

Silencing of CD86 in dendritic cells by small interfering RNA regulates cytokine production in T cells from patients with allergic rhinitis *in vitro*

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Abstract. The aim of the present study was to investigate the expression and role of the co-stimulatory molecule T-lymphocyte activation antigen CD86 (CD86) in dendritic cells (DCs) from the peripheral blood of patients with allergic rhinitis (AR) compared with those from healthy individuals. It was observed that mature DCs from the peripheral blood of patients with AR expressed high levels of the co-stimulatory molecule CD86, but not CD80, compared with healthy control subjects. CD86 expression levels in DCs decreased significantly following transfection with siRNA in a lentiviral vector. Furthermore, the level of transforming growth factor- β 1 produced by T cells co-cultured with DCs was significantly increased in the siRNA group, while interleukin (IL)-4 and IL-5 production was significantly decreased. The findings of the present study indicated that CD86 may play a pivotal role in the regulatory T cell/type 2 helper T cell imbalance in allergic inflammation.

Introduction

Allergic rhinitis (AR) is a chronic nasal mucosal inflammation characterized by infiltration and activation of a number of immune cells (1,2). Type 2 helper T (Th2) cells and regulatory T (Treg) cells play an important role in AR (3,4). A deficiency of Treg cells and higher numbers of Th2 cells are the key factors underlying the development of allergic inflammation of the nasal mucosa (5). T cells can regulate immune function by secreting cytokines and proinflammatory molecules.

Transforming growth factor (TGF)- β , interleukin (IL)-5 and IL-4 are typical cytokines secreted by Treg and Th2 cells.

Dendritic cells (DCs) are an important type of antigen-presenting cell that regulate the activation and differentiation of T cells. Antigen-activated DCs display co-stimulatory molecules, which determine whether naïve T cells differentiate into type 1 helper T (Th1), Th2 or Treg cells (6,7). A previous study demonstrated that mature DCs (mDCs) express high levels of the co-stimulatory molecules T-lymphocyte activation antigen CD80 (CD80) and CD86, which provide the signals that trigger the activation, proliferation and differentiation of T cells by interacting with CD28 (8). Despite sharing the same T cell stimulatory receptor, CD80 and CD86 induce different DC:T cell interactions through alternative pathways. CD80 may be more potent in inducing an antitumor immune response compared with CD86, while CD86 preferentially induces Th2-driven allergic responses (9,10). Vermaelen and Pauwels (11) demonstrated that airway DCs in a mouse model of asthma expressed a high level of CD86, but not CD80. Consistent with this, an allergen challenge with ovalbumin (OVA) in a murine model of airway inflammation led to the maturation of lung DCs and increased expression of CD86 (12). However, it is unclear if the upregulation of CD86 in DCs may play a critical role in the development of AR.

DCs have been shown to be important in AR. The number of DCs in the nasal mucosa of patients with AR were dramatically reduced following intranasal corticosteroid therapy (13,14). Moreover, KleinJan *et al* (15) reported that DCs in the nasal mucosa of symptomatic AR patients displayed a more mature phenotype (expressing CD86). In previous studies on AR models, mDCs have been shown to upregulate the expression of the Th2-cell cytokines IL-4, IL-5 and IL-13, and induce eosinophilic inflammation (15,16). However, the effects of the knockdown of CD86 in DCs on the differentiation and cytokine secretion by T cells in AR are not yet known.

RNA interference is an approach to gene silencing. Small interfering RNAs (siRNAs) are small non-coding RNA molecules that are complementary to the mRNA transcript of the target gene. These small RNA molecules elicit the sequence-specific degradation of a complementary mRNA target. Previous studies have reported the effects of siRNA in animal models of AR (16-22). These previous studies

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targeted CD40, signal transducer and activator of transcription 6, neurokinin-1 receptor, C-C chemokine receptor type 3, transient receptor potential cation subfamily M member 8, phosphatidylinositol-3-kinase C2 β and calcium release-activated calcium channel protein 1. In the present study, the effect of the siRNA-mediated silencing of CD86 in DCs on Treg and Th2 cell cytokine production was examined *in vitro*.

Materials and methods

Clinical specimens. Patients with AR (n=39) and healthy control subjects (n=36) participated in the present study. The patients were diagnosed based on the criteria of the Initiative on Allergic Rhinitis and its Impact on Asthma (23), including a characteristic history of paroxysmal sneezing, watery rhinorrhea, nasal obstruction, positive specific immunoglobulin E (IgE), a positive skin prick test (SPT) and nasal examination. All patients were prohibited from taking any medication within 4 weeks of the study. Patients with infectious rhinitis, an occupational or drug-induced etiology, and those with any complications, were excluded. Healthy control subjects were selected based on the following criteria: No history of allergic diseases, a negative SPT or specific IgE to common allergens (total IgE levels <100 kU/liter). In the siRNA group, DCs from patients with AR were treated with siRNA intervention. In the non-siRNA group, DCs from patients with AR were not treated with siRNA intervention. The healthy control group consisted of healthy subjects. The present study was approved by the Ethics Committee of Chongqing Medical University. Informed consent for all subjects was obtained from parents or legal guardians. All procedures strictly conformed to the principles outlined in the Declaration of Helsinki.

