

Effects of Cytokine Milieu Secreted by BCG-treated Dendritic Cells on Allergen-Specific Th Immune Response

Bacillus Calmette-Guérin (BCG) is reported to suppress Th2 response and asthmatic reaction. Dendritic cells (DCs), the major antigen-presenting cells, infections with BCG are known to result in inducing various cytokines. Thus, DCs are likely to play a role in the effects of BCG on asthma. This study aims at investigating that cytokine milieu secreted by BCG-treated DCs directly enhances allergen-specific Th1 response and/or suppresses Th2 response in allergic asthma. DCs and CD3⁺ T cells were generated from *Dermatophagoides farinae*-sensitive asthmatics. DCs were cultured with and without BCG and subjected to flow cytometric analysis. IL-12 and IL-10 were determined from the culture supernatants. Some DCs were cocultured with T cells in the presence of *D. farinae* extracts after adding the culture supernatants from BCG-treated DCs, and IL-5 and IFN- γ were determined. BCG-treated DCs enhanced significantly the expressions of CD80, CD86, and CD40, and the productions of IL-12 and IL-10. Addition of culture supernatants from BCG-treated DCs up-regulated production of IFN- γ by T cells stimulated by DCs and *D. farinae* extracts ($p < 0.05$), but did not down-regulate production of IL-5 ($p > 0.05$). The cytokine milieu secreted by BCG-treated DCs directly enhanced allergen-specific Th1 response, although did not suppress Th2 response.

Key Words : Asthma; Cytokines; Dendritic Cells; *Mycobacterium bovis*; T-lymphocytes

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Received : 23 February 2004
Accepted : 20 May 2004

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*This paper was supported by Fund (CUHRI-U-200304) of Chonnam University Hospital Research Institute of Clinical Medicine.

INTRODUCTION

Immunoglobulin-E (IgE)-mediated allergic diseases, in particular allergic asthma, are mediated primarily by Th2 lymphocytes (1). Th1 and Th2 cells reciprocally regulate each other through the cytokines they secrete (1, 2). Association of allergic disorders with the Th2 cytokine pattern raises the possibility to design prophylactic and therapeutic modalities in a way that would promote Th1 immune response and thereby down-regulate the Th2 immune response (2, 3). Mycobacteria are among the most potent inducers of Th1 response (4, 5). We have shown that bacillus Calmette-Guérin (BCG) infection suppresses the sensitivity of airway smooth muscle, airway eosinophilic inflammation, and Th2 immune response in our animal model of allergic asthma (6, 7). Recently, we have reported in our clinical trials (8, 9) that BCG vaccination improves lung function and reduces medication use in adult asthmatics.

BCG induces *Mycobacterium*-specific Th1 immune response (5) and then may suppress allergen-specific Th2 immune response which plays an important role in allergic asthma. Additionally, it is reasonable to consider that the surrounding cytokine milieu secreted by BCG-infected dendritic cells (DCs) enhances allergen-specific Th1 immune response and suppresses allergen-specific Th2 immune response. DCs, the

major antigen-presenting cells, are host cell for mycobacteria in vivo that trigger innate and acquired immunity (10). The interaction of DCs with *Mycobacterium tuberculosis* or BCG results in direct cell maturation and activation with production of IL-12 (11, 12), which plays a major role in the induction and potentiation of IFN- γ in response to BCG. (13). *Mycobacterium tuberculosis*-infected DCs are also reported to secrete IL-18 and IFN- α , which play a significant role in enhancing IFN- γ synthesis by T cells (14). Rather, DCs infection with BCG may induce IL-10 production (11), which antagonizes the effect of IL-12 (15).

Thus, the purpose of this study is to investigate that the surrounding cytokine milieu secreted by BCG-treated DCs directly enhances allergen-specific Th1 response and/or suppresses allergen-specific Th2 response in patients with *Dermatophagoides farinae*-sensitive asthma.

MATERIALS AND METHODS

Subjects

Five patients with mild-to-moderate asthma were selected on the basis of allergy to *D. farinae*, as indicated by a positive skin prick test response (wheal size > 4 mm) and specif-

ic IgE to *D. farinae* (UniCAP >11 kU/L or Multiple Allergen Simultaneous Test-Chemiluminescent Assays [MAST-CLA] class=4). They visited the Allergy Clinic at Chonnam University Hospital to get a medical certificate for current status of their asthma before they enter the military. Their mean age and asthma duration were 19.4 and 11.2 yr, respectively. The asthmatic subjects met the definition of asthma laid out by the American Thoracic Society (16). In order to enhance the chance of being rejected they ceased taking anti-asthmatic medications for some months prior to this study. They inhaled only short-acting β_2 -agonist on demand as rescue medication. The β_2 -agonist was withheld during at least an 8-hr period to the present study. We also included four nonatopic control subjects who had no symptoms of allergy or asthma. All subjects gave their informed written consent. The study was approved by the Human Ethics Review Committee of our University Hospital.

