

CD8 α ⁺ and CD8 α [−] DC subsets from BCG-infected mice inhibit allergic Th2-cell responses by enhancing Th1-cell and Treg-cell activity respectively

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The hygiene hypothesis has suggested an inhibitory effect of infections on allergic diseases, but the related mechanism remains unclear. We recently reported that DCs played a critical role in *Mycobacterium bovis* Bacille Calmette–Guérin (BCG)-mediated inhibition of allergy, which depended on IL-12 and IL-10-related mechanisms. Here, we tested the hypothesis that BCG infection could modulate the function of DC subsets, which might in turn inhibit allergic responses through different mechanisms. We sorted CD8 α ⁺ and CD8 α [−] DCs from BCG-infected mice and tested their ability to modulate Th2-cell responses to ovalbumin (OVA) using in vitro and in vivo approaches. We found that both DC subsets could inhibit the allergic Th2-cell response in both a DC:T-cell co-culture system and after adoptive transfer. These subsets exhibited different co-stimulatory marker expression and cytokine production patterns and were different in inducing Th1 and Treg cells. Specifically, we found that CD8 α ⁺ DCs produced higher IL-12, inducing higher Th1 cell response, while CD8 α [−] DCs expressed higher ICOS-L and produced higher IL-10, inducing CD4⁺CD25⁺FoxP3⁺ Treg cells with IL-10 production and membrane-bound TGF- β expression. The finding suggests that one infection may inhibit allergy by both immune deviation and regulation mechanisms through modulation of DC subsets.

Key words: Allergy · BCG · DC subsets · Hygiene hypothesis · Treg cell

Introduction

The hygiene hypothesis has been raised for more than two decades [1]. Although conflicting results have been reported, it is generally accepted that some infections, especially intracellular bacterial and parasitic infections, may have a negative impact on the development of allergic and autoimmune diseases [2–5]. In particular, a protective role for BCG infection/vaccination in the development of allergic diseases has been demonstrated by numerous epidemiological and experimental studies [6].

The mechanism behind the hygiene hypothesis is believed to be related to immune deviation and/or immune regulation

[5, 7–10]. Indeed, some bacterial infections including BCG infection have been found to alter an allergen-driven Th2-cell response to a Th1-dominated-cell response [3, 6, 11–15]. More recently, the promoting effect of infections, especially helminth parasitic infections, on Treg cells was shown to be even more relevant to the inhibitory role of infection on allergic diseases [10, 16–18]. We and other groups recently found that the adoptive transfer of DCs isolated from infected mice or those modulated with bacterial products in vitro could significantly inhibit de novo and established allergic inflammation and Th2-cell responses [19–25]. The reduction of allergic reactions and Th2-cell responses was either associated with enhanced Th1-cell responses [19] or enhanced regulatory function [22–25] or both [15, 20, 24]. In particular, we found that the mechanisms by which transferred DCs from BCG-infected mice modulate allergic reactions involve both IL-12 and IL-10-mediated mechanisms [15].

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Since IL-12 is a critical initiator for immune deviation and since IL-10 is a major cytokine for the induction of Treg cells, the data raised the possibility that DCs from BCG-infected mice may be able to induce both Treg and Th1 cells, thus inhibiting allergic reactions through immune deviation and regulation mechanisms. Since different DC subsets have been found to be different for modulating T-cell responses [26–31] and particularly we recently found CD8 α^+ and CD8 α^- DC subsets from BCG-infected mice (designated below as iCD8 α^+ and iCD8 α^- DCs respectively) produced higher IL-12 and IL-10 production respectively [32], we hypothesized that the two DC subsets were the mediator of the different allergy inhibition mechanisms. The data from the study supported this hypothesis by showing that adoptive transfer of either iCD8 α^+ or iCD8 α^- DCs inhibited OVA-induced airway mucus overproduction, eosinophilia inflammation and Th2 cytokine production, but the effect on Th1- and Treg-cell responses was different. Specifically, the transfer of iCD8 α^+ DCs which predominantly produced IL-12 enhanced Th1-cell responses, while the transfer of iCD8 α^- DCs which predominantly produced IL-10 significantly increased the frequency of Treg cells. The results suggest that BCG infection can inhibit allergy by both immune deviation and regulation through modulation of different DC subsets.

Results

iCD8 α^+ DCs and iCD8 α^- DCs showed different patterns of surface markers and cytokine profile

DC function mainly relies on surface co-stimulatory molecule expression and cytokine production. We recently reported that BCG infection induced significant expansion of CD8 α^+ DCs, which showed different patterns of surface markers and cytokine profile in C57BL/6 mice [32]. In this study we further analyzed the effect of BCG infection on the phenotype of CD8 α^+ DCs and CD8 α^- DCs in BALB/c mice because this mouse strain was used for the following experiments. The mice were infected intravenously with BCG (5×10^5 CFUs) and at 21 days post-infection, the mice were killed and splenic CD8 α^+ or CD8 α^- iDC subsets were analyzed by flow cytometry. As shown in Fig. 1A and B, CD8 α^+ DCs and CD8 α^- DCs from BCG-infected mice showed significantly higher CD80 and CD40 expression than those from naïve DC subsets. MHC-II expression was higher on CD8 α^+ DCs than CD8 α^- DCs but not relevant to infection. Interestingly, BCG infection enhanced CD86 expression on CD8 α^- DCs but not on CD8 α^+ DCs. More interestingly, the CD86 expression on iCD8 α^- DCs was significantly higher than iCD8 α^+ DCs. The results suggest that BCG infection has different influences on surface marker expression on CD8 α^+ DCs and CD8 α^- DCs subset in BALB/c mice, especially on CD86 expression.

We further used real-time PCR to analyze the mRNA messages of IL-12 and IL-10 in freshly purified iCD8 α^+ DCs and iCD8 α^- DCs (Fig. 1C). iCD8 α^+ DCs showed higher IL-12p40 mRNA message while iCD8 α^- DCs had higher IL-10 mRNA expression. While naïve DC subsets have much lower levels of mRNA for

