



The Journal of Immunology

This information is current as of January 16, 2010

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J. Immunol. 2010;184:1062-1070; originally published online Dec 9, 2009;
doi:10.4049/jimmunol.0901822
<http://www.jimmunol.org/cgi/content/full/184/2/1062>

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***Mycobacterium bovis* Bacillus Calmette-Guérin Killed by Extended Freeze-Drying Targets Plasmacytoid Dendritic Cells To Regulate Lung Inflammation**

Micheline Lagranderie,* Mohammad Abolhassani,* Jeroen A. J. Vanoirbeek,[†] Carla Lima,[‡] Anne-Marie Balazuc,[§] B. Boris Vargaftig,[¶] and Gilles Marchal*

We have previously shown that bacillus Calmette-Guérin (BCG) inactivated by extended freeze-drying (EFD) reduces airway hyperresponsiveness, whereas live and heat-killed BCG fail to do so. However, the cells involved in the protective effect and the signaling and transcriptional networks that could reprogram T cell commitment after EFD BCG treatment remained to be elucidated. We investigated whether EFD BCG targets plasmacytoid dendritic cells (pDCs) potentially involved in the polarization of regulatory T cells (Tregs) and the transcriptional factors that regulate allergic inflammation. OVA-sensitized mice were s.c. injected with EFD, live, or heat-killed BCG. We analyzed after the injection of the various BCG preparations: 1) pDCs recruited in the draining lymph nodes (day 4); 2) transcription factors involved in inflammation and T cell commitment in spleen and lungs after OVA challenge (day 28). Airway hyperresponsiveness and transcription factors were determined after *in vivo* depletion of pDCs or Tregs in EFD BCG-treated and OVA-challenged mice. EFD BCG reduced inflammation via the recruitment of pDCs polarizing the differentiation of naive CD4⁺ T lymphocytes into Tregs. *In vivo*, pDC or Treg depletion at the time of EFD BCG treatment abrogated the protection against inflammation. EFD BCG treatment upregulated Forkhead-winged helix transcription factor (Treg signature) and downregulated GATA-3 and RORγt (Th2 and Th17 signatures) more efficiently than live and heat-killed BCG. Moreover, only EFD BCG enhanced peroxisome proliferator-activated receptor γ expression and blocked NF-κB activation, cyclooxygenase expression, and p38 MAPK phosphorylation. EFD BCG reduced allergic inflammation by recruiting pDCs that promoted Tregs; EFD BCG acted as a peroxisome proliferator-activated receptor γ agonist and thus could be used in asthma and other inflammatory diseases. *The Journal of Immunology*, 2010, 184: 1062–1070.

Allergic disorders are associated with chronic inflammation characterized by eosinophilia and an increase of Th2-type cytokine production enhancing IgE synthesis (1). The incidence of asthma is lower in children vaccinated with bacillus Calmette-Guérin (BCG) (2, 3), supporting the concept that reduced exposure to microbial products during infancy leads to overexpression of allergenic responses (4, 5). In murine models, BCG induces a type 1 cytokine profile that reverses the type 2 cytokine profile characterizing atopic diseases (6–8). Our knowledge of the pathogenesis of allergic diseases has broadened to incorporate the contribution of regulatory T cells (Tregs) and Th17 cells (9, 10). We have recently shown that extended freeze-dried (EFD) BCG administered s.c. prevented airway hyperresponsiveness (AHR) and

reduced lung inflammation through IL-10 production, whereas live and heat-killed (HK) BCG did not (11). However, the cells involved in this protective effect and the mechanisms by which, after EFD BCG treatment, signaling and transcriptional networks could reprogram effector T cell lineage commitment in allergic inflammation remain to be elucidated.

Because Tregs are essential in preventing allergy (12), we first studied plasmacytoid dendritic cells (pDCs), which could be involved in the differentiation of naive T cells into Tregs. After s.c. injection of EFD BCG at the base of the tail, we detected in the inguinal draining lymph nodes (DLNs) more pDCs producing IL-10 than in those after injection of live or HK BCG. pDCs induced *in vitro* the differentiation of naive CD4⁺ T lymphocytes into CD4⁺CD25⁺FOXP3⁺ cells and played a crucial role *in vivo* because their depletion at the time of EFD BCG treatment abrogated the protective effect. In contrast to other BCG preparations, EFD BCG blocked transcriptional regulator signatures of Th2 (GATA-3) and Th17 (RORγt) cells and upregulated Forkhead-winged helix transcription factor (FOXP3) (Treg signature). Moreover, only EFD BCG enhanced peroxisome proliferator-activated receptor γ (PPAR-γ) expression and blocked pathways involved in the inflammatory process: I-κBα phosphorylation, NF-κB activation, and p38 MAPK phosphorylation.

