

***Mycobacterium bovis* Bacillus Calmette–Guérin suppresses inflammatory T_H2 responses by inducing functional alteration of TSLP-activated dendritic cells**

Takashi Yokoi¹, Ryuichi Amakawa¹, Tsutomu Tanijiri¹, Hiroyuki Sugimoto¹, Yoshitaro Torii¹, Hideki Amuro¹, Yonsu Son¹, Kenichirou Tajima¹, Yong-Jun Liu², Tomoki Ito¹ and Shirou Fukuhara¹

¹First Department of Internal Medicine, Kansai Medical University, Osaka, Japan

²Department of Immunology, Center of Cancer Immunology Research, University of Texas MD Anderson Cancer Center, Houston, TX, USA

Keywords: allergy, atopic dermatitis, asthma, IFN- γ , IL-10

Abstract

Allergic diseases such as atopic dermatitis and asthma develop as a consequence of dysregulated T_H2 responses. Recently, it has been demonstrated that interaction between dendritic cells (DCs) and thymic stromal lymphopoietin (TSLP), an IL-7-like cytokine, is essential for evoking T_H2 responses in allergy. In this study, we investigated whether *Mycobacterium bovis* Bacillus Calmette–Guérin (BCG), a strong T_H1 response-inducing adjuvant, can alter the function of DCs activated by TSLP (TSLP-DCs). We demonstrated that BCG redirects TSLP-DCs away from inducing inflammatory T_H2 cells that produce IL-4, IL-5, IL-13 and tumor necrosis factor (TNF)- α and toward regulatory T_H1 cells that produce IFN- γ and IL-10. We also demonstrated that this functional alteration of TSLP-DCs by BCG depended on both production of IL-12 from DCs and down-regulation of OX40 ligand, a member of the TNF family, on DCs. These findings suggest that BCG might be a useful adjuvant for the treatment of allergic diseases that are triggered by TSLP.

Introduction

Allergic inflammation is the result of a complex immunological cascade leading to the dysregulated production of T_H2 cell-derived cytokines such as IL-4, IL-5 and IL-13 (1–3), which in turn triggers IgE production, eosinophilia and mucus production (4–6). It has recently been proposed that thymic stromal lymphopoietin (TSLP), an IL-7-like cytokine, and myeloid dendritic cells (DCs) represent the early molecular and cellular components that trigger allergic diseases such as atopic dermatitis and asthma (7, 8). TSLP is expressed by keratinocytes of patients with atopic dermatitis, and TSLP expression is associated with Langerhans cell migration and activation *in situ* (7). Furthermore, TSLP expression is increased in airways of patients with asthma and correlated with the T_H2-attracting chemokines and disease severity (9). TSLP strongly activates human CD11c⁺ myeloid DCs without inducing T_H1-polarizing cytokines IL-12, IL-23 and IL-27 (10). And the TSLP-activated DCs instruct naive CD4⁺ T cells to differentiate into T_H2 cells. The T_H2 cells induced by TSLP-activated DCs are distinct from the classical T_H2 cells, because they produce large amounts of tumor necrosis factor (TNF)- α in addition to T_H2 cytokines IL-4, IL-5 and IL-13, but

little or no IL-10; thus, it is proposed that these unique T_H2 cells be called inflammatory T_H2 cells (7, 10). In addition to the differentiation of inflammatory T_H2 cells, TSLP-activated DCs induce the expansion of human T_H2 memory cells, contributing to the maintenance and augmentation of allergic inflammation (11). Moreover, it has recently been shown that TSLP has also a critical role in allergic inflammation in mice (12–14).

It has recently been demonstrated that OX40 ligand (OX40L) is preferentially expressed by TSLP-activated DCs and that the OX40L is a DC-derived positive signal that induces inflammatory TNF⁺IL-10[−] T_H2 cells in the absence of T_H1-polarizing cytokines (10). These findings suggest that TSLP from epithelial cells or keratinocytes induced by allergic insults activates DCs and that the activated DCs prime allergen-specific naive T cells to differentiate into inflammatory T_H2 cells via up-regulation of OX40L on DCs, which may eventually cause the induction of allergic inflammation. Furthermore, it has recently been demonstrated that OX40L plays an important role in TSLP-induced allergic inflammation in mice and non-human primates (15) as well. Therefore,

2 BCG inhibits T_H2 response induced by TSLP

interference or alteration of the function of TSLP-activated DCs that induce inflammatory T_H2 cells may constitute a rational treatment strategy for allergic diseases.

The *Mycobacterium bovis* Bacillus Calmette–Guérin (BCG) is a vaccine strain of tuberculosis that is almost non-pathogenic, yet retains the immunological properties of tuberculosis (16). And the current consensus is that BCG serves as a strong immune adjuvant via maturation of immature DCs. Infection of immature DCs with BCG up-regulates MHC class I, CD40, CD80, CD83 and CD86 (17, 18). And the BCG-activated DCs produce inflammatory cytokines such as TNF- α , IL-1 β and IL-12 (17, 18). Moreover, we previously reported that human CD11c⁺ DCs infected with BCG instruct naive CD4⁺ T cells to differentiate into IFN- γ -producing T_H1 cells (19). In the field of clinical oncology, BCG has been widely and safely used as an effective adjuvant for various cancers, including transitional carcinoma of the bladder (20–22). Based on these findings, we hypothesized that BCG could function as an immune adjuvant to alter the ability of TSLP-activated DCs that induce the pathogenic inflammatory T_H2 cell responses.

In this study, we found that BCG induced IL-12 production from DCs and inhibited up-regulation of OX40L on DCs even in the presence of TSLP. As the result of these events, BCG converted the inflammatory T_H2 cell response-inducing capacity of TSLP-activated DCs into a regulatory T_H1 -cell response-inducing one.

Methods

Isolation and culture of blood DCs

CD11c⁺ DCs were isolated from buffy coat of blood from healthy adult volunteers (Japan Red Cross Society, Osaka Blood Center), as described (23). Local Ethical Committee approval was received for the studies (Ethical Committee of Kansai Medical university: approval no. 0405) and the informed consent of all participating subjects was obtained. Briefly, the DC-enriched population (CD4⁺/CD3[−]/CD14[−] cells) was obtained from PBMC by negative and subsequent positive immunoselections (23). The CD11c⁺/lin[−]/CD4⁺ cells (CD11c⁺ DCs) were sorted by a FACS Aria® (BD Biosciences) by using allophycocyanin (APC)-labeled anti-CD11c (B-ly6), a mixture of FITC-labeled mAbs against lineage markers, CD3 (M2AB: Exalpha), CD14 (M5E2: BD Biosciences), CD15 (M5E2: BD Biosciences), CD16 (J5511: Exalpha), CD19 (HIB19: BD Biosciences) and CD56 (NCAM16.2: BD Biosciences), and APC-Cy7-labeled CD4 (RPA-T4: BD Biosciences) to reach >99% purity. CD11c⁺ DCs were cultured in Yssel's medium (Gemini Bio-Products) containing 2% human AB serum. Cells were seeded in flat-bottomed 96-well plates in the presence of 15 ng ml^{−1} of TSLP (R&D System) and/or BCG (1 MOI: EMD Biosciences) at 5 × 10⁴ cells in 200 μ l of medium per well for 24 h. In some experiments, after 24 h culture with TSLP, the DCs were washed extensively and then re-cultured with or without BCG for further 24 h.

