CD80 and CD86 knockdown in dendritic cells regulates Th1/Th2 cytokine production in asthmatic mice

JIAN-GUO LI1*, YU-MO DU1*, ZHI-DONG YAN1, JIA YAN2, YONG-XUN ZHUANSUN1, RUI CHEN1, WEI ZHANG1, SU-LING FENG1 and PI-XIN RAN3

1Department of Respiratory Medicine, Sun Yat-Sen Memorial Hospital, Institute of Respiratory Diseases, Sun Yat-Sen University, Guangzhou, Guangdong 510120; 2Department of Intensive Care Unit, Tianjin Medical University General Hospital, Tianjin 300052; 3Department of Respiratory Medicine, The First Affiliated Hospital of Guangzhou Medical College, Guangzhou, Guangdong 510120, P.R. China

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Abstract. Dendritic cells (DCs) are associated with the activation and differentiation of T helper (Th) cells. Cluster of differentiation (CD)80 and CD86, the co-stimulatory molecules highly expressed in DCs, have prominent in promoting the differentiation of Th cells toward Th2 cells. However, little is known about the effect of CD80 and CD86 knockdown on Th1/Th2 cytokine production in mature DCs (mDCs). The aim of the present study was to investigate whether small-interfering RNA (siRNA) could suppress the surface expression of CD80 and CD86 in mDCs. The effects of CD80 and CD86 knockdown in mDCs on Th1/Th2 cytokine expression were examined using an asthmatic murine model. DCs were isolated, separated and cultured in vitro. Flow cytometry was used to examine the expression of CD11c, CD80 and CD86 on the DCs. The DCs were transfected with CD80- and CD86-specific siRNA, while non-siRNA and negative siRNA controls were also designed. Then, the mRNA and protein expression levels of CD80 and CD86 were determined by reverse transcription-quantitative polymerase chain reaction and flow cytometry, respectively. The levels of interferon (IFN)-γ and interleukin (IL)-4 produced by T cells co-cultured with mDCs were measured by enzyme-linked immunosorbent assay. Substantial downregulation of CD80 and CD86 mRNA and protein levels were observed in the mDCs following transfection with siRNA. The level of IFN-γ produced by T cells co-cultured with mDCs was significantly increased in the siRNA group, while IL-4 production was significantly decreased. These results show that specific targeting of CD80 and CD86 with siRNA is able to suppress CD80/CD86 expression and consequently regulate Th1/Th2 cytokine levels by increasing IFN-γ production and decreasing IL-4 levels in an asthmatic murine model.

Introduction

Bronchial asthma is a chronic inflammatory airway disease involving many inflammatory cells and mediators. T cells, particularly T helper (Th1) and Th2, have a crucial role in the induction of airway inflammation in asthmatic patients (1). Complicated immune responses are capable of inducing Th1 deficiency and Th2 hyperactivity, which results in a Th1/Th2 imbalance. This imbalance promotes immunoglobulin (Ig)E secretion and sensitizes mastocyte and eosinophils via altered cytokine secretion and causes allergic inflammation and hyper-responsiveness of the airway (2,3). Interferon (IFN)-γ and interleukin (IL)-4 are typical cytokines of Th1 and Th2, respectively.

In patients with asthma, persistent airway inflammation is initiated by antigen presenting cells (APC), which integrate various allergens into a signal for T cells and prime the subsequent immune responses (4,5). Activation of T cells requires signals which are initiated via the TCR complex and cluster of differentiation (CD)28. Mature dendritic cells (mDCs) express high levels of the co-stimulatory molecules CD80 and CD86, which provide the signal that is required for triggering T cell activation, expansion and differentiation via interaction with CD28 (6). Previous studies have demonstrated that CD80 and CD86 levels are elevated in patients with asthma (7,8).

In previous studies investigating asthmatic models, mDCs have been shown to induce Th2 polarization, upregulate IL-4 secretion, downregulate IFN-γ production and induce eosinophilic inflammation (9,10). However, studies investigating the effects of the knockdown of CD80 and CD86 in DCs on the differentiation of and cytokine secretion by T helper cells in murine models of asthma are lacking.