Materials and Methods

Mycobacterial preparations, treatment protocols, and AHR measurement

The live BCG Pasteur strain 1173P2 was grown on Sauton medium according to the conditions used for vaccine production (13). The various BCG preparations (live BCG, HK BCG, and EFD BCG) were obtained as previously described (11).

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Received for publication June 9, 2009. Accepted for publication November 4, 2009. This work was supported by the Institut Pasteur, Paris, France.

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Abbreviations used in this paper: AHR, airway hyperresponsiveness; BAL, bronchoalveolar lavage; BCG, bacillus Calmette-Guérin; cDC, conventional dendritic cell; COX-2, cyclooxygenase-2; DLN, draining lymph node; EFD, extended freeze-dried; FOXP3, Forkhead-winged helix transcription factor; HK, heat-killed; pDC, plasmacytoid dendritic cell; PPAR-γ, peroxisome proliferator-activated receptor γ; T-bet, transcription factor T-box; Treg, regulatory T cell; RXRα, retinoid X receptor α.

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Male BP2 (6-wk-old) mice were purchased from the Centre d'Élevage Janvier (Le Genest-St Isle, France) and maintained in accordance with national guidelines for animal welfare. They were OVA-sensitized (Valeant Pharmaceuticals, Costa Mesa, CA), treated s.c. (base of the tail) with various BCG preparations, and OVA-challenged, then AHR was measured by plethysmography and by airway resistance-compliance to methacholine as previously described (11, 14). Cytokine contents in the bronchoalveolar lavage (BAL) and sera were determined using the Bio-Plex Cytokine Assay (Bio-Rad, Marnes La Coquette, France) (11), and the sensitivities of the cytokines tested were, respectively, 0.3 pg/ml (IL-5), 12 pg/ml (IL-13), 0.8 pg/ml (IL-17), and 15 pg/ml (eotaxin).

Isolation of cells for flow cytometry analysis and for in vitro cultures

Four days after s.c. injection of the various BCG preparations at the base of the tail, cells were isolated from the inguinal DLNs (15) and labeled with Abs against CD11c, CD11b, I-A^b, B220, Ly6G-Gr1, CD40, CD80, and CD86 from BD Pharmingen (San Diego, CA), DEC205 from Caltag Laboratories (Burlingame, CA), ICOS-L (eBiosciences, San Diego, CA), and PDCA-1 (Miltenyi Biotec, Bergisch Gladbach, Germany). A FACScan (BD Biosciences, San Jose, CA) was used for analytical flow cytometry. CD11c⁺ cells from the DLNs were also purified on an autoMACS (Miltenyi Biotec) according to the manufacturer's instructions and then sorted (FACStar; BD Biosciences) into CD11c⁺B220⁺ (pDCs) and CD11c⁺B220⁻ (conventional DCs [cDCs]) subtypes. These subtypes (90–95% purity) were cultured alone or cocultured in vitro with nonfractionated CD4⁺ T cells from naive mice (ratio of 1:5) without further stimulation. CD25 expression on CD4⁺ T cells cocultured with DC subsets was analyzed by flow cytometry 72 h later. The Bio-Plex Cytokine Assay (Bio-Rad) was used to measure the cytokine content of the supernatants from DC subsets (24 h) and from cocultures (72 h). The sensitivities of the cytokines tested were, respectively, 0.2 pg/ml (IL-6), 1 pg/ml (IL-10), 0.4 pg/ml (IL-12p40), and 1.2 pg/ml (IFN- γ).

