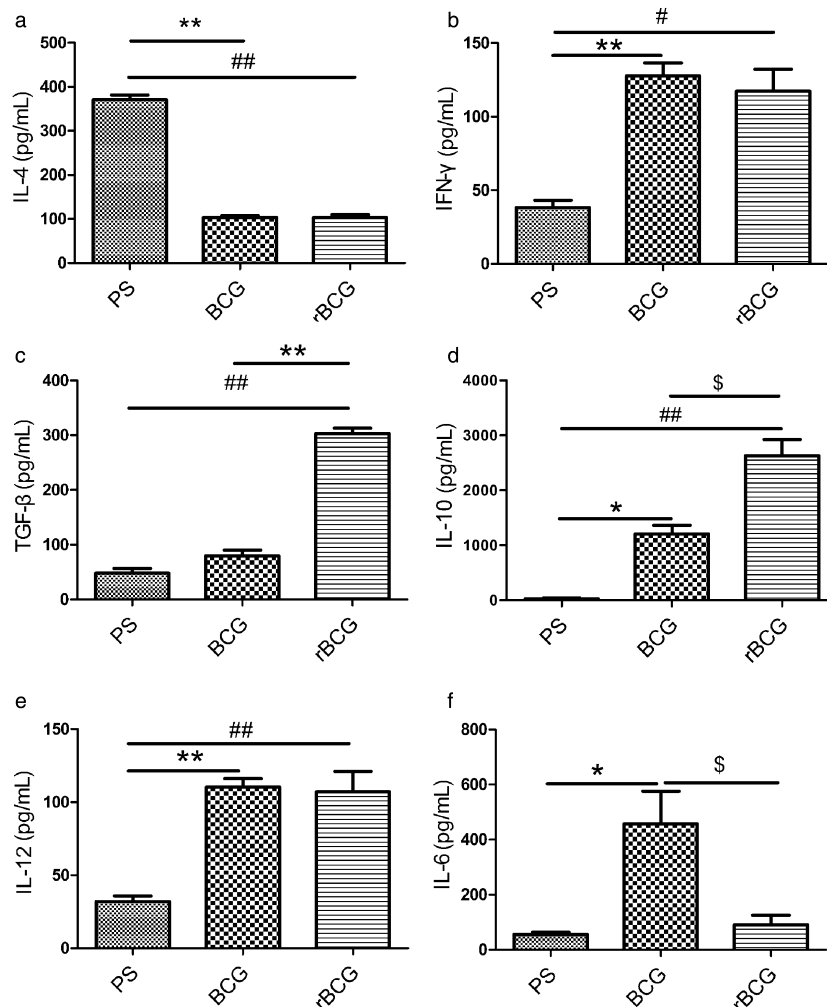


Figure 1 Cytokine profiles of stimulated bone marrow-derived dendritic cells (BMDC). BMDC was stimulated with physiological saline (PS), bacille Calmette-Guerin (BCG), Der p2 recombinant BCG (rBCG). After 72 h, the supernatants were collected and taken for cytokine analysis by enzyme-linked immunosorbent assay. Secretion levels of (a) interleukin (IL)-4, (b) interferon (IFN)- γ , (c) transforming growth factor (TGF)- β , (d) IL-10, (e) IL-12 and (f) IL-6 are shown as mean $\pm$ standard error of the mean ($n = 6$). * $P < 0.05$, ** $P < 0.01$; # $P < 0.05$, ## $P < 0.01$; \$ $P < 0.05$.

CA, USA) or TLR-9 antagonist (ODN2088, 30 μ g/mL, Invivogen) for 24 h or 1 h before Der p2 rBCG/BCG stimulation, TLR antagonists were all dissolved by dimethyl sulfoxide, while the control group was treated with the same volume of dimethyl sulfoxide. Next, CD80, CD86 and major histocompatibility complex II were detected by fluorescence-activated cell sorting. The dead cells were excluded by propidium iodide gating. The data were analysed by Cell Quest software (BD Biosciences, San Diego, CA, USA).

Real-time polymerase chain reaction detection of Notch ligand messenger RNA in DC

BMDC was divided into four groups: Der p2 rBCG, BCG and PS groups, and the fourth group was treated with OxPAPC (30 μ g/mL) 24 h before Der p2 rBCG stimulation. Real-time polymerase chain reaction was performed as described earlier.

Western blot

Western blot analysis was performed routinely, with the following primary antibodies obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA) or Abcam

(Cambridge, MA, USA): anti-TLR-2, -4 and -9 (sc-166900, sc-293072, sc-13215), anti-Dll-4(ab7280) and anti-Jag-2 (sc-28915). Anti-rabbit-immunoglobulin G (Santa Cruz Biotechnology) was used as secondary antibody.

Statistical analysis

Data were expressed as mean $\pm$ standard deviation. Statistical analyses were performed by single-factor analysis of variance for multiple groups and the Tukey test for post-hoc comparisons. A value of $P < 0.05$ was considered statistically significant.

RESULTS

Der p2 rBCG and BCG differentially modulate cytokine production in BMDC

Both Der p2 rBCG and BCG inhibited IL-4 production (Fig. 1a) and elevated interferon- γ (Fig. 1b) and IL-12 (Fig. 1e). There were no significant differences between these two groups ($P > 0.05$). However, Der p2 rBCG induced more transforming growth factor (TGF)- β and IL-10 production than BCG (Fig. 1c,d). In addition, BCG resulted in IL-6 production than Der p2 rBCG (Fig. 1f).

