

CpG-ODN inhibits airway inflammation at effector phase through down-regulation of antigen-specific T_H2-cell migration into lung

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Abstract

Allergic airway inflammation is one of the most typical characteristic features of bronchial asthma. T_H2 cells, which produce IL-4, IL-5 and IL-13, are well known as major effector lymphocytes of the inflammation. In the present work, we found that subcutaneous injection of Toll-like receptor-9-ligand, CpG-oligodeoxynucleotides (CpG-ODN), remarkably suppressed eosinophilia and mucus hyper-production in T_H2 cell-dependent airway inflammation model at the effector phase. The injection of CpG-ODN significantly blocked T_H2 cell migration into lung. The inhibitory effects of CpG-ODN were observed even when IFN- γ -deficient T_H2 cells were transferred into IFN- γ ^{-/-} mice. In contrast, the administration of neutralizing mAbs against type I cytokines such as IFN- α , IFN- β and IL-12 significantly suppressed the inhibitory effect of CpG-ODN on airway inflammation and T_H2 cell migration into the lung. We further demonstrated that the production of T_H2 chemokines, thymus and activation-regulated chemokine (TARC) and macrophage-derived chemokine (MDC), was significantly reduced by the CpG-ODN. The reduction of both TARC and MDC was also inhibited by the blockade of IFN- α , IFN- β and IL-12 with mAbs. Thus, we revealed here that IFN- α , IFN- β and IL-12, but not IFN- γ , were required for the inhibitory effect of CpG-ODN in T_H2 cell-mediated allergic airway inflammation. The present evidence strongly suggest that induction of type I cytokines would be promising therapeutic targets in T_H2-dependent allergic diseases such as bronchial asthma.

Introduction

Disruption of immune balance regulated by effector CD4⁺ T cell subsets, especially T_H1/T_H2 populations, induces various immune diseases including allergic airway inflammation. Airway hyper-responsiveness (AHR), eosinophilic airway inflammation, mucus hyper-production in airway epithelium and elevated serum level of IgE are characteristic features of bronchial asthma (1–4). It has been demonstrated that activated CD4⁺ T cells play a critical role in modulating the pathogenesis of asthma (5, 6). In particular, T_H2 cells, producing type II cytokines such as IL-4, IL-5 and IL-13, develop airway inflammation with eosinophil infiltration in allergic asthma (7, 8). In fact, the effector cytokine-producing T_H2 cells are observed in both the bronchoalveolar lavage fluid (BALF) and airway biopsies of bronchial asthma patients (5, 9–11). The migration of activated CD4⁺ T cells into the lung and the inflammatory products are closely associated with asthma symptoms and morbidity (12–14).

Previously, it has been reported that Toll-like receptor-9-ligand, CpG-oligodeoxynucleotides (CpG-ODN) remarkably activate type I immune responses (15–17). CpG-ODN enhances production of type I IFNs and IL-12 from antigen-presenting cells such as dendritic cells (DCs) and promotes the generation of IFN- γ -producing T_H1 cells *in vivo*. So far, it has been demonstrated that an administration of CpG-ODN significantly improved symptoms of asthma model at induction phase, which were established by immunization of ovalbumin (OVA) plus aluminum hydroxide [Al(OH)₃] (18–20). Recently, clinical trials using CpG-ODN have been performed for patients suffering from allergic diseases such as allergic rhinitis (21–23). Many researches have been investigating the precise mechanisms of CpG-ODN-induced inhibitory effect on the allergic airway inflammation at induction phase (24–28). However, there are still unknowns about the actions of cytokine-producing T_H2 cells at effector phase in the airway inflammation.

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We had established an allergic airway inflammation model by the adoptive transfer of T_H2 cells, which enabled us to examine the mechanisms and therapeutic strategies in T_H2 cell-mediated asthma at the effector phase (29, 30). Here, we used the allergic airway inflammation model to address the inhibitory effect of CpG-ODN on airway inflammation at effector phase. In the present paper, we revealed that *in vivo* injection of CpG-ODN remarkably inhibited the migration of T_H2 cells into lung and suppressed elevation of AHR, eosinophilia and mucus hyper-production in type I cytokine-dependent manner. We also discussed about the possible mechanisms underlying CpG-ODN-mediated prevention of T_H2 cell chemotaxis, followed by suppression of airway inflammation.

Methods

Mice

Wild-type BALB/c mice were purchased from Charles River Breeding Laboratories (Kanagawa, Japan). OVA_{323–339}-specific I-A^d-restricted TCR-transgenic mice (DO11.10) were kindly donated by Dr K. Murphy (Washington University School of Medicine, St Louis, MO, USA) (31). IFN- γ ^{–/–} BALB/c mice were kindly provided by Dr Y. Iwakura (University of Tokyo, Tokyo, Japan). All mice were maintained in specific pathogen-free conditions according to the guideline of our institute's animal department and used at 6–8 weeks.

