

# Ribavirin or CpG DNA Sequence–Modulated Dendritic Cells Decrease the IgE Level and Airway Inflammation

Dian-Jung Chiang, Yi-Ling Ye, Wei-Li Chen, Yueh-Lun Lee, Ni-Yun Hsu, and Bor-Luen Chiang

School of Medicine; Graduate Institute of Immunology, College of Medicine, National Taiwan University; and Department of Medical Research, National Taiwan University Hospital, Taipei, Taiwan, Republic of China

Asthma is an allergic disease that is characterized by the imbalance between Th1 and Th2 cells and by the predominant Th2-type immune response. In this study, we investigated the application of dendritic cell (DCs)–based immunotherapy in modulating the immune response of allergic diseases. DCs incubated with ovalbumin (OVA), OVA plus ribavirin, OVA plus CpG-oligodeoxynucleotides (ODN 1826), or OVA plus non-CpG-ODN (ODN 1745) for 48 hours were injected intravenously into four corresponding groups of BALB/c mice. All of the mice were then immunized with OVA intraperitoneally 7 days later to establish an animal model of asthma. Serum levels of OVA antibody, airway hyperresponsiveness, cell composition and cytokine levels in the bronchoalveolar lavage fluid, and cytokine profiles of spleen cells were analyzed. The data showed that ribavirin and ODN 1826 increased interleukin-12 synthesis and inhibited interleukin-10 production. ODN 1826 could also enhance the expression of B7.1, B7.2, major histocompatibility complex I, and major histocompatibility complex II molecules. Furthermore, the DCs modulated by ribavirin and ODN 1826 could downregulate the Th2-type immune response *in vivo* and could alleviate airway inflammation. This study elucidated the effect of ribavirin and CpG-ODN on DCs and demonstrated that *in vitro* modulated DCs might be a potential therapeutic approach for asthma.

**Keywords:** asthma; dendritic cells; ribavirin; CpG-ODN

Asthma is an inflammatory disease of the airways that affects 5–10% of the general population. Epidemiologic studies indicate that there has been a global increase in the incidence, morbidity, and mortality levels of asthma despite the expanding availability of medications (1). The pathophysiology of asthma is characterized by eosinophilic inflammation of the airways, bronchospasm, and hyperreactivity to nonspecific inhaled stimuli (2). Asthma has been characterized by an imbalance between Th1 and Th2 lymphocytes and by the predominant Th2-type immune response (3). Th2-type cytokines, such as interleukin (IL)-4, IL-5, and IL-10, may lead to eosinophilic and mast cell chemotaxis and activation, as well as B-cell production of IgE (4). Some studies have demonstrated that the induction of Th2-type cytokines and the proliferation of committed Th2 lymphocytes can be prevented by Th1-type cytokines, notably interferon- $\gamma$  and IL-12 (5–7).

Ribavirin, a guanosine analogue, is an active antiviral compound against many RNA and DNA viruses. Recent studies have shown that the beneficial effects of ribavirin are achieved by the significant promotion of Th1 cytokine response and the

inhibition of Th2 response (8, 9). It will be interesting to find out whether ribavirin could stimulate dendritic cells (DCs) to secrete Th1-related cytokines and decrease Th2-mediated inflammation. CpG-ODN (oligodeoxynucleotides containing immunostimulatory DNA sequence) is a bacterial DNA sequence that can trigger numerous immune responses *in vivo*. It has been reported that CpG-ODN is able to stimulate both macrophages and natural killer cells to produce cytokines that modulate T-cell cytokine production and favor the generation of Th1 responses to subsequently encountered allergens (10). DCs, as professional antigen-presenting cells, are the most efficient activators of resting T cells and have the unique capacity to activate naive T cells (11). Therefore, DCs hold a major role in the development of T cells and promote the development of Th1 or Th2 immune response toward specific antigens.