Transcription factors in in vitro cultures and in lung extracts

For in vitro study, CD4⁺ spleen cells were purified from naive mice and cocultured with pDCs or cDCs as described above, then 10⁶ cells were divided into aliquots after each culture condition. For ex vivo studies, after treatment with the various BCG preparations, we used protein extracts (70 μ g) from lung homogenates of mice treated with the various BCG preparations or depleted of either CD25⁺ cells or pDCs at the time of EFD BCG treatment and OVA challenge. The proteins extracted were resolved by 7.5% SDS-PAGE. Protein bands transferred to nitrocellulose sheets were probed with mouse monoclonal anti-FOXP3, -transcription factor T-box (T-bet), -GATA-3, -ROR γ t, -I- κ B α , -p-I- κ B- α , -p38, -p-p38, p-retinoid X receptor α (RXR α), and cyclooxygenase (COX-2) (Santa Cruz Biotechnology, Santa Cruz, CA) or β -actin mouse mAb (Ac-15 Abcam, Cambridge, U.K.). HRP-conjugated polyclonal goat anti-rabbit (DakoCytomation, Glostrup, Denmark) or goat anti-rabbit IgG (Santa Cruz Biotechnology) were used as secondary Abs. The immune complex was visualized with an enhanced chemiluminescence detection system (Amersham Biosciences, Saclay, France). For data acquisition and background normalization, we used Scan-Analyze, version 2.50, developed by Dr. Michael Eisen at Lawrence Berkeley National Laboratory, Berkeley, CA. Data were expressed as ratios of the normalized intensities of the bands (target proteins versus β -actin or phospho-protein versus native protein).

Twenty-four hours after the OVA challenge, nuclear proteins were extracted from lung homogenates after protease inhibitor treatment. Nuclear extracts (10 μ g) were tested for NF- κ B activation or PPAR- γ expression with NF- κ B p65 or PPAR- γ TransAM transcription factor assay kits (Active Motif, Carlsbad, CA) according to the manufacturer's recommendation. The limits of detection for NF- κ Bp65 and PPAR- γ were 0.5 and 1 μ g nuclear protein extract, respectively.

Depletion of pDCs and CD25⁺ cells

The anti-plasmacytoid Ab (PDCA-1 clone JF05-1C2.4.1; Miltenyi Biotec) and the isotype control (rat IgG2b) (500 μ g) or the anti-CD25 Ab (clone PC61; gift from André Herbelin and Luiza Araujo, Hôpital Necker, Paris, France) and the isotype control (rat IgG1, λ) (100 μ g) were administered i.p. at the time of EFD treatment to OVA-sensitized mice. pDCs and CD25⁺-depleted mice were OVA-challenged 28 d later. AHR was monitored as described above. To analyze the depletion efficiency in inguinal DLNs, spleens, and lungs, the numbers of pDCs (CD11c⁺B220⁺PDCA-1⁺) and Tregs CD4⁺CD25⁺ (clone 7D4 for anti-CD25 Ab) were detected by FACS analysis 24 and 96 h after the injection of the depleting Abs and 24 h after the OVA challenge (28 d after the injections).

Statistical analysis

The mean and SE for each group of six mice were calculated. The Instat package from GraphPad (San Diego, CA) was used for analysis with the Student *t* test.

Results

EFD BCG blocks NF- κ B activation and reduces lung inflammation

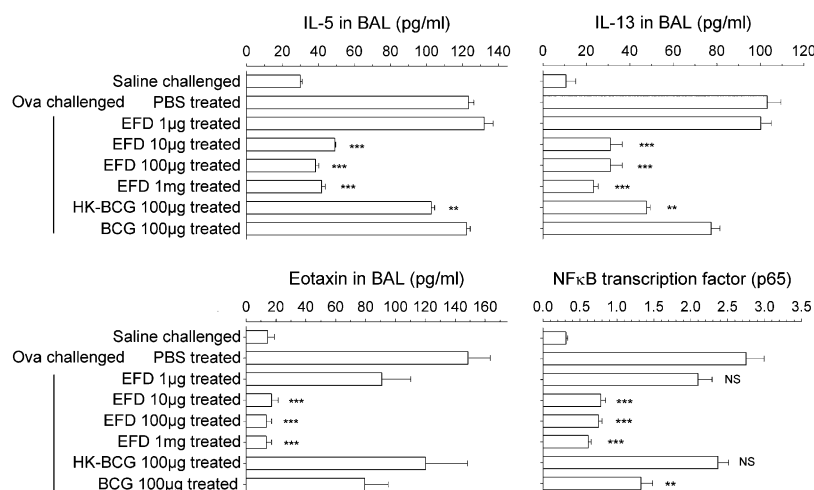
We have previously shown that only EFD BCG was able to reduce AHR, whereas live or HK BCG failed to do so (11). The translocation of NF- κ B p65 to the nucleus following the inhalation of allergens signals an inflammatory response in the lungs (16). Therefore, we measured, after OVA challenge, NF- κ Bp 65 in nuclear lung extracts of mice treated with increasing doses of EFD BCG or with a standard dose (100 μ g) of live and HK BCG. The NF- κ B activation was inhibited very significantly in lung extracts of mice treated with EFD BCG (10 μ g to 1 mg), whereas 100 μ g of live BCG reduced it to a lesser extent and HK BCG failed to block it (Fig. 1). In correlation with reduced NF- κ B activation in the lung, IL-5, IL-13, and eotaxin contents were significantly decreased in the BAL of EFD BCG-treated mice (10 μ g to 1 mg), whereas live and HK BCG treatments were less effective (Fig. 1).