Generation of DCs and T cells

Peripheral blood mononuclear cells (PBMCs) were isolated from freshly collected buffy coats by density gradient centrifugation using a Lymphoprep (Nycomed, Oslo, Norway). CD14⁺ cells were isolated from PBMCs using LS-MACS column (Miltenyi Biotec, Auburn, CA, U.S.A.), in which the purity of CD14⁺ cells was >95%. DCs were generated by culturing the CD14⁺ cells in T-25 culture flasks (Nunc, Roskilde, Denmark) with 50 ng/mL GM-CSF (LG Biochemical, Daejeon, Korea) and 50 ng/mL IL-4 (R & D Systems, Minneapolis, MN, U.S.A.) for 6 days at 1×10^6 cells/mL in RPMI 1640 (BioWhittaker, Walkersville, MD, U.S.A.) supplemented with 10% FBS (Gibco, Grand Island, NY, U.S.A.) and antibiotics consisting of 100 U/mL penicillin, 100 μ g/mL streptomycin, and 0.25 μ g/mL fungizone (BioWhittaker). CD3⁺ T cells were isolated by human T cell enrichment columns (R & D Systems), in which the purity of CD3⁺ cells was >90% and the T cells were stored in liquid nitrogen until culture.

BCG treatment of DCs

Mycobacterium bovis BCG (French strain 1173P2) was produced at Korean National Tuberculosis Association in the form of lyophilized powder. DCs (1×10^5) were treated for 40 hr with 1.5×10^5 colony forming units (CFUs) of BCG, because it has been found that the addition of BCG over 3×10^5 CFUs significantly influenced cell viability, whereas BCG below 1×10^5 CFUs did not induce any change of DC phenotypes in a previous experiment by Kim et al. (12). Some DCs were cultured without BCG treatment during the same time. Some supernatants were stored at -70°C for cytokine assays of IL-12 and IL-10 and others were added to the following DC-T cell cocultures. DCs were subjected to the following flow cytometric analysis.

Flow cytometric analysis

For immunophenotyping, DCs were resuspended in RPMI 1640 (BioWhittaker) supplemented with 10% FBS (Gibco) and incubated for 30 min at 4°C with one of the following mouse monoclonal antibodies: phycoerythrin (PE)-conjugated anti-HLA-DR; PE-conjugated anti-CD86; PE-conjugated anti-CD54; PE-conjugated anti-CD11c; fluorescein isothiocyanate (FITC)-conjugated anti-CD80; FITC-conjugated anti-CD83; FITC-conjugated anti-CD40, and FITC-conjugated anti-CD1a (PharMingen, San Diego, CA). Mouse immunoglobulin G1-PE/FITC isotype control (PharMingen) was used as a control. The fluorescence was measured on a FACS Caliber cell sorter (Becton Dickinson, San Jose, CA, U.S.A.) and analyzed with CellQuest software (Becton Dickinson).

T-DC cell cocultures

CD3⁺ T cells were thawed on the day of use. T cells (1×10^5) were cocultured with irradiated (30 Gy) autologous DCs at a ratio 10:1 for 6 days in the presence of crude protein extracts of *D. farinae* (10 μ g/mL, Yonsei University College of Medicine, Seoul, Korea). Control cultures were T cell alone or T-DC cell cocultures. In order to investigate the effect of the surrounding cytokine milieu secreted by BCG-treated DCs on *D. farinae*-specific Th1 and Th2 immune responses, 100 μ L per well of the culture supernatants from BCG-treated DCs was added at the beginning of T-DC cell cocultures in the presence of *D. farinae* extracts. Supernatants were collected from the cultures on day 5 and stored at -70°C for cytokine assays of IL-5 and IFN- γ .

Proliferation assays

T cell proliferation during the last 18 hr of 6-day cultures was quantified by [³H] thymidine uptake of cells incubated with 1 μ Ci of [methyl-³H] thymidine (Amersham Pharmacia Biotech, Buckinghamshire, U.K.). The cells were harvested onto paper filters using a Titertek Cell Harvester 530 (Flow Laboratories, Irvine, U.K.) and the radioactivity was measured using a Beckman LS 6500 multipurpose scintillation counter (Beckman Instruments, Fullerton, CA, U.S.A.). Results are presented as mean counts per minute (cpm) of triplicated cultures.