The exact effects of ribavirin and CpG-ODN on DCs are yet to be defined. In this study, we examined the effect of ribavirin or CpG-ODN on the accessory molecules and cytokine profile of DCs *in vitro*. Interestingly, both ribavirin and CpG-ODN increased IL-12 and decreased IL-10 production by the DCs. Moreover, CpG-ODN can enhance the expression of major histocompatibility complex (MHC), B7.1, and B7.2 molecules on the cell surface of DCs. *In vivo* studies further demonstrated that ribavirin or CpG-ODN–modulated DCs pulsed with allergen effectively prevent airway inflammation in a murine model of asthma.

## METHODS

### Animals

Female BALB/c mice aged 6–8 weeks were obtained from the Animal Center of the College of Medicine, National Taiwan University. The animal room was kept on a 12-hour light and dark cycle with constant temperature ( $25^{\circ}\text{C} \pm 2^{\circ}\text{C}$ ) and humidity maintained. Animal care and handling conformed to the *NIH Guide for the Care and Use of Laboratory Animals*.

### Reagents

Synthetic ODNs were purchased from Oligos Etc. (Wilsonville, OR). ODNs were phosphorothioate modified to increase their resistance to nuclease degradation. ODN with the following sequences were used (CpG motifs or altered non-CpG motifs are underlined): CpG ODN 1826, TCCATGACGTTCTGACGTT, and non-CpG ODN 1745, TCCATGAGCTTCCTGAGCTT. Ribavirin (1- $\beta$ -D-ribofuranosyl-1, 2, 4-triazole-3-carboxamide) was obtained from ICN Pharmaceuticals (Irvine, CA) and was dissolved in phosphate-buffered saline before use.

### Isolation and Modulation of DCs from Bone Marrow Cultures

Bone marrow–derived DCs (BMDCs) were generated from culture of bone marrow cells in the tibial and femoral bones of BALB/c mice as described previously (12). Bone marrow cells ( $1 \times 10^6$  cells/ml) were placed in 24-well plates in Roswell Park Memorial Institute (RPMI) medium with 5% heat inactivated fetal calf serum supplemented with recombinant murine granulocyte-macrophage colony-stimulating factor (500 U/ml) and IL-4 (1000 U/ml) (Pepro Tech Inc., Rocky Hill, NJ). For some parts of the study, BMDCs were further purified with magnetic beads conjugated with anti-CD11c antibody. BMDCs were pulsed

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Correspondence and requests for reprints should be addressed to Bor-Luen Chiang, M.D., Ph.D., Department of Medical Research, National Taiwan University Hospital, Number 1, Chang-Teh Street, Taipei, Taiwan 100, Republic of China. E-mail: gicmbor@ha.mc.ntu.edu.tw

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with various doses of ribavirin (4, 20, 100 pg/ml), ODN1826 (5 and 125  $\mu$ g/ml), or ODN1745 (5 and 125  $\mu$ g/ml) on Day 6 of the culture. On Day 8, nonadherent cells (BMDCs) and culture supernatants were collected for fluorocytometric analysis and cytokine profile assay.

The phenotypes of DCs were analyzed by flow cytometry for the expression of MHC class I, MHC class II, B7.1 (CD80), and B7.2 (CD86). The antibody for MHC class I was fluorescein isothiocyanate–anti-mouse H2<sup>a</sup>, for MHC class II was fluorescein isothiocyanate–anti-mouse K<sup>d</sup>, for B7.1 (CD80) was phycoerythrin (PE)–anti-mouse B7.1, and for B7.2 (CD86) was fluorescein isothiocyanate–anti-mouse B7.2. All of these antibodies were purchased from PharMingen (San Diego, CA).

Culture supernatants were collected on Day 8. The concentrations of IL-12 and IL-10 were determined by using PharMingen (Los Angeles, CA) and Quantikine M ELISA Kit (R&D Systems, Minneapolis, MN), respectively. The sensitivities of IL-12 and IL-10 were 30 and 15.625 pg/ml, respectively.

### Preparation of DC Immunization

BMDCs were derived and prepared as described previously (13). On Day 6, all BMDCs were pulsed with ovalbumin (OVA) antigen (500  $\mu$ g/ml) plus ribavirin (20 pg/ml) or ODN 1826 (125  $\mu$ g/ml) or ODN 1745 (125  $\mu$ g/ml) or OVA only. On Day 8, the DCs were collected and injected intravenously into different groups of mice ( $2 \times 10^5$  cells per mouse).