EFD BCG induces the recruitment of pDCs, which play a key role in the differentiation of Treg cells

Various developmental stages and subsets of DCs can contribute to the expansion and differentiation of T cells that regulate or suppress other immune T cells (17). Thus, 4 d after s.c. administration of various BCG preparations, we analyzed the DC subsets recruited to the inguinal DLNs: cDCs (CD11c⁺B220⁻) and pDCs (CD11c⁺B220⁺) displayed different morphologies (Fig. 2A) besides their markers. The number of pDCs per DLN was 2-fold higher in EFD BCG-treated mice than those in live and HK BCG-treated mice, whereas the number of cDCs was not significantly different among the various treated groups (data not shown). We then characterized both DC subsets purified 4 d after the various injections from the inguinal DLNs: 99–100% of pDCs (CD11c⁺B220⁺) expressed the marker PDCA-1 whichever the BCG preparation injected. Only pDCs purified from EFD-treated mice expressed substantial levels of ICOS-L (Fig. 2B), whereas cDCs never expressed it (data not shown). The DEC205 and CD11b markers were overexpressed on cDCs of all of the groups of mice and were expressed only on pDCs purified after EFD BCG injection (Fig. 2C). MHC class II molecules poorly expressed on pDCs from naive mice were upregulated after EFD BCG treatment, and costimulatory molecules (CD40, CD80, and CD86) were less expressed on pDCs than on cDCs (data not shown). In contrast to pDCs previously described (18), the Ly6G-Gr1⁺ marker was poorly expressed on pDCs recruited after EFD BCG injection (<5%), probably because EFD BCG induced the recruitment of a slightly different pDC subpopulation.

pDCs purified from the DLNs 4 d after the various s.c. injections at the base of the tail produced spontaneously without restimulation in vitro more IL-10, particularly after EFD BCG injection, than did cDCs, whereas cDCs produced more IL-12 p40 and IL-6, particularly after live or HK BCG injections (Fig. 3A). Naive CD4 T cells cocultured without further stimulation with pDCs purified after EFD BCG injection produced substantially more IL-10 than those cocultured with pDCs purified after live or HK BCG injections (Fig. 3B). TGF- β production was low in all of the groups (data not shown). Naive CD4 T cells cocultured with cDCs from live or HK BCG-treated mice produced more inflammatory cytokines (IFN- γ and IL-6) (Fig. 3B). It has to be noted that we have not checked cell viabilities

FIGURE 1. EFD BCG treatment reduces proinflammatory cytokines and NF- κ B p65 translocation. OVA-sensitized mice (six per group) were treated with PBS, various doses of EFD BCG (1 μ g to 1 mg), and 100 μ g of live BCG or HK BCG. Twenty-eight days after the treatment, mice were OVA-challenged, and 24 h after the challenge, IL-5, IL-13 and eotaxin were measured in the BAL fluid, and the NF- κ B p65 translocation in nuclear lung cell extracts was expressed in OD. Data are representative of two independent experiments. ** $p < 0.01$; *** $p < 0.001$; Student t test.