Generation of DCs. A total of 10 ml blood was collected from each subject. Peripheral blood mononuclear cells (PBMCs) were isolated by means of Ficoll-Plaque Plus density gradient centrifugation at 800 x g for 30 min at 4°C. After trypan blue staining, the viability (>95%) of PBMCs was detected by an exclusion assay under an optical microscope. CD14⁺ monocytes were isolated using magnetic separation (Miltenyi Biotec GmbH). Isolated CD14⁺ cells were transferred into 96-well plates, at a density of 1.0x10⁶ cells/well in 0.1 ml of complete medium, for 6 days to promote differentiation into immature DCs (imDCs). The complete medium consisted of RPMI-1640 (R&D Systems Inc.), 10% fetal calf serum (R&D Systems Inc.), 100 U/ml penicillin, 100 ng/ml streptomycin, 100 ng/ml recombinant human granulocyte-macrophage colony-stimulating factor (PeproTech, Inc.) and 50 ng/ml recombinant human IL-4 (PeproTech, Inc.). Half the volume of the old media was removed and fresh media was added every 48 h. After 6 days of culture, 100 ng/ml lipopolysaccharide (LPS; Sigma-Aldrich; Merck KGaA) was added to the cells for 24 h. The mDCs were obtained on day 7.

Transfection of DCs with CD86 siRNA in a lentiviral vector. CD86 siRNA in a lentiviral vector was purchased from Shanghai GenePharma Co., Ltd. The CD86 siRNA sequence was AGACCACATTCCTTGGATT. The component sequence of the lentivirus vector was human U6, multiple cloning sites, cytomegalovirus, green fluorescent protein (GFP), simian

virus 40 and neomycin. The expression of GFP indicated a successful transfection. Preliminary experiments to calculate the multiplicity of infection (MOI) were carried out; an MOI of 20 corresponded to a transfection efficiency of 60.2%, while an MOI of 10 corresponded to a transfection efficiency of 43.5%. Transfections were performed when DCs reached a confluency of 30-50%, according to the manufacturer's protocol. imDCs were seeded in 96-well plates and were transfected with CD86 siRNA in a lentiviral vector at an MOI of 20 on day 3 of the CD14⁺ cell culture. After 72 h, 100 ng/ml LPS was added to the DCs for 24 h. To exclude the effect of transfection reagent on CD86 expression, a preliminary experiment was carried out (data not shown). CD86 expression was determined by flow cytometry through PE-conjugated anti-human CD86 mAb as described below. The results showed that transfection with an empty lentiviral vector (Shanghai GenePharma Co., Ltd.) had no effect on CD86 expression (data not shown). To determine if siRNA had been successfully transfected into DCs, the expression of GFP was observed using fluorescence microscopy at 6 h post transfection. The transfection efficiency was determined by flow cytometry at 48 h after transfection.

Flow cytometry. DCs were stained with phycoerythrin (PE)-Cyanine5-conjugated anti-human leukocyte antigen-DR isotype (HLA-DR) mouse antibody (mAb) (eBioscience; Thermo Fisher Scientific, Inc.), PE-conjugated anti-human CD86 mAb (eBioscience; Thermo Fisher Scientific, Inc.) and FITC-conjugated anti-human CD80 mAb (eBioscience; Thermo Fisher Scientific, Inc.). A minimum of 20,000 events were collected in each analysis. DCs were analyzed using a FACScan flow cytometer (BD Biosciences). Further data analysis was performed using CellQuest software (CellQuest Pro 5.2.1, BD Biosciences).

Co-culture with CD4⁺ T cells. Peripheral CD4⁺ T cells were isolated from PBMCs using human CD4 microbeads (Miltenyi Biotec GmbH). Co-culture was performed with a DC/T cell ratio of 1:4 in 24-well plates for 7 days without any stimulating factors. The culture plate was incubated at 37°C in a humidified incubator with 5% CO₂. The supernatant and co-cultured cells were collected for the following assays. The supernatant was collected and stored at -80°C until cytokine levels were measured.