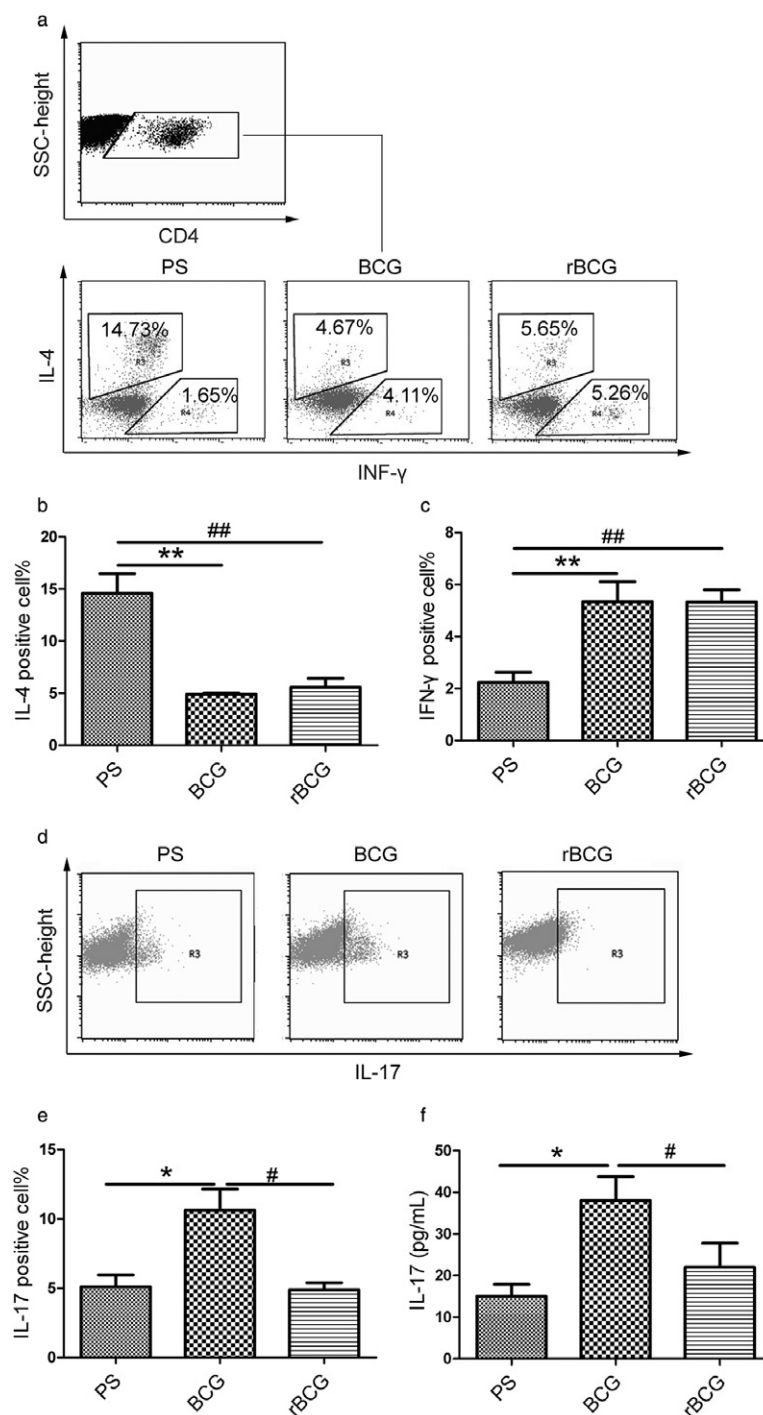


Figure 2 Interleukin (IL)-4 surface staining and interferon (IFN)- γ and IL-17 intracellular staining of CD4⁺ T cells after co-culture with stimulated bone marrow-derived dendritic cells (BMDC). BMDC was pulsed with physiological saline (PS), bacille Calmette-Guerin (BCG), Der p2 recombinant BCG (rBCG) for 72 h before co-culture with isolated CD4⁺ T cells from C57BL/6 mice. Der p2 antigen was added at the beginning of the dendritic cell (DC)-T cell co-culture. After 72 h of co-culture, cells were stained for intracellular (a-c) IL-4, IFN- γ and (d,e) IL-17 and analysed by fluorescence-activated cell sorting. (f) IL-17 of the supernatants were collected and analysis by enzyme-linked immunosorbent assay ($n = 6$). * $P < 0.05$, ** $P < 0.01$; # $P < 0.05$, ## $P < 0.01$. SSC, side scatter.

Der p2 rBCG regulates BMDC induction of Th17 differentiation

Der p2 rBCG and BCG had similar capacities to promote Th1 cell differentiation and inhibit excessive Th2 cell differentiation (Fig. 2a-c). BCG induced Th17 cell differentiation, while Der p2 rBCG did not (Fig. 2d,e). The BCG group had the greatest amount of IL-17 in the supernatants, while the Der p2 rBCG group had less, which was the same as the PS group (Fig. 2f).

Adoptive transfer with Der p2 rBCG-BMDC suppresses airway inflammation and Th17 differentiation

Der p2 rBCG-BMDC and BCG-BMDC recipients displayed significant reduction of airway inflammation and mucus secretion (Fig. 3a). The histological mucus index was less in the Der p2 BCG-BMDC group (Fig. 3b). Furthermore, Der p2 rBCG-BMDC had stronger inhibitory effects on Th17 production (Fig. 3c,d).