at the end of *in vitro* cultures; however, it has been shown recently that pDCs cultured in the same *in vitro* conditions as those in our experiment (RPMI 1640 supplemented with 10% FCS) showed 95% of cell viability (19). Maturing pDCs have the unique ability to generate Tregs through their high expression of ICOS-L (20). Indeed, we detected by FACS analysis substantial numbers (25%) of CD4⁺CD25^{high} cells only when naive CD4⁺ T cells were cocultured without further stimulation with pDCs from EFD BCG-injected mice (Fig. 3C). Although pDCs from live or HK BCG-injected mice can promote the differentiation of naive lymphocytes toward CD4⁺CD25^{high} cells, they did so to a lesser extent (9 and 5%). FOXP3 is a transcription factor involved in the development and function of CD4⁺CD25⁺ Tregs (21). After coculture without further stimulation of naive nonfractionated CD4⁺ T cells with pDCs or cDCs, we detected FOXP3 in cell extracts only when pDCs purified from mice injected with EFD BCG or live BCG were used as immunosignaling cells in the cocultures (Fig. 3D). cDCs purified from the DLNs after the various BCG injections and pDCs purified after HK BCG injection polarized not enough naive CD4 T cells toward CD4⁺CD25⁺ Tregs (5%) to be detected by Western blotting.

*pDCs are essential for *in vivo* induction of the protective regulatory immune response*

Lung pDCs played an essential role in preventing asthmatic reaction to inhaled Ags (18); thus, because shortly after injection EFD BCG induced an increase in the number of pDCs in the inguinal DLNs, we next studied the potential increase of pDCs in spleen and lungs over the long term after EFD BCG injection. As expected, the number of pDCs increased significantly 24 and 96 h after EFD BCG injection in the inguinal DLNs; however, the pDCs never increased in spleen and lungs 14 (data not shown) or 28 d postinjection and 24 h after the OVA challenge (Table I). We next addressed whether the depletion of pDCs at the time of EFD BCG treatment affected the airway inflammation. We observed 24 h after the injection of an anti-PDCA-1 Ab, concomitantly to the EFD BCG treatment, an efficient pDC depletion in inguinal DLNs, spleen, and lungs (90%). At 96 h, 68% of pDCs were still depleted in the DLNs, whereas at the end of the experiment, 24 h after the OVA challenge, the anti-PDCA-1 has a very marginal effect (Table I). However, the transient depletion of pDCs at the

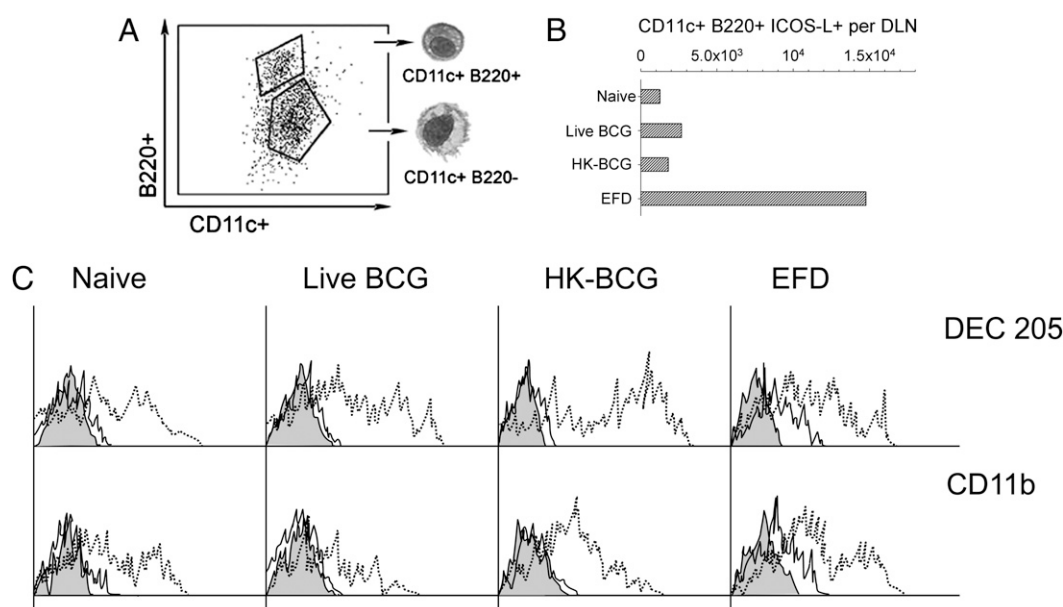


FIGURE 2. Characterization of DC subsets recruited to the inguinal DLNs. Four days after injection at the base of the tail of 100 μ g of live, HK, or EFD BCG (six mice per group), inguinal DLN cells were isolated, and pDCs (CD11c⁺B220⁺) and cDCs (CD11c⁺B220⁻) were sorted for (A) morphological study, (B) expression of ICOS-L (only on pDCs), and (C) DEC205 and CD11b expression (gray, isotype; bold line, pDCs; dotted line, cDCs). Data are representative of two independent experiments.