ELISA for IL-4, IL-5 and TGF- β 1. The levels of IL-4 (cat. no. BMS225HS), IL-5 (cat. no. BMS278INST) and TGF- β 1 (cat. nos. BMS249/4BMS249/4TEN) in the supernatant was detected using ELISA (eBioscience; Thermo Fisher Scientific, Inc.), according to the manufacturer's instructions. The limits of detection for IL-4, IL-5 and TGF- β 1 were 0.1, 10 and 8.6 pg/ml, respectively.

Reverse transcription-quantitative (RT-q)PCR analysis of forkhead box P3 (FOXP3) and GATA-binding protein 3 (GATA-3) expression. Total RNA was isolated using a Total RNA Extraction kit (BioTeke Corporation). RT was performed using a Prime Script RT Reagent kit (Takara, Biotechnology Co., Ltd.), according to the manufacturer's instructions. The sequences of the primers (Sangon Biotech, Shanghai, China) were as follows: FOXP3 forward, 5'-AGGGACCAAGAA

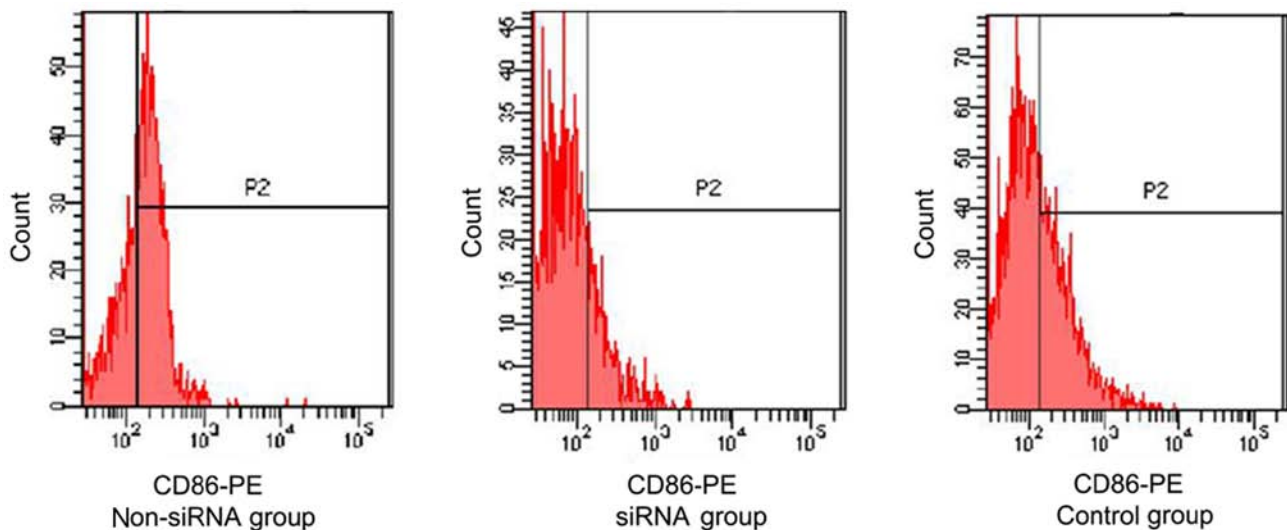


Figure 1. Expression of CD86 on dendritic cells was determined by flow cytometry. The expression levels of CD86 in the non-siRNA, siRNA and the normal control groups were 61.2 ± 15.46 , 17.2 ± 3.17 and 37.6 ± 5.93 , respectively. The CD86 expression levels in the siRNA group decreased significantly compared with those in the non-siRNA and the healthy control groups ($P < 0.05$). siRNA, small interfering RNA; CD86, T-lymphocyte activation antigen CD86; PE, phycoerythrin.

GTGAGGTTTC-3' and reverse, 5'-TGGGGTTTGTGTTGA GTGAG-3'; GATA-3 forward, 5'-AGACCACCACAACCA CACTCT-3' and reverse, 5'-GATGCCTTCCTTCTTCAT AGTCA-3'; β -actin forward, 5'-AGCGAGCATCCCCAAAG TT-3' and reverse, 5'-GGGCACGAAGGCTCATCATT-3'. The mRNA levels of FOXP3 and GATA-3 were quantified using qPCR as described previously (5). A brief introduction is as follows. Reactions were heated to 95°C for 4 min followed by 30 cycles of denaturation at 95°C for 45 sec, annealing at 55°C (FOXP3), 58°C (GATA-3), 59°C (β -actin) for 5 sec, and extension at 72°C for 10 min. All PCR reactions were performed in duplicate. To confirm the specificity of the PCR reaction, PCR products were analyzed with 3% agarose gel electrophoresis and ethidium bromide staining, followed by visualization with an ultraviolet rays transilluminator.