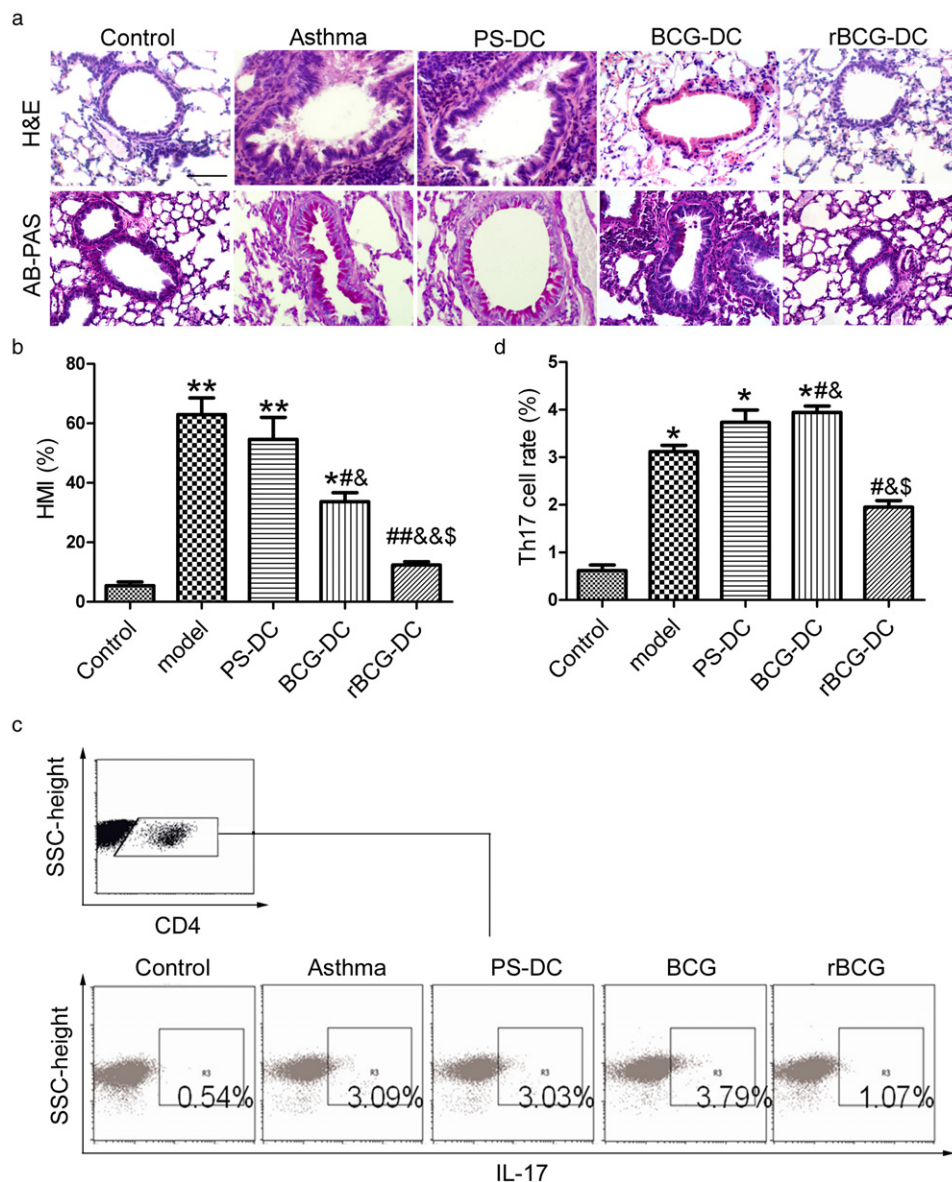


Figure 3 Adoptive transfer of Der p2 recombinant bacille Calmette-Guerin (rBCG)-bone marrow-derived dendritic cells (BMDC) suppressed airway inflammation and mucus secretion *in vivo*. The mice were sensitized with Der p2 (100 µg, intraperitoneally) absorbed with 9% potassium alum. BMDC (1×10^6) from mice immunized with Der p2 rBCG, bacille Calmette-Guerin (BCG) or physiological saline (PS) was transferred intranasally (i.n.) into the sensitized mice. Finally, the mice were challenged with Der p2 (100 µg, i.n.). The experiments were performed 48 h after the last challenge. Haematoxylin and eosin (H&E) stains for analysing airway inflammation and pathological changes. Goblet cells were stained with Alcian blue-periodic acid Schiff (AB-PAS). (a) Lungs were fixed in 10% buffered formalin, and lung sections were examined by H&E or AB-PAS staining. (b) The histological mucus index (HMI) of each group is shown. (c,d) Lung tissues were collected and minced, cells suspension was filtered through a nylon membrane and resuspended in fluorescence-activated cell sorting (FACS) buffer (phosphate buffer saline, 2% foetal calf serum, 0.05% NaN₃), and T helper 17 (Th17) cell number of every group of mice was analysed by FACS group. * $P < 0.05$, ** $P < 0.01$ versus control group; # $P < 0.05$, ## $P < 0.01$ versus model group; & $P < 0.05$, && $P < 0.01$ versus PS-DC group; \$ $P < 0.05$ versus BCG-dendritic cell (DC) group. APTI, airway pressure-time index ($n = 6$); SSC, side scatter.

The levels of IL-4, IL-5 and IL-13 of bronchoalveolar lavage fluid were less in the Der p2 rBCG-BMDC and BCG-BMDC groups (Fig. 4a-c). The levels of interferon- γ in the Der p2-rBCG-BMDC and BCG-BMDC groups were all greater than those in the positive control group and PS-BMDC group (Fig. 4d). Der p2 rBCG-BMDC could enhance

IL-10 more effectively than BCG-BMDC (Fig. 4e). In addition, TGF- β was also significantly greater in the Der p2 rBCG-BMDC group, but there was no change in the BCG-BMDC group (Fig. 4f). The levels of IL-6 and IL-17 were less in the Der p2 rBCG-BMDC group compared with the asthma model group (Fig. 4g,h).