In the present study, co-stimulatory T-cell activation signals were blocked by the suppression of CD80 and CD86 molecule.
expression on DCs using small interfering RNA (siRNA), and
the effects of CD80 and CD86 knockdown on the expression
levels of the Th1/Th2 typical cytokines IFN-γ and IL-4 were
evaluated. Thus, the potential of CD80 and CD86 as targets for
the application of RNA interference (RNAi) in the therapeutic
targeting of asthma were investigated.

Materials and methods

Animals. A total of 20 healthy specific-pathogen-free grade
BALB/c mice (6-8 weeks; mean weight, 18±2 g) were
purchased from the Center of Experimental Animals of Sun
Yat-Sen University (Guangzhou, China). Experiments were
performed according to protocols approved by the Animal
Studies Committee of Sun Yat-Sen University.

Asthma models. A total of 20 mice were randomly assigned to two
groups: i) the asthmatic group, and ii) the normal control group.
In the asthmatic group, each mouse was sensitized to ovalbumin
(OVA; Sigma-Aldrich, St. Louis, MO, USA) intraperitoneally on
days 1, 14 and 21 with 100 µg OVA that was emulsified in 20 mg
alum (Guangzhou Chemical Reagent Co., Guangzhou, China).
Subsequently, the mice were exposed to an aerosol challenge of
5% OVA for 30 min/day in airtight containers (with dimen-
sions of 50x50x50 cm) on days 28-34. In the normal control
group, mice were sensitized and challenged as above with an
equivalent amount of saline solution instead of the OVA protein
solution. At 24 h after the last challenge, mice were sacrificed
by an approved cervical dislocation procedure conducted by
skilled and fully trained personnel. The lungs were removed and
fixed by cervical dislocation 24 h after the final
challenge.

Separation of bone marrow-derived DCs. All mice were sacri-
ficed by cervical dislocation 24 h after the final challenge. Bone
marrow was flushed from the femurs and tibiae with RPMI-1640
culture medium (Gibco; Thermo Fisher Scientific, Inc.,
Waltham, MA, USA). Following centrifugation at 250 x g for
5 min, cells were treated with red blood cell (RBC) lysis buffer
(CWBio Co., Ltd., Beijing, China), washed with phosphate-buff-
ered saline (PBS), centrifuged at 250 x g for 5 min and cultured
in RPMI-1640 supplemented with recombinant mouse granu-
locyte macrophage colony-stimulating factor (rmGM-CSF;
Peprotech, Inc., Rocky Hill, NJ, USA; 10 ng/ml) and rmIL-4
(Peprotech; 10 ng/ml) were used in turn. After 6 days of culture,
1 µg/ml lipopolysaccharide (LPS; Sigma-Aldrich) was added.
and the non-adherent mDCs were harvested on day 7. DCs
were cultured in a 24-well tissue culture plate at a density of 1x10
cells/well on the day prior to transfection. To prepare lipid-siRNA complexes, 3 µl Lipofectamine 2000 was incubated in 50 µl Opti-MEM
(Gibco; Thermo Fisher Scientific, Inc.) at room temperature
for 5 min, and 12 µl of the indicated siRNA was concurrently combined with 50 µl Opti-MEM. The diluted Lipofectamine
2000 and siRNA were subsequently mixed and incubated for a further 20 min at room temperature for complex forma-
tion. Subsequently, the complex was incubated with the DCs in
a 24-well plate at 37°C in a 5% humidified CO2 in air
atmosphere for 6 h. When cotransfection was performed, equivalent amounts of CD80 siRNA: Lipofectamine 2000 and
CD86 siRNA: Lipofectamine 2000 complexes were added to
each well. FAM-scrambled-siRNA was used as the negative
control in order to determine the transfection efficiency. Three
groups of transfected DCs were established: In the non-siRNA
group, only Lipofectamine 2000 was added, without any
siRNA being added to the DCs. In the siRNA group, mDCs
cotransfected by CD80- and CD86-specific siRNA. In the
negative siRNA group, mDCs were transfected by non-specific
non-targeting FAM-siRNA, which has no homology with the
targeted RNAs. Experiments were performed in triplicate for
each sample. Transfection efficiency was determined using
fluorescence microscopy (Nikon Eclipse Ti, Nikon Corporation)
and detected by flow cytometry.

