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Dendritic Cells from Mycobacteria-Infected Mice Inhibits Established Allergic Airway Inflammatory Responses to Ragweed via IL-10- and IL-12-Secreting Mechanisms

Laura Bilenki, Xiaoling Gao, Shuhe Wang, Jie Yang, Yijun Fan, Xiaobing Han, Hongyu Qiu, and Xi Yang

Previous studies have demonstrated that *Mycobacterium bovis* bacillus Calmette-Guerin (BCG) infection can inhibit de novo and established allergen-induced asthma-like responses. The aim of this study was to examine the role of dendritic cells (DCs) in BCG infection-mediated inhibition of established allergy to a common environmental allergen—ragweed. The results showed that adoptive transfer of DCs from BCG-infected mice (DC[BCG]), in contrast to DCs from naive mice (DC[naive]), significantly inhibited established allergic airway eosinophilia and mucus overproduction. The inhibitory effect was correlated with alterations of allergen-driven cytokine and chemokine production as well as VCAM-1 expression in the lung. Flow cytometric analysis showed higher surface expression of CD8 α and costimulatory markers in DC(BCG) than in DC(naive). Moreover, DC(BCG) produced significantly higher levels of IL-10 and IL-12 and expressed higher levels of TLRs than did DC(naive). Furthermore, blockade of IL-10 or IL-12 significantly reversed the inhibitory effect of DC(BCG) on established allergic airway inflammation and Th2 cytokine responses. These findings suggest that DCs play a crucial role in infection-mediated inhibition of established allergic responses, and IL-10 and IL-12 production by these DCs may be a major mechanism for the inhibition. *The Journal of Immunology*, 2010, 184: 000–000.

It is evident from a variety of studies undertaken in a range of geographic areas that the prevalence of allergic diseases, particularly atopic asthma, has increased dramatically over the past two to three decades and are spreading among populations living a Western lifestyle (1). This allergy epidemic has been attributed to various factors, such as pollution, changes of diet, allergen exposure, and more recently, overly increased hygiene (2–4). The so called hygiene hypothesis proposes that the reduced rate of exposure to infectious agents, especially in developed areas, is directly related to the increase of allergic diseases, such as allergic asthma, atopic eczema, and rhinitis, because certain infections have an inhibitory effect on allergic reactions (5–7). According to this hypothesis, changing interactions between humans and microbial agents caused by overly increased hygiene

can alter the balance between Th1- and Th2-like immune responses, thereby predisposing one to atopic diseases (8–10). More recently, a mechanism of T cell unresponsiveness caused by tolerogenic DCs and regulatory T (Treg) cells has been implicated for explaining the modulating effect of certain infections on allergy (11–14).

Dendritic cells (DCs) have been demonstrated to be crucial in the regulation of immune responses to Ags. They can preferentially skew naive Th cell differentiation into Th1- or Th2-like cells (15). As a result, DCs have been further characterized into two functional subsets, namely type 1 (DC1) and type 2 (DC2) cells (16–18). IL-12, which is mainly produced by DC1-like cells, has been shown to be particularly important for Th1-like T cell differentiation. It was also found that the CD8 α molecule is often, though not always, seen on the surface of DC1-like cells in mice (19). Interestingly, it has been reported that the balance between DC1 and DC2 cells in the periphery differs among atopic and nonatopic individuals (20, 21). Alternatively, the importance of IL-10-producing DCs in inducing Treg cells and causing T cell unresponsiveness has been well established (22–24).

Previous studies have demonstrated that mycobacterial infection can inhibit de novo and established airway allergic responses in a murine model, characterized by reduced allergen-driven Th2-like cytokine production and airway eosinophilic inflammation (25–28). The cellular and molecular basis for the infection-mediated inhibition of de novo and established allergy remains unclear. Because a key point in determining Th cell differentiation is the interaction between DCs and T cells, we hypothesized that DCs play a central role in *Mycobacterium bovis* bacillus Calmette-Guérin (BCG) infection-mediated inhibition of allergic asthma-like reactions. To test this hypothesis, we performed the current study using an adoptive transfer approach to examine the ability of freshly isolated DCs from BCG-infected mice (DC[BCG]) in redirecting established allergic response to ragweed (RW), a common environmental allergen that afflicts allergic individuals on a seasonal basis. Our

Immune Regulation of Allergy Research Group, Department of Medical Microbiology and Department of Immunology, University of Manitoba, Winnipeg, Manitoba, Canada

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Address correspondence and reprint requests to Dr. Xi Yang, Department of Medical Microbiology and Infectious Diseases, Faculty of Medicine, University of Manitoba, 1730 William Avenue, Winnipeg, MB, Canada R3E 0W3. E-mail address: yangxi@cc.umanitoba.ca

Abbreviations used in this paper: alum, Al(OH)₃; BAL, bronchoalveolar lavage; BCG, bacillus Calmette-Guerin; DC, dendritic cell; DC(BCG), dendritic cells from BCG-infected mice; DC(naive), dendritic cells from naive mice; HMI, histologic mucus index; i.n., intranasal(ly); MLN, mediastinal lymph node; RW, ragweed; Treg, regulatory T.

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results demonstrate that DC(BCG), unlike DCs from naive mice (DC[naive]), are capable of inhibiting established allergic response induced by RW. The modulating effect was associated with a switch of allergen-driven cytokine production and reduced VCAM-1 and eotaxin expression. Comparative analysis of DC(BCG) and DC(naive) showed that the former had higher expression of costimulatory molecules, TLRs, and higher production of IL-10 and IL-12. More importantly, the blockade of IL-10 or IL-12 using neutralizing Abs significantly reduced the inhibitory effect of DC(BCG) on established allergic responses.

Materials and Methods

Animals

Female C57BL/6 mice (7–10 wk old) were bred and maintained at the University of Manitoba Central Animal Facility (Winnipeg, Manitoba, Canada). Animals were used in accordance with the guidelines issued by the Canadian Council on Animal Care and the protocol was approved by the University of Manitoba animal ethical use committee.

BCG infection and DC isolation and characterization

Naive mice were inoculated intravenously with 2×10^5 CFUs of *Mycobacterium bovis* BCG as previously described (25, 26). Twenty-one days postinfection, spleens were aseptically removed and DCs were isolated using MACS (Miltenyi Biotech, Auburn, CA) CD11c columns according to the manufacturer's instructions as described (29). Spleens were digested with Collagenase D (Boehringer Mannheim Biochemicals, Germany), and single cells suspensions were prepared in PBS with 0.5% BSA. MACS CD11c microbeads were used for positive selection of CD11c⁺ cells using the column. Purified DCs were used for adoptive transfer or analyzed for expression of cell surface markers including CD8 α , CD80, CD86, CD40, and MHC class II using a FACSCaliber II and CellQuest software (BD Biosciences, San Jose, CA). Fluorescence-conjugated mAbs with appropriate isotype controls were purchased from BD Pharmingen (San Diego, CA). Purity of isolated DCs was between 94 and 97%, based on flow cytometric analysis of CD11c⁺ cells.