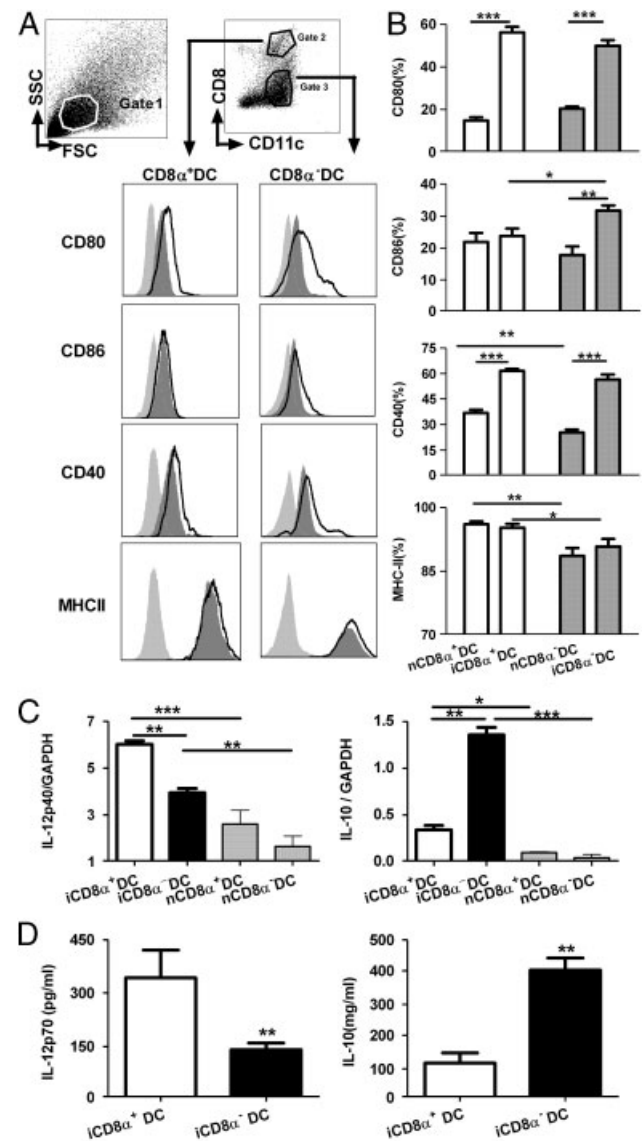


Figure 1. Differential surface co-stimulatory marker expression and cytokine profiles of iDC subsets. Balb/c mice were infected i.v. with BCG (5×10^5 CFUs). On day 21 after infection, DCs were isolated from the spleen of BCG-infected or naïve mice using CD11c microbeads as described in Materials and Methods. (A) Cells were stained for surface molecules (CD80, CD86, CD40, MHC class II), and analyzed using flow cytometry. Light shaded area, isotype control; dark shaded area, naïve DC subset; solid line, iDC subset. (B) Surface marker expression by DC subsets from naïve and BCG-infected mice. (C) iCD11c $^+$ CD8 α^+ and iCD11c $^+$ CD8 α^- subsets were sorted by flow cytometry as described in Materials and Methods. The expression of mRNA of IL-12 and IL-10 in the iCD8 α^+ DC and iCD8 α^- DCs was measured by real-time PCR. The ratio of copies of IL-12 or IL-10 to GAPDH is shown as mean $\pm$ SEM of $n=3$ pooled from three independent experiments. (D) The sorted DC subsets were cultured for 72 h. IL-12p70 and IL-10 protein content in the supernatant of iDC subset cultures were measured by ELISA. (B, D) Data are shown as mean $\pm$ SD of $n=3$ and are representative of three independent experiments. * $p<0.05$, ** $p<0.01$, *** $p<0.001$, one-way ANOVA.

both cytokines. To further confirm the difference in IL-12 and IL-10 production by DC subsets, the purified DC subsets were cultured in complete medium for 72 h to test cytokine production.

The testing of culture supernatants showed same pattern of cytokine differences as message measurement (Fig. 1D). DC subsets isolated from naive mice showed undetectable IL-12 and IL-10 production. Therefore, BCG infection indeed had influence on DC subsets which were different in the expression of surface co-stimulatory markers and cytokine production.

iCD8 α^+ DCs are more effective than iCD8 α^- DCs inhibiting the allergic response

Our previous works have demonstrated that mycobacterial infection can inhibit allergic responses induced by ovalbumin (OVA) [13], and adoptive transfer of iDCs is capable of modulating allergic responses [15]. In the present study, we intended to further examine the functional relevance of iDC subsets in the inhibition of allergic reactions by BCG infection. DC donor mice were infected intravenously with BCG (5×10^5 CFUs) and at 21 days post-infection, the splenic CD8 α^+ or CD8 α^- iDC subsets were isolated and adoptively transferred to naive recipient mice at 2 h before the mice were sensitized intraperitoneally (i.p.) with OVA and alum. The mice were challenged intranasally (i.n.) with OVA at 14 days after OVA sensitization. The data showed that the recipients of either iCD8 α^+ DC or iCD8 α^- DC subsets mounted significantly reduced airway inflammation than the control mice (PBS) measured by total infiltrating cells (Fig. 2A) and eosinophils (Fig. 2B), while the reduction was more dramatic in the recipients of iCD8 α^+ DCs. The adoptive transfer of CD8 α^+ DC or CD8 α^- DC subsets from naive mice (nCD8 α^+ DC or nCD8 α^- DC) had no significant effect on the allergic responses (Fig. 2A and B). H&E staining of the lung tissues (Fig. 2C) showed that a dramatic decrease of eosinophilic infiltration in alveolar, peribronchial and perivascular areas in the recipients of iCD8 α^+ DC and iCD8 α^- DC subsets especially those received iCD8 α^+ DCs compared with the control mice without DC transfer. Moreover, the mucus staining by the periodic acid-schiff method showed less mucus production in the iDC recipients than control mice (Fig. 2C). Histological mucus index (HMI), a quantitative method for measuring mucus production, showed that the percentage of mucinous airway epithelium was significantly low in the recipients of iCD8 α^+ DCs and iCD8 α^- DCs (Fig. 2D). Moreover, the recipients of iCD8 α^+ DC and iCD8 α^- DC subsets showed significantly lower serum OVA-specific IgE responses (Fig. 2E). The results suggest that iCD8 α^+ DC and iCD8 α^- DC subsets, but not nCD8 α^+ DCs or nCD8 α^- DCs, can inhibit airway allergic reactions and that iCD8 α^+ DC subset is more efficient in mediating this inhibition.

Adoptive transfer of iCD8 α^+ DCs increases Th1 cytokine production

To explore the molecular basis for the altered allergic inflammation in the iCD8 α^+ DC and iCD8 α^- DC recipients, we further examined the allergen-driven cytokine production. As shown in

Fig. 3A, the recipients of iCD8 α^+ DCs and iCD8 α^- DCs showed significantly lower OVA-driven Th2 cytokine (IL-4 and IL-5) production than control mice without DC transfer in the culture of draining LNs cells or direct measurement of BALF cytokines (BAL). The reduction of Th2 cytokines was more significant in iCD8 α^+ DC recipients. Notably, although the recipients of iCD8 α^+ DCs showed dramatically higher allergen-driven IFN- γ production, the IFN- γ production in the recipients of iCD8 α^- DCs was similar to control mice. The data demonstrate that adoptive transfer of either iDC subsets reduced allergen-driven Th2 cytokine production but only iCD8 α^+ DCs can enhance Th1 cytokine production. Since the Th1 and Th2-related cytokines can be produced by multiple cells, we did further CD4 $^+$ T-cell intracellular cytokine staining to confirm the changes in Th1 and Th2 cells. As shown in Fig. 3B, both iCD8 α^+ DC and iCD8 α^- DC recipients showed lower percentage of IL-4-producing CD4 $^+$ T cells, while the recipients of iCD8 α^+ DCs showed higher percentage of IFN- γ -producing CD4 $^+$ T cells than control mice. Therefore, adoptive transfer of either iCD8 α^+ DCs or iCD8 α^- DCs indeed inhibited Th2 cells but only iCD8 α^+ DCs enhanced Th1 cells, implying that the two iDC subsets may inhibit allergen-driven Th2 cells by different mechanisms.