Western blot analysis of FOXP3 and GATA3 expression. Proteins were extracted using RIPA (Thermo Fisher Scientific, Inc.) from control co-cultured cells or following transfection with empty vector or CD86-siRNA containing vector. The protein levels of FOXP3 and GATA-3 were determined by western blot analysis as described previously (5). The dilution of FOXP3 monoclonal antibody (cat. no. 14-7979-82, eBioscience; Thermo Fisher Scientific, Inc.) was 1:1,000. The dilution of GATA3 Monoclonal Antibody (cat. no. 66400-1-IG, eBioscience; Thermo Fisher Scientific, Inc.) was 1:2,000. The dilution of Goat anti-Mouse IgG (H+L) Cross-Adsorbed Secondary Antibody (cat. no. G-21040, Invitrogen; Thermo Fisher Scientific, Inc.) was 1:2,000. The incubation temperature of the primary antibody was 4°C and the incubation duration was overnight. The incubation temperature of the secondary antibody was room temperature and the incubation duration was 1 h. Data were analyzed with Quantity One software, version 4.52 (Bio-Rad Laboratories, Inc.).

Statistical analysis. Each experiment was performed a minimum of five times. All results are presented as the

mean \pm SD. Data were analyzed by one-way ANOVA followed by Bonferroni post-hoc test using SPSS v.11.5 software (SPSS, Inc.). $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Expression of mDC surface molecules. The expression of CD80, CD86 and HLA-DR on mDCs was analyzed using flow cytometry. There were no significant differences in the expression of CD80 and HLA-DR on mDCs between the AR and the healthy control groups (data not shown). However, mDCs from the AR group exhibited a significantly higher expression level of CD86 compared with the control group ($P < 0.05$; Fig. 1). CD83 is an important surface antigen expressed by mDCs. To verify that mDCs had been induced, CD83 expression levels on mDCs were analyzed by flow cytometry in a preliminary experiment. The mDCs were found to exhibit a significantly higher CD83 expression level compared with the imDCs (data not shown).

Effects of siRNA on surface molecule expression in DCs. At 6 h after CD86 siRNA transfection, the efficiency of transfection of the DCs was assessed by fluorescence microscopy and the transfection efficiency was determined by flow cytometry. DCs were successfully transfected with CD86 siRNA using a lentiviral vector. As shown in Fig. 2, the transfection efficiency of CD86-siRNA was 60.2%, as determined by assessing the number of GFP-positive cells. At 96 h after transfection, the expression of CD86 on DCs was determined by flow cytometry. CD86 expression levels were significantly lower in the siRNA group (17.2 ± 3.17) compared with those in the non-siRNA (61.2 ± 15.46) and control (37.6 ± 5.93) groups. The difference was statistically significant ($P < 0.05$; Fig. 1). However, the CD80 expression levels in the non-siRNA, siRNA and control groups were 85.45 ± 17.81 , 83.79 ± 14.27 and $86.36 \pm 18.96\%$, respectively; the differences were not significant ($P > 0.05$; data not shown).