Analyses of DCs

To analyze the expression of co-stimulatory molecules, the cultured DCs were stained with FITC-labeled anti-CD40

(5C3: BD Biosciences), CD80 (BB-1: Ancell) or CD86 (2331: BD Biosciences) and then analyzed by a FACScan® (BD Biosciences). The production of cytokines in the culture supernatants was determined by ELISA 24 h later (kits for IL-12 p40 + p70 and TNF- α were purchased from Endogen).

Analyses of OX40L expression

Reverse transcription (RT) reactions were performed with SuperScript RT II. The DNA resulting from each RT reaction was then subjected to PCR. The temperature profiles of the PCR were as follows: an initial denaturation step at 94°C for 5 min; followed by 36 cycles at 94°C for 1 min, 55°C for 1 min and 72°C for 30 s; and then a final elongation step at 72°C for 7 min. The sequences of primers were as follows: (for OX40L) forward 5'-CCCAGATTGTGAAGATGGAA-3' and reverse 5'-GCCTGGTTTTAGATATTGCC-3', and (for β -actin) forward 5'-CTGGAACGGTGAAGGTGACA-3' and reverse 5'-AAGGGACTTCCTGTAACAATGCA-3'. To evaluate surface OX40L expression, CD11c⁺ DCs freshly isolated or after being cultured at different time points were stained with PE-labeled anti-OX40L mAb (Ancell) or with an isotype-matched control mAb and then were analyzed by a FACScan® (BD Biosciences).

DC–T cell co-culture

CD4⁺CD45RA⁺ naive T cells (purity >99%) were isolated by using CD4⁺ T cell Isolation Kit II (Miltenyi Biotec) followed by cell sorting (as a CD4⁺CD45RA⁺CD45RO[−] fraction). After 24 h of culture under different conditions, CD11c⁺ DCs were washed twice (by centrifugation at 800 r.p.m. for 8 min) to remove any cytokines and BCG and then co-cultured with 2 × 10⁴ freshly purified allogenic naive CD4⁺ T cells (DC to T ratio, 1:5) in round-bottomed 96-well culture plates for 7 days. In some experiments, CD11c⁺ DCs pre-cultured with TSLP were co-cultured with naive CD4⁺ T cells in the presence or absence of BCG for 7 days. Yssel's medium containing 2% human AB serum was used for the T cell cultures. We used the following reagents for culture conditions: anti-OX40L mAb (ik-5: 50 μ g ml^{−1}), anti-IL-12 antibody (AF-219-NA, R&D Systems: 1 μ g ml^{−1}) and recombinant soluble OX40L (200 ng ml^{−1}, R&D Systems). Mouse IgG2a and Goat IgG (R&D Systems) were used as controls.

Analyses of T cell cytokine production

After 7 days of DC–T cell co-culture, the primed CD4⁺ T cells were collected and washed. For detection of cytokine production in the culture supernatants, the T cells were re-stimulated with plate-bound anti-CD3 (OKT3, 5 μ g ml^{−1}) and soluble anti-CD28 (1 μ g ml^{−1}) at a concentration of 10⁶ cells ml^{−1} for 24 h. The levels of IL-4, IL-5, IL-10, IL-13, TNF- α and IFN- γ were measured by ELISA (all kits from R&D Systems). For intracellular cytokine production, the primed CD4⁺ T cells were re-stimulated with 50 ng ml^{−1} of phorbol myristate acetate plus 2 μ g ml^{−1} of ionomycin for 6 h. Brefeldin A (10 μ g ml^{−1}) was added during the last 2 h. The cells were stained with the combination of PE-labeled mAbs to IL-4, IL-13 or TNF- α and FITC-labeled anti-IFN- γ (all from BD Biosciences) using FIX and PERM kit (CALTAG).

Statistical analysis

The paired Student's *t*-test was used for statistical analysis with a StatView statistical program (Abacus Concepts). Differences were considered significant when tied *P*-values were <0.05 .

Results

BCG strongly activates immature myeloid DCs

To evaluate the effects of BCG on human $CD11c^+$ DC activation, we first analyzed the ability of BCG to stimulate DCs to express co-stimulatory molecules and to secrete cytokines. TSLP strongly activated DCs to express surface CD40, CD80 and CD86 (Fig. 1A), in accord with previous report (10). On the other hand, BCG alone or the combination of TSLP plus BCG comparatively induced the maturation of

DCs. In agreement with the previous study (7), TSLP showed a poor ability to induce DCs to secrete T_H1 -inducing cytokine, IL-12 (Fig. 1B) and $TNF-\alpha$ (data not shown). By contrast, we found that BCG induced DCs to produce large amount of IL-12, even in the presence of TSLP.

BCG inhibits up-regulation of OX40L on DCs induced by TSLP

TSLP has recently been reported to induce OX40L on DCs. OX40L plays a central role in the induction of inflammatory T_H2 responses (10). Therefore, we next investigated whether BCG represses OX40L expression on DCs activated by TSLP. As shown in Fig. 2(A), TSLP activated DCs to express OX40L, and the expression level was increased until 72 h, as was previously reported (10). However, the addition of BCG suppressed the up-regulation of OX40L induced by