Reagents

Recombinant murine IL-2 was kindly donated by Dr T. Sawada (Shionogi Pharmaceutical Institute Co., Osaka Japan). Recombinant murine IL-4 was purchased from PeproTech EC Ltd (London, UK). PE-conjugated anti-IL-4 mAb (11B11), PE-conjugated anti-IL-5 mAb (TRFK5), FITC-conjugated anti-IFN- γ mAb (XMG1.2), PE-Cy5-conjugated anti-CD4 mAb (GK1.5) were purchased from BD PharMingen (San Diego, CA, USA). Anti-OVA_{323–339}-specific TCR mAb was purified from ascites fluid of mice intra-peritoneally (i.p.) inoculated with KJ1.26 hybridoma cells in our laboratory and conjugated with FITC for some experiments. Neutralizing mAb against IFN- α was purchased from PBL Biomedical Laboratories and anti-IFN- β mAb was kindly donated by Dr F. Okano (Toray Institute Co., Kanagawa, Japan). Neutralizing mAbs against IFN- γ (R4-6A2) and IL-12 (C15.1 and C15.6), donated by Dr G. Trinchieri (Wistar Institute of Anatomy and Biology, Philadelphia, PA, USA), were purified from ascites fluid of mice in our laboratories. Recombinant OVA_{323–339} peptide was kindly supplied by Dr H. Tashiro (Fujiya Co. Ltd, Hadano, Japan). OVA protein and β -methacholine chloride (Mch) were purchased from Sigma-Aldrich Japan (Tokyo, Japan). Phosphorothioate-stabilized CpG-ODN 1668 (5'-TCCATGACGTTTCCTGATGCT-3') was purchased from Hokkaido System Science (Hokkaido, Japan). ISOGEN RNA extraction reagent was purchased from Nippon Gene (Tokyo, Japan). Superscript II RT and oligo (dT)_{12–18} primer was purchased from Invitrogen (Carlsbad, CA, USA).

Generation of OVA-specific T_H2 cells

CD4⁺CD45RB⁺ naive T_H cells were isolated from DO11.10 TCR-transgenic mouse spleen cells using cell sorter (FACSaria; BD Bioscience, San Jose, CA, USA). OVA-specific

T_H2 cells were generated from purified naive T_H cells with 5 μ g ml^{–1} OVA peptide (OVA_{323–339}), IL-2 (100 U ml^{–1}), IL-4 (1 ng ml^{–1}), anti-IL-12 mAbs (10 μ g ml^{–1}; C15.1 and C15.6) and anti-IFN- γ mAb (10 μ g ml^{–1}; R4-6A2) in the presence of mitomycin C-treated spleen cells. The cells were re-stimulated with OVA_{323–339} peptide under the same conditions at 48 h. For evaluation of the T_H2 differentiation, these cells were analyzed by intracellular staining with FITC-conjugated anti-IFN- γ and PE-conjugated anti-IL-4 or anti-IL-5 mAb at day 8 as previously reported (29). After washing out the factors at 9–11 days of culture, the generated T_H2 cells (2×10^7 cells per mouse) were transferred intravenously (i.v.) into syngeneic BALB/c mice.

Induction of allergic airway inflammation in T_H2 cell-transferred mice

A model of allergic airway inflammation was established by the adoptive transfer of T_H2 cells as described previously (29). Briefly, OVA-specific T_H2 cells (2×10^7 cells in 0.2 ml PBS) were injected into the tail vein of normal recipient BALB/c mice. One day after the transfer, mice were daily exposed with aerosolized OVA protein (2 wt/vol % in PBS), which was generated by a nebulizer (OMRON NE-U07 nebulizer; Omron Corporation) driven at 1.0 ml min^{–1} atomization of OVA protein solution, for 30 min during consecutive 3 days. CpG-ODN (50 micrograms per mice) was subcutaneously (s.c.) injected at 30 min before the first OVA inhalation. Twenty-four hours after the third OVA exposure, pulmonary function was tested and the lung histological examinations were carried out for the mice.

Measurement of AHR

AHR was measured by Mch-induced airflow obstruction as reported previously (32). Briefly, the subjected mice were placed into whole-body plethysmographs (Buxco Electronics, Troy, NY, USA) interfaced with computers using differential pressure transducers. Measurements were done for respiratory rate, tidal volume and enhanced pause. Airway resistance was expressed as Penh = [(Te/0.3Tr) – 1] $\times$ (2Pef/3Pif), where Penh = enhanced pause, Te = expiratory time (seconds), Tr = relaxation time (seconds), Pef = peak expiratory flow (milliliters) and Pif = peak inspiratory flow (milliliters per second). Increasing doses of Mch were administered by nebulization (for 3 min), and Penh were calculated over the subsequent 3 min.

Histology

After measuring AHR, the lungs and tracheas were gently lavaged three times with 1 ml PBS containing 0.1% BSA. The cells in BALF were collected and stained with hematoxylin and peroxidase, and the morphology differentials were evaluated based on the staining characteristics. For mucus observation, the lungs were perfused with 10% buffered formalin, and the sections were stained with hematoxylin and periodic acid-Schiff (PAS).

FACS

Cells in the BALF were stained with PE-Cy5-conjugated anti-CD4 mAb and FITC-conjugated anti-OVA_{323–339}-specific

TCR mAb (KJ1.26), and then double-positive cells were detected by flow cytometry as transferred OVA-specific T_H2 cells.

ELISA

The cytokine and chemokine levels in the BALF were measured by OptEIA™ mouse IL-4, IL-5 and IFN- γ (BD PharMingen) and Quantikine® mouse IL-13, thymus and activation-regulated chemokine (TARC) and macrophage-derived chemokine (MDC) (R&D Systems, Minneapolis, MN, USA) ELISA kit according to the manufacturer's directions.

Reverse transcription-PCR analyses

Lungs were immediately removed after measurement of AHR. Total RNA was extracted from the lung tissues with ISOGEN reagent and cDNA was prepared by reverse transcription with Superscript II RT and oligo (dT)_{12–18} primer. Genes for Gob5, MUC5AC and β -actin were amplified by PCR with the corresponding primers. After the amplification, PCR products were separated by electrophoresis in 1% agarose gels containing ethidium bromide and visualized by UV light illumination. Primer sequences were as follows: Gob5 (forward: 5'-GGGAAAGCTGCAGGATGGAATC-3', reverse: 5'-TATACCGCCCCCTACAGGAAGTCTAA-3'); MUC5AC (forward: 5'-CAGCCGAGAGGAGGGTTTGATCT-3', reverse: 5'-AGTCTCTCTCCGCTCCTCTCAAT-3') and β -actin (forward: 5'-GTGATGGTGGGAATGGGTCAG-3', reverse: 5'-TTTGATGTCACGCACGATTTC-3').