Reverse transcription-quantitative polymerase chain reaction
(RT-qPCR). To evaluate CD80 and CD86 mRNA expression
levels following transfection, RT-qPCR was performed. Primer
sequences (Table II) were designed according to GenBank and
synthesized by DaAn Gene Co., Ltd. of Sun Yat-Sen University
(Guangzhou, China). At 24 h post-transfection, the total RNA
of 1x10
DCs was extracted using TRIzol reagent (Invitrogen;
Thermo Fisher Scientific, Inc.) and reverse transcribed and
amplified using QuantiTect SYBR Green RT-PCR kit (Qiagen
GmbH, Hilden, Germany) in a Roche LightCycler 480
(Roche Diagnostics, Basel, Switzerland). The amplifications
were carried out according to the manufacturer's protocol
for the QuantiTect SYBR Green RT-PCR kit (Takara, Japan).
Amplification conditions were 40 cycles of 93°C for 3 min,
93°C for 30 sec, 55°C for 45 sec and 72°C for 45 sec. Every
sample was administered to each of three wells. Relative gene
expression levels were calculated using the quantification cycle
(Cq) method with normalization to glyceraldehyde-3-phosphate
dehydrogenase (GAPDH) as the reference gene using 2
expression.

Flow cytometry. To detect the positive expression rates of CD80
and CD86 on the DCs following transfection, flow cytometry
was performed on the MHC II/CD11c gate for CD80 and
CD86. Six hours after transfection, DCs were washed twice
with PBS and incubated with fluorescently-labeled antibody at
4°C for 30 min. Subsequently, the cells were washed again with
PBS and fixed with 10 g/l paraformaldehyde. The following
anti-mouse monoclonal antibodies were used: PE-anti-MHC II, FITC-anti-CD11c, PE-anti-CD80, and FITC-anti-CD86, as mentioned above. All flow cytometric analyses were performed using IgG isotypic controls.

**T-cell separation.** Spleens were removed after the mice had been euthanized by cervical dislocation. T cells were separated using Mouse Lymphocyte Separation Medium according to the manufacturer's protocol (Dakewe Biotech Co., Ltd., China). The cell density was adjusted to 1x10^9/l prior to further processing.

**Mixed lymphocyte reaction (MLR).** The asthmatic murine bone marrow-derived DCs (1x10^4/well) and healthy T cells (1x10^5/well) were co-cultured in 96-well plates at a 1:10 ratio. The co-culture systems were divided into three groups: i) The non-siRNA group, ii) the siRNA group, and iii) the negative siRNA group, with DCs from the corresponding groups as described above. Next, OVA was added to each well to a final concentration of 10 mg/l in a total volume of 200 µl. The cells were incubated at 37˚C in a 5% humidified CO_2 in air atmosphere for 72 h.

**Enzyme-linked immunosorbent assays (ELISAs).** After 3 days of co-culture, the supernatant was collected. IFN-γ and IL-4 levels were analyzed using ELISA kits specific for IFN-γ and IL-4 (Dakewe Biotech Co., Ltd.) according to the manufacturer's instructions. Absorbance values were read at 450 nm using a Multiskan MK3 (Thermo Fisher Scientific, Inc.).

**Statistical analysis.** Statistical analyses were performed with SPSS software, version 13.0 (SPSS, Inc., Chicago, IL, USA). Data are presented as the mean ± standard deviation (SD). Examinations were carried out in triplicate for each mouse. Statistical comparisons between groups were performed using one-way analysis of variance, and comparisons within a group were performed using Student's t-test. Differences among groups were considered statistically significant when P<0.05.

**Results**

**Asthmatic model.** The 20 healthy SPF-grade BALB/c mice were assigned to asthmatic and normal control groups, with 10 mice in each. All mice were evaluated in the final analysis without experimental animal loss. According to the lung tissue pathology, lung sections from the OVA-immunized mice showed clear airway inflammation with peribroncholar and perivascular infiltrates. These infiltrates consisted predominantly of eosinophils and lymphocytes, and sections showed bronchial mucosa and smooth muscle thickening, increased mucus secretion, airway epithelial cell shedding, airway stenosis, and inflammatory cells scattered in the lung interstitium. No significant pathological changes were observed in lung sections from the normal control group. Representative histopathological data are shown in Fig. 1.