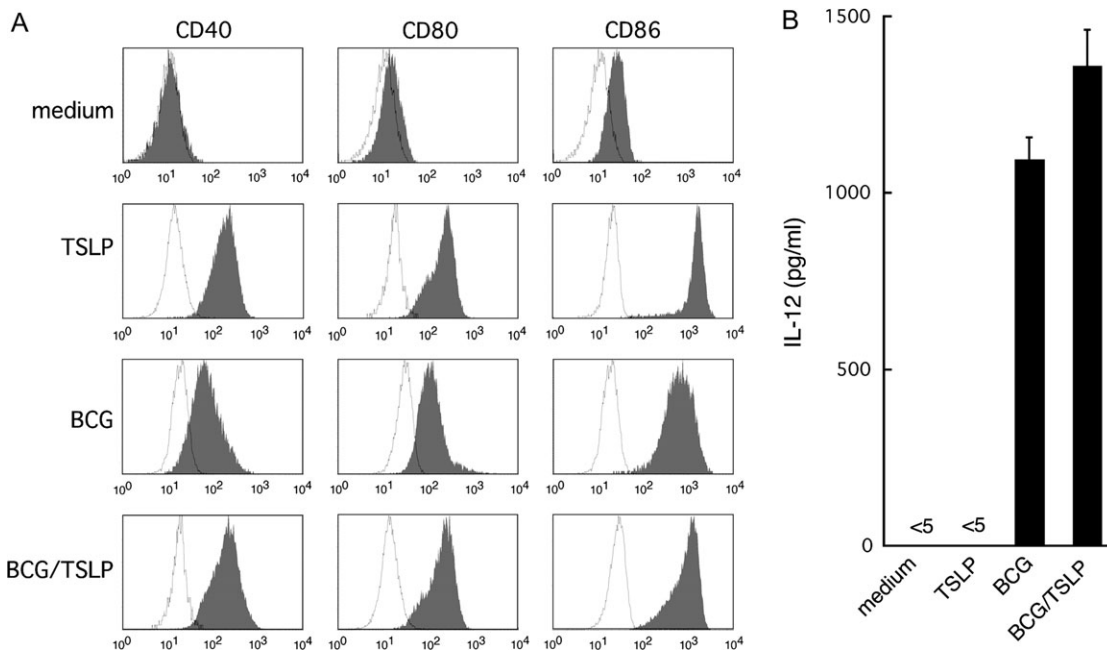


Fig. 1. BCG activates immature myeloid DCs. DCs were cultured with the indicated stimuli for 24 h. (A) DCs were stained with FITC-conjugated anti-human CD40, CD80, CD86 and isotype control. The staining profiles of anti-CD40, CD80, CD86 mAbs and isotype-matched control are shaded and open areas, respectively. (B) Culture supernatants were collected and the cytokine levels were analyzed by ELISA. Data are means $\pm$ SEM of three independent experiments.

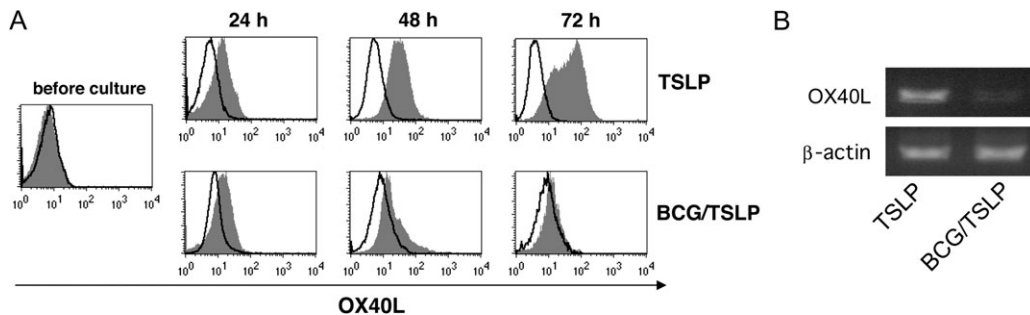


Fig. 2. Inhibition of OX40L expression on TSLP-DCs by BCG. Under culture with different stimuli, OX40L expression on activated DCs was monitored at different time points by flow cytometry (A) and analyzed at 72 h by RT-PCR (B). The staining profiles of anti-OX40L mAb and isotype-matched control are shaded and open areas, respectively. Data are representative of three independent experiments.

4 BCG inhibits T_H2 response induced by TSLP

TSLP during these 72 h. This result was confirmed at the mRNA level by RT-PCR (Fig. 2B).

BCG treatment on TSLP-DCs redirects from inflammatory T_H2 to regulatory T_H1 responses through IL-12 induction and OX40L inhibition

Because BCG has been implicated in triggering T_H1 responses by affecting DCs (19), we investigated whether BCG can counteract the role of TSLP in the induction of the inflammatory T_H2 responses that trigger the immune cascade of allergy. Naive $CD4^+$ T cells were cultured for 7 days with allogenic DCs pre-treated with TSLP alone (TSLP-DCs) or TSLP plus BCG (BCG/TSLP-DCs), and the cytokine production by the primed $CD4^+$ T cells was then examined using an intracellular cytokine staining method (Fig. 3A) and ELISA analysis of the culture supernatants following re-stimulation with anti-CD3 and anti-CD28 mAbs (Fig. 3B). TSLP-DCs induced naive $CD4^+$ T cells to differentiate into $IL-10^-$ inflammatory T_H2 cells, as previously shown (7), whereas BCG/TSLP-DCs increased the frequency of IFN- γ -producing T cells (Fig. 3A). Notably, some of these IFN- γ -producing T cells concomitantly expressed IL-10, suggesting so-called $IL-10^+$ IFN- γ^+ regulatory T_H1 cells (10). In ELISA analyses, $CD4^+$ T cells primed by TSLP-DCs produced large amounts of IL-4, IL-5, IL-13 and TNF- α but low amounts of IL-10 and IFN- γ . Meanwhile, BCG/TSLP-DCs significantly decreased the production of IL-4, IL-5 IL-13 and TNF- α and concomitantly increased the production of IL-10 and IFN- γ by T cells (Fig. 3B). The next question was whether these $IL-10^+$ IFN- γ^+ T_H cells have the suppressive function. Both $CD4^+CD45RO^-CD25^-$ T cells and inflammatory T_H2 cells induced by TSLP-DCs underwent strong proliferation in responses to the allostimulation. On the other hand, $IL-10^+$ IFN- γ^+ T_H cells induced by BCG/TSLP-DCs were relatively anergic and have the ability to suppress bystander $CD4^+$ T cell proliferation (Fig. 4). And the regulatory function of $IL-10^+$ IFN- γ^+ T_H cells (regulatory T_H1 cells) induced by BCG/TSLP-DCs was partially inhibited by addition of anti-IL-10 antibodies. These findings suggest that BCG redirect TSLP-DCs function from inducing inflammatory T_H2 cells to functional regulatory T_H1 cells.

Because BCG has been demonstrated to be a strong inducer of IL-12 from DCs (Fig. 1B) and BCG suppressed the up-regulation of OX40L expression on TSLP-DCs (Fig. 2), we hypothesized that the BCG effect of converting the inflammatory T_H2 cell responses mediated by TSLP-DCs into regulatory T_H1 responses requires both T_H1 -inducing cytokine IL-12 and the repression of inflammatory T_H2 -inducing molecule OX40L expression. To test this hypothesis, we added neutralizing anti-IL-12 antibodies and/or soluble recombinant OX40L (rOX40L) to the co-culture of $CD4^+$ T cells and BCG/TSLP-DCs. We found that addition of anti-IL-12 antibodies inhibited the generation of IFN- γ -producing T_H1 cells but did not promote the generation of T_H2 cells producing IL-4, IL-5 and IL-13 (Fig. 3A and B). Moreover, anti-IL-12 antibodies decreased the frequency of the $IL-10$ /IFN- γ double-producing T_H cells (Fig. 3A).