### In Vivo Experimental Design

Four groups of mice were immunized with DCs pulsed with OVA only (group 1), DCs pulsed with OVA and ribavirin (group 2), DCs pulsed with OVA and ODN 1826 (group 3), or DCs pulsed with OVA and ODN 1745 (group 4), respectively.

On Days 7 and 21, all groups of mice were immunized intraperitoneally with 10  $\mu$ g of OVA antigen. The mice were challenged by OVA protein aerosol (0.1% OVA normal saline solution) in a closed chamber using an ultrasonic nebulizer (DeVilbiss, Somerset, PA) three times from Day 36 to Day 38. The output of the nebulizer was 0.3 ml/min, and the produced particles had a size range of 0.5 to 5  $\mu$ m.

### Serum Ab Assay

Serum samples were collected from the retro-orbital venous plexus 7 days after the intraperitoneal immunization with OVA antigen. The OVA-specific IgE, IgG1, and IgG2a serum antibody titers were determined by ELISA.

### Measurement of Airway Hyperresponsiveness and Bronchoalveolar Lavage Study

Twenty-four hours after the last aerosol exposure, the airway resistance of mice was measured by a single-chamber, whole-body plethysmograph (Buxco Electronics, Inc., Troy, NY) as previously described (7). Airway resistance was expressed as Penh (enhanced pause). Increasing doses of methacholine were administered by nebulization for 3 minutes, and Penh were measured over the subsequent 3 minutes.

The lungs were lavaged immediately via the trachea with  $3 \times 1$  ml of Hanks' balanced salt solution, which is free of ionized calcium and magnesium. The total cells in the bronchoalveolar lavage (BAL) were resuspended in 1 ml of Hanks' balanced salt solution, and the total cells were determined by hemocytometer. Cytoцентрифугed preparations were stained with Liu's stain for differential cell counts. A minimum of 200 cells was counted and classified as macrophages, lymphocytes, neutrophils, or eosinophils.

### BAL Fluid and Splenocyte Cytokine Measurement

The levels of IL-12 and IL-5 in the BAL fluid supernatants were evaluated using an appropriate ELISA kit (R&D). These assays have a threshold of detection of 30 and 2 pg/ml, respectively.

The splenocytes ( $1 \times 10^7$  per well) were stimulated with 10  $\mu$ g/ml of OVA in a 48-well microtiter plate for 48 hours at 37°C. The level of IL-5 in the supernatants was evaluated by sandwich ELISA (PharMingen, Los Angeles, CA). The sensitivity for IL-5 determination was 2 pg/ml.

### Statistical Analysis

The data of cytokine secretion by DCs were analyzed by chi-square test. Significance levels were set at a p value of 0.05. The Student's

t test was used to determine the level of difference in the serum antibody assay, airway hyperresponsiveness measurement, BAL study, antigen-specific proliferation study, and splenocytes cytokine assay. Values for all measurements were expressed as mean  $\pm$  SEM. Significance levels were set at a p value of 0.05.

## RESULTS

### Effect of Ribavirin or CpG-ODN on the Phenotype of DCs

To determine whether the ribavirin or CpG motif can modulate the development of DCs *in vitro*, we compared the phenotype of DCs pulsed with different doses of ribavirin, CpG-ODN (ODN 1826), or non-CpG-ODN (ODN 1745) for 48 hours. The data demonstrated that approximately 77% of BM-DCs expressed CD11c molecule after 8 days of culture (Figure 1A). The purity of CD11c+ DCs can reach 95% after further isolation with magnetic bead conjugated with anti-CD11c antibody (Figure 1A). The data showed that CpG-ODN (ODN 1826) increased the presentation of B7.1, B7.2, and MHC class I and class II molecules on the cell membrane of DCs (Figures 1B). Non-CpG-ODN (ODN 1745)