**Cell surface molecule expression by mDCs.** The expression levels of CD11c, CD80 and CD86 on the mDC surfaces were detected by fluorescence-activated cell sorting (FACS). mDCs from the asthmatic group expressed CD11c at a level comparable with that in the normal control group; no significant difference was found between the two groups (P>0.05). However, in comparison with the normal control group, the asthmatic group showed significantly higher CD80 and CD86 expression levels (P<0.05; Fig. 2).

**Transfection of mDCs.** The CD80- and CD86-specific siRNA constructs were successfully transferred into the mDCs. The transfected mDCs were observed under a fluorescence microscope 6 h after transfection. The transfection efficiency of siRNA detected by FACS was ~75% (Fig. 3).
mRNA and protein expression of CD80 and CD86 by mDCs following transfection. The mRNA and protein expression levels of CD80 and CD86 in the transfected mDCs were detected by flow cytometry prior to transfection. The CD80 and CD86 mRNA expression levels detected by RT-qPCR indicated that CD80 mRNA expression levels in the non-siRNA, siRNA and negative siRNA groups were 2.09±0.46, 0.60±0.17, and 2.04±0.93, respectively, and the CD86 mRNA expression levels were 3.58±0.20, 0.91±0.48, and 2.83±0.83, respectively. The data provided by FACS indicated that the CD80 protein positive expression rates in the non-siRNA, siRNA and negative siRNA groups were 82.45±15.80, 30.79±7.07 and 81.83±10.07%, respectively, and the CD86 protein positive expression rates were 89.45±10.22, 27.29±6.99, and 87.66±11.74%, respectively. The mRNA expression level and protein positive expression rates exhibited comparable results. There was no significant significance between the non-siRNA group and the negative siRNA group (P>0.05). CD80 and CD86 expression at the mRNA and protein levels in the siRNA group decreased significantly compared with that in the non-siRNA and negative siRNA groups (P<0.05;...
Figs. 4 and 5). This demonstrates that siRNA-targeted interference can significantly suppress CD80 and CD86 mRNA and protein expression levels.

**IFN-γ and IL-4 secretion by T cells co-cultured with mDCs.** After 72 h of co-culture, IFN-γ and IL-4 levels in the supernatant of the mDC and T-cell co-culture system were detected by ELISA. IFN-γ expression was significantly increased in the siRNA group (132.73±25.04 pg/ml), as compared with the non-siRNA and negative siRNA groups (72.56±26.30 and 80.21±24.42 pg/ml, respectively; P<0.05), whereas no significant differences were detected between the non-siRNA and negative siRNA groups (P>0.05). IL-4 expression levels were significantly decreased in the siRNA group (93.04±23.13 pg/ml), as compared with the non-siRNA and negative siRNA groups (150.69±29.50 and 163.19±25.36 pg/ml, respectively; P<0.05), whereas no significant differences were detected between the non-siRNA and negative siRNA groups (P>0.05; Fig. 6).

**Discussion**

Bronchial asthma is a chronic inflammatory airway disease involving a variety of inflammatory cells, including mast cells, eosinophils, lymphocytes and other cell components (14-17). Th1/Th2 imbalance is a key factor contributing to asthma severity (18). APCs, including DCs, macrophages and B cells (19), play a crucial role in the stimulation of T cells (3). Among those, the DC is the most powerful APC, contributing to primary and secondary immune responses, including allergic immunity. Mature DCs (mDCs) with high levels of expression of the co-stimulatory molecules CD80 and CD86 can activate T cells, while immature dendritic cells (iDCs) with a low level of expression of CD80 and CD86 suppress the T-cell response and induce immune tolerance (20,21). DCs in patients with asthma have been demonstrated to be hyperactive (22).