Allergen sensitization and challenge

Mice were immunized and treated as described (25, 26, 30). Mice were initially sensitized i.p. with 100 μ g of RW extract (Hollister-Stier Canada Co., Toronto, Canada) in 2mg Al(OH)₃ (alum) adjuvant. On day 14 post-sensitization, mice were challenged intranasally (i.n.) with 150 μ g RW (40 μ l). Twelve days after RW challenge, mice were injected i.v. or i.n. with DCs (2×10^6 cells/mouse) isolated from BCG-infected or naive mice (see below). In designated experiments, the recipients were treated with i.p. injection of 1 mg purified neutralizing anti-IL-10 (SXC1) and anti-IL-12 Ab (C17.8; provided by Dr. David Gray, University of Edinburgh, Edinburgh, U.K.) immediately after DC transfer. Two hours after DC transfer, mice were rechallenged i.n. with RW (150 μ g). Mice were euthanized 6–8 d later and analyzed for allergic and immune responses to RW.

Bronchoalveolar lavage and blood differential cell counts

Mouse tracheas were cannulated and lungs were washed twice with 1 ml of sterile PBS to collect bronchoalveolar lavage (BAL) fluids. Fluids were centrifuged immediately and cell pellets were resuspended to prepare BAL smears. The slide was air-dried, fixed with ethanol, and stained with the Fisher Leukostat Stain Kit (Fisher Scientific, Nepean, Ontario, Canada). Numbers of monocytes, lymphocytes, and eosinophils per 200 cells were counted based on their morphology and staining characteristics. For analyzing blood leukocytes, blood smears from mice were prepared and stained for leukocytes using a Hema 3 Stain Set (Fisher Scientific) containing a cell fixative and eosin Y stain. For measuring eotaxin-1, the BAL fluid supernatants were tested using ELISA as described (30).

Histopathologic analysis

Lungs of RW-treated mice with DC transfer from either infected or naive mice were collected and fixed in 10% buffered formalin. Lung tissues were embedded, sectioned, and stained by H&E as described (25, 26). For analysis of mucus production, bronchial mucus and mucus-containing goblet cells were stained by periodic-acid Schiff staining kit (Sigma-Aldrich, St. Louis, MO) and quantified as a histologic mucus index (HMI) as previously described (31). The HMI represents the ratio of the

area of mucus-positive epithelium over the total area of bronchial epithelium. VCAM-1 was measured by immunohistochemical analysis and the intensity was scored as described (25).

Cell culture and cytokine determination

Mice were euthanized at day 6–8 following the last i.n. RW challenge and examined for cytokine production patterns by both spleen and lymph node cells. Spleens and draining mediastinal lymph nodes were aseptically removed, and single-cell suspensions were cultured as previously described (25, 26). Spleen and lymph node cells were cultured at a concentration of 7.5×10^6 cells/ml and 5.0×10^6 cells/ml, respectively, in the presence or absence of RW Ag (0.1 mg/ml). The complete culture medium was RPMI 1640 containing 10% heat-inactivated FBS, 25 μ g/ml gentamycin, 2 mM L-glutamine, and 5×10^{-5} 2-ME (Kodak, Rochester, NY). Duplicate cultures were established from the spleen and lymph node cells of individual mice in each group. Culture supernatants were harvested at 72 h for the measurement of cytokines. For determination of spontaneous cytokine production, DCs from BCG-infected or control mice were plated at a concentration of 2.5×10^6 cell per well in 48-well culture plates and incubated at 37°C for 72 h. Cytokines in the supernatants of spleen, lymph node, and DC cultures were analyzed by ELISA using purified (capture) and biotinylated (detection) Abs as previously described (30). Abs purchased from BD Pharmingen were used for ELISAs measuring IL-4, IL-5, IL-9, IL-10, IL-12, and IFN- γ . IL-13 was determined using paired Abs from R&D Systems (Minneapolis, MN).

Ab analysis

ELISAs were used for determination of RW-specific IgE, IgG1, and IgG2a Abs. Sera were examined for RW-specific IgG1 and IgG2a using biotinylated goat anti-mouse IgG1 or goat anti-mouse IgG2a Abs purchased from Southern Biotechnology Associates (Birmingham, AL), as described previously (25, 26). For determination of RW-specific IgE, sera were incubated twice with a 50% slurry of protein G-Sepharose (Pharmacia, Uppsala, Sweden) to remove most of the serum IgG1 and then measured for IgE using biotinylated anti-mouse IgE Ab purchased from BD

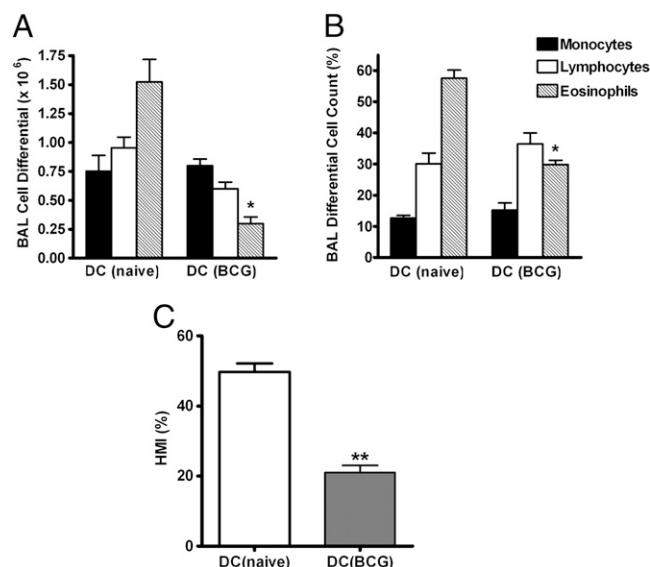


FIGURE 1. Mice treated with DCs from BCG-infected mice during established RW allergy show decreased pulmonary eosinophilic infiltration induced by RW. Mice were sensitized i.p. with 100 μ g RW in alum. The mice were challenged i.n. with 150 μ g RW at day 14 following RW sensitization. Mice were injected i.v. with DC(BCG) or DC(naive) from mouse spleens and rechallenged with 150 μ g RW i.n. Mice were sacrificed at day 6 post last RW-challenge. Pulmonary cellular infiltration was examined by differential cell counts of the BAL fluid cells. The absolute number (A) and percentage (B) of each type of infiltrating cells are shown. C, Lungs were fixed in 10% buffered formalin and the lung sections were examined for mucus and goblet cells by means of periodic acid-Schiff staining. HMI in each group is shown. Each group was composed of four mice. Data represent one of three independent experiments. * $p < 0.05$; ** $p < 0.01$, DC(naive) versus DC(BCG) groups.