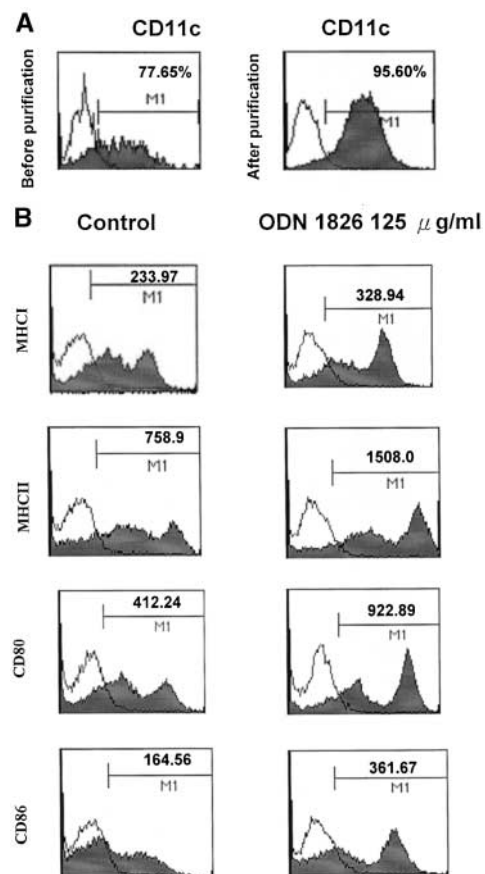


**Figure 1.** The phenotypes of dendritic cells (DCs) incubated with different doses of ribavirin, oligodeoxynucleotides (ODN) 1826, or ODN 1745 for 48 hours. The DCs cultured with culture medium alone served as the control. The result suggested that the expression of CD11c molecules was approximately 77% and 95% before and after magnetic beads conjugated with anti-CD11c antibody purification respectively (A). ODN 1826 can increase the mean fluorescence intensity (MFI) levels of CD80 (B7.1), CD86 (B7.2), major histocompatibility complex (MHC) I, and MHC II molecules on DCs cell membrane (B). In addition, there was no difference in the MFI expression of these accessory molecules when stimulated with ODN 1725 or ribavirin. Representative data from one of four experiments are shown.

or ribavirin does not have any modulatory effect on the phenotype. The MHC or B7 expression is similar to that of the control group (Figures 1B).

#### Effect of Ribavirin or CpG-ODN on the Cytokine Secretion of DCs

To determine whether ribavirin or CpG-ODN can affect the cytokine production of DCs, we compared the cytokine concentrations in the supernatants of DCs cultured in different culture conditions. It is clear that ribavirin enhanced the production of IL-12 (Figure 2A) but inhibited the production of IL-10 by the DCs (Figure 2B). ODN 1826 also enhanced the production of IL-12, and this effect was more prominent in high doses (Figure 3A). It was clear that the stimulatory effect of ODN 1826 was dose dependent. On the other hand, ODN 1826 tended to decrease IL-10 production by DCs without statistical significance (Figure 3B). In contrast, non-CpG-ODN (ODN 1745) did not have any effect on the levels of IL-12 or IL-10 (Figure 3).

#### *In Vitro* Modulated DC Downregulation of Th2-type Antibody Response *In Vivo*

*In vitro* modulated BMDCs were injected intravenously into mice, and an animal model of asthma was induced by OVA sensitization afterward. The data demonstrated that both the ribavirin and CpG motif-modulated DCs can strongly decrease the production of OVA-specific IgE (Figure 4A) in serum and enhance the production of OVA specific IgG2a (Figure 4C). It also slightly decreased the production of OVA-specific IgG1 (Figure 4B).