Cytokine assays

The concentrations of IL-12, IL-10 (R & D Systems), IL-5, and IFN- γ (Endogen, Woburn, MA, U.S.A.) were determined by using commercially available enzyme-linked immunosorbent assay (ELISA) kits. The standard curves were generated by the standards of known cytokine content provided. Sensitivities were 0.5 pg/mL for IL-12, 3.9 pg/mL for IL-10, 2 pg/mL for IL-5, and 2 pg/mL for IFN- γ .

Statistical analysis

Data were expressed as mean \pm SEM. The differences between the two groups and within the same group were analyzed with the Mann-Whitney *U* test and the Wilcoxon's signed-rank test, respectively. A $p < 0.05$ value was considered statistically significant.

RESULTS

DCs activation by BCG and IL-12 and IL-10 productions by BCG-treated DCs

Activated DCs are known to express high levels of co-stimulatory molecule and secrete inflammatory cytokines (17). We tested the effect of BCG on DC activation. DCs from asthmatics were cultured with or without BCG, and the cell-surface marker expression was examined by flow cytometry after staining with various antibodies. DCs cultured with BCG showed a significant increase in expression of the co-stimulatory molecules CD40 ($p < 0.05$), CD80 ($p < 0.05$), and CD86 ($p < 0.05$) compared with those cultured without BCG. The expression of the MHC class II molecule HLA-DR ($p = 0.08$) and the DC-specific marker CD83 ($p = 0.08$) tended to increase in BCG-treated DCs compared with untreated DCs (Fig. 1). These data suggests that BCG treatment is sufficient to induce DCs to mature into an 'activated' phenotype.

In parallel, we tested the effect of BCG treatment on IL-12 and IL-10 cytokine productions by DCs from asthmatics.

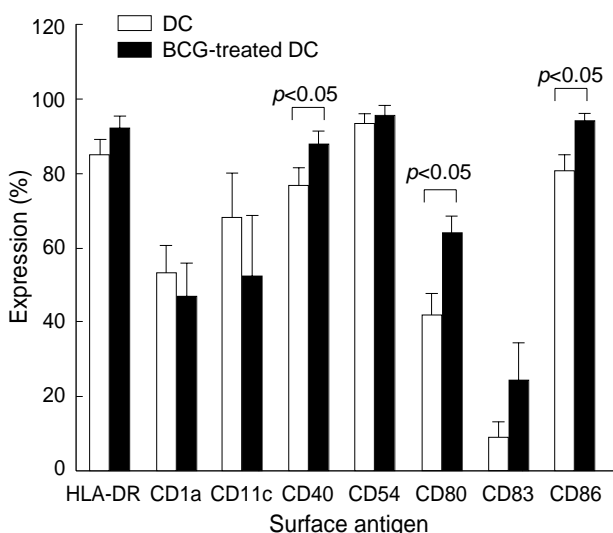


Fig. 1. Comparisons of surface antigen expression between untreated DCs and BCG-treated DCs from asthmatics. Expression represents the percentage of positive cells on FACS analysis after staining with the corresponding antibodies. Data are expressed mean \pm SEM. Statistical significance was determined by using the Wilcoxon's signed-rank test.

The quantities of IL-12 ($p < 0.05$) and IL-10 ($p < 0.05$) were significantly higher in the culture supernatant from BCG-treated DCs than in that from untreated DCs (Fig. 2).

T cell proliferation and IL-5 and IFN- γ productions by T cells stimulated by DCs and *D. farinae* extracts

In asthmatics, T cell proliferations of T-DC cell cocultures were significantly increased in the presence of *D. farinae* extracts compared with the absence of *D. farinae* extracts ($p < 0.05$, Fig. 3). In contrast, in nonatopic controls, T cell proliferations of T-DC cell cocultures in the presence of *D. farinae* extracts were similar to those in the absence of *D. farinae* extracts ($p > 0.05$, data not shown). This indicates that T cell proliferation from asthmatics is induced by autologous DCs in a pattern of *D. farinae*-specific response.