Statistical analyses

All experiments were repeated at least three times. Mean values and standard deviation were calculated for data from representative experiment and are shown in the figures. Significant differences in the results were determined by the Student's *t*-test. The $P < 0.05$ was considered as significant in the present experiments.

Results

CpG-ODN inhibited airway inflammation at effector phase through down-modulation of T_H2 cell migration into lung

In order to address the inhibitory effects of CpG-ODN on allergic airway inflammation, we used our established T_H2 cell-dependent allergy model (29). OVA-specific T_H2 cells were adoptively transferred into wild-type BALB/c mice. After subcutaneous injection of CpG-ODN, the mice were inhaled with OVA. Eosinophils were mainly detected in the BALF and the total cell number was significantly increased compared with control mice (Fig. 1A). IL-5 level was also notably increased (Fig. 1B) and severe mucus hyper-production was observed in the airway epithelium after the third OVA inhalation (Fig. 1C). The s.c. injection of CpG-ODN remarkably suppressed the airway eosinophilia, up-regulation of IL-5 in the lung and mucus hyper-production in the airway epithelium (Fig. 1A–C). We examined mRNA expression levels of Gob5 and MUC5AC in the lung, which were related to mucus hyper-production. The elevated gene expressions were clearly reduced by the CpG-ODN injection, which were consistent with the results of histology (Fig. 1C). We also

found that the administration of CpG-ODN completely blocked the migration of OVA-specific T_H2 cells into the lung (Fig. 1D). In these conditions, production of IL-4 but not IFN- γ in BALF, elevated by the adoptive transfer of T_H2 cells, was significantly reduced in the CpG-ODN-treated mice (Fig. 1E). Furthermore, CpG-ODN administration resulted in the suppression of AHR elevated by the adoptive transfer of OVA-specific T_H2 cells (Fig. 1F). These findings strongly suggested that CpG-ODN administration would inhibit T_H2 cell-dependent allergic airway inflammation at effector phase through the blockade of antigen-specific T_H2 cell migration, which resulted in the reduction of IL-4 level in the lung.

CpG-ODN suppressed T_H2 cell-mediated allergic airway inflammation in IFN- γ -independent manner

Next, we investigated the mechanisms underlying CpG-ODN-induced inhibition of T_H2 cell-dependent airway inflammation. Because CpG-ODN powerfully activates type I immunity, we evaluated whether IFN- γ is involved in its inhibitory effects. OVA-specific IFN- $\gamma^{-/-}$ T_H2 cells were generated from IFN- $\gamma^{-/-}$ DO11.10 naive CD4⁺ T cells and i.v. injected into IFN- $\gamma^{-/-}$ BALB/c mice. After OVA inhalation, the mice transferred with IFN- $\gamma^{-/-}$ T_H2 cells showed severe eosinophilia (Fig. 2A), elevation of IL-5 level (Fig. 2B), mucus hyper-production with up-regulation of Gob5 and MUC5AC mRNA expressions (Fig. 2C) and elevation of AHR (Fig. 2D). Surprisingly, we found that CpG-ODN administration significantly inhibited the allergic airway inflammation even in the IFN- γ -deficient condition. Moreover, CpG-ODN administration totally inhibited migration of IFN- $\gamma^{-/-}$ T_H2 cells into the lung and elevation of IL-4 production in BALF (Fig. 2E and F). Although similar numbers of total lymphocytes appeared in the BALF of both CpG-ODN-injected group and the untreated one (Fig. 2A), the number of transferred IFN- $\gamma^{-/-}$ T_H2 cells (CD4⁺KJ1.26⁺), which accounted for 20% of the total infiltrating CD4⁺ T cells and 1–2% of the total lymphocytes, was significantly reduced by the *in vivo* administration of CpG-ODN. Furthermore, we confirmed that the host-derived CD4⁺ T cells did not produce T_H2 cytokines such as IL-4 and IL-5 in response to OVA antigen (data not shown). From these findings, we considered not only that host CD4⁺ T cells would not influence the present experimental airway inflammation but also that blocking effect of CpG-ODN on the migration of transferred T_H2 cells would be caused in IFN- γ -independent manner.

IFN- α , IFN- β and IL-12 are involved in the inhibitory effects of CpG-ODN on T_H2 cell migration into lung and allergic airway inflammation

As described above (Fig. 2), a typical type I cytokine, IFN- γ , induced by CpG-ODN was not crucial for the inhibition of the allergic airway inflammation in our model. Therefore, we next examined which factors were required for the inhibitory effects of CpG-ODN. As it has been demonstrated that CpG-ODN induced type I cytokines including IFN- α , IFN- β and IL-12 as well as IFN- γ (15, 16), we examined the pathogenesis of airway inflammation in the presence of neutralizing mAbs against IFN- α , IFN- β and IL-12 to evaluate their involvement in the CpG-ODN-induced inhibitory mechanisms.