When we added both rOX40L and anti-IL-12 antibodies in DCs-T cell co-culture, we found that T_H2 cells producing IL-4, IL-5 and IL-13 were restored and the generations of $IL-10$ -producing T_H cells (both $IL-10$ single producers and $IL-10$ /IFN- γ double producers) were almost completely inhibited (Fig. 3A

and B). These results suggest that the ability of BCG to redirect from inflammatory T_H2 cell differentiation induced by TSLP-DCs to regulatory T_H1 cell differentiation depends on both IL-12 production from DCs and repression of OX40L on DCs.

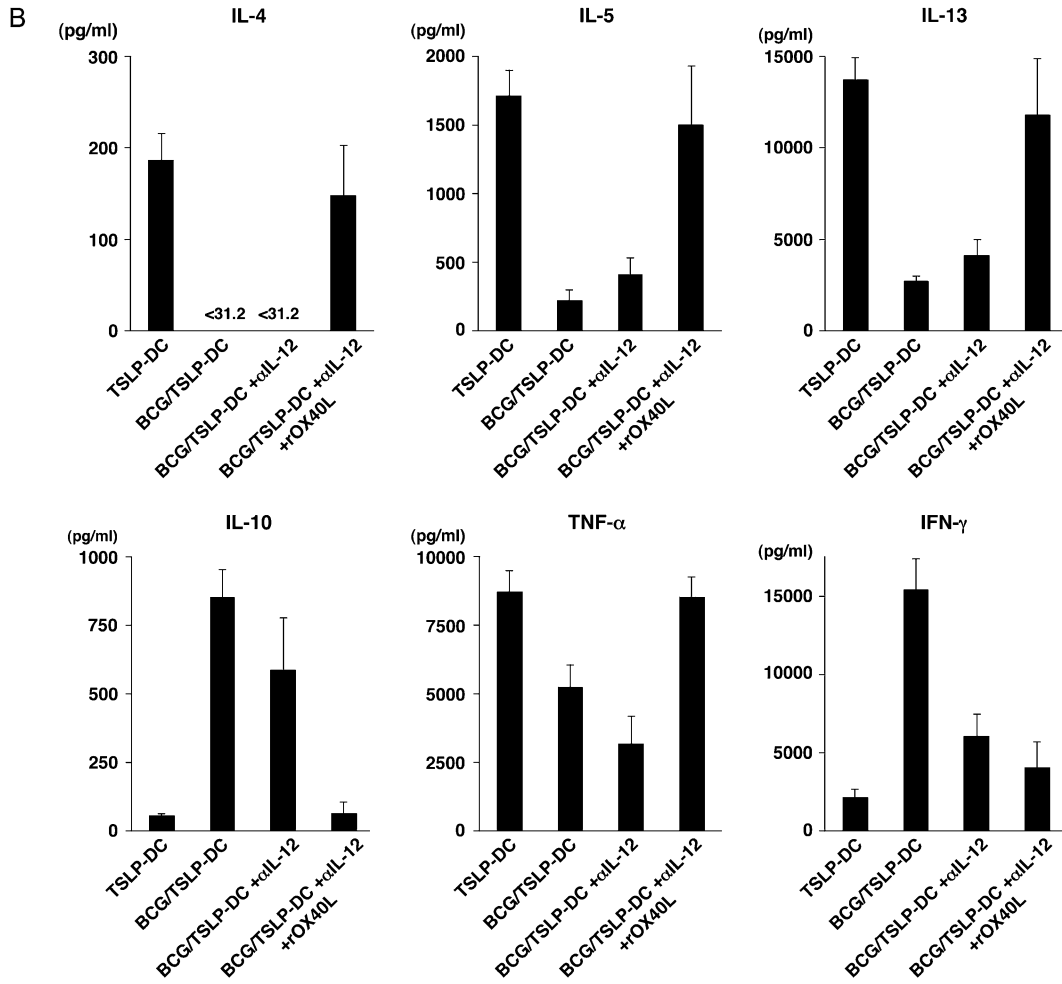
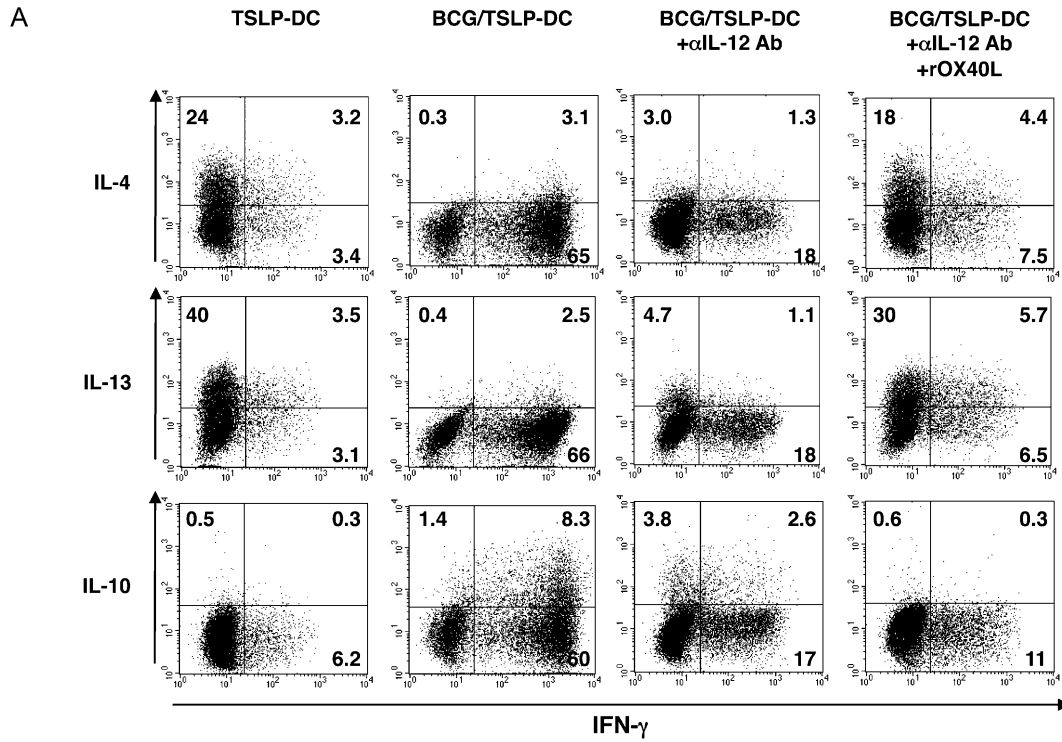
Addition of BCG afterward into DC-T cell co-culture also promotes T_H1 differentiation

To further assess whether BCG also has the ability to counteract the function of DCs that are already activated and matured by TSLP, DCs were pre-treated with TSLP first for 24 h and then BCG was added afterward into the culture of naive $CD4^+$ T cells and TSLP-DCs. The addition of BCG into the DC-T cell co-culture also significantly inhibited the generation of T_H2 cells producing IL-4, IL-5 and IL-13, and concomitantly promoted the generation of IFN- γ -producing T_H1 cells (Fig. 5A and B). However, productions of IL-10 and TNF- α were not significantly changed in this setting. Next, to test whether BCG can down-regulate the increased expression of OX40L induced by TSLP, DCs were pre-treated with TSLP first and then BCG was added afterward into the culture for further 24 h. As shown in Fig. 5(C), BCG did not decrease the expression of OX40L that was already up-regulated by TSLP. On the other hand, large amount of IL-12 was induced from DCs even after sequential stimulation with TSLP first and then BCG (data not shown).