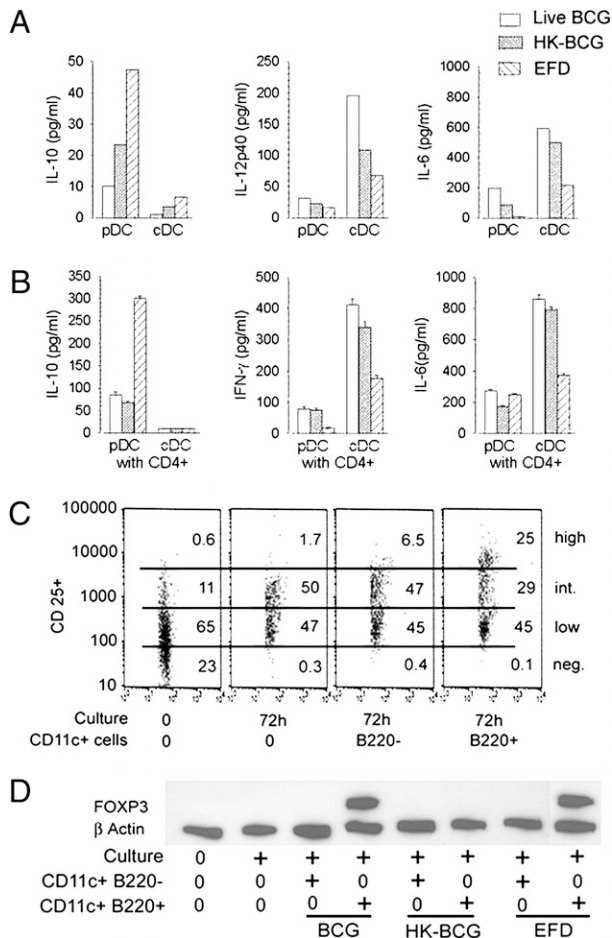


FIGURE 3. pDCs recruited after EFD treatment drive naive T cells toward Tregs. Four days after injection at the base of the tail of 100 μ g of live, HK, or EFD BCG (15 mice per group), pDC and cDC subsets sorted from the inguinal DLNs cells were (A) cultured alone (24 h) and (B) cocultured (72 h) with nonfractionated naive CD4⁺ T cells (ratio of 1:5) for cytokine determination in the supernatants. After coculture (C), the CD4⁺CD25⁺ T cells were analyzed by flow cytometry, and (D) the expression of FOXP3 in cell extracts was measured by Western blot. Data are representative of two independent experiments.

time of EFD BCG injection was sufficient to abrogate the protection conferred by EFD BCG as assessed by significantly higher Penh values (Fig. 4A) and the presence of more inflammatory cells in the

BAL of EFD BCG-treated and pDC-depleted mice than in those receiving only EFD BCG (Fig. 4B). Twenty-four hours after the OVA challenge, the number of cDCs expressing CD11c, MHC class II, and CD11b markers was significantly increased in the lung tissue of PBS-treated mice ($782,000 \pm 152,600$) and of those pDCs depleted at the time of EFD BCG treatment ($736,750 \pm 110,900$). Mice receiving only EFD BCG have a similar number of cDCs as the control, $194,500 \pm 23,600$ and $180,000 \pm 24,200$, respectively.

PPAR- γ activation blocks NF- κ B p65-mediated gene transcription and inflammation (22), and FOXP3, T-bet, and GATA-3 transcription factors are involved, respectively, in the regulation of inflammation and Th1 and Th2 immune responses. When we analyzed, in the nuclear lung cell extracts 24 h after the challenge, the balance between the activities of NF- κ B p65 and PPAR- γ , we found a reduced level of NF- κ B p65 associated with enhanced PPAR- γ expression only after EFD BCG treatment (Fig. 4C). Depletion of pDCs at the time of EFD BCG treatment inhibited these effects. We observed higher levels of FOXP3 and T-bet in lung cell extracts of EFD BCG-treated mice and lower levels of GATA-3 than in those of PBS-treated mice; when pDCs were depleted, EFD BCG treatment failed to regulate the immune deviation associated with asthma (Fig. 4D). Similar data were found in spleen cell extracts (data not shown).