4 CpG-ODN blocked T_H2 -cell migration into lung

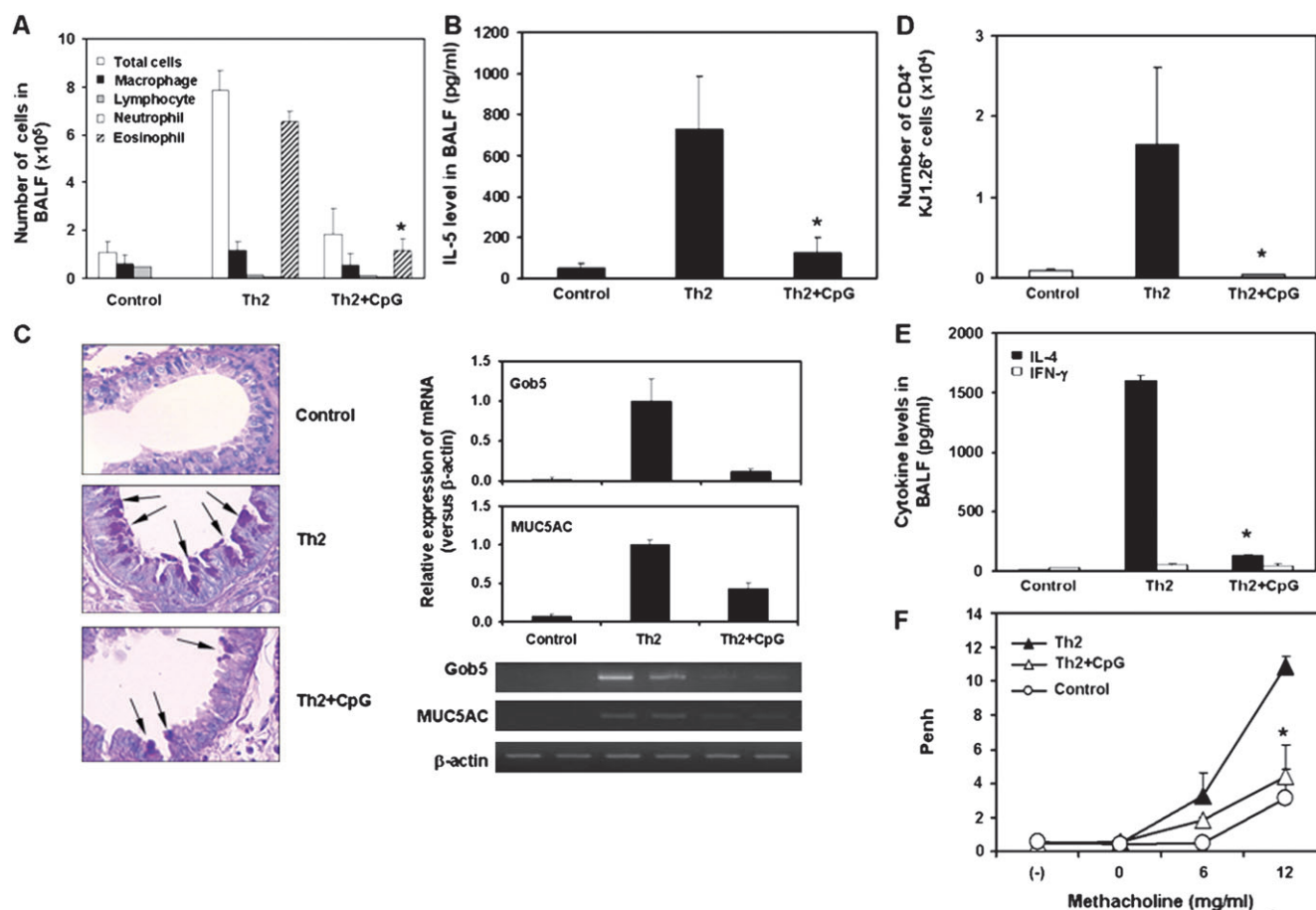


Fig. 1. CpG-ODN inhibited airway inflammation at effector phase through down-modulation of T_H2 -cell migration into lung. OVA-specific T_H2 cells were generated from naive $CD4^+$ T cells derived from DO11.10 mice. The generated T_H2 cells (2×10^7) were i.v. injected into wild-type BALB/c mice. One day after the transfer, the mice were s.c. injected with CpG-ODN (50 μ g) and exposed to OVA protein solution (2 wt/vol %) by a nebulizer for 30 min. At 24 h after three consecutive OVA inhalations, the mice were subjected to the following analysis. (A) Inflammatory cells accumulated in BALF were stained with hematoxylin peroxidase. Differentials of macrophages, lymphocytes, neutrophils and eosinophils were judged according to the morphology and stained characteristics. (B) IL-5 cytokine levels in BALF were measured by ELISA at 6 h after the final OVA inhalation. (C) Lungs were perfused with 10% buffered formalin and stained with hematoxylin and PAS for observation of mucus hyperproduction. Expression levels of both Gob5 and MUC5AC mRNA in lung were evaluated for T_H2 -transferred and T_H2 plus CpG-ODN-injected mice by reverse transcription-PCR, respectively. (D) OVA-specific T_H2 ($CD4^+KJ1.26^+$) cells in BALF were detected by flow cytometry. Each number of the T_H2 cells was evaluated for control, T_H2 -transferred and T_H2 plus CpG-ODN-injected mice after final OVA inhalation. (E) IL-4 and IFN- γ cytokine levels in BALF were measured by ELISA at 6 h after the final OVA inhalation. (F) AHR of basal values (–) or the response to nebulized PBS (0) and to two concentrations of methacholine (6 and 12 mg ml⁻¹) were measured with whole-body plethysmograph. One experimental group was composed of four mice ($n = 4$). Similar results were obtained in three separate experiments and representative data are shown in the figures. Control experiment was performed for only transferred T_H2 cells without inhalation of OVA protein. Asterisk means significant difference from CpG-ODN-untreated mice ($P < 0.05$).