Table I. Differential blood cell count in DC(naive) versus DC(BCG)-treated mice ($\times 10^3$ cells/mm³)

	DC(Naive)	DC(BCG)	No Transfer
Monocytes	5.68 $\pm$ 0.342	3.44 $\pm$ 1.950	6.23 $\pm$ 0.782
Lymphocytes	7.92 $\pm$ 1.650	2.72 $\pm$ 0.354	7.63 $\pm$ 0.399
Neutrophils	6.24 $\pm$ 0.323	2.04 $\pm$ 0.149	4.44 $\pm$ 0.342
Eosinophils	2.31 $\pm$ 0.021	0.90 $\pm$ 0.043 ^a	1.94 $\pm$ 0.321

Mice were treated with DC(naive), DC(BCG), or no DC transfer after ragweed sensitization and challenge as described in Fig. 1. Peripheral blood samples were counted for leukocyte differentials at day 6 following the last ragweed challenge. Data are presented as mean $\pm$ SD of each group, representing one of three independent experiments with similar results.

^a $p < 0.05$, compared with the groups receiving DC(naive) or no DC transfer.

Pharming. The treatment with protein G-Sepharose allowed the removal of $\sim 95\%$ of total IgG1 without affecting the concentration of IgE.

Semiquantitative and real-time RT-PCR analysis

For analysis of the expression of TLR gene expression in isolated DCs, RT-PCR was performed as described (30). Total cellular RNA was extracted from DCs followed by ethanol precipitation. The first-strand cDNA was synthesized from 1.2 μ g RNA in a final volume of 15 μ l using M-MLV reverse transcriptase (Invitrogen) and oligo (dT) primer. One microliter of cDNA was used for each PCR reaction. One microliter of cDNA was used

for each PCR reaction in a total volume of 20 μ l. The reaction condition for PCR was as follows: one cycle at 95°C for 5 min; 32 cycles (for TLR2) or 35 cycles (for TLR4 and TLR9) at 95°C for 1 min, at 55°C for 1 min, and 72°C for 1 min. β -Actin was used as a loading control. PCR products were run on a 1% agarose gel containing 0.1 mg/ml ethidium bromide. Image analysis was performed using Gel Doc 2000 gel documentation system (Bio-Rad, Hercules, CA) and quantified using Scion Image software (Scion Corporation, Frederick, MD). The specific primers for TLR2, TLR4, TLR9, and β -actin were as described (32). IL-10 and IL-12 messages in the DC preparation were measured by real time RT-PCR as described (24). The ratio of the copies of IL-10 with GAPDH and the ratio of IL-12 with GAPDH are shown in Fig. 7C.

Statistical analysis

Ab titers (ELISA) were converted to logarithmic values and analyzed using the unpaired Student *t* test. Differential cellular counts, total IgE, and cytokine production levels were analyzed using the unpaired Student *t* test.

Results

Adoptive transfer of DCs from BCG-infected mice to RW-sensitized/challenged mice inhibits airway eosinophilia and mucus overproduction

We have demonstrated previously that mycobacterial infection can inhibit established allergic inflammatory responses induced by allergen (25). Because DCs are key APCs capable of directing Th cell differentiation, we further analyzed the effect of DC(BCG) on the asthma-like reactions induced by RW in mice with established allergy. Mice were first sensitized (i.p.) and subsequently challenged i.n. with RW, then administered DC(BCG) i.v., and further challenged with RW. Control mice received the same RW treatment, but were administered DC(naive). The results showed that mice receiving DC(BCG) exhibited significantly less cellular infiltration into the lung than the controls (Fig. 1A), especially in eosinophils (1.6×10^6 versus 0.28×10^6 ; $p < 0.001$). The proportion of eosinophils in the total infiltrating cells was significantly less in DC(BCG) recipients than DC(naive) recipients (Fig. 1B). In addition, histologic analyses also showed remarkable differences in cellular infiltration into the lung of the two groups. DC(BCG) recipients displayed significantly less infiltration of eosinophils in the bronchial submucosa, alveolar, and perivascular sheaths than in DC(naive) recipients. In addition, the levels of goblet cell hyperplasia and mucus production in the DC(BCG) recipients were significantly lower than in those receiving DC(naive) (Fig. 1C). The results indicate that

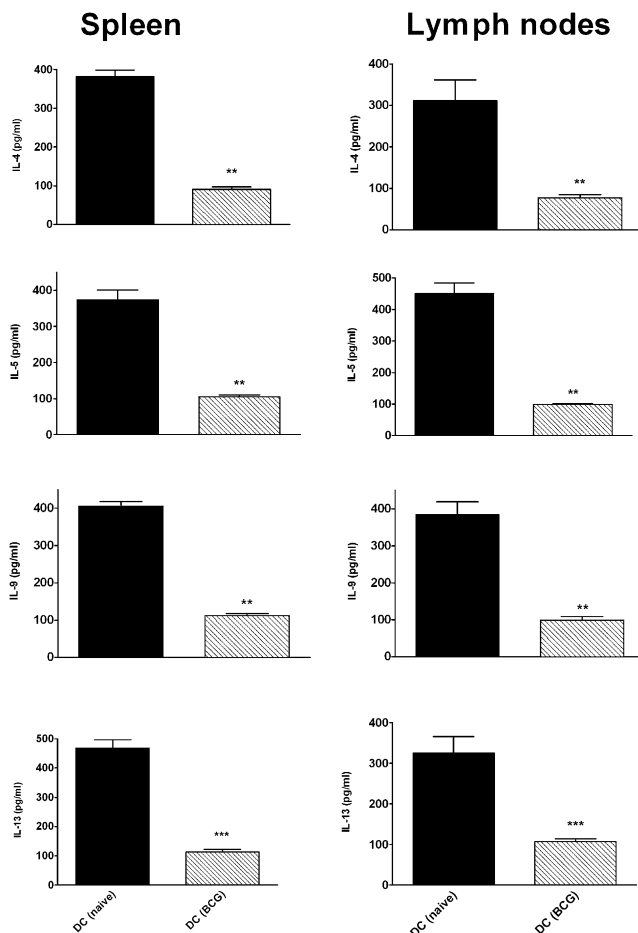


FIGURE 2. Dramatic reduction in allergy-driven Th2 cytokine production in mice treated with DCs from BCG-infected mice during established RW allergy. Mice (four mice per group) were treated as described in the legend to Fig. 1. When the mice were killed at day 6 post-RW rechallenged, spleen and MLN cells were cultured for 72 h in the presence of RW. The culture supernatants were tested for Th2 cytokines by ELISA. Data are the means $\pm$ SD of each group. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. One representative experiment of three independent experiments is shown.