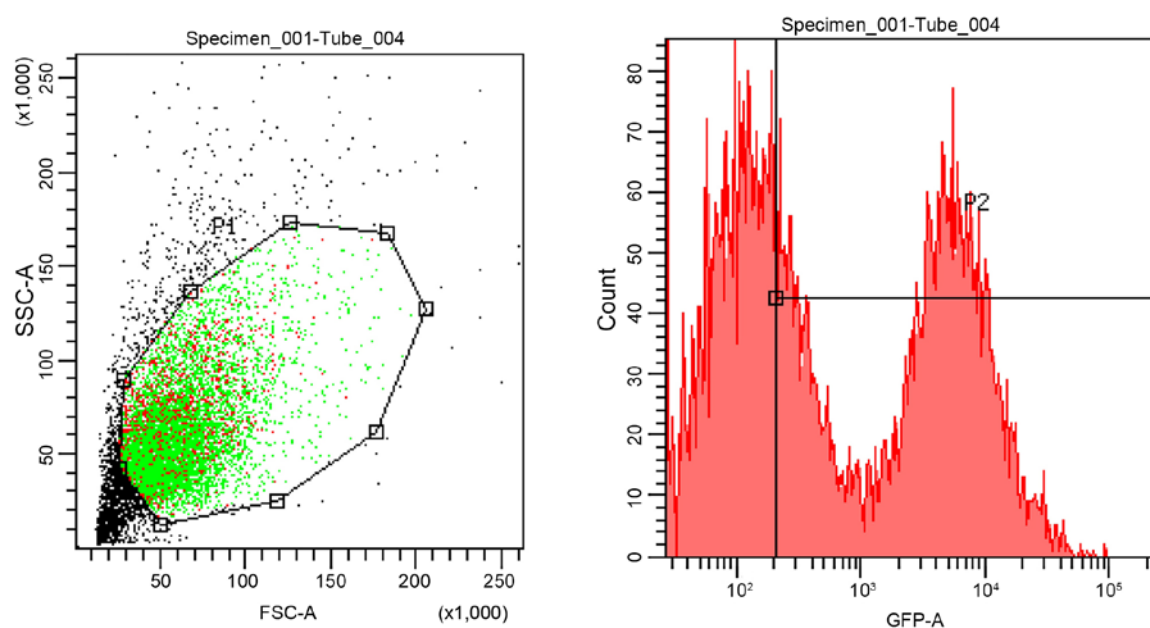


Figure 2. Transfection efficiency was determined by flow cytometry. A multiplicity of infection of 20 corresponded to a transfection efficiency of 60.2%. SSC, side scatter; FSC, forward scatter; GFP, green fluorescent protein.

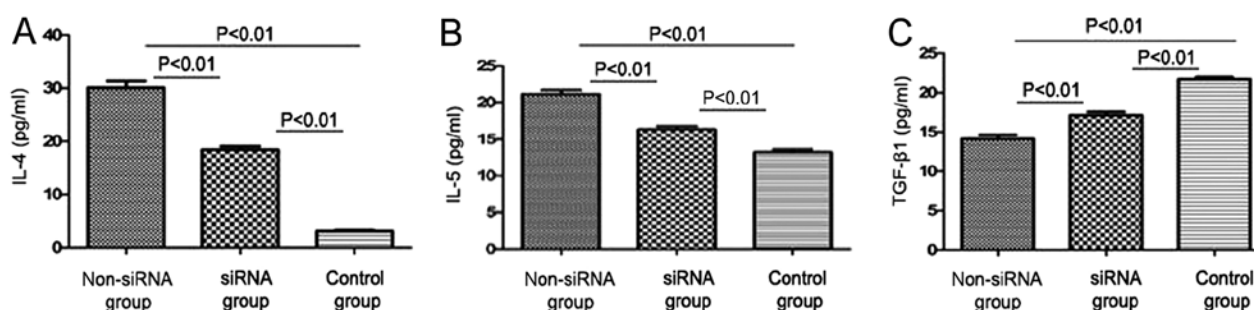


Figure 3. Levels of IL-4, IL-5 and TGF- β 1 in the supernatant of DC:CD4⁺ T cell co-culture. The levels of (A) IL-4 and (B) IL-5 and (C) TGF- β 1 were determined using ELISA. IL-4 and IL-5 levels were significantly lower, but the level of TGF- β 1 was significantly higher after T-lymphocyte activation antigen CD86 knockdown in patients with AR compared with the non-siRNA AR group. Data are presented as the mean \pm SD. IL, interleukin; TGF- β 1, transforming growth factor- β 1; DCs, dendritic cells; AR, allergic rhinitis; siRNA, small interfering RNA.

IL-4, IL-5 and TGF- β 1 secretion by T cells co-cultured with DCs. The expression levels of IL-4 and IL-5 were significantly lower in the supernatant of the co-cultured cells in the siRNA-treated group (19.07 ± 0.58 and 15.9 ± 0.51 pg/ml, respectively) compared with those in the non-siRNA group (30.23 ± 1.37 and 22.04 ± 0.78 pg/ml, respectively); the differences were statistically significant ($P < 0.01$; Fig. 3A and B). Moreover, IL-4 and IL-5 expression levels were significantly higher in the non-siRNA and siRNA groups compared with those in the control group ($P < 0.01$; Fig. 3A and B). The expression of TGF- β 1 in the supernatant of the co-cultured cells was significantly higher following transfection with siRNA targeting CD86 (16.22 ± 0.48 pg/ml) compared with that in the non-siRNA group (14.27 ± 0.51 pg/ml). The difference was statistically significant ($P < 0.01$; Fig. 3C).

FOXP3 and GATA3 mRNA expression by T cells co-cultured with DCs. The expression level of FOXP3 mRNA was higher in the siRNA-treated group (0.541 ± 0.027) compared with that in the non-siRNA group (0.412 ± 0.035 ; $P < 0.01$; Fig. 4),

whereas GATA3 mRNA expression was significantly lower in the siRNA group (0.196 ± 0.013) compared with that in the non-siRNA group (0.305 ± 0.029). The difference was statistically significant ($P < 0.01$; Fig. 4).

FOXP3 and GATA3 protein expression by T cells co-cultured with DCs. The expression level of the FOXP3 protein was considerably higher in the siRNA group (0.619 ± 0.035) compared with that in the non-siRNA group (0.217 ± 0.021 ; $P < 0.01$; Fig. 5). However, the GATA3 protein expression level in the siRNA group (0.317 ± 0.027) was significantly lower compared with that in the non-siRNA group (0.917 ± 0.046 ; $P < 0.01$; Fig. 5). These results were consistent with those of the RT-qPCR analysis.