iCD8 α^+ DCs promote the differentiation of naïve allergen-specific CD4 $^+$ T cells to the Th1-cell direction

To more directly determine the capacity of iCD8 α^+ DC and iCD8 α^- DC subsets in directing allergen-specific T-cell responses, we co-cultured the different iDC subsets with naïve OVA-specific CD4 $^+$ T cells. As showed in Fig. 4A and B, a significantly lower percentage of OVA-specific CD4 $^+$ T cells developed to IL-4-producing cells when co-cultured with iCD8 α^+ DCs than those cultured with iCD8 α^- DCs. In contrast, a significantly high percentage of CD4 $^+$ T cells became IFN- γ -producing cells when co-cultured with iCD8 α^+ DCs (Fig. 4A and B). Measurement of cytokine proteins in the supernatants of the co-culture system showed similar pattern of Th2 and Th1 cytokine production (Fig. 4C).

Since we had found different pattern of cytokine production by the iDC subsets (Fig. 1), we further tested the role of IL-12 and IL-10 which were predominantly produced by iCD8 α^+ DCs and iCD8 α^- DCs, respectively, in influencing T-cell responses using neutralizing mAbs in the DC:CD4 $^+$ T-cell co-culture system. The results showed that in the iCD8 α^+ DC:T-cell co-culture system, the blockade of IL-12 led to dramatic increase of IL-4 cytokine production (>10-fold), parallel with a significant reduction of IFN- γ production (Fig. 4C), while the blockade of IL-10 in this system had a much milder effect (2.5-fold). In contrast, the blockade of IL-12 in the co-culture of iCD8 α^- DC:T cells had no significant effect on IL-4 and IFN- γ production, but the blockade of IL-10 led to significant increase of IL-4. The results suggest that the higher IL-12 production by iCD8 α^+ DCs is critical for the development of antigen-specific Th1 cells which is likely the major mechanism for inhibiting allergic Th2-cell responses while

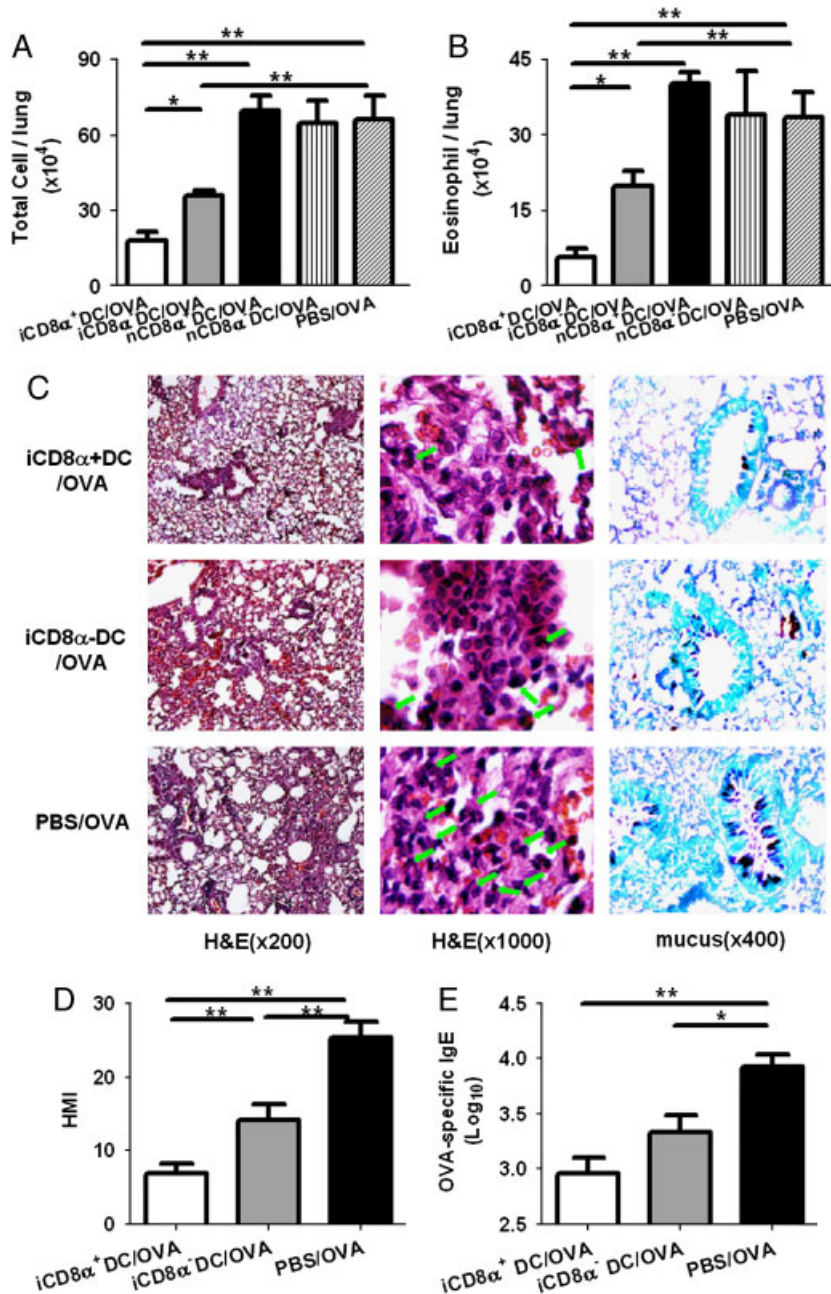


Figure 2. The effect of adoptive transfer of iDC subsets on airway inflammation and mucus production in the lung. CD11c $^+$ CD8 α^+ and CD11c $^+$ CD8 α^- DC subsets were sorted from spleens of naive mice or BCG-infected mice by flow cytometry and injected i.v. to recipient mice (5×10^5 cells in 200 μ L PBS). Two hours after the cell transfer, mice were sensitized i.p. with 2 μ gOVA (in alum) followed by intranasal challenged 50 μ g OVA (in 40 μ L PBS) at day 14 days after sensitization. Control mice received the same OVA sensitization and challenge but only pretreated with PBS instead of DCs transfer. At day 7 after intranasal OVA challenge lungs were analyzed for inflammation, mucus production and serum IgE responses. (A, B) Cells in the bronchoalveolar lavage (BAL) fluids were counted and the cell differential was detected by staining with Fisher Leukostat Stain kit. (A) The absolute number of total infiltrating cells and (B) eosinophils in the different groups are shown. (C) Lung tissue sections (5 μ m) were stained with H&E for inflammation (left and middle columns) or periodic acid-schiff (PAS) for mucus production (right column). Green arrows indicate eosinophils in the lung tissue (middle column). (D) Mucus-producing epithelium was quantified as histological mucus index (HMI) as described in Materials and Methods. (E) OVA-specific serum IgE levels in different groups are shown. (C) One experiment representative of four independent experiments is shown. (A, B, D, E) Data are shown as the mean $\pm$ SD of $n = 4$ and are representative of three experiments. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$, one-way ANOVA.