In parallel, we analyzed the cytokine pattern in the supernatants from T-DC cell cocultures in the presence of *D. farinae* extracts. IL-5 concentration was higher in asthmatics than in nonatopic controls (325.2 ± 87.2 pg/mL vs. 181.6 ± 110.2 pg/mL, $p = 0.33$), although not significant. However, IFN- γ concentration in asthmatics was similar to that in nonatopic controls ($1,605.1 \pm 423.0$ pg/mL vs. $1,509.4 \pm 574.1$ pg/mL).

Up-regulation of *D. farinae*-specific IFN- γ production after addition of culture supernatants from BCG-treated DCs to T-DC cell cocultures

In order to test the effects of the cytokine milieu secreted by BCG-treated DCs on *D. farinae*-specific Th1 and Th2 response in asthmatics, we analyzed the cytokine pattern in the supernatants collected from T-DC cell cocultures in the

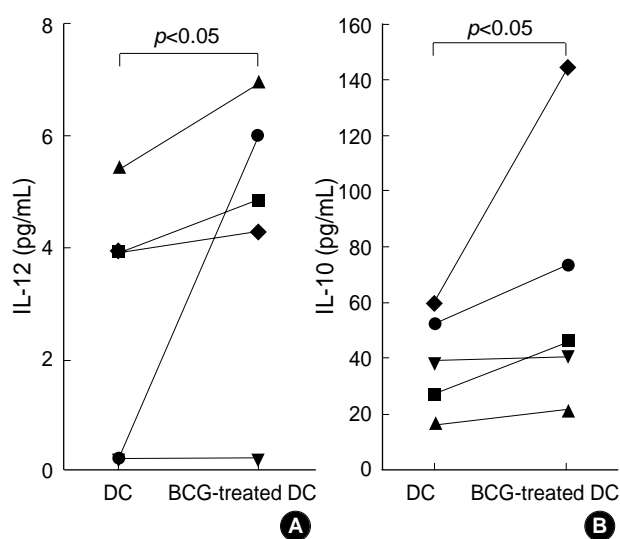


Fig. 2. Comparisons of IL-12 (A) and IL-10 (B) productions between untreated DCs and BCG-treated DCs from asthmatics. Statistical significance was determined by using the Wilcoxon's signed-rank test.

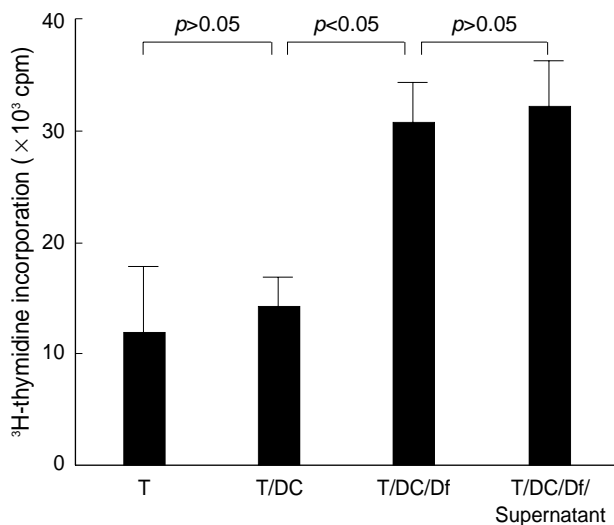


Fig. 3. Comparisons of T cell proliferations among T cell alone (T), T-DC coculture (T/DC), T-DC coculture in the presence of *D. fariniae* extracts (T/DC/Df), and T-DC coculture in the presence of *D. fariniae* extracts after adding the culture supernatant from BCG-treated DC (T/DC/Df/Supernatant) in *D. fariniae*-sensitive asthmatics. Statistical significance was determined by using the Wilcoxon's signed-rank test.

presence of *D. fariniae* extracts after adding the culture supernatants from BCG-treated DCs. The addition of the culture supernatant from BCG-treated DCs significantly up-regulated the production of IFN- γ by T cells, with IFN- γ levels being $2,538.1 \pm 461.8$ pg/mL and $1,605.1 \pm 423.0$ pg/mL in the presence or absence of the culture supernatant from BCG-treated DC, respectively ($p < 0.05$). On the other hand, the addition of the culture supernatant from BCG-treated DCs did not down-regulate the production of IL-5, with IL-5 levels being 445.1 ± 160.7 pg/mL and 325.2 ± 87.2 pg/mL in the presence or absence of the culture supernatant from BCG-treated DCs, respectively (Fig. 4). Additionally, the addition of the culture supernatant from BCG-treated DCs did not affect T cell proliferation of T-DC cell cocultures in the presence of *D. fariniae* extracts (Fig. 3).