Discussion

In the present study, the mDCs of patients with AR were found to express high levels of the co-stimulatory molecule CD86. Following transfection with CD86 siRNA using a lentiviral

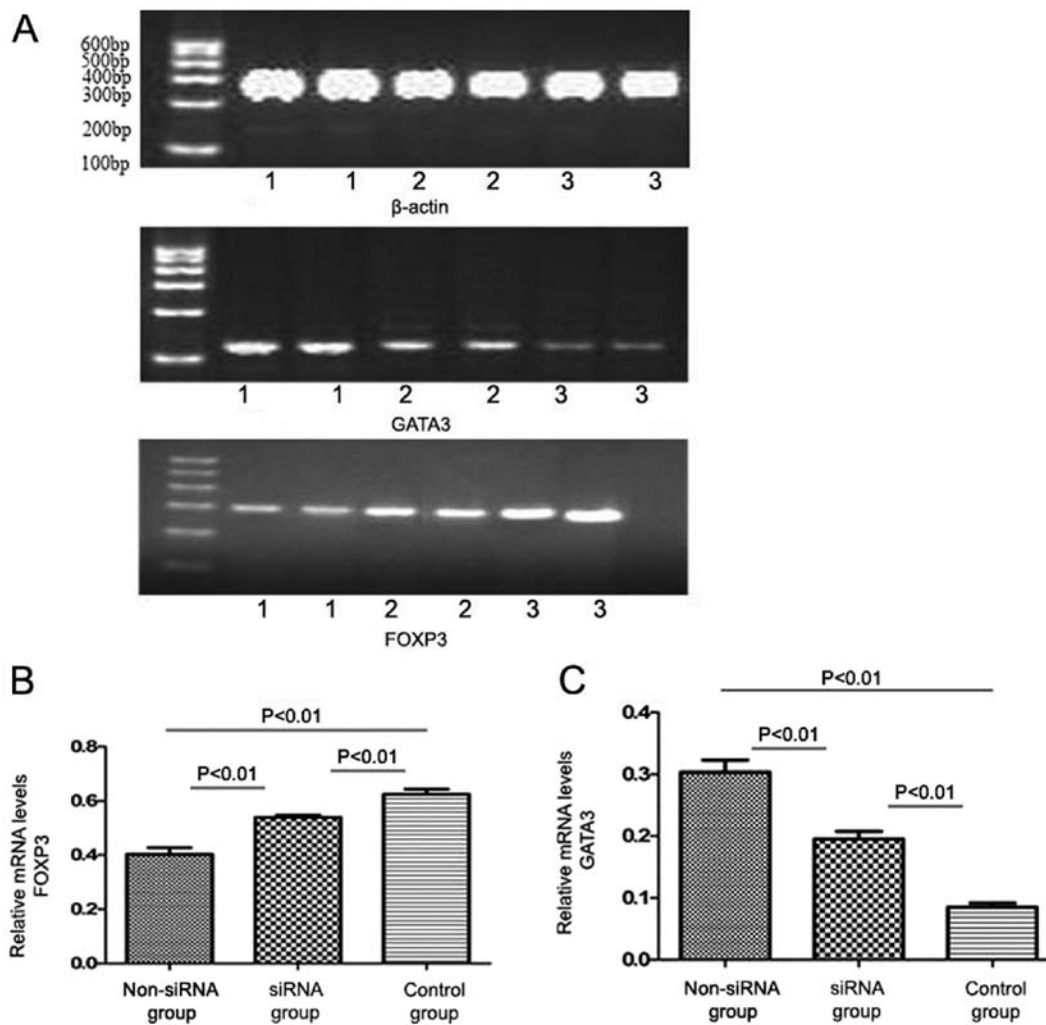


Figure 4. Expression of FOXP3 and GATA3 mRNA in dendritic cell/CD4⁺ T cell co-culture. (A) Expression levels of FOXP3 and GATA3 mRNA were determined using reverse transcription-quantitative PCR. (B) The expression of FOXP3 mRNA was higher in the siRNA-treated group compared with that in the non-siRNA treated group. (C) GATA3 mRNA expression was lower in the siRNA group compared with that in the non-siRNA treated group. Lane 1, non-siRNA group; 2, siRNA group; 3, control group. FOXP3, forkhead box P3; GATA3, GATA-binding protein 3.

vector, the expression levels of CD86 on DCs were markedly reduced. CD86 siRNA-transfected DCs induced Th2 cell deficiency and Treg cell hyperactivity. These results indicated that CD86 plays a potentially important role in the regulation of the Treg/Th2 cell imbalance in allergic inflammation.