Depletion of CD25⁺ cells at the time of EFD BCG injection suppresses the protective effect of the treatment

We have shown (Fig. 3C, 3D) that pDCs purified from the inguinal DLNs after EFD BCG injection polarized in vitro naive T cells toward Tregs expressing FOXP3, and in Fig. 4D, we observed high FOXP3 expression in the lung extracts of EFD BCG-treated and OVA-challenged mice. Thus, we studied by flow cytometry shortly and over the long term after EFD BCG injection the number of Tregs in various organs. Ninety-six hours after EFD BCG injection, the number of Tregs increased significantly in the DLNs (8-fold) and slightly in the spleens (1.3-fold), whereas 28 d after the EFD BCG injection the number of Tregs was significantly increased in the spleens and lungs (3-fold) (Table II). To confirm the role of CD4⁺CD25⁺ in the immunoregulation of the lung inflammation, CD25⁺ cells were depleted at the time of EFD BCG treatment; 24 and 96 h after the injection of an anti-CD25 Ab, the number of Tregs was significantly decreased in all of the the organs studied; and after the OVA challenge in spleens and lungs, >50% of CD25 cells were still depleted (Table II). The AHR was measured 24 h after the OVA challenge by whole-body plethysmography and by measurement of dynamic resistance-compliance.

Table I. Number of pDCs (CD11c⁺B220⁺PDCA-1⁺) in inguinal DLNs, spleens, and lungs of BP2 mice depleted or not depleted with anti-PDCA-1 Ab

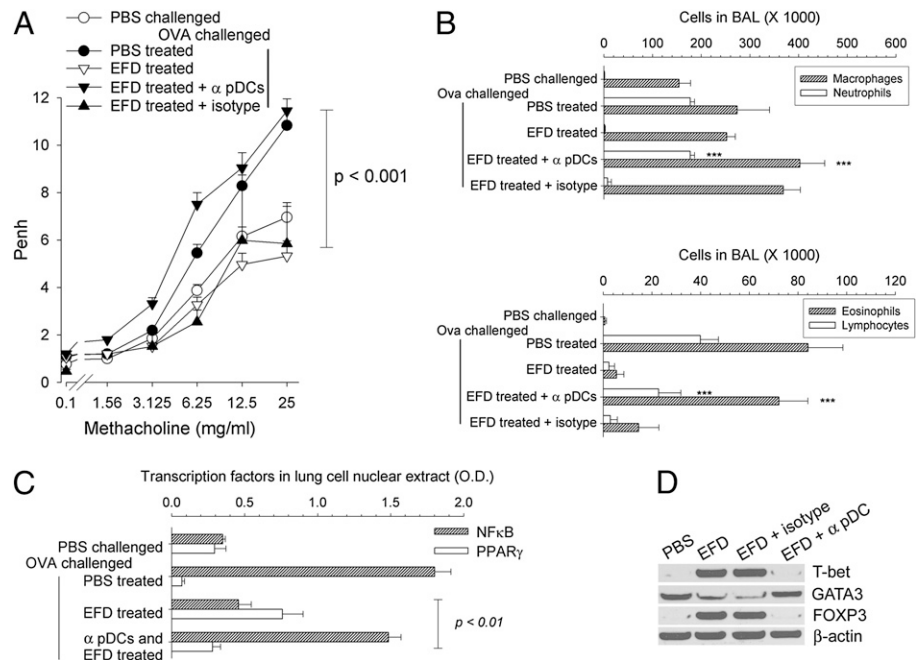
No. pDCs $\times 10^4$	Naive	PBS	EFD	EFD + Isotype	EFD + Anti-PDCA-1	(%) ^a
24 h						
Inguinal DLNs	0.61 ± 0.03	0.59 ± 0.02	1.12 ± 0.08	0.95 ± 0.06	0.09 ± 0.008	91.1
Spleen	11.31 ± 0.88	10.93 ± 0.93	10.15 ± 0.83	12.09 ± 0.68	0.97 ± 0.05	92.0
Lungs	1.09 ± 0.12	1.07 ± 0.09	0.97 ± 0.17	1.05 ± 0.11	0.11 ± 0.01	89.6
96 h						
Inguinal DLNs	0.62 ± 0.04	0.70 ± 0.03	2.46 ± 0.11	2.52 ± 1.46	0.82 ± 0.02	68.0
Spleen	11.04 ± 0.43	10.38 ± 0.62	10.49 ± 0.37	10.43 ± 0.57	5.28 ± 0.23	49.4
Lungs	1.02 ± 0.14	1.04 ± 0.07	1.09 ± 0.15	1.11 ± 0.12	0.49 ± 0.04	55.3
28 d ^b						
Inguinal DLNs	0.82 ± 0.24	0.62 ± 0.2158	0.79 ± 0.08	0.70 ± 0.06	0.62 ± 0.05	11
Spleen	12.39 ± 0.58	12.79 ± 0.39	10.76 ± 0.39	14.29 ± 0.60	12.87 ± 0.33	10
Lungs	0.99 ± 0.08	1.91 ± 0.18	1.69 ± 0.13	1.60 ± 0.14	1.80 ± 0.27	0