Each or the combined mAbs were (i.p.) injected into mice at 30 min before the first and the second OVA inhalations. As a result, we found that the inhibitory effects of CpG-ODN on eosinophilia, the level of IL-5 in BALF and the mucus hyperproduction with Gob5 and MUC5AC mRNA expressions were significantly suppressed by the administration of mAbs (Fig. 3A–C). Moreover, the administration of the neutralizing mAbs significantly reduced the inhibitory effects of CpG-ODN on the migration of OVA-specific T_H2 cells into lung (Fig. 3D). These results suggest that type I cytokines such as IFN- α , IFN- β and IL-12 induced by CpG-ODN is associated with the inhibitory effects on T_H2 cell-mediated allergic airway inflammation.

Blockade of CpG-ODN-induced IFN- α , IFN- β and IL-12 restores the reduction of T_H2 cytokine and chemokine productions in lung

Finally, we investigated the mechanisms as to how CpG-ODN administration inhibited T_H2 cell migration into lung in the present allergic airway inflammation model. To address the function of IFN- α , IFN- β and IL-12 induced by CpG-ODN, we examined effects of the neutralizing mAbs on the production of T_H2 cytokines and chemokines in the lung. Similar to the results of T_H2 cell migration (Fig. 3D), CpG-ODN-induced reduction of IL-4 and IL-13 levels was partially but significantly suppressed by the combined injection of mAbs against IFN- α , IFN- β and IL-12 (Fig. 4A). Moreover,