The co-stimulatory molecule CD86 is prominent in allergic inflammation, including asthma and atopic dermatitis (24-26). Despite sharing the same T cell stimulatory receptor, CD86 appears to induce more potent allergic responses compared with CD80. Wong *et al* (24) reported that steroid- and non-steroid-treated asthmatic patients expressed higher levels of plasma CD86, but not CD80, compared with control subjects. The expression of CD86 on airway DCs was increased gradually following continuous stimulation by allergens (11). In another animal model of asthma, a continuous allergen challenge induced the maturation of lung DCs and an increased expression of CD86 (12). It was demonstrated that T cell activation is closely associated with increased levels of CD86, but not CD80, on the surface of DCs during late-phase airway allergic response using siRNA targeting CD86 (25). It has been shown that the local application of CD86 siRNA targeting cutaneous DCs improved

allergic skin inflammation (26). In the present study, mDCs from the peripheral blood of patients with AR were found to express higher levels of CD86, but not CD80, compared with the healthy control group. A similar observation in DCs was described by KleinJan *et al* (15). DCs in the nasal mucosa of symptomatic AR patients expressed higher levels of CD86, which was associated with Th2-driven allergic responses. Together with the study by KleinJan *et al* (15), the results of the present study suggested that mDCs express high levels of CD86, which may play an active role in allergic inflammation. Therefore, CD86 molecules on DCs may represent a novel therapeutic target for AR.

The role of the co-stimulatory molecule CD86 in the differentiation and activation of T cells was also investigated in the present study. The data in the present study demonstrated that T cells co-cultured with DCs produced higher levels of TGF- β 1 and lower levels of IL-4 and IL-5 in the siRNA group compared with those in the non-siRNA and healthy control group. The expression of GATA-3 was lower and that of FOXP3 higher at the mRNA and protein levels in the siRNA-treated group compared with in the non-siRNA and healthy control group. These results indicated that