Discussion

Historically, the study of allergy has predominantly been focused on the effector phase of the classical immune cascade of allergy (1–6). Reflecting this situation, conventional drugs such as corticosteroids and chemical mediator antagonists, and newly exploited drugs such as anti-IgE antibodies (24) and soluble IL-4 receptor α -chains (25), basically target the effector cells or factors involved in the downstream steps of allergic inflammation. On the other hand, it has long remained an enigma how T_H2 responses are induced in allergy, although DCs have been thought to play a critical role in upstream steps of allergy (26, 27). Recently, it has been demonstrated that the interaction between epithelial cell-derived TSLP and immature $CD11c^+$ myeloid DCs is a master switch of allergic inflammation, and TSLP-activated DCs dictate the fate of naive $CD4^+$ T cells: differentiation into inflammatory T_H2 cells (7). Therefore, the interface between TSLP and DCs has emerged as a novel target for the treatment of allergic diseases (28, 29).

In this study, we have demonstrated that BCG has the ability to counteract the TSLP function of inducing inflammatory T_H2 cells *in vitro*. Actually, BCG profoundly suppressed the T_H2 -inducing capacity of TSLP-DCs and instead endowed them with a regulatory T_H1 -inducing capacity. $IL-10$ -producing $CD4^+$ type 1 regulatory T cells were originally isolated from patients with severe combined immunodeficiency who had undergone successful HLA-mismatched bone marrow transplantation (30, 31) and also $IL-10^+$ IFN- γ^+ T_H cells generated from naive $CD4^+$ T cells *in vitro* are T regulatory type 1 cells (32, 33). Indeed, BCG/TSLP-DCs instructed naive $CD4^+$ T cells to differentiate into IFN- γ /IL-10 double-producing T cells, which remained in a relatively anergic state and have the inhibitory function for bystander



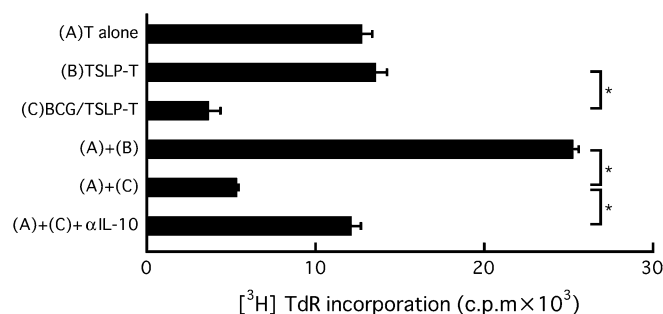


Fig. 4. BCG treatment induces the functional regulatory T_H1 cells. $CD4^+ CD25^-$ T cells were used as responders, and primed T cells co-cultured with TSLP-DCs and BCG/TSLP-DCs for 7 days were used as suppressors. $CD4^+ CD25^-$ T cells alone (A), TSLP-DC-primed T cells alone (B), BCG/TSLP-DC-primed T cells alone (C) and mixtures of T cell populations were cultured for another 5 days with irradiated allogeneic monocyte-derived DCs as stimulators. Ratio of responders to suppressors is 1 to 1. Proliferation was assessed by [³H]thymidine ([³H]TdR) incorporation and error bars represent the SEM for triplicate wells. Statistical significance was determined using paired Student's *t*-test (* $P < 0.005$).

T cell proliferation. Thus, the IFN- γ /IL-10 double-producing T cells induced by BCG/TSLP-DCs may represent regulatory T_H1 cells. In light of the classical paradigm of allergic immune cascade (1–6), the functional conversion of TSLP-DCs by BCG from inducing inflammatory T_H2 to regulatory T_H1 responses would presumably lead to the suppression of the T_H2 -dependent downstream effector phase of allergy, such as IgE production by B cells, eosinophilia, the release of chemical mediators from mast cells and mucous production *in vivo*. Notably, BCG exhibited its unique function not only in the priming stage of DCs by TSLP but also in the later stage, even after the completion of DC maturation by TSLP. This seems to be a great advantage when BCG is clinically applied for the treatment of allergic diseases that are triggered by TSLP, because BCG might possibly not only block the initiation of allergy but also repress the established allergic inflammation at the level of TSLP-DCs.

The functional conversion of TSLP-DCs by BCG was shown to depend on two major molecular events. First, BCG strongly inhibited the expression of OX40L expression by TSLP-DCs and thus shut down the inflammatory T_H2 response with promoting IL-10 production from T cells. Second, BCG triggered TSLP-DCs to produce large amounts of IL-12, which together with the down-regulation of OX40L expression switched the function of TSLP-DCs from inducing the inflammatory T_H2 response to the regulatory T_H1 response. Indeed, IL-12 alone has been shown to instruct human $CD4^+$ T cells to produce IL-10 together with IFN- γ (34). Our findings are in accord with the report that OX40L-induced inflammatory T_H2 cell differentiation depends on both the lack of IL-12 and the presence of OX40L, as OX40L loses the ability to trigger inflammatory T_H2 cell differentiation in the

presence of IL-12 (10). Although BCG did not have the ability to decrease the expression of OX40L that was already up-regulated by TSLP, BCG, even when added afterward, induced the production of great amount of IL-12 from TSLP-DCs (data not shown). In this situation in which OX40L is still expressed on DCs, IL-12 induced by BCG may be functionally dominant over OX40L, eventually inducing functional conversion from inflammatory T_H2 responses to T_H1 responses. However, IL-10 and TNF- α productions appear to be unaffected because of unsuppressed OX40L expression. Overall, BCG overcame the function of TSLP, a master switch of allergy, at the level of DCs by creating a unique environment in which IL-12 strictly dominates over OX40L function.

As for the signaling cascade through which BCG convert the function of TSLP-DCs, it is interesting to note that the cell wall skeleton of BCG (BCG-CWS), a major cellular component of BCG, is a ligand for Toll-like receptor (TLR)2 and TLR4 (35–37). Indeed, some reports demonstrated that TLR2 and/or TLR4 had a protective effect in allergen-induced lung inflammation (38–40). However, how the engagement of TLR2 and/or TLR4 is involved in the functional conversion of TSLP-DCs remains to be elucidated because the molecular mechanism of OX40L expression in DCs including the regulation of the promoter region of OX40L gene is not well understood.