DISCUSSION

In the present study, the production of IFN- γ from T cells stimulated by DCs and *D. fariniae* extracts was increased following the addition of the culture supernatant obtained from BCG-treated DCs in *D. fariniae*-sensitive asthmatics, while IL-5 production was not decreased. This finding suggested that the surrounding cytokine milieu secreted by BCG-treated DCs directly enhanced allergen-specific Th1 response, although did not suppress allergen-specific Th2 response.

The present study showed that BCG treatment induced the maturation and activation of DCs and the production of IL-12 from DCs. The production of IL-12, which is thought

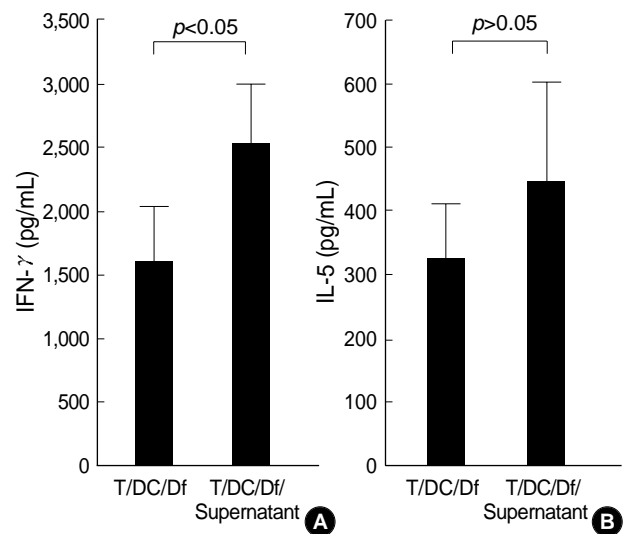


Fig. 4. Comparisons of IFN- γ (A) and IL-5 (B) productions by T cells from *D. fariniae*-sensitive asthmatics between T-DC coculture in the presence of *D. fariniae* extracts (T/DC/Df) and T-DC coculture in the presence of *D. fariniae* extracts after adding the culture supernatant from BCG-treated DC (T/DC/Df/Supernatant). Statistical significance was determined by using the Wilcoxon's signed-rank test.

to be a prime initiator of Th1 response (18), may contribute mainly to the direct enhancement of allergen-specific Th1 response in our study. Additionally, DC infection with *Mycobacterium tuberculosis* or BCG results in inducing another cytokines, such as IL-1 (11), IL-10 (11), IL-18 (14), TNF- α (11, 12), and IFN- α (14). IL-18 and IFN- α have been also reported to have a significant role in enhancing Th1 response by inducing T cell IFN- γ production and the expression of Th1-type cytokine receptors (14, 19). IL-18 and IFN- α are likely to contribute to the direct enhancement of allergen-specific Th1 response, although they were not determined in our study. We also found that the production of IL-10 by BCG-treated DCs was enhanced. Because IL-10 may inhibit T cell IFN- γ production by suppressing IL-12 synthesis (15), the enhancing effect of IL-12, IL-18, and IFN- α on allergen-specific Th1 response could be down-regulated by IL-10. However, this is not the case in our study, suggesting that a dynamic interplay among the various cytokines is responsible for net IFN- γ production.

It is well known that Th1 and Th2 cells reciprocally regulate each other through the cytokines they secrete (1, 2). It is possible that the allergen-specific Th1 cytokine enhanced by the cytokine milieu secreted by BCG-treated DCs may suppress the production of allergen-specific Th2 cytokine, which is instrumental in initiating and sustaining the allergic inflammation in asthma. IL-4 down-modulates the expression of the IL-12R β chain on T cells. This down-modulation leads to the generation of Th2 cells, since they are no longer responsive to IL-12-mediated signaling (20). However, the presence of IFN- γ may inhibit this IL-4-induced

effect. Therefore, allergen-specific Th2 memory cells may produce Th1 cytokines when activated in the presence of IL-12, a potent IFN- γ -inducing protein (21), before the more malleable resting memory T cells differentiate into end-stage effector cells (22) in which Th2 effector cells lose IL-12 receptor function (23). However, the enhanced allergen-specific Th1 response failed to do that in this in vitro study. One of the possible explanations can be that the polarized memory and/or end-stage effector allergen-specific Th2 cells might have lost the expression of functional IL-12 receptors. This can be supported by a recent study (24) showing that the cytokine profile of allergen-specific Th2 cells from atopic individuals is not inhibited when the allergen is presented by DCs in the presence of exogenous recombinant human IL-12. Another can be that the allergen-specific Th1 cytokine enhanced directly by the cytokine milieu secreted by BCG-treated DCs is not sufficient to suppress the allergen-specific Th2 response. Indeed, *Mycobacterium* species induce potent *Mycobacterium*-specific Th1 response (4, 5), which may also contribute to the inhibition of allergen-specific Th2 responses. Therefore, it is more probable that the administration of BCG in vivo suppresses the allergen-specific Th2 response, via both the allergen-specific Th1 response by the cytokine milieu secreted by BCG-infected DC and the *Mycobacterium*-specific Th1 response.