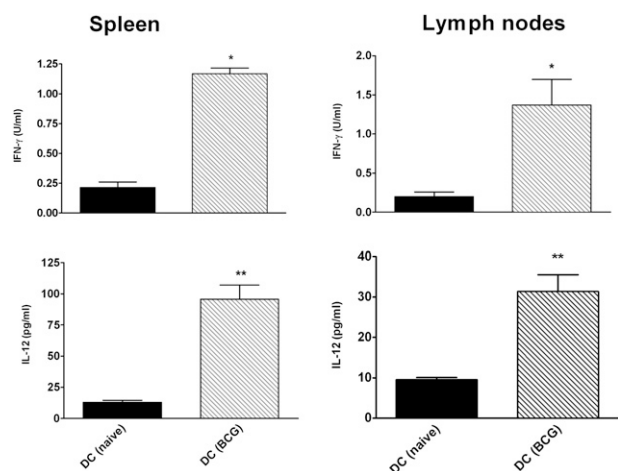


FIGURE 3. Significant increase in IFN- γ and IL-12 production in mice administered DCs from BCG-infected mice with established RW allergy. Seventy-two-hour culture supernatants of spleen and draining lymph node cells as described in Fig. 2 were determined for IFN- γ and IL-12 by ELISA. Data are presented as the means $\pm$ SD of each group. One representative of three independent experiments is shown. * $p < 0.05$; ** $p < 0.01$.

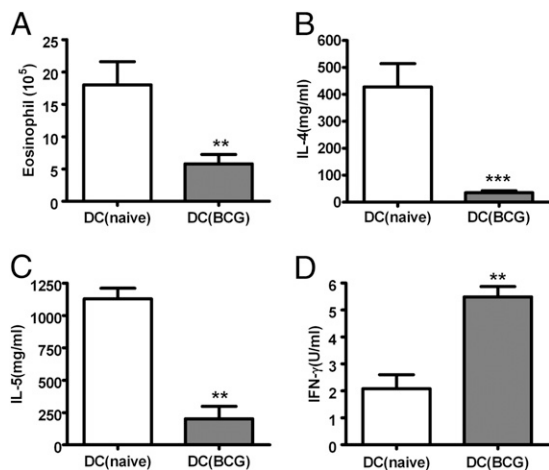


FIGURE 4. Intranasal transfer of DCs from BCG-infected mice also inhibits pulmonary eosinophilic inflammation and Th2 cytokine responses in established RW allergy. Mice were sensitized i.p. with 100 μ g RW in alum. The mice were challenged i.n. with 150 μ g RW at day 14 following RW sensitization. At day 12 after the first RW challenge, mice were administered i.n. with 2×10^6 DC(BCG) or DC(naive) and rechallenged with RW i.n. Mice were sacrificed at day 6 after the last RW challenge for analysis of lung inflammation and Ag-driven cytokine production. **A**, Pulmonary eosinophilic infiltration. **B–D**, Cells prepared from draining lymph nodes were cultured for 72 h with stimulation of RW and cytokine levels in the culture supernatants were detected by ELISA. Each group was composed of four mice. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

DCs taken from BCG-infected mice are able to inhibit established airway eosinophilic inflammation and mucus overproduction elicited by rechallenge with the RW allergen.

Adoptive transfer of DCs from BCG-infected mice results in reduced circulating eosinophils following RW rechallenge

To investigate whether the reduced allergen-induced airway eosinophilia observed in the recipients of DC(BCG) was associated with alterations in peripheral blood eosinophils, we further tested the effects of adoptively transferred DC(BCG) to mice with established RW allergy on systemic eosinophilia. As seen in Table I, the mice receiving DC(BCG) showed significantly less increase in blood

FIGURE 5. Mice that were administered DCs from BCG-infected mice during established RW allergy displayed significant decrease in RW-specific IgE, IgG1, and total serum IgE. Mice (four mice per group) were treated as described in Fig. 1 and were bled 6–8 d after RW rechallenge. The RW-specific IgE, IgG1, IgG2a, and total IgE in the sera collected after RW treatment were determined as described in *Materials and Methods*. ELISA titers for RW-specific Abs were converted to log 10 and presented as the means $\pm$ SEM. Pooled data for three experiments are shown. * $p < 0.05$.

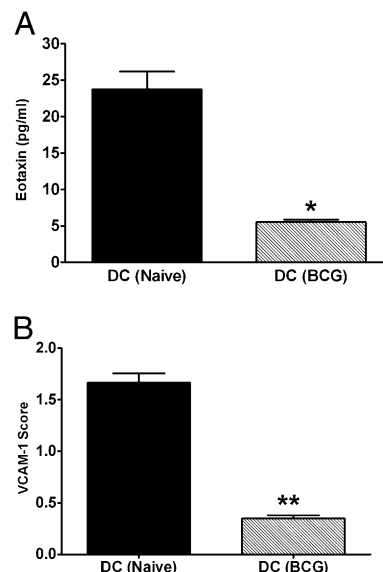
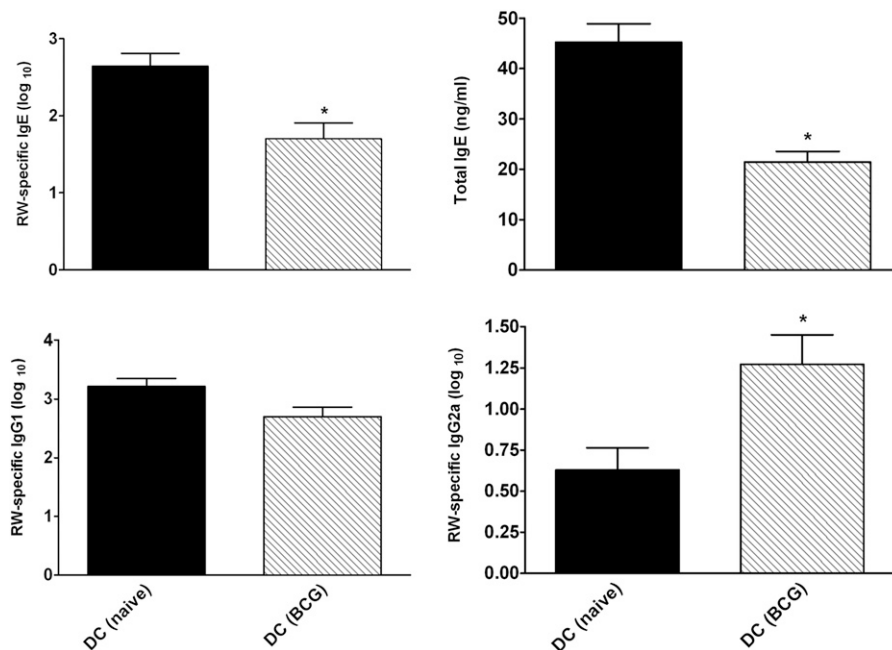