There is a series of clinical and experimental evidence to support the efficacy of BCG in allergy. For example, *in vivo* administration of BCG to allergic patients resulted in a decrease of IgE levels (41, 42). In murine models, infection in the lung with BCG prevented allergen-induced airway eosinophilia and the development of airway hyperreactivity (43, 44) and administration of BCG or *M. bovis* suppressed IgE response in ovalbumin-sensitized newborn mice (45, 46). However, the precise molecular and cellular mechanism underlying the BCG effect has not clearly been demonstrated in those studies. Recently, it has been shown that BCG activated V α 14 NK T cells to express high levels of IL-21, which preferentially induces apoptosis in B ϵ cells, eventually suppressing allergic response (47). On the other hand, our current study findings differentially delineate a novel mechanism underlying the BCG-mediated suppression of allergy: BCG redirects TSLP-activated DCs away from inducing inflammatory T_H2 to regulatory T_H1 immune response.

Our findings also seem to provide a clue for the understanding of the underlying mechanism of the 'hygiene hypothesis' (48), which was originally formulated by Strachan ~20 years ago. The hygiene hypothesis postulates that the increased prevalence of allergies is due to the reduced exposure to infections owing to improved hygiene in the developed nations over the last 4 decades (48). And since the first report on Japanese school children (49), numerous epidemiological studies have demonstrated an inverse association between infection with *Mycobacterium tuberculosis* or BCG vaccination and decreased prevalence of allergic

Fig. 3. BCG treatment on TSLP-DCs converts T_H2 into T_H1 differentiation through both IL-12 induction and OX40L repression. Naive $CD4^+$ T cells were cultured for 7 days with DCs pre-treated with TSLP alone or TSLP plus BCG. Anti-IL-12 antibody and/or soluble rOX40L were added in DC-T cell co-culture. (A) Cytokine production by $CD4^+$ T cells was analyzed intracellularly by flow cytometry. Percentages of the respective cytokine-producing T cells are indicated. (B) Cytokine production was measured in supernatants after re-stimulation with anti-CD3 and anti-CD28 mAbs for 24 h by ELISA. Data are means $\pm$ SEM of three independent experiments.

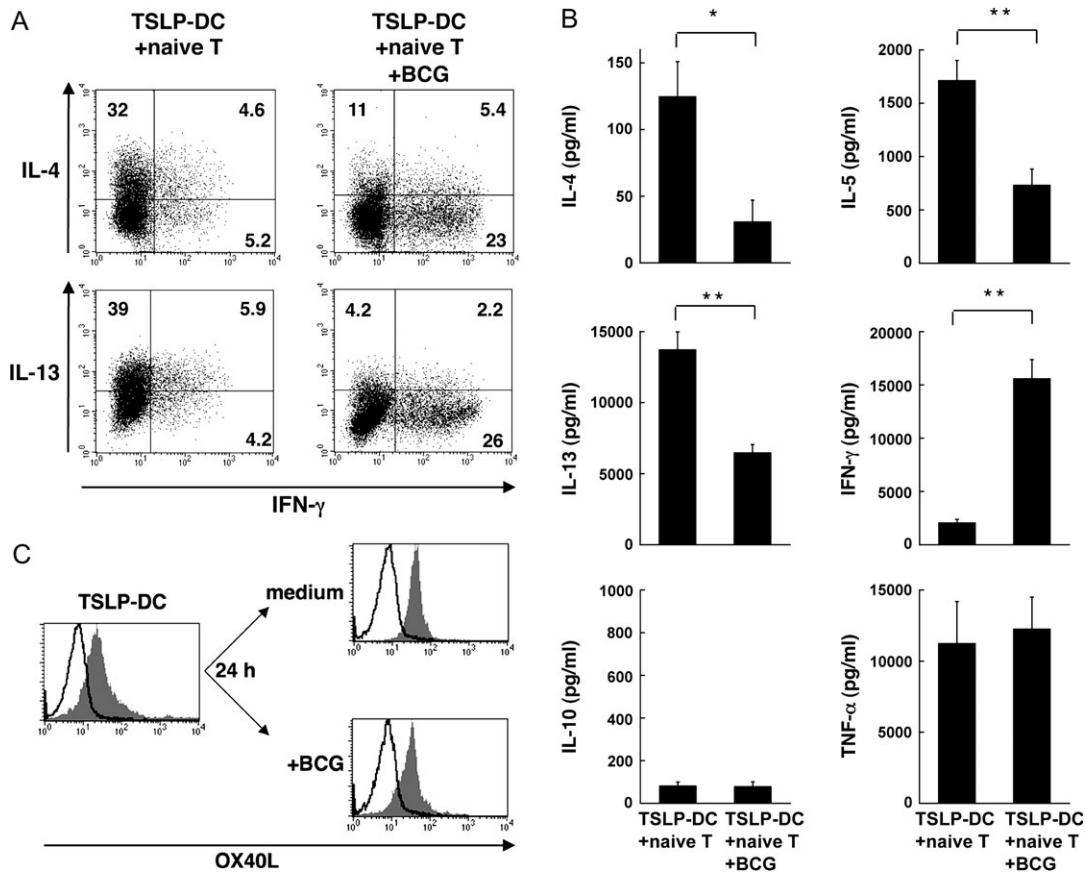


Fig. 5. Addition of BCG afterward into DC-T co-culture results in inhibition of T_H2 differentiation. DCs were pre-treated with TSLP for 24 h and were then cultured for 7 days with naive $CD4^+$ T cells. BCG was added at the beginning of DC-T cell co-culture. (A) Cytokine production by $CD4^+$ T cells was analyzed intracellularly by flow cytometry. Percentages of the respective cytokine-producing T cells are indicated. (B) Cytokine production was measured in supernatants after re-stimulation with anti-CD3 and anti-CD28 mAbs for 24 h by ELISA. Data are means $\pm$ SEM of three independent experiments. Statistical significance was determined using paired Student's *t*-test (* P < 0.05, ** P < 0.01). (C) After culture with TSLP for 24 h, DCs were then stimulated with BCG for further 24 h. OX40L expression on the DCs was analyzed by flow cytometry. The staining profiles of anti-OX40L mAb and isotype-matched control are shaded and open areas, respectively. Data are representative of three independent experiments.

illnesses (50–53), favoring the hygiene hypothesis, although several studies refuted such a relationship (54, 55). The findings of this study that BCG redirects TSLP-DCs from inducing inflammatory T_H2 response to regulatory T_H1 response may support the hypothesis and provide a novel molecular and cellular explanation for the hypothesis at least in the context of TSLP and BCG.