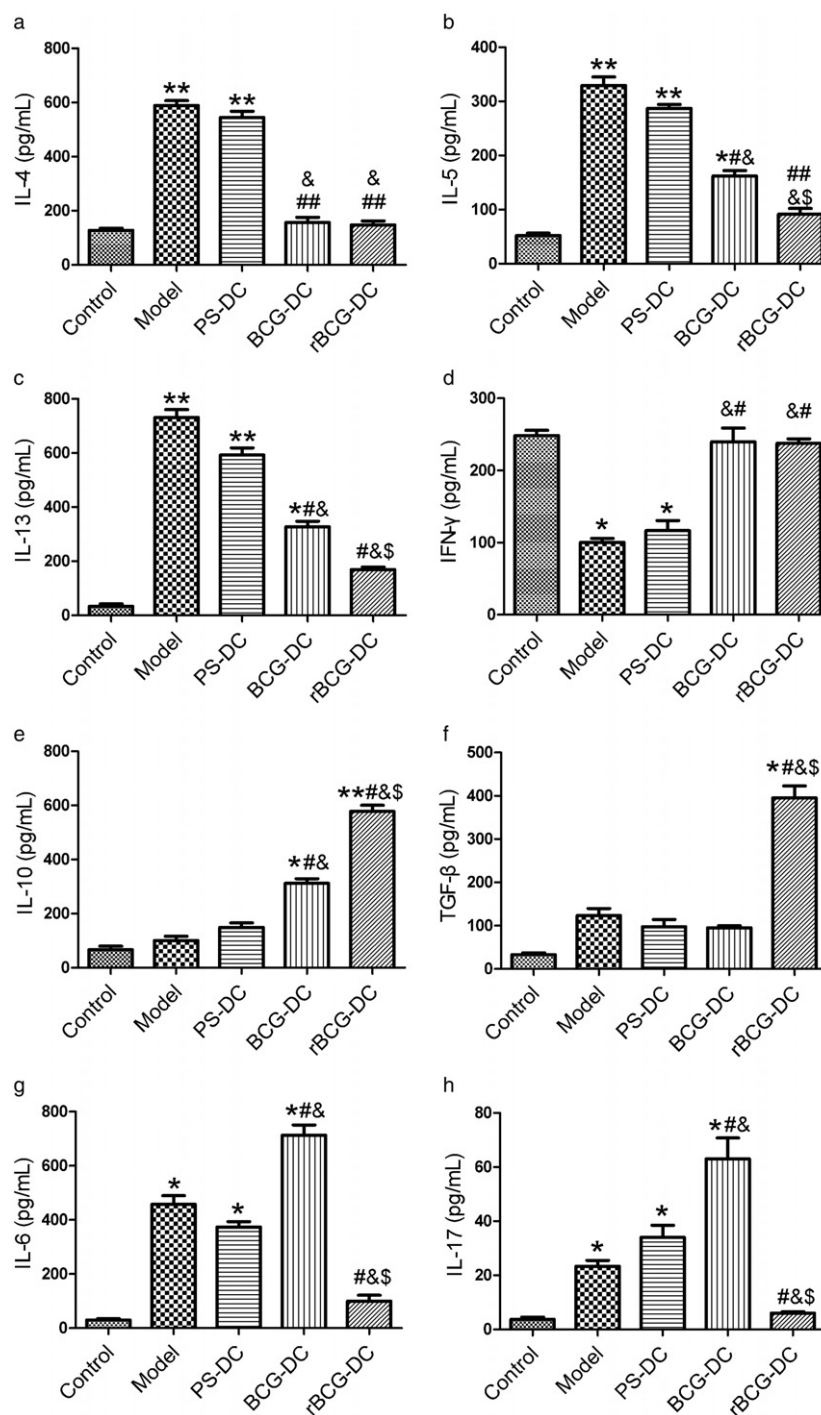


Figure 4 Bronchoalveolar lavage fluid was collected 48 h after the last challenge. The concentrations of (a) interleukin (IL)-4, (b) IL-5, (c) IL-13, (d) interferon (IFN)- γ , (e) IL-10, (f) transforming growth factor (TGF)- β , (g) IL-6 and (h) IL-17 were assayed by enzyme-linked immunosorbent assay. Both Der p2 recombinant bacille Calmette-Guerin (rBCG)-bone marrow-derived dendritic cells (BMDC) and bacille Calmette-Guerin (BCG)-BMDC suppressed the production of T helper 2 (Th2) cytokines (IL-4, 5, 13), but only Der p2 rBCG-BMDC induced immunoregulatory molecules TGF- β and suppress Th17 ($n = 6$). * $P < 0.05$, ** $P < 0.01$ versus control group; # $P < 0.05$, ## $P < 0.01$ versus model group; & $P < 0.05$ versus physiological saline (PS)-dendritic cell (DC) group; \$ $P < 0.05$ versus BCG-DC group.

Der p2 rBCG-infected BMDC regulates the transcription factor Th17

The messenger RNA level of ROR γ t was much less in lung tissues of the Der p2 rBCG-BMDC-group mice than in those of the asthma model, PS-BMDC and BCG-BMDC groups (Fig. 5a). However, it was greater in the BCG-BMDC group than in the asthma model and PS-BMDC groups. In addition, the relative ratio of ROR γ t messenger RNA was also less in the Der p2 rBCG-BMDC group after MLR (Fig. 5b).

Der p2 rBCG activated BMDC through TLR

Western blot analysis showed that Der p2 rBCG-BMDC expressed great levels of TLR-2 and TLR-4 than the PS-BMDC group, while there was no significant difference in the TLR-9 level. Conversely, TLR-9 was elevated in the BCG-BMDC group (Fig. 6).

Der p2 rBCG-BMDC expressed greater levels of major histocompatibility complex II (Fig. 7a,b), CD80 (Fig. 7c,d) and CD86 (Fig. 7e,f) than control DC. Furthermore, TLR-2 and TLR-4 antagonists could

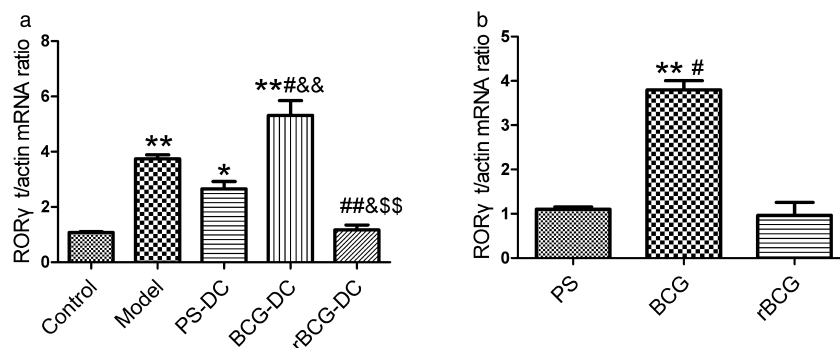


Figure 5 Cells were collected, and the retinoic acid-related orphan receptor γ t (ROR γ t), the vital transcription factor signature of T helper 17 (Th17), messenger RNA (mRNA) level was determined by real-time polymerase chain reaction. (a) *In vivo*; RNA was extracted from every group of mice lung tissue. * $P < 0.05$, ** $P < 0.01$ versus control group; # $P < 0.05$, ## $P < 0.01$ versus model group; & $P < 0.05$, && $P < 0.01$ versus physiological saline (PS)-dendritic cell (DC) group; \$\$\$ $P < 0.01$ versus bacille Calmette-Guerin (BCG)-DC group. (b) *In vitro*, cells were collected from the mixed lymphocyte reaction system ($n = 6$). ** $P < 0.01$ versus PS group vs BCG group; # $P < 0.05$ recombinant BCG (rBCG) group versus BCG group.