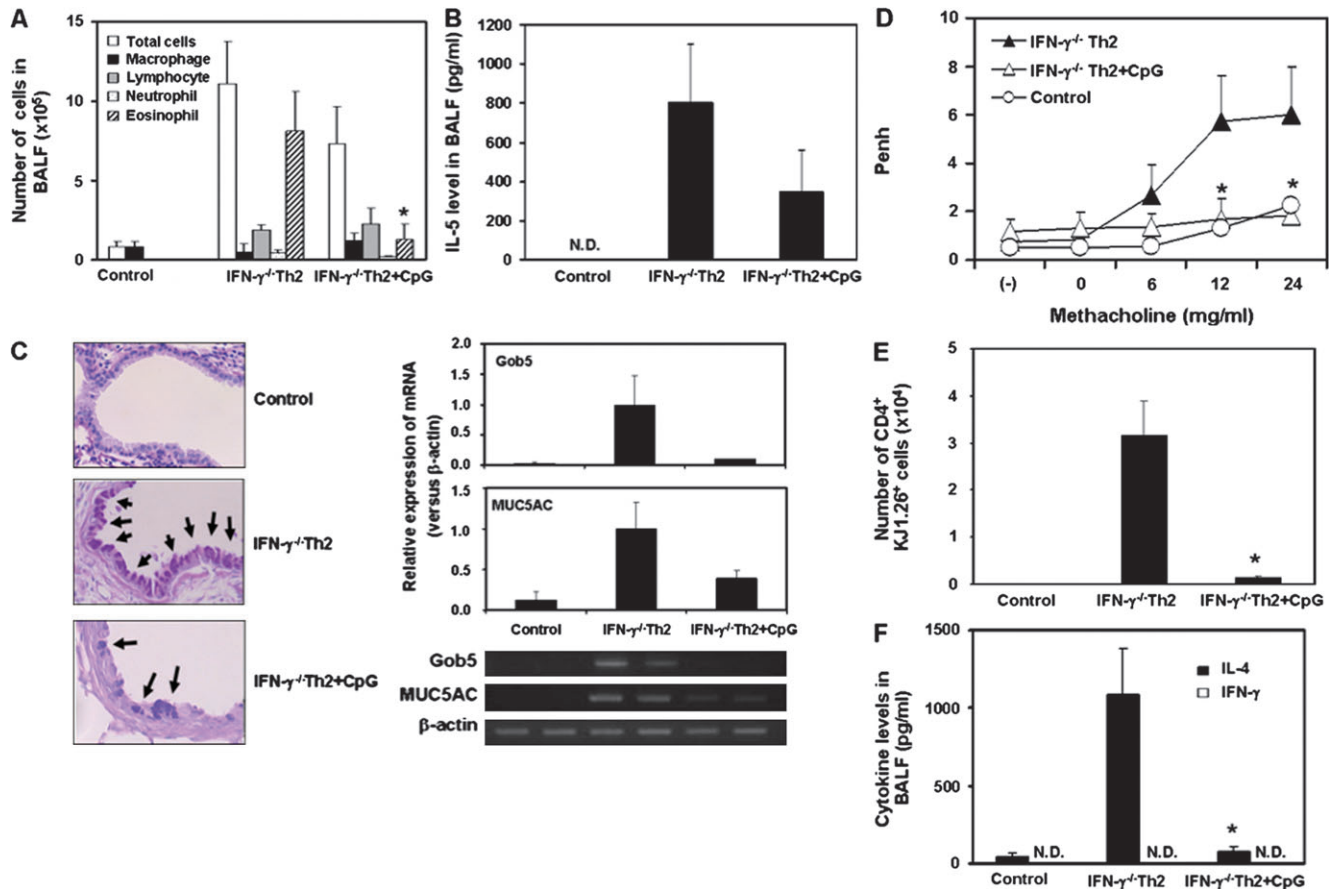


Fig. 2. CpG-ODN suppressed T_H2 cell-mediated allergic airway inflammation in IFN- γ -independent manner. OVA-specific IFN- $\gamma^{-/-}$ T_H2 cells were generated from naive CD4⁺ T cells derived from IFN- $\gamma^{-/-}$ DO11.10 mice. The generated IFN- $\gamma^{-/-}$ T_H2 cells (2×10^7) were i.v. injected into IFN- $\gamma^{-/-}$ BALB/c mice. One day after the IFN- $\gamma^{-/-}$ T_H2 cells transfer, IFN- $\gamma^{-/-}$ BALB/c mice were treated by the same methods as described in Fig. 1. (A) Inflammatory cells accumulated in BALF were stained with hematoxylin peroxidase. Differentials of macrophages, lymphocytes, neutrophils and eosinophils were judged according to the morphology and stained characteristics. (B) IL-5 cytokine levels in BALF were measured by ELISA at 6 h after the final OVA inhalation. (C) Lungs were perfused with 10% buffered formalin and stained with hematoxylin and PAS for mucus hyper-production. Expression levels of both Gob5 and MUC5AC mRNA in lung were evaluated for IFN- $\gamma^{-/-}$ T_H2 -transferred and IFN- $\gamma^{-/-}$ T_H2 plus CpG-ODN-injected mice by reverse transcription-PCR, respectively. (D) AHR of basal values (–) or the response to nebulized PBS (0) and to three concentrations of methacholine (6, 12, 24 mg ml⁻¹) were measured with whole-body plethysmograph. (E) OVA-specific IFN- $\gamma^{-/-}$ T_H2 (CD4⁺KJ1.26⁺) cells in BALF were detected by flow cytometry. Each number of the IFN- $\gamma^{-/-}$ T_H2 cells was evaluated for control, IFN- $\gamma^{-/-}$ T_H2 -transferred and IFN- $\gamma^{-/-}$ T_H2 plus CpG-ODN-injected mice after OVA inhalation. (F) IL-4 and IFN- γ cytokine levels in BALF were measured by ELISA at 6 h after the final OVA inhalation. One experimental group was composed of four mice ($n = 4$). Similar results were obtained in three separate experiments and representative data are shown in the figures. Control experiment was performed for only transferred IFN- $\gamma^{-/-}$ T_H2 cells without inhalation of OVA protein. Asterisk means significant difference from CpG-ODN-untreated mice ($P < 0.05$).

production of T_H2 chemokines, TARC and MDC, was also attenuated by the administration of CpG-ODN at the same time (Fig. 4B). These data suggest that CpG-ODN-induced type I cytokine storm including IFN- α , IFN- β and IL-12 would be involved in the suppression of T_H2 cytokine production by the transferred T_H2 cells. And the suppressions caused the subsequent attenuation of T_H2 chemokine production such as TARC and MDC, which were essential for antigen-specific T_H2 cell migration and accumulation into lung.

Discussion

Type I/type II immune balance is closely related with various immunological diseases including allergy. Many investigators have revealed that type II immunity is responsible for allergic immune responses and the subsequent pathogene-

sis of allergic inflammation diseases (33, 34). In modern lifestyle, the prevalence of allergies is steadily increasing due to reduced exposures to microbial components. The proposed allergy-preventing potential of these factors is no more present in sufficient qualities and/or quantities, which leads to an imbalance of the immune system with a predisposition to the development of allergic disorders (35).

CpG-ODN, which is a powerful activator of type I immune responses, has been expected as a promising immunomodulator for preventing T_H2 -dominant diseases such as allergic rhinitis, pollen allergy, atopic dermatitis and bronchial asthma (21–23). It has been reported that CpG-ODN treatment decreased elevation of AHR, eosinophil infiltration, mucus hyper-production, levels of IgE and airway remodeling in various bronchial asthma models using alum-OVA immunized mice (18–20, 24–28).