FIGURE 6. Dramatic reduction of eotaxin and VCAM-1 in the lungs of the mice treated with DCs from BCG-infected mice during established RW allergy. Mice were treated as described in Fig. 1. **A**, Eotaxin-1 levels in BAL fluids were determined by ELISA as described (30). **B**, Frozen lung sections (10 μ M) were stained for VCAM-1 expression using a DAKO Envision Staining Kit (Dako, Carpinteria, CA) and the intensity of VCAM-1 expression was semiquantified as described (25). Data are presented as mean $\pm$ SD. One representative experiment of two independent experiments is shown. * $p < 0.05$; ** $p < 0.01$.

eosinophils than in those receiving DC(naive). The results, together with the data shown above, demonstrate that DCs taken from BCG-infected mice can inhibit the allergic eosinophilia at both local and systemic levels.

Adoptively transferred DCs from BCG-infected mice alters established, allergen-driven Th2-like cytokine responses

To explore the molecular basis for the alteration of allergic responses observed in the recipients of DC(BCG), we examined the cytokine and chemokine profiles of both spleen and draining lymph node cells (mediastinal lymph nodes [MLNs]) in the

recipients of DCs with established allergy. As shown in Figs. 2 and 3, upon restimulation with the RW allergen, both spleen and MLN cells from recipients of DC(BCG) produced significantly lower levels of Th2-related cytokines (Fig. 2), namely IL-4, IL-5, IL-9, and IL-13, but higher levels of IL-12 and IFN- γ (Fig. 3), in comparison with the recipients of DC(naive).

To examine whether the local delivery of DC(BCG) had the same effect on allergic inflammation and cytokine responses as systemic delivery, we transferred DCs i.n. also. As shown in Fig. 4, the recipients of i.n. delivered DC(BCG) also showed significant reduction in eosinophilia and Ag-driven Th2 cytokine production.

We further examined the effect of the adoptively transferred DCs on established IgE responses. Both RW-specific and total serum IgE were analyzed. The results showed that, inconsistent with their significantly reduced Th2 cytokine production, mice receiving DC (BCG) showed significantly lower levels of both RW-specific and total IgE production compared with those receiving DC(naive) (Fig. 5). In addition, the results showed a significant increase of RW-specific IgG2a in DC(BCG) recipients. The results demonstrate that adoptive transfer of DCs from infected mice can inhibit established allergen-driven Th2 cytokine production and IgE responses, which are the bases for allergic inflammations.

Eotaxin production and VCAM-1 expression in the airway are inhibited by adoptive transfer of DCs from BCG-infected mice

Apart from the powerful efficacy of Th2 cytokines in the development, recruitment, and activation of eosinophils into the

allergic airways, chemokine (eotaxin) and adhesion molecules also play a significant role in this process. We tested the effect of the transfer of DC(BCG) on eotaxin-1 production and VCAM-1 expression in the airway of the DC recipients with established allergy. As shown in Fig. 6A, the recipients of DC(BCG) showed significantly lower levels of eotaxin in the BAL fluids than those received DC(naive). Similarly, although high density of VCAM-1 expression on airway endothelial cells was observed in mice that received DC(naive), faint or undetectable VCAM-1 expression was observed in those received DC(BCG) (Fig. 6B). The results suggest that suppression of eotaxin production and VCAM-1 expression in the airway may also be an important mechanism by which DCs from BCG-infected mice inhibit airway eosinophilic inflammation.

DCs from BCG-infected mice display altered expression of surface markers, TLRs, and production of cytokines compared with those from naive mice

To further investigate the basis for DC(BCG) in modulating allergen-driven cytokine production patterns and allergic reactions in the recipients, we examined the expression of various cell surface molecules and TLR messages and the production of cytokines by these DCs. As seen in Fig. 7A, DC(BCG) expressed significantly higher levels of CD8 α , CD80, CD86, and CD40 than DC(naive), both in percentage of positive cells and mean fluorescence intensity (MFI). RT-PCR analysis of the messages for TLRs showed that DC(BCG) expressed significantly higher TLR-2, -4 and -9 than did DC(naive) (Fig. 7B). In addition, quantitative RT-