#### *In Vitro* Modulated DCs on the Airway Hyperresponsiveness of Mice

We next explored whether the ribavirin or CpG-ODN-modulated DCs can play a protective role against airway hyperactivity in the murine model of asthma. Twenty-four hours after the last OVA aerosol exposure, all groups of mice were subjected to the methacholine stimulation test. The mice, which were treated with ribavirin-modulated DCs, had a significant decrease in airway hyperactivity, as assessed by their response to increasing doses of inhaled methacholine (Figure 5). CpG-ODN-modulated DCs also decreased airway hyperresponsiveness in

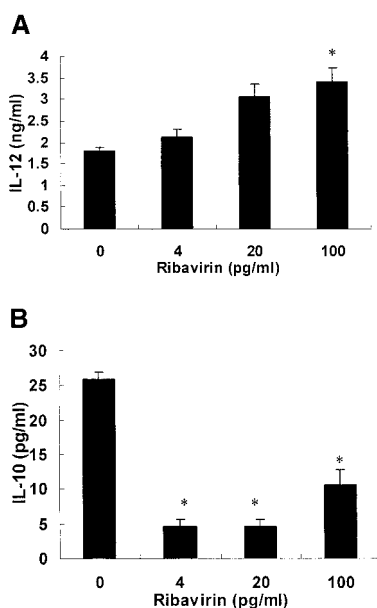


**Figure 2.** The interleukin (IL)-12 (A) and IL-10 (B) levels in DCs incubated with different doses of ribavirin. The DCs cultured with culture medium only served as the control. The dose of ribavirin was from 4 to 100 pg/ml. Ribavirin could stimulate the production of IL-12 from DCs but inhibited the production of IL-10. Representative data from one of three experiments are shown. Values were expressed as mean  $\pm$  SEM. \*The difference is statistically significant as compared with the control group ( $p < 0.05$ ).

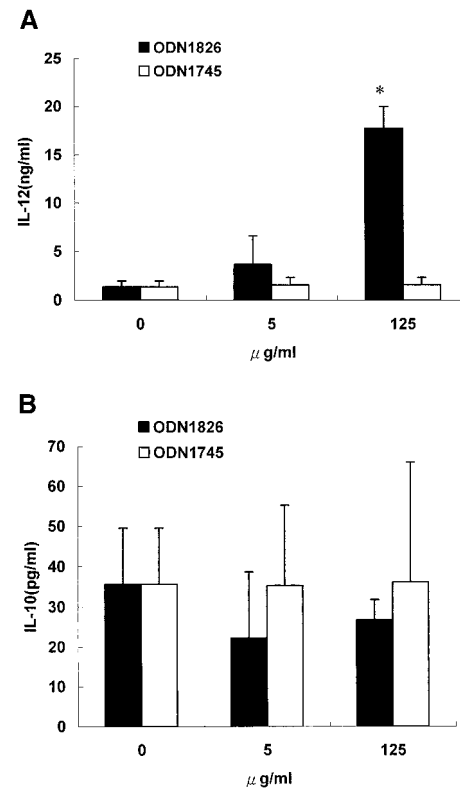


**Figure 3.** The IL-12 (A) and IL-10 (B) levels in different groups of DCs treated with different doses of ODN 1826 or ODN 1745. The dose of ODN 1826 or ODN 1745 was from 5 and 125  $\mu$ g/ml, respectively. The stimulatory effect of ODN 1826 on IL-12 production is dose dependent, but it could inhibit the production of IL-10 from DCs. Representative data from one of three experiments are shown. Values were expressed as mean  $\pm$  SEM. \*The difference is statistically significant as compared with the control group ( $p < 0.05$ ).

mice. In contrast, mice treated with non-CpG-ODN had a similar airway hyperactivity as those of the control group (Figure 5).

#### Cellular Composition in the BAL Fluid

To evaluate further whether the *in vitro* modulated DCs can modulate the recruitment of inflammatory cells in the airway, the differential cell counts in BAL fluid were determined with Liu's stain. The data showed that the ribavirin- or CpG-ODN-modulated DCs can downregulate the numbers of eosinophils and increased macrophages in BAL fluid (Figure 6). On the other hand, DCs treated with non-CpG-ODN did not have any immunomodulatory effects on the composition of inflammatory cells in BAL fluid.

#### Cytokine Levels in the Bronchoalveolar Lavage Fluid and Splenocyte

The IL-12 and IL-5 expression in the BAL fluid derived from each group was measured by ELISA. The IL-12 expression increased in ribavirin-treated groups (Figure 7A). The level of IL-12 also tended to increase in CpG-ODN group, although without statistical significance. IL-5 expression is not obviously different between each group (data not shown).