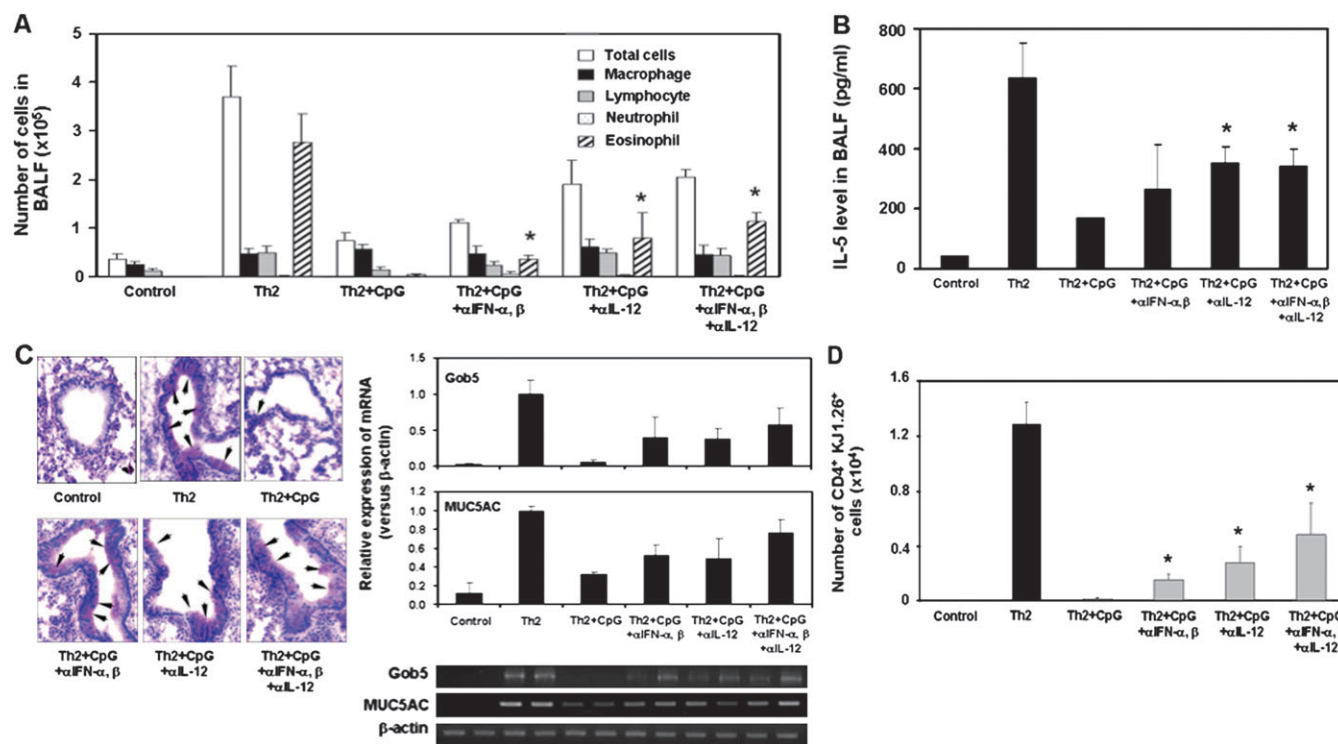


Fig. 3. IFN- α , IFN- β and IL-12 are involved in the inhibitory effects of CpG-ODN on T_H2 cell migration into lung and allergic airway inflammation. OVA-specific T_H2 cells were generated from naive CD4⁺ T cells derived from DO11.10 mice. The generated T_H2 cells (2×10^7) were i.v. injected into wild-type BALB/c mice. One day after the transfer, the mice were s.c. injected with CpG-ODN (50 μ g) and exposed to OVA protein solution (2 wt/vol %) by a nebulizer for 30 min. Anti-IFN- α mAb (100 μ g) plus anti-IFN- β mAb (250 μ g), anti-IL-12 mAb (500 μ g; C15.1 and C15.6) and anti-IFN- α mAb and anti-IFN- β mAb plus anti-IL-12 mAb were i.p. injected at 30 min before the first and the second OVA inhalations. At 24 h after the third OVA exposure, the mice were subjected to the following analysis. (A) Inflammatory cells accumulated in BALF were stained with hematoxylin peroxidase. Differentials of macrophages, lymphocytes, neutrophils and eosinophils were judged according to the morphology and stained characteristics. (B) IL-5 cytokine levels in BALF were measured by ELISA at 6 h after the final OVA inhalation. (C) Lungs were perfused with 10% buffered formalin and stained with hematoxylin and PAS for observation of mucus hyper-production. Expression levels of both Gob5 and MUC5AC mRNA in lung were evaluated for each group of mice by reverse transcription-PCR, respectively. (D) OVA-specific T_H2 (CD4⁺KJ1.26⁺) cells in BALF were detected by flow cytometry. Each number of the T_H2 cells was evaluated for control, T_H2 -transferred and T_H2 plus CpG-ODN-injected mice. One experimental group was composed of four mice ($n = 4$). Similar results were obtained in three separate experiments and representative data are shown in the figures. Control experiment was performed for only transferred T_H2 cells without inhalation of OVA protein. Asterisk means significant difference from CpG-ODN-injected mice ($P < 0.05$).

In the present work, we demonstrated that the administration of CpG-ODN remarkably inhibited eosinophilia, mucus hyper-production and T_H2 cytokine production via the blocking effect on migration of T_H2 cells into lung (Fig. 1). Although IFN- γ was highly produced by CpG-ODN-stimulated DCs and macrophages directly or by activated NK cells indirectly (15, 16, 22), we found that CpG-ODN inhibited allergic airway inflammation in IFN- γ -independent manner (Fig. 2). However, it is still controversial whether the CpG-ODN-induced type I IFNs such as IFN- α and IFN- β were involved in the general inhibitory effects of CpG-ODN on various airway inflammation models. Here, using neutralizing mAbs, we demonstrated at least that type I cytokines including IFN- α , IFN- β and IL-12 were involved in the inhibitory effects on T_H2 cell migration (Fig. 3D).

Previous papers indicated that TARC and MDC, which were downstream products of T_H2 cytokines, recruited the activated T_H2 cells into the lung (36, 37). In the present T_H2 cell-mediated allergic airway inflammation model, the CpG-ODN administration significantly suppressed the elevation of

T_H2 chemokine levels in IFN- α -, IFN- β - and IL-12-dependent manner (Fig. 4B). These data suggested that (i) CpG-ODN initially induced type I cytokine storm including IFN- α , IFN- β and IL-12, (ii) the type I cytokines reduced IL-4 and IL-13 production of T_H2 cells, (iii) the reduced type II cytokines suppressed TARC and MDC production and (iv) the suppression of the type II chemokines caused the blockade of T_H2 cell chemotaxis into the lung, which resulted in the inhibition of allergy airway inflammation. Alternatively, CpG-ODN-induced type I cytokines may first down-modulate the function of T_H2 cells to migrate into lung, which causes the inhibition of sequential cascades (the production of type II cytokines and chemokines) essential for the induction of eosinophilia and mucus hyper-production in the lung.

In the present work, the CpG-ODN-mediated inhibitory effect was partially blocked by the blockade of IFN- α , IFN- β and IL-12. It has been demonstrated that CpG-ODN induced various pro-inflammatory cytokines such as tumor necrosis factor (TNF)- α and IL-6 and anti-inflammatory cytokines including IL-10 in addition to type I cytokines (22).