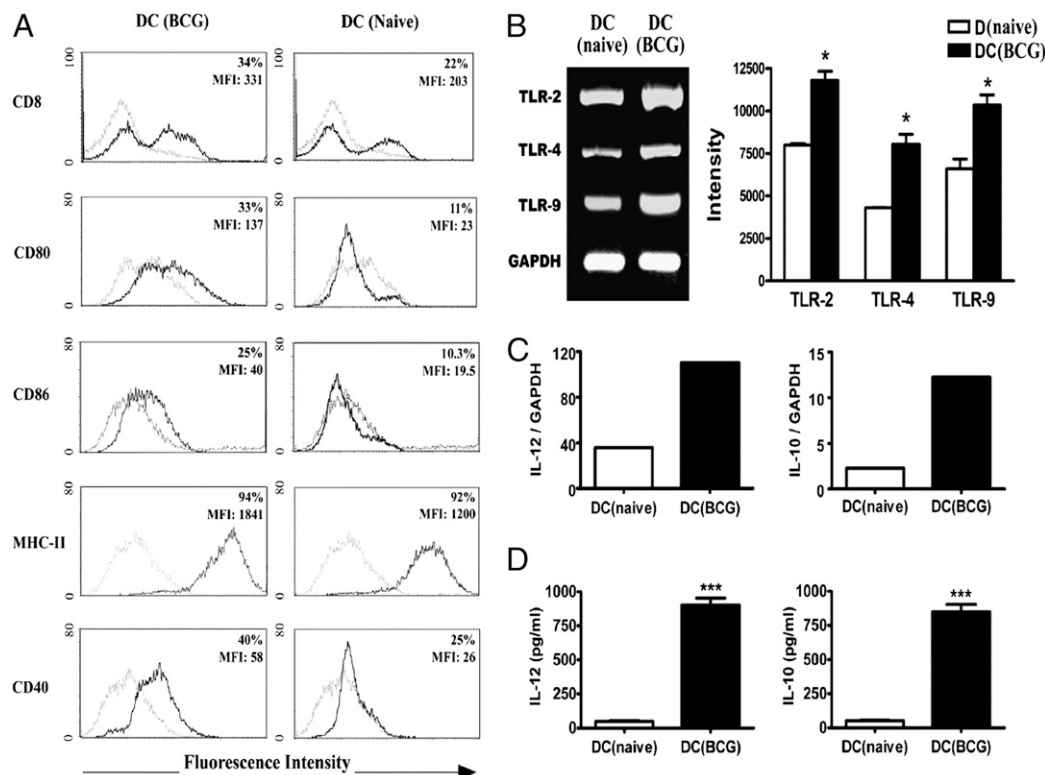


FIGURE 7. Characterization of DCs isolated from BCG-infected and naive mice. C57BL/6 mice were infected i.v. with 5×10^5 CFU BCG and sacrificed at day 21 postinfection. Splenic DCs were isolated using CD11c-coated MACS beads from BCG infected and naive mice. *A*, The expression levels of surface markers were analyzed by flow cytometry. The percentage (upper) and MFI (lower) are shown at the right upper corners. *B*, RT-PCR was performed to measure the mRNA messages of TLR-2, TLR-4, TLR-9, and GAPDH. The average of the intensity of the bands in different groups is shown on the right. *C*, The expression levels of IL-10 and IL-12 were measured by real-time PCR. The ratio of the copies of IL-10 with GAPDH and the ratio of IL-12 with GAPDH, respectively, are shown. *D*, 1×10^6 freshly isolated DC from BCG-infected mice or naive mice were cultured in complete media at 37°C for 72h in the presence of heat-killed BCG. The supernatants of DC culture were collected and IL-10 and IL-12 proteins were detected by ELISA. Pooled data for three experiments are presented. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; comparison between DC(BCG) versus DC(naive).

PCR analysis of freshly isolated DCs showed that DC(BCG) expressed dramatically higher IL-10 and IL-12 messages than did DC(naive) (Fig. 7C). Consistently, DC(BCG), when placed in culture, produced significantly higher levels of both IL-12 and IL-10 in comparison with DC(naive) (Fig. 7D). The results demonstrate that DCs from BCG-infected and naive mice are different in regard to the cellular marker expression and cytokine production, which may be the basis for the functional differences of these DCs.

Neutralization of IL-10 or IL-12 results in reversal of the inhibitory effect of DCs from BCG-infected mice on established allergy

To confirm the role of IL-12 and IL-10 produced by DC(BCG) in the inhibition of allergic reactions, we injected (i.p.) anti-IL-10 and anti-IL-12 neutralizing mAbs to the recipients of DC(BCG) with established allergy to RW, immediately after DC transfer. The results showed that mice delivered DC(BCG) in conjunction with anti-IL-10, or anti-IL-12 mAb exhibited significant reversal of the inhibitory effect of the DCs on airway allergic reactions, including eosinophil infiltration and mucus overproduction (Fig. 8). Consistently, the allergen-driven Th2 cytokine (IL-4, IL-5, IL-9, and IL-13) production in the Ab-treated mice was also significantly reversed (Fig. 9). Anti-IL-12 mAb treatment reduced IFN- γ

production in the DC(BCG) recipients, whereas anti-IL-10 mAb had little effect on IFN- γ production (Fig. 9). Of note, anti-IL-10 or anti-IL-12 Ab treatment of the recipients of DC(naive) did not show significant effect on airway inflammation and Th2 cytokine production induced by RW sensitization and challenge (Figs. 8, 9). The data indicate that the higher IL-10 and IL-12 production by DC(BCG) contributes significantly to the cell's ability to redirect the *in vivo* allergic responses induced by RW allergen.

We next tested whether the blockade of both IL-10 and IL-12 in the DC(BCG) recipients can further reverse the inhibitory effect of DC(BCG) on eosinophilic inflammation and Th2 cytokine responses. As shown in Fig. 10, the combined neutralization of IL-10 and IL-12 did not show further reversal effect. Interestingly and understandably, although neutralization of IL-10 led to increased IFN- γ production, the coneutralization of IL-10 and IL-12 actually reduced IFN- γ production (Fig. 10C). Therefore, the roles played by the different regulatory cytokines are complicated and are involved in a network interaction.

Discussion

In the current study, using an adoptive transfer approach, we showed that DCs play a pivotal role in mycobacteria-mediated modulation of the allergic responses to RW, a common environmental allergen. We found that adoptive transfer of DCs isolated from infected mice was capable of reducing the established Th2-like cytokine responses and airway eosinophilic inflammations as well as mucus overproduction induced by RW, which was dependent on IL-10 and IL-12 production by DCs. This is a novel finding, because previously reported data have demonstrated an inhibitory role of BCG infection in *de novo* and established allergy models (25–28), the present data extend toward the cellular and molecular basis of the infection mediated inhibition of allergy.

The mechanism by which transferred DCs from BCG-infected mice mediate inhibitory activity to allergic reaction appears to influence allergen-specific CD4 T cell differentiation. It is clear that the recipients of DC(BCG) showed Th1-like allergen-driven cytokine production instead of Th2-like responses. Although it was not directly examined, the transferred DCs may present allergen directly to RW-specific T cells and/or modulate the function of the existing DCs in the recipients. The cytokine microenvironment generated by the DCs from BCG-infected mice can lead to an overall decrease in the development of the established Th2 phenotype and an increase of Th1-like RW-specific T cells. Indeed, our data showed that the adoptive transfer of DC(BCG) switched allergen-specific T cell cytokine pattern from established Th2 responses to Th1 phenotype. Specifically, our results showed that spleen and MLN cells from mice that received DC(BCG) produced significantly lower levels of allergen-driven IL-4, IL-5, IL-9, and IL-13, but higher levels of IFN- γ and IL-12, compared with mice that received DC(naive).

In addition to identifying the critical role of DCs in mycobacterial infection-mediated inhibition of allergy, the current study also provides some insight into the molecular basis by which DCs from infected mice modulate allergen-specific T cell responses. It has been previously reported that the ability of DCs to modulate T cell responses is largely dependent on their cytokine patterns and costimulatory molecules (33, 34). Our data showed that DC(BCG) expressed significantly higher levels of CD8 α , CD80, CD86 and CD40 and produced remarkably higher levels of IL-12 and IL-10 than DC(naive). More importantly, we found that the blockade of either IL-12 or IL-10 significantly reversed the inhibitory effect of DC(BCG) on established allergic responses. Because numerous