By studying the cytokine concentration in the supernatant of splenocytes culture, we tried to clarify if *in vitro* modulated DCs can affect the immune response through the alteration of cytokine production in splenocytes. It was shown that ODN 1826

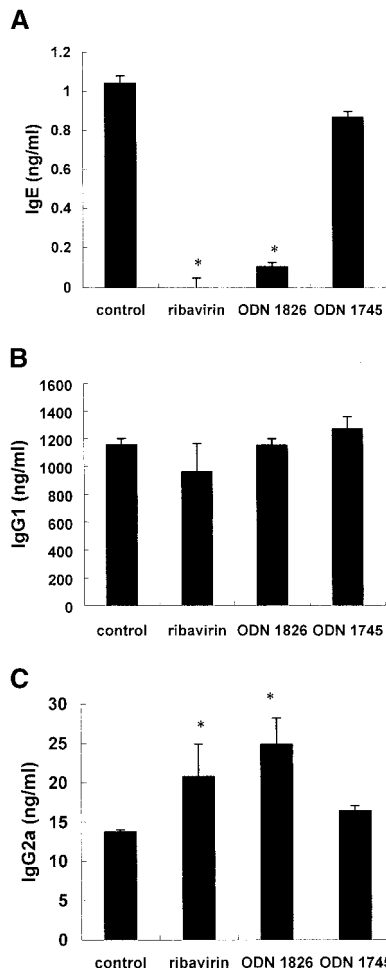


**Figure 4.** Serum ovalbumin (OVA)-specific antibody levels. Ribavirin modulated DCs can effectively decrease the production of OVA-specific IgE (A) in serum and enhance the production of OVA specific IgG2a (C). It also slightly decreased the production of OVA specific IgG1 (B). ODN 1826 modulated DCs can also significantly downregulate the synthesis of OVA specific IgE in serum (A). Furthermore, ODN 1826 also stimulated the synthesis of OVA specific IgG2a in serum (C), but their effect on OVA-specific IgG1 is not prominent (B). Each group included five mice. Values were expressed as mean  $\pm$  SEM. Representative data from one of two experiments are shown. \*The difference is statistically significant as compared with the control group ( $p < 0.05$ ).

and ribavirin modulated DCs could downregulate the synthesis of IL-5 in mice splenocytes (Figure 7B).

## DISCUSSION

Allergic asthma is now regarded as a T-cell-mediated disorder in which sensitized individuals develop eosinophilic airway in-

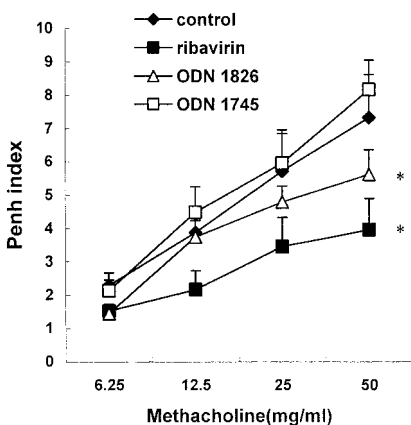


**Figure 5.** The airway hyperresponsiveness in mice treated with different preparations of DCs. The mice treated with ribavirin or CpG-ODN-modulated DCs had a significant decrease in airway hyperreactivity in mice. In contrast, the mice that were treated with non-CpG-ODN had similar airway hyperreactivity as the control group. Each group included five mice. Values were expressed as

mean  $\pm$  SEM. Representative data from one of two experiments are shown. \*The difference is statistically significant as compared with the control group ( $p < 0.05$ ).