the higher IL-10 production by iCD8 α^- DCs may be the major mechanism for inhibiting Th2-cell responses.

iCD8 α^- DCs induced more IL-10 producing OVA-specific CD4 $^+$ T cells than iCD8 α^+ DCs

Having shown the major role of IL-10 production by iCD8 α^- DCs in inhibiting IL-4 production by OVA-specific CD4 $^+$ T cells, we further examined the capacity of this DC subset in inducing T-cell IL-10 production. We co-cultured CD4 $^+$ T cells isolated from naïve DO11.10 mice with iCD8 α^+ DCs or iCD8 α^- DCs for 48 h

and performed intracellular IL-10 staining for T cells. As shown in Fig. 5A and B, co-culture with iCD8 α^- DCs, compared with co-culture with iCD8 α^+ DCs, led to significantly higher percentage of IL-10-producing allergen-specific CD4 $^+$ T cells. Significantly higher levels of IL-10 were also found in the co-culture with iCD8 α^- DCs than iCD8 α^+ DCs (Fig. 5C). In combination with the data on Th1 cytokine analysis in vivo and in vitro (Figs. 3 and 4), the results suggest that iCD8 α^+ DCs may modulate allergic Th2 cells mainly by enhancing immune deviation (change Th1/Th2 balance) while iCD8 α^- DCs may inhibit the Th2-cell response mainly through induction of IL-10-producing Treg cells and/or by directly inhibiting Th2 cells.

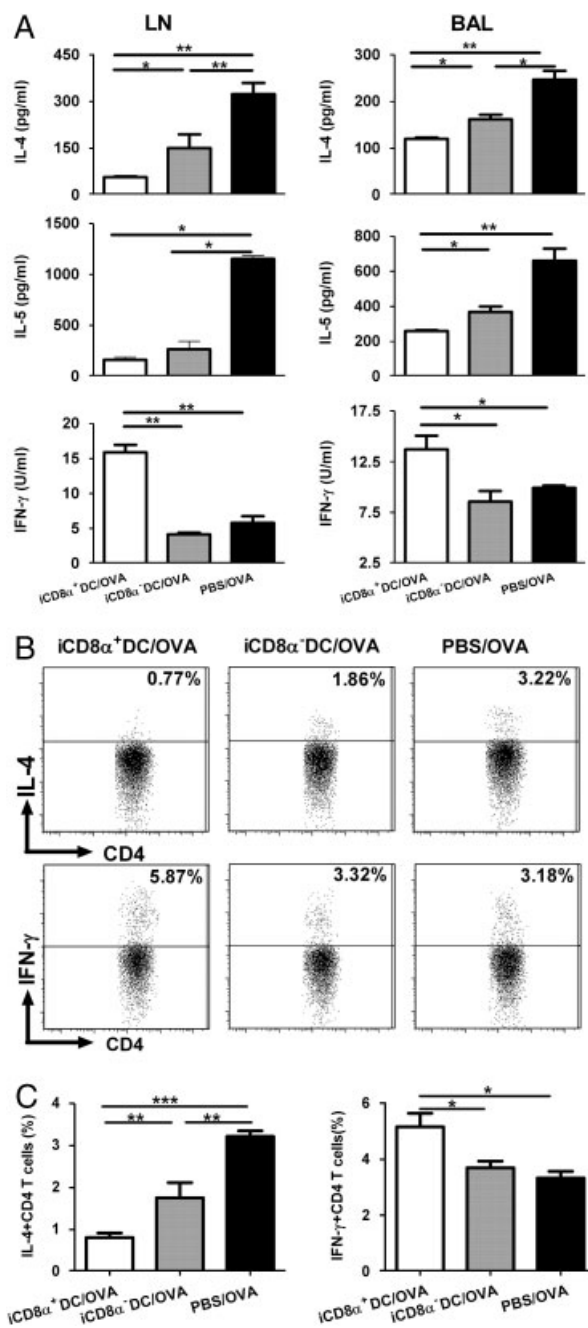


Figure 3. Effect of iDC subset transfer on allergen-driven cytokine production by draining LN cells and in the BAL. Recipients ($n = 4$ /group) of iDC subsets were sensitized and challenged with OVA as described in Fig. 2. At day 7 after OVA challenge, draining lymph nodes were collected and cells (5×10^6 cells/mL) were cultured for 72 h with re-stimulation of OVA. (A) Cytokines in the culture supernatants of draining LNs (left) and in BAL (right) were measured by ELISA. (B) Cells from draining LNs were restimulated with PMA/ionomycin for 6 h and Brefeldin A was added during the last 3 h incubation. Cells were gated on CD3 ϵ^+ CD4 $^+$ T cells and evaluated for IFN- γ and IL-4 production by flow cytometry. Data shown are representative of three independent experiments. (C) The frequencies of IL-4-producing (left) and IFN- γ -producing (right) CD4 $^+$ T cells in draining LNs of each group are shown. Data are presented as mean $\pm$ SD of $n = 4$ and are representative of three independent experiments. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$, one-way ANOVA.

iCD8 α^- DCs promoted Treg cell responses with higher TGF- β expression and IL-10 production

To directly examine the capacity of iCD8 α^- DCs in inducing Treg cells in vivo, we adoptively transferred iCD8 α^- DCs and iCD8 α^+ DCs and examined IL-10 production by cells in draining LNs and the development of CD4 $^+$ CD25 $^+$ Foxp3 $^+$ T cells following OVA sensitization and challenge. Similar to that observed in the co-culture experiments, the adoptive transfer of iCD8 α^- DCs, but not iCD8 α^+ DCs, significantly increased IL-10 production in vivo (data not shown). In fact, the transfer of iCD8 α^- DCs inhibited, instead of increased, IL-10 production. More specific analysis showed significantly enhanced CD4 $^+$ CD25 $^+$ Foxp3 $^+$ Treg cells in the recipients of iCD8 α^- DCs, but not iCD8 α^+ DCs (Fig. 6A and B).