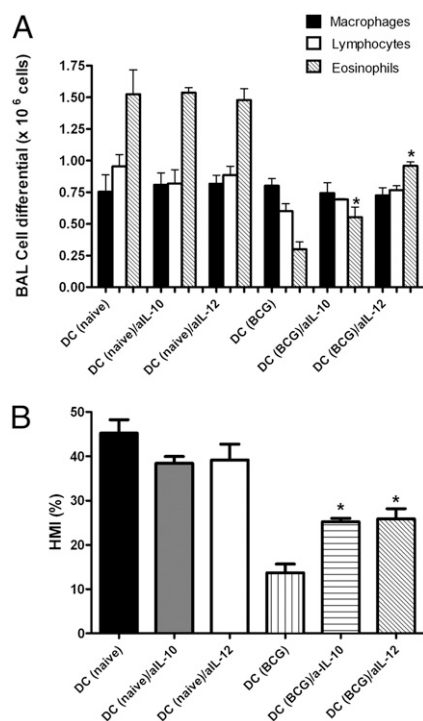


FIGURE 8. Anti-IL-10 or anti-IL-12 treatment significantly reduced the inhibitory effect of DC(BCG) on allergic airway inflammation and mucus production. Mice (four mice per group) were sensitized (i.p.) with 100 μ g RW in alum and were challenged i.n. with 150 μ g RW at day 14. Twelve days after RW challenge, the mice were injected i.v. with DC (BCG) or DC(naive). Immediately following DC adoptive transfer, mice were administered i.p. with 1 mg/ml anti-IL-10 or anti-IL-12 Ab and subsequently (2 h after transfer) rechallenged with 150 μ g RW i.n. Six days after re-challenge, mice were sacrificed. **A**, Differential infiltrating cells in the BAL fluids were counted. **B**, Mucus-secreting goblet cells were examined by periodic-acid Schiff staining. HMI was calculated based on the percentage of the mucus-positive area over the total area of the airway epithelium. Data represents means $\pm$ SD. One representative experiment of three independent experiments is shown. * p < 0.05; comparison between DC(BCG) recipients with and without Ab treatment.

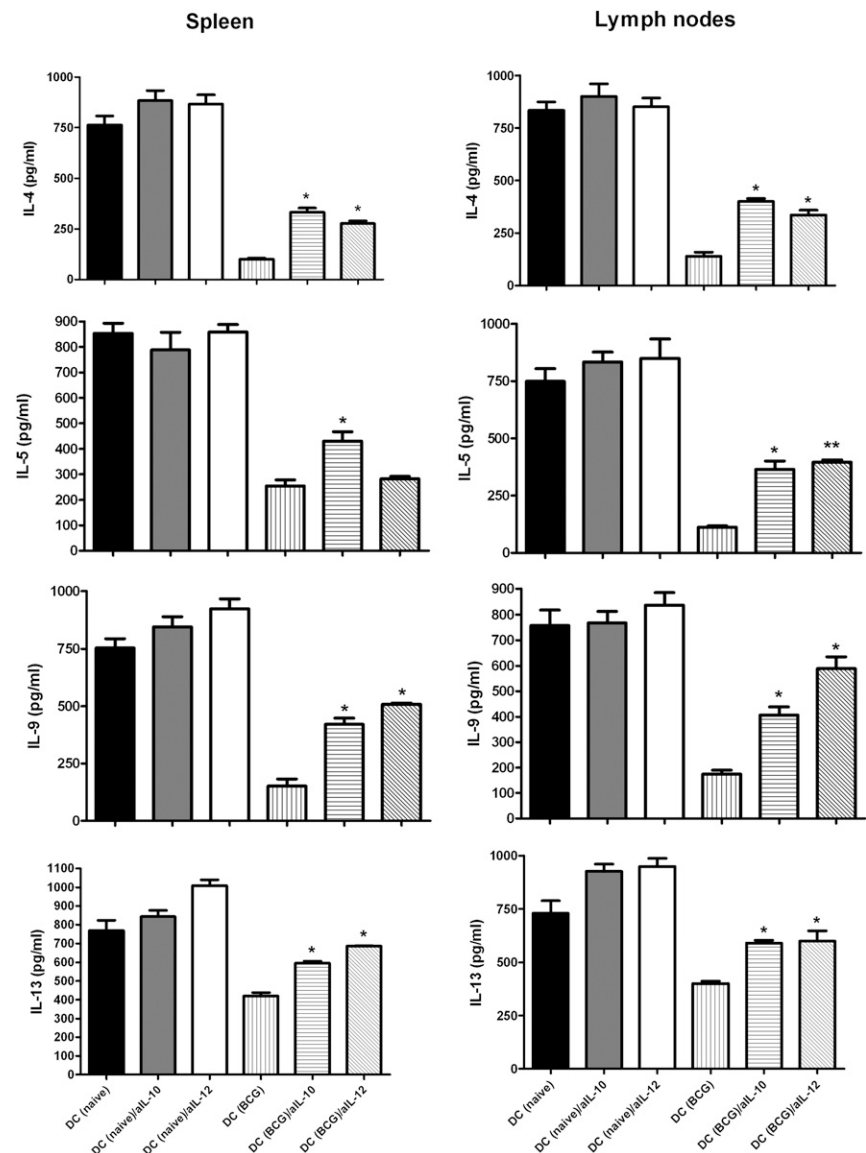


FIGURE 9. Anti-IL-10 or anti-IL-12 treatment significantly reduced the inhibitory effect of DC(BCG) on Th2 cytokine responses. Mice were treated as described in Fig. 8. When the mice were sacrificed at 6 d after re-challenge, the spleen and MLN cells were cultured in the presence of RW (0.1 mg/ml). Culture supernatants were harvested at 72 h for testing cytokines by ELISA. Data are presented as the means $\pm$ SD of each group. One representative experiment of three independent experiments is shown. * $p < 0.05$; ** $p < 0.01$; comparison between DC (BCG) recipients with and without Ab treatment.

reports have shown differential IL-12 and IL-10 production patterns of functional DC subsets, the higher production of both IL-12 and IL-10 by DC(BCG) might suggest that the DCs from BCG infected mice are composed of heterogeneous DC subpopulations that inhibit allergic reactions through different mechanisms. For example, one subpopulation can be higher IL-12 producers (DC1-like cells) that can enhance the development of Th1 cells, thus inhibiting the development or expansion of allergen-specific Th2 cells, whereas another population can produce higher IL-10 (tolerogenic DC or regulatory DC), leading to unresponsiveness of allergen-specific Th2 cells or the development of Treg cells. Therefore, the mechanism the DCs induced by BCG infection may not only include DC1-like cells but also IL-10 producing tolerogenic DCs. In line with this thought, a differential effect of anti-IL-12 and anti-IL-10 mAb treatment on allergen-driven IFN- γ production was observed in the recipients of DC(BCG) (Fig. 9). Thus, the reduced IFN- γ production in anti-IL-12-treated mice may partially explain the reversal effect of this treatment on the inhibition of allergy by DC(BCG) as a shift in Th1/Th2 balance. However, the treatment with anti-IL-10 mAb, which had little effect on IFN- γ production, also showed a reversal of the inhibitory effect. Therefore, an IFN- γ -independent, IL-10-dependent, tolerogenic mechanism might operate in this scenario.