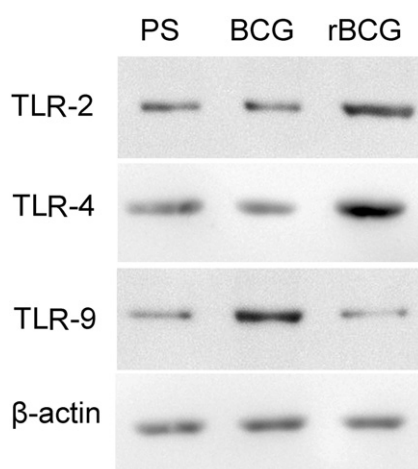


Figure 6 To investigate the molecular basis of Der p2 recombinant bacille Calmette-Guerin (rBCG) in modulating dendritic cell (DC) allergen-driven cytokine production patterns and allergic reactions, Western blot was performed to measure the protein level of toll-like receptor (TLR)-2, TLR-4, TLR-9 and β -actin. Der p2 rBCG-bone marrow-derived dendritic cells (BMDC) expressed significantly greater levels of TLR-2 and TLR-4 than the physiological saline (PS)-BMDC group. Conversely, TLR-9 was elevated in the bacille Calmette-Guerin (BCG)-BMDC group. The data of average of the intensity of the bands in different groups were not shown. Western blotting was performed, and TLR-2, -4 and -9 expression values were normalized to β -actin ($n = 6$).

inhibit these changes effectively, while the TLR-9 antagonist could not. Conversely, TLR-9 antagonists could inhibit these changes effectively, while TLR-2 and TLR-4 antagonists did not achieve this effect in the BCG group (Fig. 7a–e).

Der p2 rBCG regulates Notch ligand expression in BMDC through TLR

Both quantitative polymerase chain reaction and Western blot results showed that after stimulation of Der p2 rBCG, jagged-2 expression in BMDC was

reduced ($P < 0.05$). Dll-4 in BMDC was elevated by Der p2 rBCG and BCG, while the expression of Dll-1 and Dll-3 was not changed (Fig. 8). TLR-2 and TLR-4 antagonists inhibited these changes.

DISCUSSION

In previous studies, we found that Der p2 rBCG could inhibit allergen-induced airway inflammation by regulating DC phenotypes and functions.⁴ However, whether Der p2 rBCG regulates Th17 cells remains to be determined. We used adoptive transfer methods to demonstrate that Der p2 rBCG could suppress Der p2-triggered Th17 differentiation possibly by downregulating jagged-2 and elevating Delta-like-4.

Th17 is characterized by producing IL-17 (or IL-17A), IL-17F and IL-22. IL-17 is involved in a variety of pathological and pathophysiological changes of asthma.^{12–20} Many studies have shown that IL-17 levels are significantly elevated in asthmatic patients' samples compared with healthy individuals.^{21–24} In this study, we found that Der p2 rBCG-treated BMDC had a more potent effect on inhibiting airway inflammation in asthma compared with BCG-treated BMDC. Fluorescence-activated cell sorting analysis showed that the ratio of Th17 cells in the Der p2 rBCG-BMDC group was much less than that in the asthma group, but it was greater in the BCG-BMDC group.

Several kinds of BCG vectors can influence Th17 differentiation. Lagranderie *et al.* found that BCG inactivated by extended freeze-dried reduces airway hyperresponsiveness, partly through downregulation of ROR γ t (Th17 signatures), but BCG cannot.⁵ In our studies, Der p2 rBCG-treated BMDC could inhibit Der p2 antigen-induced Th17 overproduction. IL-6 induces Th17 differentiation potently.²⁵ Freches *et al.* found that there is a significant elevation of IL-6 and TNF- α in the spleen and lungs after BCG immunization.²⁶ IL-6 induces naive Th0 cell polarization to Th17 by activating the signal transducers and transcription activators 3 signalling pathway and then transcriptional activation of ROR γ t.²⁷ In the present study,