Since surface expressed TGF- β and the production of IL-10 have been found to be related to the function of Treg cells [33, 34], we further examined CD4 $^+$ T cells from the recipients of iCD8 α^- DCs, the only DC subset inducing CD4 $^+$ CD25 $^+$ Foxp3 $^+$ Treg cells, in the expression of TGF- β and production of IL-10. As shown in Fig. 7, the transfer of iCD8 α^- DCs significantly enhanced the percentage of surface-TGF- β -expressing CD4 $^+$ T cells and IL-10-producing T cells. Due to technique limitations, we were unable to directly examine TGF- β expression and IL-10 production in CD4 $^+$ CD25 $^+$ Foxp3 $^+$ Treg cells, but the consistency of these parameters in the separated analyses suggest the possible induction of TGF- β -expressing and IL-10-producing CD4 $^+$ CD25 $^+$ Foxp3 $^+$ Treg cells by iCD8 α^- DCs although other types of TGF- β expressing and/or IL-10-producing Treg cells may also be induced.

iCD8 α^- DCs but not iCD8 α^+ DCs expressed ICOS-L

The above results strongly suggest a tolerogenic nature of iCD8 α^- DCs. Since our and other recent studies have shown an important role of ICOS-L expression on the tolerogenic function of DCs [20, 35], we further examined ICOS-L expression by the iCD8 α^- DCs and iCD8 α^+ DCs. RT-PCR analysis showed a clear ICOS-L message expression in the iCD8 α^- DCs but not iCD8 α^+ DCs (Fig. 8A). Moreover, flow cytometric analysis of these DC subsets showed significant surface expression of ICOS-L in the iCD8 α^- DCs but not iCD8 α^+ DCs (Fig. 8B). In addition, comparison of ICOS-L expression by CD8 α^- DCs from naïve and BCG-infected mice showed a promoting effect of BCG infection on the percentage of ICOS-L-positive CD8 α^- DCs (Fig. 8C) and the density of ICOS-L expression on these DCs (Fig. 8D). The data showed significant expression of ICOS-L on CD8 α^- DCs which was enhanced by BCG infection, was consistent with the above observed Treg cell promoting effect of this DC subset, thus likely a molecular basis for its tolerogenic function in addition to its higher IL-10 production.

Discussion

In this study, we have demonstrated that both iCD8 α^+ and iCD8 α^- DCs, but not nCD8 α^+ and nCD8 α^- DCs, can inhibit the

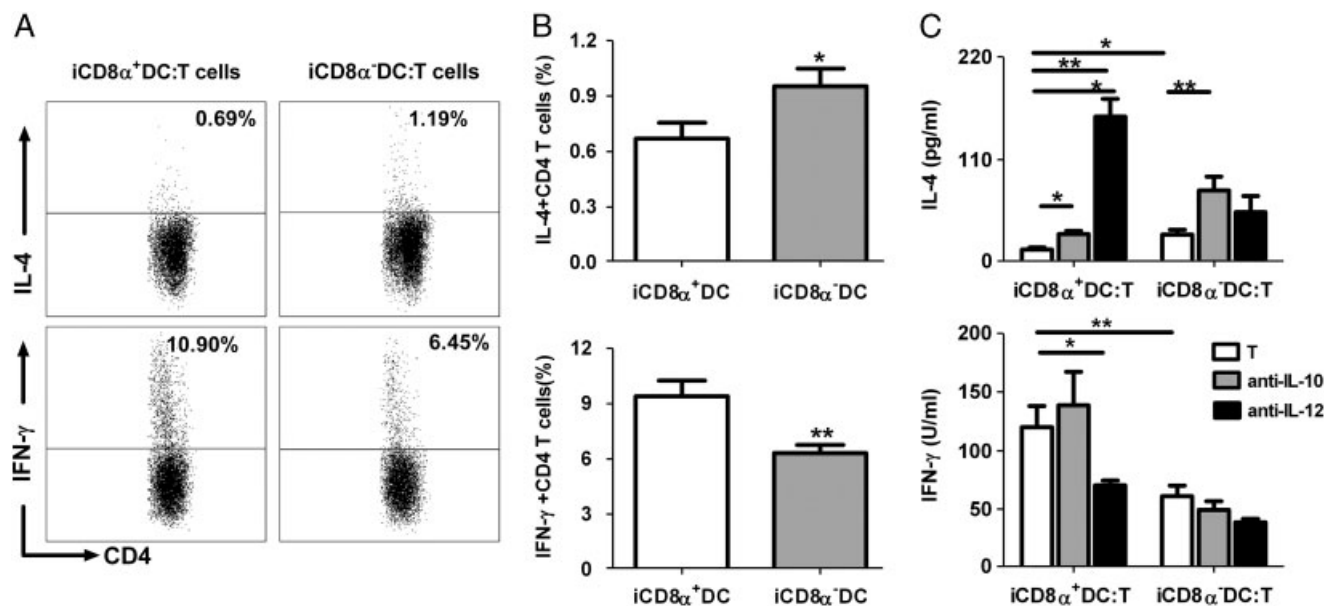


Figure 4. Different ability of iDC subsets to direct IL-4 and IFN-γ production by naïve OVA-specific CD4⁺ T cells and the contribution of IL-10 and IL-12. OVA peptide-specific CD4⁺ T cells isolated from naïve DO11.10 transgenic mice were co-cultured with freshly isolated iDC subsets (DC:T ratio, $5 \times 10^5:5 \times 10^6$) with OVA stimulation (0.1 mg/mL). (A) After 48 h co-culture, CD4⁺ T cells were analyzed for intracellular IFN-γ or IL-4 by flow cytometry. (B) The frequencies of IL-4 and IFN-γ-producing CD4⁺ T cells are shown. (C) The OVA-specific CD4⁺ T cells were co-cultured with iDC subsets with OVA stimulation in the absence or presence of anti-IL-10 or anti-IL-12 mAb for 72 h as described in *Materials and Methods*. The levels of IFN-γ and IL-4 in culture supernatants were determined by ELISA. (B, C) Data are shown as mean±SD of $n = 3$ and are representative of three independent experiments with similar results. * $p < 0.05$, ** $p < 0.01$, one-way ANOVA.

development of allergic airway inflammation in BALB/c mice, suggesting a critical role of BCG infection in modulating the function of different DC subsets. More importantly we found the two DC subsets from BCG-infected mice inhibited allergic reactions through different mechanisms. Specifically, iCD8α⁺ DCs inhibited airway eosinophilic inflammation mainly through switching Th2-dominant allergen-driving CD4⁺ T-cell response to Th1-dominant response while iCD8α⁻ DCs inhibited the allergic reactions mainly via increasing IL-10 production and generating regulatory T cells. Indeed, we found that more CD4⁺ T cells isolated from naïve OVA TCR transgenic mice (DO11.10) co-cultured with iCD8α⁺ DCs became IFN-γ-producing Th1 cells (Fig. 4) while the same CD4⁺ T cells tended to develop into IL-10-producing Treg cells (Fig. 5) when co-cultured with iCD8α⁻ DCs. Moreover, adoptive transfer of either iDC subset significantly inhibited the allergic airway eosinophilic inflammation and mucus over-production, IgE production and Th2 cytokine (IL-4 and IL-5) responses induced by OVA sensitization and challenge. Notably, the adoptive transfer of iCD8α⁺ DCs led to significant increase of IFN-γ in the local tissues (lung) and its production by cells from dLNs following OVA-specific re-stimulation. In contrast, the adoptive transfer of iCD8α⁻ DCs significantly enhanced the level of IL-10 in the local tissue and, more interestingly, CD4⁺CD25⁺Foxp3⁺Treg cell responses. The strong capacity of the iCD8α⁻ DCs in inducing Treg cells and tolerance was confirmed by the enhanced induction of IL-10-producing and membrane TGF-β-expressing CD4⁺ T cells (Fig. 7). These results generated from both in vitro and in vivo studies confirmed our

previous reports on the important role of DCs in infection-mediated inhibition of allergic responses and further demonstrated the involvement of variable mechanisms used by different DC subsets in the inhibition of allergy. At the same time, it should be pointed out that the infection route with BCG in this study (i.v.) is not the same with most natural mycobacterial infections which happen in lung. The reason to choose the i.v. infection model is because DC subsets in the spleen is better characterized. Future studies on lung infection models and humans would be important for testing the relevance of the finding.