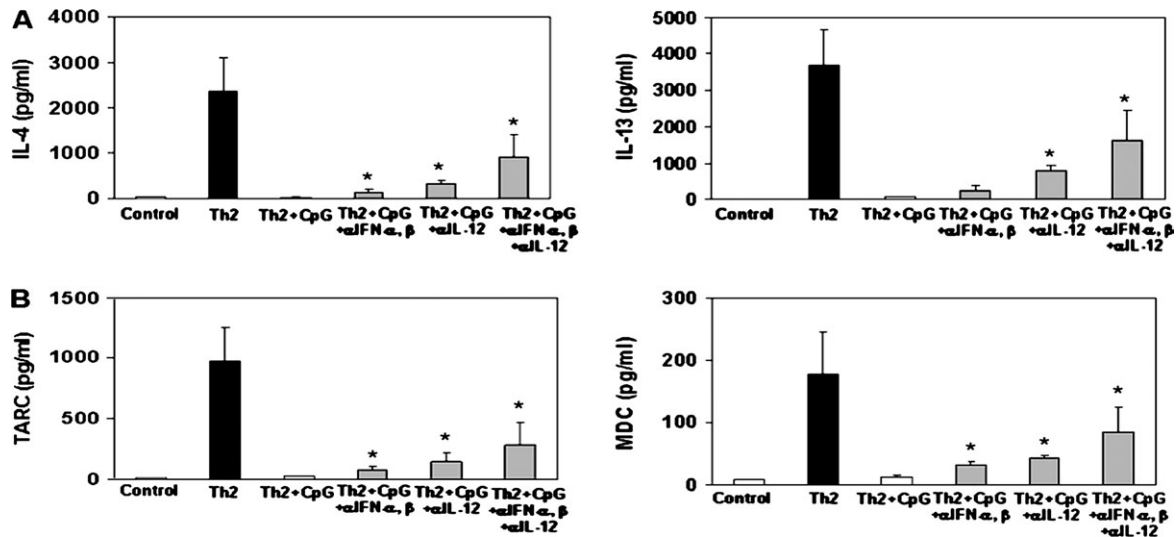


Fig. 4. IFN- α , IFN- β and IL-12 induced by CpG-ODN suppressed the production of T_H2 chemokines, TARC and MDC during T_H2 cell-mediated allergic airway inflammation. OVA-specific T_H2 cells were generated from naive CD4⁺ T cells derived from DO11.10 mice. The generated T_H2 cells (2×10^7) were i.v. injected into wild-type BALB/c mice. One day after the transfer, the mice were s.c. injected with CpG-ODN (50 μ g) and exposed to OVA protein solution (2 wt/vol %) by a nebulizer for 30 min. Anti-IFN- α mAb (100 μ g) plus anti-IFN- β mAb (250 μ g), anti-IL-12 mAb (500 μ g; C15.1 and C15.6) and anti-IFN- α mAb and anti-IFN- β mAb plus anti-IL-12 mAb were i.p. injected at 30 min before the first and the second OVA inhalations. At 6 h after the third OVA exposure, IL-4 and IL-13 cytokine (A) or TARC and MDC chemokine (B) levels in BALF were measured by ELISA. One experimental group was composed of three mice ($n = 3$). Similar results were obtained in three separate experiments and representative data are shown in the figures. Control experiment was performed for only transferred T_H2 cells without inhalation of OVA protein. Asterisk means significant difference from CpG-ODN-injected mice ($P < 0.05$).

We confirmed that *in vivo* injection of anti-TNF- α mAb did not inhibit the suppression of T_H2 cell migration induced by CpG-ODN (data not shown). Therefore, the other factors might contribute to the inhibitory effect of CpG-ODN on the airway inflammation.

We further observed that CCR4, a receptor of both TARC and MDC, mRNA expression level of the transferred T_H2 cells in mediastinal lymph nodes did not change between CpG-ODN-untreated and -treated mice (data not shown). Thus, the blockade of T_H2 cell migration might be responsible for the reduction of TARC and MDC by CpG-ODN.

Many investigators have reported that a lot of patients with bronchial asthma developed AHR to bronchoconstrictor, inflammatory cell infiltration and elevation of T_H2 cytokine levels in BALF (7, 8). Moreover, TARC and MDC are well-known T_H2 chemokines, which are up-regulated in the airway of atopic asthma patients following allergen challenge (38, 39). Therefore, it has been expected that CpG-ODN would give a novel therapeutic strategy for bronchial asthma through the action on upstream of allergic immune responses including T_H2 cell chemotaxis into lung.

CpG-ODN contains characteristic unmethylated cytosine and guanine component array, which could be found in virus and microbes. The CpG-ODN administration could dramatically change the type II-dominant immunity to type I immunity by causing type I cytokine storm. The present findings suggest that type I cytokines triggered by microbial components, CpG-ODN, play a crucial role for the regulation of T_H2 cell migration into lung, which is a critical step for the production of both T_H2 cytokines and T_H2 chemokines during T_H2 -dependent allergic airway inflammation. Thus, CpG-

ODN will become a promising immunomodulator applicable to the therapy of T_H2 -dependent allergic disorders such as asthma.

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Abbreviations

AHR	airway hyper-responsiveness
BALF	bronchoalveolar lavage fluid
CpG-ODN	CpG-oligodeoxynucleotides
DC	dendritic cell
i.p.	intra-peritoneally
i.v.	intravenously
MDC	macrophage-derived chemokine
Mch	β -methacholine chloride
OVA	ovalbumin
PAS	periodic acid-Schiff
s.c.	subcutaneously
TARC	thymus and activation-regulated chemokine
TNF	tumor necrosis factor

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