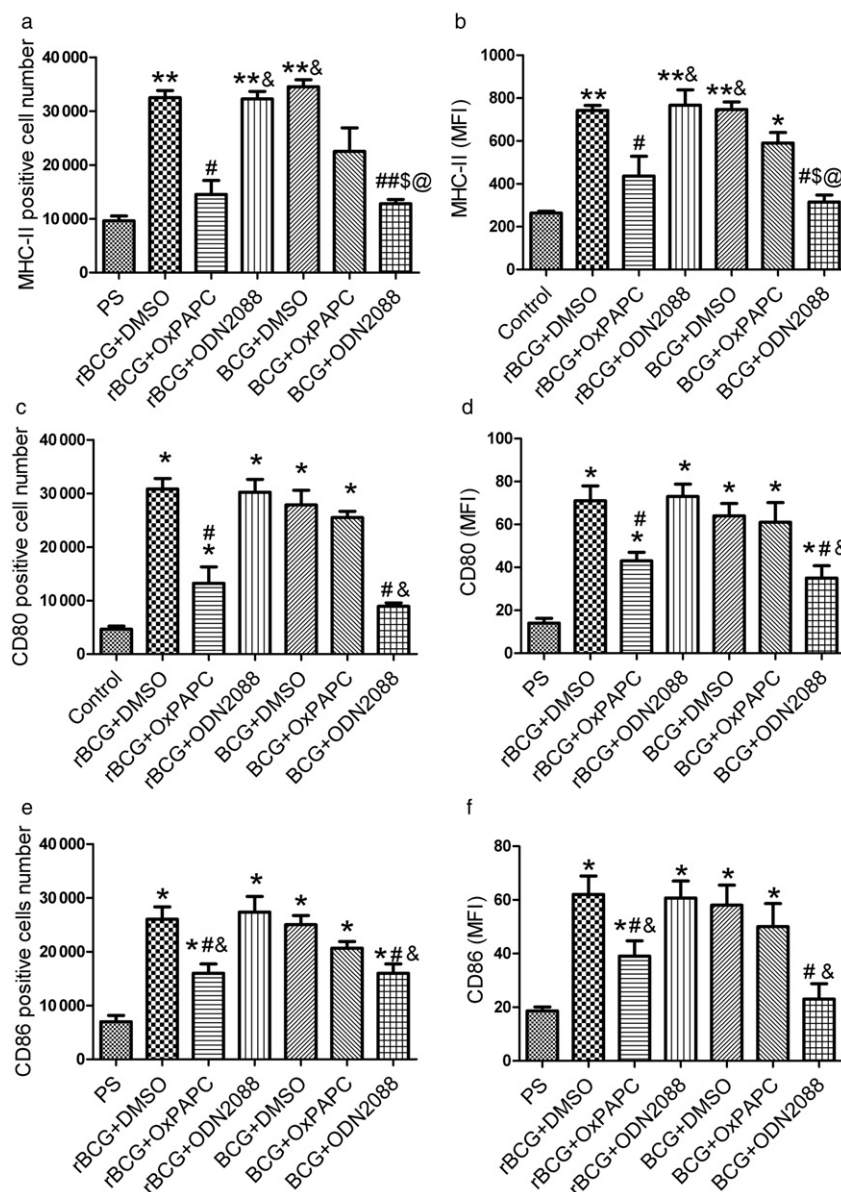


Figure 7 Bone marrow-derived dendritic cells (BMDC) were treated with toll-like receptor (TLR)-2/TLR-4 or TLR-9 antagonist for 24 h or 1 h before Der p2 recombinant bacille Calmette-Guerin (rBCG) or bacille Calmette-Guerin (BCG) stimulation, while the control group was treated with dimethyl sulfoxide (DMSO). The expression levels of (a) major histocompatibility complex (MHC)-II, (b) CD80 and (c) CD86 in BMDC were analysed by fluorescence-activated cell sorting. The results are shown as mean $\pm$ standard error of the mean ($n = 6$). * $P < 0.05$, ** $P < 0.01$ versus control group; # $P < 0.05$, ## $P < 0.01$ versus rBCG + DMSO group; & $P < 0.05$ versus BCG-DMSO group; \$ $P < 0.05$ versus rBCG + ODN2088; @ $P < 0.05$ versus BCG + OxPAPC group.

BMDC stimulated with BCG secreted a high level of IL-6. Moreover, there was a greater ratio of Th17 cells in the BCG-BMDC group than in the control group. The level of IL-6 in bronchoalveolar lavage fluid was also greater.

Different BCG vectors have different biological effects. Chatterjee *et al.* have provided evidence that H37Rv, which secretes early secretory antigenic target 6, promotes Th17 cell responses, and this effect depends on TLR-2/MyD88 signalling and involves IL-6 and TGF- β production by DC.²⁸ TGF- β is also required for Th17 cell differentiation. It has been pointed out that at lower concentrations, together with pro-inflammatory factor IL-6 or IL-21, TGF- β promotes Th17 cell differentiation. However, at higher concentrations, TGF- β favours suppression of Th17 differentiation.²⁹ Different immunological effects between Der p2 rBCG and BCG may be related to their

different molecular structures. BCG, as a mycobacterial vector, interacts with DC mainly through TLR.³⁰ Although there were no differences in the degree of DC activation between BCG and Der p2 rBCG, BCG could elevate IL-6 more than Der p2 rBCG. BMDC isolated from TLR-9-deficient mice treated with BCG expressed lower levels of IL-6 than the same cells isolated from wild-type mice.³¹ TLR-9 seemed to be required for IL-6 generation induced by BCG. We found that the Der p2 rBCG-BMDC group expressed significantly greater levels of TLR-2 and TLR-4 than the PS-BMDC group, while there was no significant difference for TLR-9. Conversely, TLR-9 was elevated in the BCG-BMDC group. Using different TLR antagonists further confirmed that Der p2 rBCG activated DC through TLR-2 and TLR-4, and BCG through TLR-9. Combined with previous studies, we believe that due to the molecular structure differences, Der