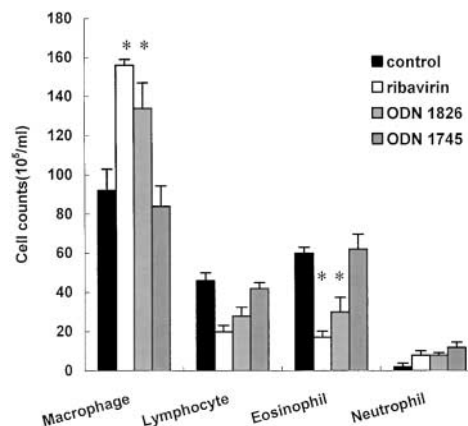


**Figure 6.** The cell compositions in bronchoalveolar lavage fluid (BALF) were analyzed. Ribavirin and ODN 1826-modulated DCs can downregulate the infiltration of eosinophils in the airways. In contrast, ODN 1745-modulated DCs cannot affect the recruitment of inflammatory cells in the airway. Each group included five mice. Values were expressed as mean  $\pm$  SEM. Representative data from one of two experiments are shown. \*The difference is statistically significant as compared with the control group ( $p < 0.05$ ).

flammation in response to inhaled allergen. The DC is the most potent antigen-presenting cell that can activate naive T cells by the MHC-antigen complex and adjacent costimulatory factors (14, 15). Previous studies have demonstrated that intratracheal injection of DCs are potent in inducing asthma-like diseases by recruiting lymphocytes to the lung-draining lymph nodes and by stimulating Th2 responses (16–19). Allergen-pulsed DCs without any modulation can induce marked airway eosinophils infiltration and inflammation (16, 17, 19). In addition, it has been demonstrated that pulmonary DCs can produce significant amount of IL-10 when exposure to allergen (20). Other studies showed that DC-depleted mice have no eosinophilic airway inflammation and goblet cell hyperplasia induced by OVA (21).

These studies suggest that DCs are critical in developing a specific immune response in the lungs and DC pulsed with antigen can promote the selection of a stronger Th2 response (22–24). Furthermore, distinct populations of DC, with the capacity to induce differential priming for Th1 or Th2 cells, have been identified (25, 26). It was also reported in several murine models that the pulmonary environment promotes a preferential Th2-type immune response (27–29). Whether or not this local immune preference is achieved through T-cell priming by distinct subsets of DCs in the lungs needs more studies. It will be interesting to study whether *in vitro* modulated DCs can affect the allergic immune response and prevent airway inflammation.

Ribavirin has been found to be active against various RNA and DNA viruses, such as respiratory syncytial virus, murine immunodeficiency virus, hepatitis B virus, and hepatitis C virus (30, 31). However, the real role of ribavirin in immune modulation is still not defined. Previous studies have reported that ribavirin may modulate the T helper (Th1/Th2) subset balance in hepatitis B and C virus-specific immune responses (32). Ribavirin has been found to increase the level of IL-12 both *in vitro* and *in vivo* (9).

To investigate the effect of ribavirin further, we examined whether ribavirin can affect the development and function of DCs *in vitro*. The study showed that ribavirin had minimal effects on the expression of B7.1, MHC I, and MHC II on the cell surface of DCs. However, the data suggested that ribavirin could

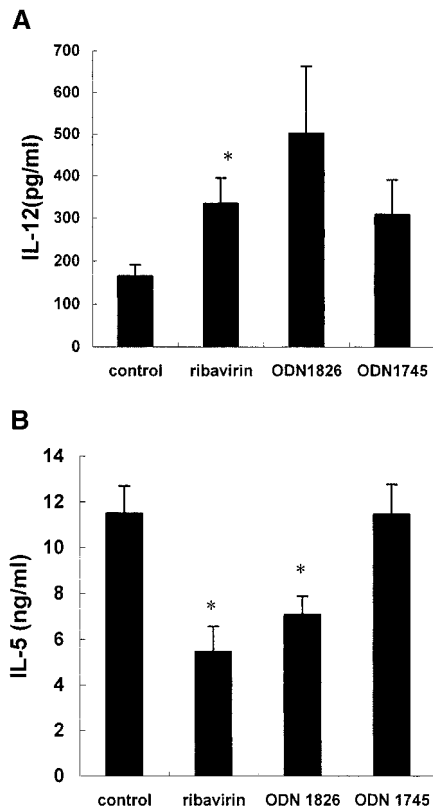


**Figure 7.** The expression levels of cytokines in BALF and splenocyte in each group of mice. (A) The IL-12 level was significantly higher in BALF of ribavirin group compared with that of control group. (B) Cytokine level produced by OVA-stimulated splenocytes. The data suggested that ribavirin and ODN 1826 modulated DCs reduced IL-5 production of splenocytes in mice. Each group included five mice. Values were expressed as mean  $\pm$  SEM. Representative data from one of two experiments are shown. \*The difference is statistically significant as compared with the control group ( $p < 0.05$ ).