The finding on the involvement of different mechanisms related to DC subsets has implication on understanding the mechanism of hygiene hypothesis. Notable debates are currently ongoing on the mechanisms of hygiene hypothesis, especially for the modulating effect on allergen-driven Th2-cell responses by infections. The major controversy is whether the modulating effect is mediated by immune deviation (Th2 switching to Th1) or by immune regulation (tolerance and Treg-cell development) [5, 7]. Numerous reports have shown the involvement of either mechanism in various infections. In the present study, we showed that both mechanisms can operate in a single type of infection and this coordination can be done by DC subsets. Indeed, the iCD8α⁺ DCs mainly enhance allergen-driven Th1 response, thus modulating the Th2 response through immune deviation while the iCD8α⁻ DCs mainly induced Treg cells, thus reducing Th2 via immune regulation and tolerance. Therefore, both mechanisms are valid and are important for the inhibition of allergy by infections. On the other hand, in this BCG infection model, that CD8α⁺

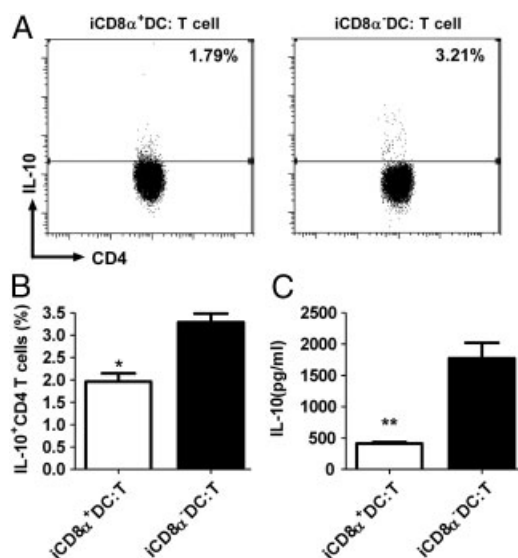


Figure 5. iCD8 α^- DCs are more efficient at promoting IL-10-producing CD4 $^+$ T cells than iCD8 α^+ DCs. OVA peptide-specific CD4 $^+$ T cells isolated from naïve DO11.10 transgenic mice were co-cultured with freshly isolated iCD11c $^+$ CD8 α^+ and iCD11c $^+$ CD8 α^- DC subsets (DC:T ratio, 5×10^5 : 5×10^6) with OVA stimulation (0.1 mg/mL). (A) After 48 h co-culture, cells were collected and stimulated with PMA/ionomycin for 8 h, Brefeldin A was used in the last 4 h. CD4 $^+$ T cells were analyzed for intracellular IL-10 production by flow cytometry. Dot plots are representative of $n=3$. (B) The percentage of IL-10-producing CD4 $^+$ T cells in co-culture are shown. (C) The 72 h co-culture supernatants of each group were tested for IL-10 protein levels by ELISA. (B, C) The data are shown as mean $\pm$ SD of $n=3$ and are representative of two independent experiments with similar results. * $p<0.05$, ** $p<0.01$, one-way ANOVA.

DCs were more powerful than CD8 α^- DCs in inhibiting allergic Th2-cell responses and airway inflammation. Therefore, although BCG infection can inhibit allergy through both mechanisms, immune deviation is likely a more dominant mechanism than immune regulation in this intracellular bacterial infection. This is consistent with numerous previous reports showing significantly enhanced allergen-driven Th1-cell response in BCG infected/vaccinated mice and humans [6, 11–15]. This is also consistent with the nature of mycobacterial infections which are found to mainly induce Th1-cell type responses [13, 14, 36]. Notably, however, *Mycobacterium vaccae* infection has been reported to induce Treg cells which are inhibitory for allergic responses [23]. Our data demonstrated that even for an infection which mainly induces a Th1-cell response, it is still able to modulate immune response through multiple mechanisms, for which different DC subsets likely play a critical role for modulating the respective mechanisms.

Our data identified several characteristics of iCD8 α^- DCs which might be related to their tolerogenic function. First, the iCD8 α^- DCs produce higher levels of IL-10 than iCD8 α^+ DCs. This was demonstrated by quantitative RT-PCR of freshly isolated DCs and ex vivo culture of these cells. The importance of IL-10 production for the function of tolerogenic DCs has been found in many studies [37, 38]. Second, iCD8 α^- DC expressed ICOS-L on their surface, which was significantly increased following BCG infection. It has

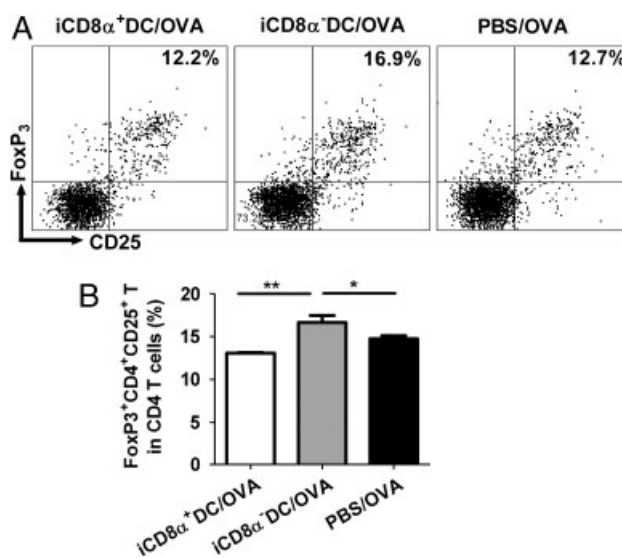


Figure 6. Adoptive transfer of iCD8 α^- DCs, but not iCD8 α^+ DCs, enhances CD4 $^+$ CD25 $^+$ Foxp3 $^+$ T cells following OVA sensitization and challenge. Mice were treated with iDC subsets and OVA as described in the legend to Fig. 2. At day 7 following intranasal OVA challenge, 2×10^6 draining LN cells were stained for expression of CD3e, CD4, CD25 and Foxp3 and analyzed by flow cytometry as described in Materials and Methods. (A) Representative dot plots of Foxp3 $^+$ CD25 $^+$ cells as a percentage of total CD3e $^+$ CD4 $^+$ T cells are shown. (B) A summary of the frequency of CD4 $^+$ CD25 $^+$ Foxp3 $^+$ T cells in draining LNs. Data are shown as mean $\pm$ SD of $n=3$ and are representative of three independent experiments with similar results. * $p<0.05$, ** $p<0.01$, one-way ANOVA.