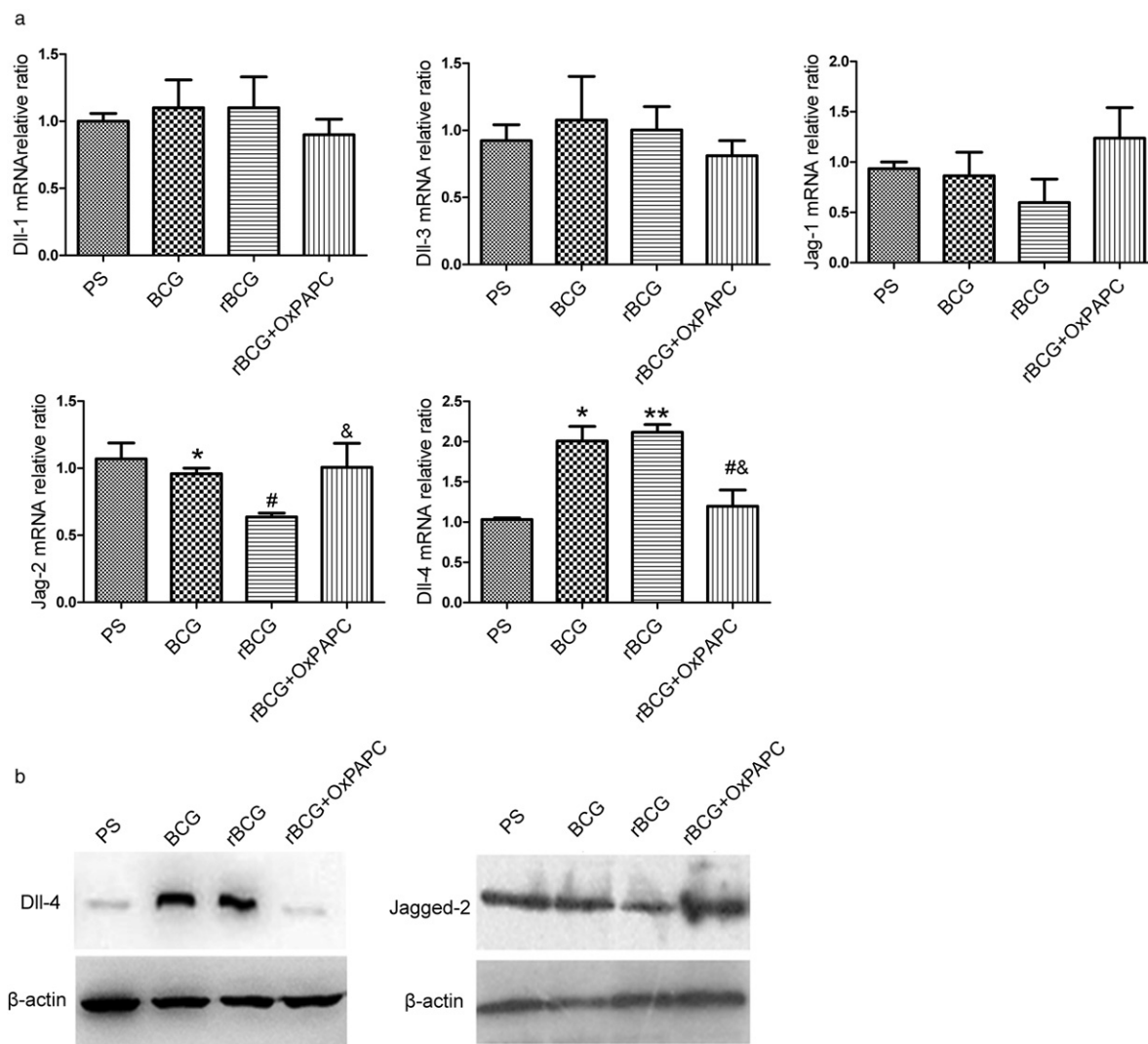


Figure 8 Bone marrow-derived dendritic cells (BMDC) were treated with physiological saline (PS), Der p2 p2 recombinant bacille Calmette-Guerin (rBCG) or bacille Calmette-Guerin (BCG) for 72 h, and the fourth group of BMDC was treated with OxPAPC (30 μ g/mL) 24 h before Der p2 rBCG stimulation. (a) All groups of dendritic cell (DC)'s RNA were collected, and the Notch ligands messenger RNA (mRNA) levels were determined by real-time polymerase chain reaction; (b) Western blotting was performed, and Dll-4, Jag-2 expression values were normalized to β -actin. Data were presented as mean $\pm$ standard error of the mean ($n = 6$). * $P < 0.05$, ** $P < 0.01$ versus PS group; # $P < 0.05$ versus BCG group; & $P < 0.05$ versus rBCG group.

p2 rBCG and BCG interact with DC through different TLR. In turn, different TLR trigger different levels of IL-6. Finally, TLR has different capacities for inducing TH17 *in vivo* and *in vitro*.

The Notch signalling pathway is an important pathway that regulates development with high conservation between species. It is involved in the interaction between DC and T cells. But, the effects produced by the different Notch receptors and ligands are different. The Delta-like family of ligands can promote naïve T cells to differentiate into Th1, while jagged-2 promotes Th2.³² According to the literature,³¹ mycobacterial infection can affect the levels of Notch receptor ligands in DC. BCG can recognize TLR-9 on DC and affect the expressions of IL-6 and Delta-like-4 in DC through the MyD88-dependent

pathway, promoting Th17 differentiation. Our results showed that both Der p2 rBCG and BCG could increase the messenger RNA and protein level of Delta-like-4. It is worth noting that Der p2 rBCG was more efficient in suppressing jagged-2 expression than BCG; and TLR-2 and TLR-4 antagonists could effectively reverse this function. In addition, Notch signalling is also involved in the regulation of Treg differentiation. Fu *et al.* found that although the frequency of Treg was intact in recombination signal binding protein-Jk-knockout recipients, their ability to suppress effector T cell responses was significantly dampened in recombination signal binding protein-Jk-deficient mice.³³ Furthermore, Kared *et al.* has reported that jagged-2-expressing hematopoietic progenitors promote regulatory T cell expansion in

the periphery through Notch signalling.³⁴ Possibly, Der p2 rBCG may affect the differentiation and function of Treg by changing the Notch ligands of DC and ultimately restore Th1/Th2 balance.

In summary, our findings emphasize the potent immunomodulatory functions involved in asthma and lay a foundation for future clinical rBCG vaccine research and development.

Acknowledgement

This work was supported by the National Natural Science Foundation of China (30971305).