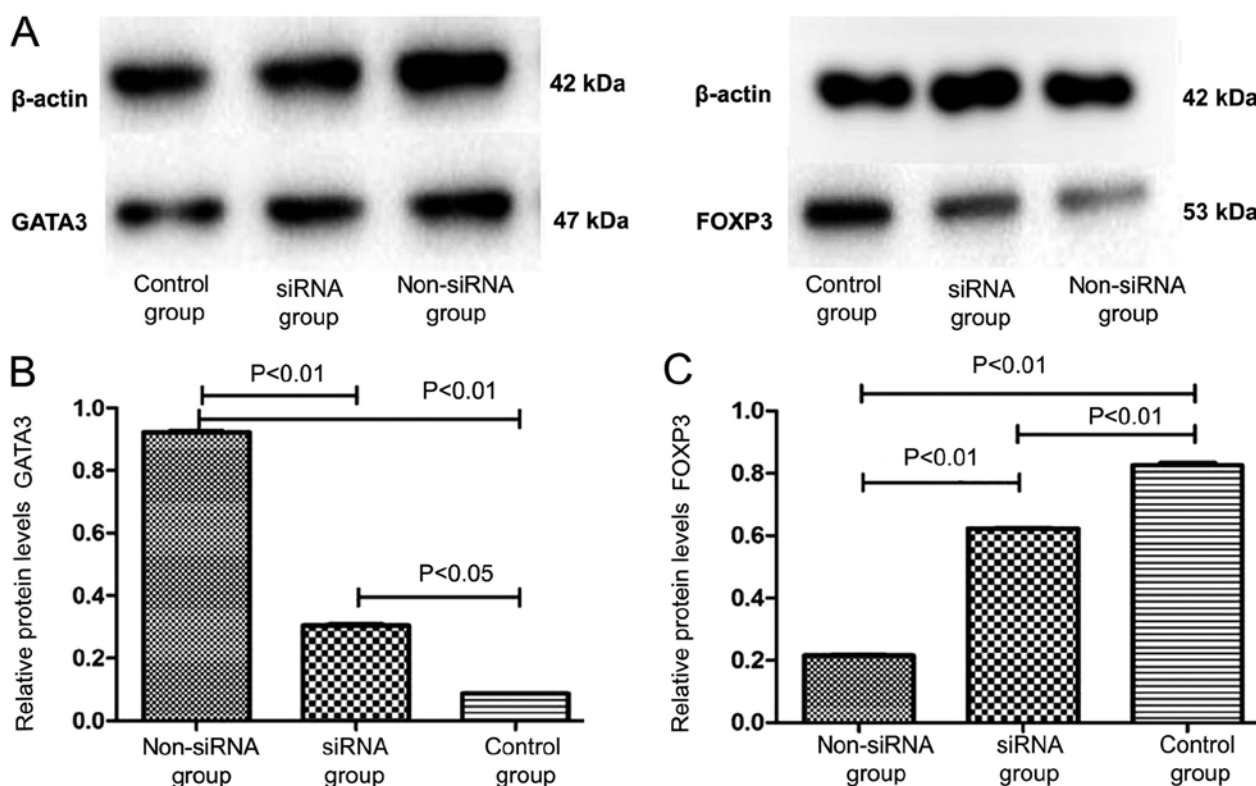


Figure 5. Expression of FOXP3 and GATA3 protein in dendritic cell/CD4⁺ T co-culture. T cell co-culture was investigated by western blot analysis. (A) Western blot analysis for GATA3 and FOXP3 protein. (B) GATA3 protein expression was lower in the siRNA group compared with that in the non-siRNA group. (C) FOXP3 protein expression was higher in the siRNA group compared with that in the non-siRNA group. FOXP3, forkhead box P3; GATA3, GATA-binding protein 3; siRNA, small interfering RNA.

CD86-silenced DCs may regulate the Treg/Th2 cell imbalance in allergic inflammation. There are two aspects to the effect of CD86-silenced DCs on T cells: Th2 deficiency and Treg hyperactivity. CD86-silenced DCs decrease the number of IL-4- and IL-5-producing Th2 cells. The presence of CD86 on DCs is required for Th2 cell differentiation and activation (27). Previous studies using animal models of asthma have demonstrated that anti-CD86 mAb treatment, but not anti-CD80 mAb treatment, effectively inhibited antigen-specific IgE and Th2 cytokine production (27-29). In addition, the blockade of CD86 may suppress airway hyper-responsiveness and Th2-driven allergic responses in allergic mice (30). The present study also demonstrated that CD86-silenced DCs did not promote T cell activation or production of IL-4 and IL-5, whereas CD86-silenced DCs increased the number of TGF- β 1-producing Treg cells. CD86 plays a crucial role in DC maturation (5). Immature or semi-mature DCs induce immune tolerance (31,32). The mAb against CD3 on DCs increased the number of antigen-specific Tregs in autoimmune diabetes (33). Knockdown of CD40 using siRNA in OVA-exposed DCs induced the generation of allergen-specific Treg cells, which suppressed allergic responses *in vivo* (16). The present study demonstrated that the expression of FOXP3 at the mRNA or protein level was higher in the CD86 siRNA-treated group compared with that in the control group. These results suggested that the effects of CD86-silenced DCs on allergic inflammation are closely associated with the regulation of the Treg/Th2 cell imbalance. Using CD86 siRNA-treated DCs to skew the immune

response from Th2 cells towards Treg cells may be a viable therapeutic approach to the treatment of AR.

To the best of our knowledge, the present study is the first to introduce an effective method for inhibiting the upregulation of CD86 on DCs from patients with AR using siRNA. An advantage of siRNA is the ability to specifically knock down a designated gene without directly affecting the expression of any other mRNAs. CD86 expression on DCs decreased significantly following siRNA treatment, whereas HLA-DR and CD80 expression exhibited little or no change.

The present study had some limitations. For example, Treg hyperactivity does not necessarily mean an increase in function, it may also be due to an increase in the quantity of Treg cells. The increased expression of FOXP3 at the mRNA and protein level does not indicate amplification of the Treg function or population; changes in function need to be verified using mixed lymphocyte reaction experiments. Additionally, the data provided in the present study are from *in vitro* experiments and no disease model was used. These points will be addressed in future studies.

In conclusion, the findings of the present study suggested that mDCs from patients with AR express high levels of the co-stimulatory molecule CD86, but not of CD80. CD86 expression in DCs was specifically reduced using a lentiviral vector expressing siRNA. CD86 siRNA-treated DCs altered the Treg/Th2 cell balance. The findings from the present study indicated that CD86 may play an important role in the pathogenesis of allergic inflammation and may be a novel target for RNA interference that could be used in the treatment of AR.

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Availability of materials and data

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

RS made substantial contributions to the experimental design, generation and transfection of DCs, co-culture of cells, acquisition of data and analysis of data. YY and ZG were responsible for the collection of clinical specimens and the ELISA to determine the expression levels of cytokines. CZ and XT were responsible for flow cytometry. WK and PW carried out RT-qPCR and western blotting analysis of FOXP3 and GATA3 expression levels. In addition, XT was also involved in drafting the manuscript and revising it critically for important intellectual content. All the authors have read and approved the final version of this manuscript.

Ethics approval and consent to participate

The present study was approved by the Ethics Committee of Chongqing Medical University. Informed consent was obtained from the parents or legal guardians of all subjects. All procedures strictly conformed to the principles outlined in the Declaration of Helsinki.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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