enhance the production of IL-12 and inhibit the synthesis of IL-10 of DCs *in vitro*. However, a rather higher amount of IL-12 might also directly inhibit IL-10 production of DCs. More studies are needed to elucidate the real mechanism of ribavirin. It is suggested that ribavirin may mount a Th1-type immune response by promoting a Th1-type milieu of cytokine production from DCs. The *in vivo* study further demonstrated that ribavirin-modulated DCs can effectively decrease allergen-specific IgE and consequently airway inflammation.

CpG motifs were initially identified and isolated from mycobacterial DNA (33), as they were found to appear at a higher frequency in many pathogenic bacteria and viruses even though they were underrepresented in the vertebrate genome. It has been demonstrated that the infection of mice with *Mycobacterium bovis*-Bacillus Calmette-Guerin can suppress allergen induced airway eosinophilia (34). Thus, natural exposure to CpG-ODN through infection during the maturation of the immune system may play an important role in veering the immune response away from an allergic reaction after allergen challenge.

Immunostimulatory CpG-ODN has a wide range of effects on inflammation and immune responses (35–37). It has been documented that CpG-ODN can decrease airway inflammation in murine model of asthma (15, 38, 39). Most of these actions of CpG-ODN have been associated with the induction of a Th1 immune response. It can induce the T cell to develop into a Th1

cell by the secretion of different milieu of cytokines and affect the expression of B7.1 or B7.2 on the cell surface. To evaluate whether its ability to induce the immune response is through the DCs, we examined the phenotype and cytokine production of DCs cultured with CpG-ODN. In this study, we demonstrated that CpG-ODN can modulate the differentiation and function of DCs *in vitro* through the enhancement of MHC (MHC I and MHC II) and the presentation of costimulatory factors B7.1 (CD80) on the cell membrane. Furthermore, CpG-ODNs can also stimulate the production of IL-12 and inhibit the level of IL-10 of DCs. By affecting the maturation of DCs, CpG-ODN could induce a Th1-type immune response toward a specific antigen. As shown in our animal model of asthma, it is not only the systemic exposure of CpG-ODN but also the exposure of CpG-ODN to DCs alone that might shape the immune response.

Interestingly, the modulated DCs can activate a stronger Th1 response in mice, as assessed by the antigen-specific immunoglobulin subtypes. Furthermore, the modulated DCs can also exert a protective effect against airway hyperresponsiveness and airway hyper eosinophilia. The immune system deviation achieved by modulated DCs can treat the allergic response to a specific antigen. Although we have demonstrated that CpG-ODN and ribavirin may direct the immune response toward the Th1 direction by modulating DCs, it is still unclear how they affect the DCs *per se*. In recent studies, it was suggested that CpG-ODN might act through TLR9 receptors and the nuclear factor- $\kappa$ B pathway to influence DCs (40). In addition, adaptor protein MyD88 has been implicated in the signal pathway of CpG-ODN stimulation (41). However, the exact mechanisms underneath demand more studies.

The study demonstrated that DCs modulated with ribavirin or CpG-ODN *in vitro* can decrease allergen-specific IgE levels and alleviate airway inflammation. The knowledge of how ribavirin and CpG-ODN modulate the development of DCs and the immune system will enable us to formulate a preventive vaccine against asthma and provide an alternative to alleviate the severity of atopic diseases in the future.

**Conflict of Interest Statement:** D-J.C. has no declared conflict of interest; Y-L.Y. has no declared conflict of interest; Y-L.C. has no declared conflict of interest; Y-L.L. has no declared conflict of interest; N-Y.H. has no declared conflict of interest; B-L.C. has no declared conflict of interest.

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