been reported that expression of ICOS-L on DCs is important for the maintenance of immune homeostasis. ICOS/ICOS-L signaling is essential for IL-10-producing tolerogenic DCs to induce T-cell anergy [39]. Without ICOS-L co-stimulation by DCs, IL-10 failed to influence the differentiation and cytokine production by CD4 $^+$ T cells [39]. Moreover, several studies have shown the importance of ICOS/ICOS-L signaling in Treg-cell responses [40–42]. The co-expression/production of ICOS-L and higher levels of IL-10 by the iCD8 α^- DCs shown in the present study provided a molecular basis for the synergistic effect in inducing allergic Th2 cell anergy and Treg cells. In this respect, the iCD8 α^+ DC showed a quite different pattern in phenotype, displaying higher IL-12 production and lower IL-10 production. The contribution of the differently produced cytokines by the different DC subsets in inducing Th1-cell response and suppressing allergic Th2-cell response was confirmed in the co-culture experiments with neutralization of IL-10 and IL-12 activity, respectively (Fig. 4). Moreover, it was found that CD8 α^+ DCs from both naïve and BCG infected mice did not express ICOS-L, shown by RT-PCR and flow cytometric analyses, demonstrating a selective influence of the infection on ICOS-L expression on CD8 α^- DCs. Indeed, blockade of ICOSL in the co-culture of CD4 $^+$ T cells from OVA immunized mice with CD8 α^- DCs significantly increased the proportion of IL-4-producing CD4 $^+$ T cells (data not shown). This sharp contrast in cytokine production and ICOS-L expression provided a basis for the difference of these DC subsets in inducing different type of T cells, particularly Treg and Th1 cells. Another interesting finding on

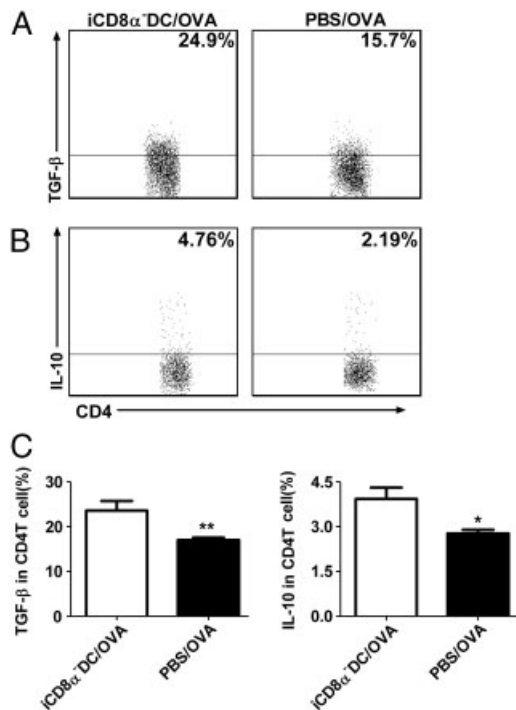


Figure 7. Lung CD4⁺CD3 ϵ ⁺ T cells in the recipients of iCD8 α ⁻ DCs express higher membrane-bound TGF- β and produce more IL-10 than control mice following OVA exposure. Mice were pre-treated with iCD8 α ⁻ DCs or PBS followed by OVA sensitization and challenge as described in the legend to Fig. 2. Lungs were collected at day 7 after challenge and digested by collagenase XI. (A) 2×10^6 cells were stained for expression of CD3 ϵ , CD4, and TGF- β and analyzed by flow cytometry. (B) Intracellular IL-10 production by lung CD4⁺ T cells was evaluated by stimulating lung mononuclear cells with PMA/ionomycin, permeabilizing the cells with cytopermabilization buffer as described in *Materials and Methods* and staining for intracellular IL-10 production. (C) The frequencies of CD3 ϵ ⁺CD4⁺ T cells expressing TGF- β and producing IL-10 in mice that received CD8 α ⁻ DCs and those of the control group are shown. Data are shown as mean $\pm$ SD of $n = 3$ and are representative of two independent experiments with similar results. * $p < 0.05$, ** $p < 0.01$, one-way ANOVA.

surface molecules is the difference of the DC subsets in surface CD86 expression. Unlike CD80 and CD40 molecules which were significantly enhanced in levels in both iCD8 α ⁻ and iCD8 α ⁺ DCs, the expression of CD86 was only increased in iCD8 α ⁻ DCs (Fig. 1). The preferential increase of CD86 by iCD8 α ⁻ DCs might also contribute the suppressive function of these DCs on allergic Th2-cell responses. Notably, it has been reported that CD86 expression controls the suppressive function of DCs in mycobacterial infection [43]. Moreover, although having been found to be important for the induction of Th1-cell responses in numerous studies, CD40/CD40L signaling is also critical for inducing IL-10 production by tolerogenic DCs [44]. Therefore, some co-stimulatory molecules may be preferentially important for a subset of T-cell response, such as ICOS/ICOS-L signaling particularly for Treg cells, while others for multiple T-cell subsets, such as CD80 and CD40 signaling for both Th1 and Treg cells, depending on the expression/production of other molecules by a particular DCs. Further study on the relevance of individual molecules and, more importantly, the combination of these molecules in the induction of different T-cell