Recently, Arkwright and David (25) have reported that intradermal administration of a killed *Mycobacterium vaccae* suspension is associated with improvement in the severity of the atopic dermatitis in children. We have also reported in two successive clinical trials (8, 9) that single or repeated BCG vaccinations improve lung functions in asthmatics, being indicative of the therapeutic effects of BCG on asthma. Based on the data from the present in vitro study, it may be inferred that IFN- γ production from T cells enhanced by the surrounding cytokine milieu produced by BCG-infected DC contributes to the increased lung function. It has been reported that IFN- γ can induce nitric oxide production (26), which causes dilatation of the airway smooth muscle in patients with allergic asthma and reverses bronchoconstriction (27, 28) and also reduces airway hyperresponsiveness (28, 29). We have also shown that the change in IFN- γ produced by peripheral blood T cells has a positive relationship with the change in morning peak expiratory flow rate in asthmatics (30). However, as mentioned above, it is possible that strong Th1 response generated in vivo by both the cytokine milieu secreted by BCG-infected DCs and the *Mycobacterium*-specific T cell response may suppress Th2 response and then improve lung function. Besides, IL-10 from BCG-treated DCs may also contribute to the therapeutic effects of BCG on asthma. Although IL-10 is known to inhibit Th1 response (15), IL-10 therapy might alleviate allergic inflammation by inhibiting different steps involved in the accumulation of eosinophils into target tissues and be useful in controlling asthma (31). Recently, Zuany-Amorim et al. (32) have demon-

strated that regulatory T cells generated by mycobacteria treatment may inhibit the allergic inflammation, suggesting that mechanism other than simple changes in the balance between Th1 and Th2 response may be responsible for the effect of BCG treatment on asthma. Because IL-10 production by DCs has been reported to develop the regulatory T cells (33), IL-10 from BCG-treated DC might induce the regulatory T cells in the present study, for which a further investigation is needed.

In the present study, although the concentration of IL-5 in supernatants from T-DC cell cocultures stimulated by *D. fariniae* extracts was higher in asthmatics than in nonatopics, the significant Th2 cytokine profile was not observed in asthmatics. It is possible that T cell stimulation may not sufficient to induce the significant Th2 profile. Indeed, no more stimulation was used in our study, while T cells were restimulated twice more with newly generated allergen-pulsed DCs in a study of Bellinghausen et al. (34) showing that the significant Th2 cytokine profile was induced in T helper cells from atopics after stimulation with autologous allergen-pulsed DCs.

An endotoxin or unknown mitogen-like factor contamination of the *D. fariniae* extracts or the culture supernatant from BCG-treated DCs is likely to affect the *D. fariniae*-specific Th1 and Th2 immune response, although the factors were not determined in the present study. However, our finding that T cell proliferation of T-DC cell cocultures did not differ according to the presence or absence of *D. fariniae* extracts in nonatopic controls might indicate that the factors were unlikely to be contained in the *D. fariniae* extracts. Also, it was unlikely that the factors were contained in the culture supernatant based on our observation that the addition of the culture supernatant from BCG-treated DCs did not affect T cell proliferation of T-DC cell cocultures in the presence of *D. fariniae* extracts, as shown in Fig. 3. Additionally, the possible reason for the observation might be the counterbalance effect of IL-12 and IL-10 in the culture supernatant on allergen-specific T cell proliferation. It has been demonstrated that IL-12 enhances allergen-specific T cell proliferation (21) but IL-10 inhibits the T cell proliferation (35).

In summary, the surrounding cytokine milieu secreted by BCG-treated DCs may directly enhance allergen-specific Th1 immune response in allergic asthma, which may be one of the various mechanisms of beneficial effects of BCG on asthma. Furthermore, the present study may provide a potential basis for an immunomodulation therapy using BCG-treated DC as a new treatment of asthma.

ACKNOWLEDGEMENTS

We are indebted to Young-A Koh for her expert technical assistance.

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