REFERENCES

- Riffo-Vasquez Y, Spina D. Role of cytokines and chemokines in bronchial hyperresponsiveness and airway inflammation. *Pharmacol. Ther.* 2002; **94**: 185–211.
- Steinman L. A brief history of T (H)17, the first major revision in the T(H)1/T(H)2 hypothesis of T cell-mediated tissue damage. *Nat. Med.* 2007; **13**: 139–45.
- Ou-Yang H-F, Han X-P, Zhao F *et al.* The role of bone marrow-derived adult stem cells in a transgenic mouse model of allergic asthma. *Respiration* 2012; **83**: 74–80.
- Qu S-Y, Ou-Yang H-F, He Y-L *et al.* Der p2 recombinant BCG targets dendritic cells to inhibit allergic airway inflammation in a mouse asthma model. *Respiration* 2013; **85**: 49–58.
- Lagranderie M, Abolhassani M, Vanoirbeek JA *et al.* *Mycobacterium bovis* bacillus Calmette-Guérin killed by extended freeze-drying targets plasmacytoid dendritic cells to regulate lung inflammation. *J. Immunol.* 2010; **184**: 1062–70.
- Chen X, Zhang M, Liao M *et al.* Reduced Th17 response in patients with tuberculosis correlates with IL-6R expression on CD4⁺ T Cells. *Am. J. Respir. Crit. Care Med.* 2010; **181**: 734–42.
- Artavanis-Tsakonas S, Rand MD, Lake RJ. Notch signaling: cell fate control and signal integration in development. *Science* 1999; **284**: 770–6.
- Amsen D, Antov A, Flavell RA. The different faces of Notch in T-helper-cell differentiation. *Nat. Rev. Immunol.* 2009; **9**: 116–24.
- Shi JR, Li Y, Qi HW *et al.* Construction and identification of the *E. coli*-BCG shuttle vector expressing lipoprotein Der p2 on cell wall of *Mycobacterium vaccae*. *Xi Bao Yu Fen Zi Mian Yi Xue Za Zhi* 2003; **19**: 132–5.
- Inaba K, Inaba M, Romani N *et al.* Generation of large numbers of dendritic cells from mouse bone marrow cultures supplemented with granulocyte/macrophage colony-stimulating factor. *J. Exp. Med.* 1992; **176**: 1693–702.
- Bilenki L, Gao X, Wang S *et al.* Dendritic cells from mycobacteria-infected mice inhibits established allergic airway inflammatory responses to ragweed via IL-10- and IL-12-secreting mechanisms. *J. Immunol.* 2010; **184**: 7288–96.
- Barczyk A, Pierzchala W, Sozanska E. Interleukin-17 in sputum correlates with airway hyperresponsiveness to methacholine. *Respir. Med.* 2003; **97**: 726–33.
- Kaminska M, Foley S, Maghni K *et al.* Airway remodeling in subjects with severe asthma with or without chronic persistent airflow obstruction. *J. Allergy Clin. Immunol.* 2009; **124**: 45–51.e1–e4.
- Milovanovic M, Drozdenko G, Weise C *et al.* Interleukin-17A promotes IgE production in human B cells. *J. Invest. Dermatol.* 2010; **130**: 2621–8.
- Saitoh T, Kusunoki T, Yao T *et al.* Role of interleukin-17A in the eosinophil accumulation and mucosal remodeling in chronic rhinosinusitis with nasal polyps associated with asthma. *Int. Arch. Allergy Immunol.* 2010; **151**: 8–16.
- Agache I, Ciobanu C, Agache C *et al.* Increased serum IL-17 is an independent risk factor for severe asthma. *Respir. Med.* 2010; **104**: 1131–7.
- Schwarzenberger P, Huang W, Ye P *et al.* Requirement of endogenous stem cell factor and granulocyte-colony-stimulating factor for IL-17-mediated granulopoiesis. *J. Immunol.* 2000; **164**: 4783–9.
- Schwarzenberger P, La Russa V, Miller A *et al.* IL-17 stimulates granulopoiesis in mice: use of an alternate, novel gene therapy-derived method for in vivo evaluation of cytokines. *J. Immunol.* 1998; **161**: 6383–9.
- Cai XY, Gommoll CP, Justice L *et al.* Regulation of granulocyte colony-stimulating factor gene expression by interleukin-17. *Immunol. Lett.* 1998; **62**: 51–8.
- Laan M, Prause O, Miyamoto M *et al.* A role of GM-CSF in the accumulation of neutrophils in the airways caused by IL-17 and TNF- α . *Eur. Respir. J.* 2003; **21**: 387–93.
- Doe C, Bafadhel M, Siddiqui S *et al.* Expression of the T helper 17-associated cytokines IL-17A and IL-17F in asthma and COPD. *Chest* 2010; **138**: 1140–7.
- Song C, Luo L, Lei Z *et al.* IL-17-producing alveolar macrophages mediate allergic lung inflammation related to asthma. *J. Immunol.* 2008; **181**: 6117–24.
- Molet S, Hamid Q, Davoine F *et al.* IL-17 is increased in asthmatic airways and induces human bronchial fibroblasts to produce cytokines. *J. Allergy Clin. Immunol.* 2001; **108**: 430–8.
- Chakir J, Shannon J, Molet S *et al.* Airway remodeling-associated mediators in moderate to severe asthma: effect of steroids on TGF- β , IL-11, IL-17, and Type 1 and Type 3 collagen expression. *J. Allergy Clin. Immunol.* 2003; **111**: 1293–8.
- Akdis CA, Akdis M. Mechanisms and treatment of allergic disease in the big picture of regulatory T cells. *J. Allergy Clin. Immunol.* 2009; **123**: 735–46.
- Freches D, Romano M, Korf H *et al.* Increased pulmonary tumor necrosis factor α , interleukin-6 (IL-6), and IL-17A responses compensate for decreased gamma interferon production in anti-IL-12 autovaccine-treated, *Mycobacterium bovis* BCG-vaccinated mice. *Clin. Vaccine Immunol.* 2011; **18**: 95–104.
- Yamashita T, Iwakura T, Matsui K *et al.* IL-6-mediated Th17 differentiation through ROR γ t is essential for the initiation of experimental autoimmune myocarditis. *Cardiovasc. Res.* 2011; **91**: 640–8.
- Chatterjee S, Dwivedi VP, Singh Y *et al.* Early secreted antigen ESAT-6 of *Mycobacterium tuberculosis* promotes protective T helper 17 cell responses in a toll-like receptor-2-dependent manner. *PLoS Pathog.* 2011; **7**: e1002378.
- Zhou L, Lopes JE, Chong MM *et al.* TGF- β -induced Foxp3 inhibits T(H)17 cell differentiation by antagonizing ROR γ t function. *Nature* 2008; **453**: 236–40.
- Hope JC, Thom ML, McCormick PA *et al.* Interaction of antigen presenting cells with mycobacteria. *Vet. Immunol. Immunopathol.* 2004; **100**: 187–95.
- Ito T, Schaller M, Hogaboam CM *et al.* TLR9 regulates the mycobacteria-elicited pulmonary granulomatous immune response in mice through DC-derived Notch ligand delta-like 4. *J. Clin. Invest.* 2009; **119**: 33–46.
- D'Souza B, Miyamoto A, Weinmaster G. The many facets of Notch ligands. *Oncogene* 2008; **27**: 5148–67.
- Fu T, Zhang P, Feng L *et al.* Accelerated acute allograft rejection accompanied by enhanced T-cell proliferation and attenuated Treg function in RBP-J deficient mice. *Mol. Immunol.* 2011; **48**: 751–9.
- Kared H, Adle-Biassette H, Foïs E *et al.* Jagged2-expressing hematopoietic progenitors promote regulatory T cell expansion in the periphery through notch signaling. *Immunity* 2006; **25**: 823–34.

Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Table S1 Sequences of primers used.

Supplementary Information

Der p2 recombinant BCG priming of bone marrow-derived dendritic cells suppresses Der p2-induced Th17 function in a mouse model of asthma

Shuo-Yao Qu^{1*}, Hai-Feng Ou-yang^{1*}, Ya-Long He², Zhi-Kui Li¹, Jie-Ran Shi¹,
Li-Qiang Song¹, Chang-Gui Wu^{1¥}

Table S1

Gene	Primer sequence
ROR-γt	Forward GTACATAGAAAGAGACGTGACTCCT; Reverse GAGTTGACCCTCACGGAATGGTCCA.
jagged-1	Forward CAGTCCTAAGCATGGGTCTTG; Reverse GCATAGCCAGGATGGACAGATA.
jagged-2	Forward, TTCCAGTGCATGCCTACA; Reverse, GTGCTCGGTGGCTCTTCT;
Dll-1	forward, GCCAGCGTGAGAAGGACATCTC; reverse, CTCGGATATGACGTACCGACTG;
Dll-3	forward, GAGACACCCAGGTCCTTTGA; reverse, CAGTGGCAGATGTAGGCAGA;
Dll-4	forward, AACTGCCCTTCAATTTACCT; reverse, GCTGGTTTGCTCATCCAATAA;
β-actin	Forward CATCCGTAAAGACCTCTATGCCAAC; Reverse ATGGAGCCACCGATCCACA.

TLR2	forward, GCTCCAGGTCTTTCACCTCTAT; reverse, TCCAGCAGGAAAGCAGACTCGCTTA;
TLR4	forward, GGAAGCTTGAATCCCTGCATAGAG; Reverse, TCCACATGTACTAGGTTCGTCAG;
TLR9	Forward, AACCTGCGGCAGCTGAACCTCAA; Reverse, AGTTCAGTGTATGGAGAGAGCTGGATGGAGGG-3'.