In conclusion, we have demonstrated that BCG inhibited the function of TSLP by inducing the production of IL-12 from DCs and by down-regulating OX40L on DCs. Thus, BCG is a promising immune adjuvant for the prevention and treatment of allergic diseases that stem from TSLP, although many issues remain to be addressed for the clinical use of BCG, such as optimal timing of BCG administration, dose and route of delivery.

Funding

Ministry of Education, Culture, Sports, Science and Technology of Japan (RR2002) (recipient: 1st Department of Internal Medicine, Kansai Medical University); Takeda Science Foundation (recipient: Kansai Medical University).

Acknowledgements

The authors thank Tsuneyasu Kaisho, Yasuyuki Ishii and Masaru Taniguchi for scientific advice. The authors have no conflicting financial interests.

Abbreviations:

APC	allophycocyanin
BCG	Bacillus Calmette-Guérin
DC	dendritic cell
OX40L	OX40 ligand
rOX40L	recombinant OX40L
RT	reverse transcription
TLR	Toll-like receptor
TNF	tumor necrosis factor
TSLP	thymic stromal lymphopoietin

References

1. Mosmann, T. R. and Coffman, R. L. 1989. $TH1$ and $TH2$ cells: different patterns of lymphokine secretion lead to different functional properties. *Annu. Rev. Immunol.* 7:145.
2. Abbas, A. K., Murphy, K. M. and Sher, A. 1996. Functional diversity of helper T lymphocytes. *Nature* 383:787.

3. Constant, S. L. and Bottomly, K. 1997. Induction of Th1 and Th2 CD4+ T cell responses: the alternative approaches. *Annu. Rev. Immunol.* 15:297.
4. Renaud, J. C. 2001. New insights into the role of cytokines in asthma. *J. Clin. Pathol.* 54:577.
5. Holgate, S. T. 2000. Science, medicine, and the future. Allergic disorders. *Br. Med. J.* 320:231.
6. Busse, W. W. and Lemanske, R. F. Jr 2001. Asthma. *N. Engl. J. Med.* 344:350.
7. Soumelis, V., Reche, P. A., Kanzler, H. *et al.* 2002. Human epithelial cells trigger dendritic cell mediated allergic inflammation by producing TSLP. *Nat. Immunol.* 3:673.
8. Gilliet, M., Soumelis, V., Watanabe, N. *et al.* 2003. Human dendritic cells activated by TSLP and CD40L induce proallergic cytotoxic T cells. *J. Exp. Med.* 197:1059.
9. Ying, S., O'Connor, B., Ratoff, J. *et al.* 2005. Thymic stromal lymphopoietin expression is increased in asthmatic airways and correlates with expression of Th2-attracting chemokines and disease severity. *J. Immunol.* 174:8183.
10. Ito, T., Wang, Y. H., Duramad, O. *et al.* 2005. TSLP-activated dendritic cells induce an inflammatory T helper type 2 cell response through OX40 ligand. *J. Exp. Med.* 202:1213.
11. Wang, Y. H., Ito, T., Wang, Y. H. *et al.* 2006. Maintenance and polarization of human TH2 central memory T cells by thymic stromal lymphopoietin-activated dendritic cells. *Immunity* 24:827.
12. Yoo, J., Omori, M., Gyarmati, D. *et al.* 2005. Spontaneous atopic dermatitis in mice expressing an inducible thymic stromal lymphopoietin transgene specifically in the skin. *J. Exp. Med.* 202:541.
13. Al-Shami, A., Spolski, R., Kelly, J., Keane-Myers, A. and Leonard, W. J. 2005. A role for TSLP in the development of inflammation in an asthma model. *J. Exp. Med.* 202:829.
14. Zhou, B., Comeau, M. R., De Smedt, T. *et al.* 2005. Thymic stromal lymphopoietin as a key initiator of allergic airway inflammation in mice. *Nat. Immunol.* 6:1047.
15. Seshasayee, D., Lee, W. P., Zhou, M. *et al.* 2007. *In vivo* blockade of OX40 ligand inhibits thymic stromal lymphopoietin driven atopic inflammation. *J. Clin. Invest.* 117:3868.
16. Kaufmann, S. H. and van Embden, J. D. 1993. Tuberculosis: a neglected disease strikes back. *Trends Microbiol.* 1:2.
17. Demangel, C., Bean, A. G., Martin, E., Feng, C. G., Kamath, A. T. and Britton, W. J. 1999. Protection against aerosol *Mycobacterium tuberculosis* infection using *Mycobacterium bovis* Bacillus Calmette Guerin-infected dendritic cells. *Eur. J. Immunol.* 29:1972.
18. Kim, K. D., Lee, H. G., Kim, J. K. *et al.* 1999. Enhanced antigen-presenting activity and tumour necrosis factor- α -independent activation of dendritic cells following treatment with *Mycobacterium bovis* bacillus Calmette-Guerin. *Immunology* 97:626.
19. Uehira, K., Amakawa, R., Ito, T. *et al.* 2002. Dendritic cells are decreased in blood and accumulated in granuloma in tuberculosis. *Clin. Immunol.* 105:296.
20. Alexandroff, A. B., Jackson, A. M., O'Donnell, M. A. and James, K. 1999. BCG immunotherapy of bladder cancer: 20 years on. *Lancet* 353:1689.
21. Ransom, J. H., Pelle, B. A., Hubers, H., Keynton, L. M., Hanna, M. G. Jr. and Pomato, N. 1993. Identification of colon-tumor-associated antigens by T-cell lines derived from tumor-infiltrating lymphocytes and peripheral-blood lymphocytes from patients immunized with an autologous tumor-cell/bacillus Calmette-Guerin vaccine. *Int. J. Cancer* 54:734.
22. Vermorken, J. B., Claessen, A. M., van Tinteren, H. *et al.* 1999. Active specific immunotherapy for stage II and stage III human colon cancer: a randomised trial. *Lancet* 353:345.
23. Ito, T., Inaba, M., Inaba, K. *et al.* 1999. A CD1a+/CD11c+ subset of human blood dendritic cells is a direct precursor of Langerhans cells. *J. Immunol.* 163:1409.
24. Presta, L. G., Lahr, S. J., Shields, R. L. *et al.* 1993. Humanization of an antibody directed against IgE. *J. Immunol.* 151:2623.
25. Borish, L. C., Nelson, H. S., Lanz, M. J. *et al.* 1999. Interleukin-4 receptor in moderate atopic asthma. A phase I/II randomized, placebo-controlled trial. *Am. J. Respir. Crit. Care Med.* 160:1816.
26. Holt, P. G. 1993. Macrophage: dendritic cell interaction in regulation of the IgE response in asthma. *Clin. Exp. Allergy* 23:4.
27. Lambrecht, B. N., De Veerman, M., Coyle, A. J., Gutierrez-Ramos, J. C., Thielemans, K. and Pauwels, R. A. 2000. Myeloid dendritic cells induce Th2 responses to inhaled antigen, leading to eosinophilic airway inflammation. *J. Clin. Invest.* 106:551.
28. Liu, Y. J. 2006. Thymic stromal lymphopoietin: master switch for allergic inflammation. *J. Exp. Med.* 203:269.
29. Holgate, S. T. 2007. The epithelium takes centre stage in asthma and atopic dermatitis. *Trends Immunol.* 28:248.
30. Bacchetta, R., Bigler, M., Touraine, J. L. *et al.* 1994. High levels of interleukin 10 production *in vivo* are associated with tolerance in. *J. Exp. Med.* 179:493.
31. Groux, H., O'Garra, A., Bigler, M. *et al.* 1997. A CD4+ T-cell subset inhibits antigen-specific T-cell responses and prevents. *Nature* 389:737.
32. Levings, M. K., Sangregorio, R., Galbiati, F., Squadrone, S., de Waal Malefyt, R. and Roncarolo, M. G. 2001. IFN- α and IL-10 induce the differentiation of human type 1 T regulatory. *J. Immunol.* 166:5530.
33. Kawamura, K., Kadowaki, N., Kitawaki, T. and Uchiyama, T. 2006. Virus-stimulated plasmacytoid dendritic cells induce CD4+ cytotoxic regulatory T. *Blood* 107:1031.
34. Gerosa, F., Paganin, C., Peritt, D. *et al.* 1996. Interleukin-12 primes human CD4 and CD8 T cell clones for high production of both interferon- γ and interleukin-10. *J. Exp. Med.* 183:2559.
35. Tsuji, S., Matsumoto, M., Takeuchi, O. *et al.* 2000. Maturation of human dendritic cells by cell wall skeleton of *Mycobacterium bovis* bacillus Calmette-Guerin: involvement of toll-like receptors. *Infect. Immun.* 68:6883.
36. Uehori, J., Matsumoto, M., Tsuji, S. *et al.* 2003. Simultaneous blocking of human Toll-like receptors 2 and 4 suppresses myeloid dendritic cell activation induced by *Mycobacterium bovis* bacillus Calmette-Guerin peptidoglycan. *Infect. Immun.* 71:4238.
37. Heldwein, K. A., Liang, M. D., Andresen, T. K. *et al.* 2003. TLR2 and TLR4 serve distinct roles in the host immune response against *Mycobacterium bovis* BCG. *J. Leukoc. Biol.* 74:277.
38. Akdis, C. A., Kussebi, F., Pulendran, B. *et al.* 2003. Inhibition of T helper 2-type responses, IgE production and eosinophilia by synthetic lipopeptides. *Eur. J. Immunol.* 33:2717.
39. Revets, H., Pynaert, G., Grooten, J. and De Baetselier, P. 2005. Lipoprotein I, a TLR2/4 ligand modulates Th2-driven allergic immune responses. *J. Immunol.* 174:1097.
40. Patel, M., Xu, D., Kewin, P. *et al.* 2005. TLR2 agonist ameliorates established allergic airway inflammation by promoting Th1 response and not via regulatory T cells. *J. Immunol.* 174:7558.
41. Cavallo, G. P., Elia, M., Giordano, D., Baldi, C. and Cammarota, R. 2002. Decrease of specific and total IgE levels in allergic patients after BCG vaccination: preliminary report. *Arch. Otolaryngol. Head Neck Surg.* 128:1058.
42. Barlan, I. B., Tukenmez, F., Bahceciler, N. N. and Basaran, M. M. 2002. The impact of *in vivo* Calmette-Guerin Bacillus administration on *in vitro* IgE secretion in atopic children. *J. Asthma* 39:239.
43. Erb, K. J., Holloway, J. W., Soback, A., Moll, H. and Le Gros, G. 1998. Infection of mice with *Mycobacterium bovis*-Bacillus Calmette-Guerin (BCG) suppresses allergen-induced airway eosinophilia. *J. Exp. Med.* 187:561.
44. Herz, U., Gerhold, K., Gruber, C. *et al.* 1998. BCG infection suppresses allergic sensitization and development of increased airway reactivity in an animal model. *J. Allergy Clin. Immunol.* 102:867.
45. Bakir, M., Tukenmez, F., Bahceciler, N. N., Barlan, I. B. and Basaran, M. M. 2000. Heat-killed *Mycobacterium bovis*-bacillus Calmette Guerin-suppressed total serum IgE response in ovalbumin-sensitized newborn mice. *J. Asthma* 37:329.
46. Tukenmez, F., Bahceciler, N. N., Barlan, I. B. and Basaran, M. M. 1999. Effect of pre-immunization by killed *Mycobacterium bovis* and vaccae on immunoglobulin E response in ovalbumin-sensitized newborn mice. *Pediatr. Allergy Immunol.* 10:107.
47. Harada, M., Magara-Koyanagi, K., Watarai, H. *et al.* 2006. IL-21-induced Bepsilon cell apoptosis mediated by natural killer T cells suppresses IgE responses. *J. Exp. Med.* 203:2929.