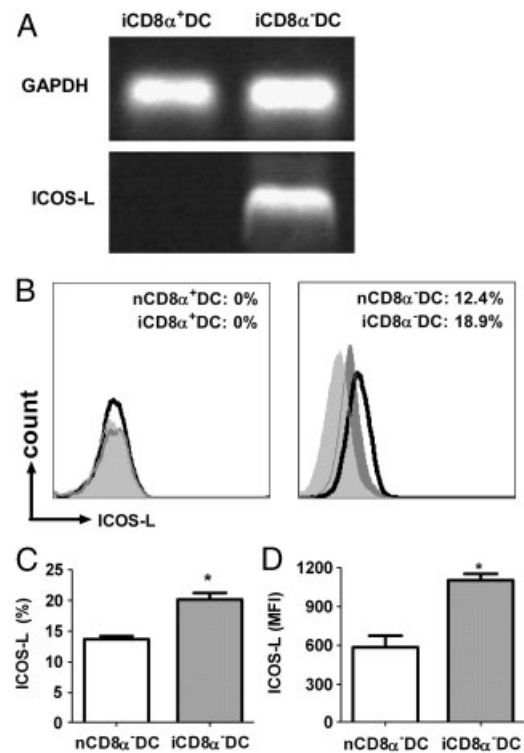


Figure 8. BCG infection enhances ICOS-L expression on iCD8 α ⁻ DCs but not on iCD8 α ⁺ DCs. Mice were infected with BCG intravenously. At 21 days after infection, mice were sacrificed and spleen DCs were analyzed for ICOS-L expression by RT-PCR and flow cytometry. (A) iCD8 α ⁻ DC and iCD8 α ⁺ DC subsets were sorted by flow cytometry and semi-quantitative RT-PCR was performed to detect the mRNA levels of ICOS-L. (B) Total CD11c⁺ DCs were isolated by MACS column and triple-stained for expression of CD11c, CD8 α and ICOS-L. Cells were gated on double positive (left, CD11c⁺CD8 α ⁺) and single positive (right, CD11c⁺CD8 α ⁻) cells. Representative flow histograms are shown. Light shaded areas, isotype control; Dark shaded areas, naive DCs; solid lines, DC subsets isolated from BCG infected mice. (C) The percentages of ICOS-L-positive CD8 α ⁻ DCs in naive and BCG-infected mice are shown. (D) The mean fluorescence intensity (MFI) of ICOS-L on CD8 α ⁻ DCs in naive and BCG-infected mice. Data are shown as mean $\pm$ SD of $n = 3$ and are representative of two independent experiments with similar results. * $p < 0.05$, one-way ANOVA.

subsets, particularly the induction of Treg cells, would be very helpful for understanding the mechanisms by which different DC subsets from infected mice inhibit allergic Th2-cell reactions.

In summary, our data have demonstrated the co-existence of immune deviation and regulatory mechanisms for the modulating effect on Th2-cell allergic reactions by an intracellular bacterial infection. Moreover, we have shown the role of different DC subsets in infection-mediated inhibition of allergy in determining the initiation of the different inhibition mechanisms. This study has provided new insight into the mechanism of hygiene hypothesis. Further study on the relative contribution and interaction of the different mechanisms in modulation of allergic diseases mediated by different types of infections, and the cellular and molecular basis for the induction and maintenance of the different mechanisms in vivo will be helpful for better understanding immune regulation and for developing new preventive and therapeutic strategies for allergic and autoimmune diseases.

Materials and methods

Adoptive transfer of DC subsets and OVA sensitization and challenge

Female Balb/c (6–8 wk) mice were infected with BCG (5×10^5 CFUs) through the lateral tail vein injection. Twenty-one days later, the spleens were aseptically collected and whole DCs were enriched using a MACS (Miltenyi Biotech, Auburn, CA) CD11c beads column. Freshly enriched DCs were further sorted by a flow cytometer to CD8 α^+ DC and CD8 α^- DC subsets based on CD11c and CD8 α markers as described [45]. The purity of DC subsets were >98%. For adoptive transfer experiments, 5×10^5 DC subset cells in 200 μ L sterile protein-free PBS were injected intravenously to recipient mice. Control mice were treated with 200 μ L PBS. 2 h after DC subset transfer, recipient mice were sensitized intraperitoneally (i.p.) with 2 μ g OVA in alum followed by intranasally (i.n.) challenge with 50 μ g OVA (40 μ L) at 14 days later. Mice were sacrificed 7 days later for analysis of airway inflammation and immune responses as described [15, 20].

Histological analysis

Lung tissues were routinely fixed in 10% buffered formalin, embedded, sectioned and stained with hematoxylin and eosin (H & E) as previously described [14]. Slides were examined for pathological changes by light microscopy [46]. Bronchial mucus and mucus-containing goblet cells within airway bronchial epithelium were stained by a periodic-acid Schiff (PAS) staining kit (Sigma) as described [46]. The mucus secretion was quantified by histological mucus index (HMI), which represents the percentage of the area of mucus-positive epithelium (Goblet cells) in the total area of airway epithelium, using Image-Pro Plus software (Media Cybernetics) [14].

Isolation of CD4 $^+$ T cell and DC subsets – T-cell co-culture

Naive CD4 $^+$ T cells were isolated from the spleens of DO11.10 OVA peptide-specific TCR- $\alpha\beta$ transgenic mice (Balb/c background) using a MACS LS CD4 positive selection column (Miltenyi Biotech). The purified CD4 $^+$ T cells (5×10^6 cells/well) were co-cultured with DC subsets isolated from BCG-infected mice (5×10^5 cells/well) with OVA (0.1 mg/mL) stimulation as described [20, 21]. For CD4 T cell intracellular IFN- γ and IL-4 staining, cells were collected at 48 h. For intracellular IL-10 staining, the cells were collected at 72 h of co-culture. For testing cytokines in the culture supernatants, the supernatants were collected at 72 h and tested by ELISA. In designated experiments, anti-IL-12 or anti-IL-10 mAb (PharMingen, San Diego, CA, USA) was added to the co-culture wells at a final concentration of 5 μ g/mL to block endogenous IL-12 or IL-10 activities.

Intracellular cytokines and Foxp3 measurement

For intracellular cytokine analysis, cells isolated from the draining LNs, lung mononuclear cells and cells collected from DC:T-cell co-culture system were stimulated with PMA (50 ng/mL, Sigma) and ionomycin (1 μ g/mL, Sigma) and incubated for 6 h in complete RPMI-1640 medium at 37°C. For the last 3 h incubation, Brefeldin A (Sigma) was added to accumulate cytokines intracellularly as described [21]. Cells were then stained with fluorescence-conjugated antibodies for cell surface markers, including FITC-anti-CD3 ϵ , PE-anti-CD4 and PE-Cy7-anti-CD25. For Foxp3 expression measurement, the cells from draining LNs were collected and stained for cell surface markers (CD3 ϵ , CD4, CD25) without stimulation as described by the manufactory instruction. After surface marker staining, cells were permeabilized and stained intracellularly with a specific allophycocyanin-conjugated antibody for specific cytokine (IFN- γ , IL-4, and IL-10 from eBiosciences) or Foxp3 (eBiosciences) or corresponding isotype control Abs in permeabilization buffer (BD PharMingen).

Statistic analysis

One-way ANOVA (one-way analysis of variance) was used to determine statistic significance of difference among groups and further Newman–Keulse test was used to compare any two groups for statistic difference.

Acknowledgements: X. G. was a PhD trainee in of the CIHR National Training Program in Allergy and Asthma and recipient of Manitoba Health Research Council/Manitoba Institute of Children Health Graduate Studentship. X. Y. is the Canada Research Chair in Infection and Immunity. This work was supported by a grant from Canadian Institutes of Health Research (CIHR) to X.Y.

Conflict of interest: The authors declare no financial or commercial conflict of interest.

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Abbreviations: BAL: broncho alveolar lavage · Foxp3: forkhead box P3

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Received: 8/6/2011
 Revised: 8/9/2011
 Accepted: 13/10/2011
 Accepted article online: 18/10/2011