Interestingly, Stock et al. (35) recently reported that *Listeria monocytogenes*, when used as an adjuvant, induced a CD8 α + DC population that produced both IL-10 and IL-12 and induced the so-called Th1-like Treg cell. Unlike the regular regulatory T cells, this Th1-like Treg cell produced both IL-10 and IFN- γ and expresses ICOS, Foxp3, and T-bet—a combination of the features of regulatory and type-1 T cells. It remains unclear whether a similar regulatory mechanism exists in our experimental model, but the higher CD8 α expression and IL-10/IL-12 production by DC(BCG) suggest the likelihood. Obviously, further studies to specifically address this question and to address the role of DC subsets in BCG-mediated inhibition of allergic responses may lead to a better understanding of the mechanisms by which DCs modulate allergic responses and the influence of microbial agents on DC functions. Moreover, because the DCs from infected mice likely carry some BCG Ags, it is important to test whether these Ags also contribute to the change of DC function.

In addition to the change in cytokine patterns, our data demonstrate significant alterations in chemokine production and adhesion molecule expression related to eosinophil recruitment into the lung. Recipients of DCs from BCG-infected mice showed reduced levels of eotaxin in the lungs of allergen-treated mice. This is important because eotaxin is a key chemokine in eosinophil

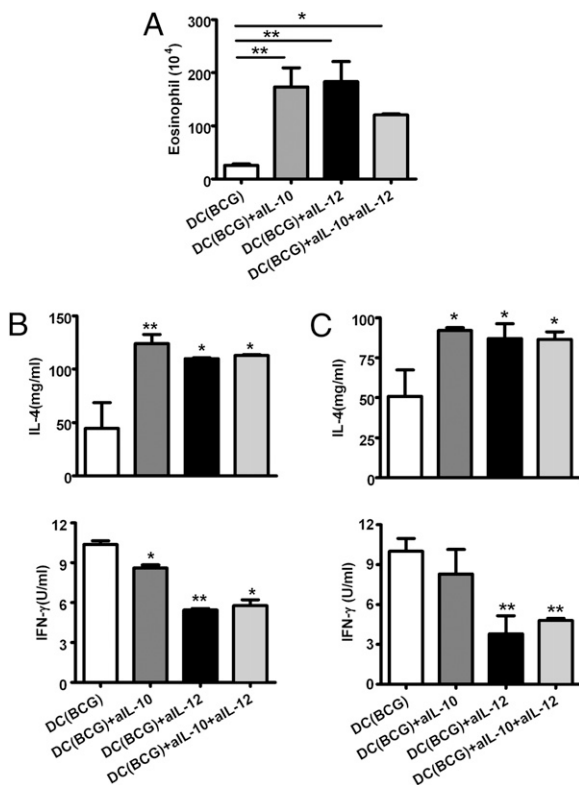


FIGURE 10. Conneutralization of both IL-10 and IL-12 failed to further reverse the inhibitory effect of DC(BCG) on allergic responses compared with individual cytokine neutralization. Mice (four mice per group) were sensitized and challenged with RW as described in Fig. 8. The mice were injected i.v. with DC(BCG) or DC(naive) and immediately following DC adoptive transfer, mice were administered i.p. with 1 mg/ml of anti-IL-10 or anti-IL-12 Ab or both and subsequently (2 h after transfer) rechallenged i.n. with RW. Six days after rechallenge, mice were sacrificed. **A**, The infiltrating eosinophils in the BAL fluids were counted. **B**, Spleen cells were cultured in the presence of RW (0.1 mg/ml). Cytokine levels in the culture supernatants and BAL fluids (**C**) were tested by ELISA. Data are presented as the means $\pm$ SD of each group. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; comparison between DC(BCG) recipients without Ab treatment and those with either anti-IL-10 or anti-IL-12 or both anti-IL-10 and anti-IL-12 Ab treatment.

recruitment and an inducer of eosinophil degranulation via CCR3 expressed on the eosinophil cell surface, which may also contribute to eosinophilic inflammation in the airway (36). Moreover, this study showed that adoptive transfer of DC(BCG) could inhibit RW-induced VCAM-1 expression on pulmonary vascular endothelium. VCAM-1 has been reported to be critical in the interaction between vascular endothelial cells and eosinophils into the airways through interactions between VCAM-1 and VLA-4 (37, 38). Because IL-4 plays an important role in the expression of VCAM-1 (39), and because IL-4 production in the mice that received DC(BCG) was significantly lower than in those that received DC(naive), the mechanism by which DC(BCG) inhibited RW-induced VCAM-1 expression might be the reduction of IL-4 production by CD4 T cells.

The finding on the effectiveness of adoptive transfer of DC(BCG) in redirecting established IgE responses is particularly encouraging. Notably, although numerous studies have shown the inhibitory effect of bacterial infections and bacterial products administration on de novo or established allergic inflammation, the treatments often failed to inhibit established allergen-specific IgE responses (25, 27, 28). This finding is consistent with earlier reports showing that it is difficult to inhibit established IgE responses (40). However, our

data show that the adoptive transfer of DCs from infected mice could effectively inhibit well-established IgE responses. The reason for this discrepancy is likely because we tested the role of a particular cell type (i.e., DCs), whereas the previous studies tested an outcome of interactions among different cells in infected mice (25, 26). Therefore, it is possible that the reason for the observed unchanged or even increased allergen-specific IgE production in the infected mice might actually reflect the effect of infection on other cells. For example, some reports have shown that BCG infection can activate NK T cells, which are capable of producing large amounts of IL-4 (41). Our own study also showed that NK T cells play an important role in IgE responses in mouse models (42, 43). More recently, it was reported that basophils could function as APCs to promote Th2 responses (44–46). Because patients with tuberculosis show significant basophil accumulations (47), it is likely that BCG infection can also promote basophil response; this might be a reason for enhanced IgE response in the infected mice after allergen exposure. Therefore, the data tell us at least two things: the use of potent APCs, such as DCs, may be able to alter established IgE responses, and the influence of infection on allergy is through multiple cell types. One caution for interpreting the data from this study is that the DCs used in this study are from normal mice with BCG infection. In a clinical scenario, the DCs from allergic or atopic individuals may be different from those of healthy individuals after mycobacterial infection. Therefore, directly addressing this question is important in determining to which degree the study reflects the real mechanism underlying the modulating effect of infection on allergy and asthma.

This study has demonstrated a pivotal role played by DCs in infection-mediated modulation of established allergic reactions induced by RW allergen. The alteration of DCs mediated by microbial agents, such as BCG, in cytokine production, as well as costimulatory molecules and pattern recognition receptors, may contribute to the critical role of DCs in infection-mediated inhibition of allergic reactions. Further studies investigating the function of DC subsets generated by infection could provide more insights into the mechanisms by which DCs and microbial agents modulate allergic responses in human diseases.

Disclosures

The authors have no financial conflicts of interest.

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