48. Strachan, D. P. 1989. Hay fever, hygiene, and household size. *Br. Med. J.* 299:1259.
49. Shirakawa, T., Enomoto, T., Shimazu, S. and Hopkin, J. M. 1997. The inverse association between tuberculin responses and atopic disorder. *Science* 275:77.
50. Obihara, C. C., Kimpen, J. L., Gie, R. P. *et al.* 2006. *Mycobacterium tuberculosis* infection may protect against allergy in a tuberculosis endemic area. *Clin. Exp. Allergy* 36:70.
51. Gruber, C., Meinschmidt, G., Bergmann, R., Wahn, U. and Stark, K. 2002. Is early BCG vaccination associated with less atopic disease? An epidemiological study in German preschool children with different ethnic backgrounds. *Pediatr. Allergy Immunol.* 13:177.
52. Garcia-Marcos, L., Suarez-Varela, M. M., Canflanca, I. M. *et al.* 2005. BCG immunization at birth and atopic diseases in a homogeneous population of Spanish schoolchildren. *Int. Arch. Allergy Immunol.* 137:303.
53. Aaby, P., Shaheen, S. O., Heyes, C. B. *et al.* 2000. Early BCG vaccination and reduction in atopy in Guinea-Bissau. *Clin. Exp. Allergy* 30:644.
54. Alm, J. S., Lilja, G., Pershagen, G. and Scheynius, A. 1997. Early BCG vaccination and development of atopy. *Lancet* 350:400.
55. Ehrlich, R. I., White, N., Norman, R. *et al.* 2005. Wheeze, asthma diagnosis and medication use: a national adult survey in a developing country. *Thorax* 60:895.