**Mycobacterium bovis** Bacillus Calmette–Guérin suppresses inflammatory Th2 responses by inducing functional alteration of TSLP-activated dendritic cells

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**Abstract**

Allergic diseases such as atopic dermatitis and asthma develop as a consequence of dysregulated Th2 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 Th2 responses in allergy. In this study, we investigated whether *Mycobacterium bovis* Bacillus Calmette–Guérin (BCG), a strong Th1 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 Th2 cells that produce IL-4, IL-5, IL-13 and tumor necrosis factor (TNF)-α and toward regulatory Th1 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 Th2 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 Th2-attracting chemokines and disease severity (9). TSLP strongly activates human CD11c⁺ myeloid DCs without inducing Th1-polarizing cytokines IL-12, IL-23 and IL-27 (10). And the TSLP-activated DCs instruct naive CD4⁺ T cells to differentiate into Th2 cells. The Th2 cells induced by TSLP-activated DCs are distinct from the classical Th2 cells, because they produce large amounts of tumor necrosis factor (TNF)-α in addition to Th2 cytokines IL-4, IL-5 and IL-13, but little or no IL-10; thus, it is proposed that these unique Th2 cells be called inflammatory Th2 cells (7, 10). In addition to the differentiation of inflammatory Th2 cells, TSLP-activated DCs induce the expansion of human Th2 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** Th2 cells in the absence of Th1-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 Th2 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,
interference or alteration of the function of TSLP-activated DCs that induce inflammatory Th2 cell may constitute a rational treatment strategy for allergic diseases.

The *Mycobacterium bovis* Bacillus Calmette–Guerin (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-1β 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 Th2 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 Th2 cell response-inducing capacity of TSLP-activated DCs into a regulatory Th2 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 (CD45/C0+ /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: Exalpa), CD14 (M5E2: BD Biosciences), CD15 (M5E2: BD Biosciences), CD16 (J551: Exalpa), 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 μl 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 FACSscan® (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'-CCCAGATTGTGAAGTGAGGA-3' and reverse 5'-GCTGTTTTATGATGTTGCC-3', and (for β-actin) forward 5'-CTGGAGCCGTCGAGGTCA-3' and reverse 5'-AAGGACTCTCTGTAAACTGCA-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 FACSscan® (BD Biosciences).

**DC–T cell co-culture**

CD4+CD45RA+ naïve 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 allogeneic naïve 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 naïve 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 μg ml⁻¹), anti-IL-12 antibody (AF-219-NA, R&D Systems: 1 μg 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 μg ml⁻¹) and soluble anti-CD28 (1 μg 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 μg ml⁻¹ of ionomycin for 6 h. Brefeldin A (10 μg 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-INF-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 Th1-inducing cytokine, IL-12 (Fig. 1B) and TNF-α (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 Th2 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 TSLP during these 72 h. This result was confirmed at the mRNA level by RT-PCR (Fig. 2B).

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 ± 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.
**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. Naïve 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 naïve 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-\(\gamma\)-producing T cells (Fig. 3A). Notably, some of these IFN-\(\gamma\)-producing T cells concomitantly expressed IL-10, suggesting so-called IL-10\(^{+}\) IFN-\(\gamma\)^- 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-\(\alpha\) but low amounts of IL-10 and IFN-\(\gamma\). Meanwhile, BCG/TSLP-DCs significantly decreased the production of IL-4, IL-5 IL-13 and TNF-\(\alpha\) and concomitantly increased the production of IL-10 and IFN-\(\gamma\) by T cells (Fig. 3B). The next question was whether these IL-10\(^{+}\) IFN-\(\gamma\)^- T 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-\(\gamma\)^- T 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-\(\gamma\)^- T\(_{h1}\) 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 redirects 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 40X40L 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-\(\gamma\)-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-\(\gamma\) 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-\(\gamma\) 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 after ward 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 naïve 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-\(\gamma\)-producing T\(_{h1}\) cells (Fig. 5A and B). However, productions of IL-10 and TNF-\(\alpha\) 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 \(\alpha\)-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 Th2 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 naïve 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...
BCG inhibits T_{h}2 response induced by TSLP
Indeed, BCG/TSLP-DCs instructed naive CD4+ T cells to produce IL-12 together with IFN-γ (34). Our findings are in accord with the report thatOX40L-induced inflammatory Th2, cell differentiation depends on both the lack of IL-12 and the presence of OX40L, as OX40L loses the ability to trigger inflammatory Th2 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 Tn1 responses to Th1 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 promotor 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 Vα14 NK T cells to express high levels of IL-21, which preferentially induces apoptosis in B2 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 Th2 to regulatory Th1 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...
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 Th2 response to regulatory Th1 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.

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Abbreviations
APC allophycocyanin
BCG Bacillus Calmette–Gue´rin
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
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