CD80 and CD86 are two types of protein that are expressed on the APC surface, and which work in tandem to provide co-stimulatory signals necessary for T-cell activation and survival. Previous studies have shown that the expression levels of the co-stimulatory molecules CD80 and CD86 on the mDC surface are closely associated with Th2-cell reaction and airway inflammation (23,24). In asthmatic patients, mDCs that highly express CD80 and CD86 can activate T cells, while immature dendritic cells (iDCs) with a low level of expression of CD80 and CD86 suppress the T-cell response and induce immune tolerance (20,21). DCs in patients with asthma have been demonstrated to be hyperactive (22).
the promotion of Th2-cell activation (26-28). The first signal is the formation of antigen-MHC complexes on the mDC surface that bind specifically with the T-cell receptor-CD3 receptor complex on T-cell surfaces, and the second signal is co-stimulatory molecule expression and functional activation on the mDC surfaces that specifically bind to receptors on naïve T cells; the two signals form a co-stimulatory pathway (29). It has also been suggested that there is a third signal (30), as certain cellular molecules produced by DCs, such as thymic stromal lymphopoietin (TSLP), affect the direction of Th-cell differentiation. However, among all the aforementioned signals, the CD80/CD86-CD28 co-stimulatory pathway is the most classic and important. Previous research has indicated that the CD80/CD86-CD28 co-stimulatory pathway may be an effective target for asthma treatment by demonstrating that blocking the CD80/CD86 co-stimulatory pathway by monoclonal antibody approaches can inhibit inflammation in asthmatic mice (25). In addition, suppressing the CD80/CD86 co-stimulatory pathway using antiense oligonucleotides can suppress airway hyperactivity (31).

RNAi is a gene-silencing phenomenon whereby endogenous- or exogenous-specific double-stranded RNAs trigger the degradation of homologous mRNA and induce the loss of corresponding functional phenotypes. Since the technique was first discovered in 1998 by Fire et al (32), the technique has undergone further development to attain a high degree of specificity and efficiency. The therapeutic application of RNAi technology is a topic that has been attracting high levels of interest in basic medical and clinical research in recent years. The ability of RNAi to inhibit virulent gene expression has been widely used to treat a variety of diseases (33-35). Also, a number of studies have reported that RNAi can be used in DCs to diagnose and treat bronchial asthma (36-39). Darcan-Nicolaisen et al (40) discovered that the two major signs of allergic asthma in the OVA-induced asthma mouse model, which are airway inflammation and hyperactivity, were significantly ameliorated by RNAi-mediated suppressor of cytokine signaling 3 (SOCS3) of RNAi nose drops. Moriwaki et al (41) demonstrated that siRNA-mediated suppressor of cytokine signaling 3 (SOCS3) gene silencing could suppress the airway reactivity and eosinophilic infiltration that was induced by allergic stimulation in asthmatic mice. Zheng et al (42) found that the application of siRNA was able to inhibit tyrosine protein kinase (TPK) gene expression in the DCs of asthmatic mice, repressing the functional capability of DCs as antigen-presenting cells, thereby inhibiting T-cell activation and differentiation. However, studies concerning the effects of siRNA-mediated CD80 and CD86 knockdown in DCs on T-cell differentiation in asthmatic mice are lacking. In the present study, CD80 and CD86 mRNA and protein expression in murine bone marrow-derived DCs was successfully decreased with CD80- and CD86-targeting siRNA, which verified the efficiency of RNAi.

In the present study, an asthmatic mouse model was used that was established according to previously reported methods (43). The results indicated that the mDCs obtained from the asthmatic group exhibited increased CD80 and CD86 expression levels, which implies that the CD80/CD86 capacity may be heightened in asthmatic patients. Following transfection of the mDCs with CD80- and CD86-targeted siRNA, the mRNA expression levels and protein positive expression rates of CD80 and CD86 were significantly decreased, confirming the inhibitory effect that the siRNA approach had on the co-stimulatory molecules CD80 and CD86 at the transcriptional and translational levels. In the supernatant from the co-culture of mDCs and T cells, RNAi induced an increase in IFN-γ expression and a reduction of IL-4 levels, indicating that decreasing the expression of the co-stimulatory molecules CD80 and CD86 in mDCs weakened the CD80/CD86-CD28 co-stimulatory pathway in asthmatic mice. RNAi also affected the expression of Th1/Th2 cytokines, indicating that the original Th1/Th2 imbalance was changed and, consequently, immune tolerance was induced. These findings indicate that CD80 and CD86 may be potential targets for RNAi application in asthma treatment, and provide a new avenue for the gene therapy of asthma.

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References