BP2 mice were OVA-sensitized and PBS- or EFD BCG-treated as described in *Materials and Methods*. One group received either isotype or anti-PDCA-1 Abs at the time of EFD BCG treatment. The number of pDCs (CD11c⁺B220⁺PDCA-1⁺) was detected after flow cytometry analysis of DLN, spleen, and lung cells recovered 24, 48 h, and 28 d after the treatment.

^aThe percentage of pDC depletion after PDCA-1 Ab treatment was calculated versus the EFD BCG treated, isotype-receiving group.

^bTwenty-eight days after the treatments and 24 h after the OVA challenge.

FIGURE 4. pDC depletion abrogates the protective effect of EFD BCG. OVA-sensitized and EFD BCG-treated mice, pDC-depleted or not depleted at the times of the treatment (six mice per group), were PBS- or OVA-challenged 28 d later. (A) Twenty-four hours after the challenge, Penh values were recorded after increasing doses of methacholine. (B) Inflammatory cells numbers in the BAL fluid. (C) NF- κ B p65 translocation and PPAR- γ expression were measured in nuclear lung cell extracts of six mice per group. (D) T-bet, GATA-3, and FOXP3 transcription factor expression in lung cell extracts from OVA-challenged mice. *** $p < 0.001$; Student t test.



EFD BCG-treated and CD25⁺-depleted mice had after the OVA challenge similar Penh values to those of the PBS-treated mice (Fig. 5A). EFD BCG-treated mice receiving or not receiving the isotype control, and therefore not CD25⁺-depleted, were fully protected. The inhibitory effect of EFD BCG treatment on AHR observed only in nondepleted mice was confirmed by resistance-compliance (Fig. 5A). There were in EFD BCG and CD25⁺-depleted mice significantly more inflammatory cells in the BAL (Fig. 5B) and more NF- κ B p65 and less PPAR- γ in lung cell nuclear extracts (Fig. 5C) than in the EFD BCG-treated mice. We observed an upregulation of FOXP3 and T-bet and reduced expression of GATA-3 in lung cells extracts from EFD BCG-treated mice, with or without isotype control, and not after depletion of CD25⁺ cells (Fig. 5D). Similar data were found in spleen cell extracts (data not shown).

EFD regulates transcription factors that influence Th2 and Th17 differentiation and inflammation

Allergic asthma is considered to be a Th2-dominant inflammatory disease of the lungs; however, IL-17 also participates in inflammation by promoting neutrophil influx into airways (23). After the OVA challenge, IL-17 was not detected in BAL fluids, but we found sig-

nificantly less IL-17 in sera of EFD BCG-treated mice than in that of PBS-treated mice. On the contrary, IL-17 was not reduced in sera of live and HK BCG-treated mice (Fig. 6A). The expression of ROR γ t, the transcription factor signature of the Th17 immune response, was reduced only in lung extracts of EFD BCG-treated mice, and only EFD BCG reduced GATA-3 and enhanced FOXP3 and T-bet (Fig. 6B).

The MAPK cascade is one of the important signaling pathways in immune responses, and p38 MAPK is involved in mucus cell metaplasia (24). The expression of inflammatory cytokines is regulated by NF- κ B (25) after degradation of I- κ B α and translocation of NF- κ B p65 to the nucleus. To understand the mechanisms by which EFD BCG, but not live or HK BCG, blocks NF- κ B p65 and regulates lung inflammation, we investigated the effects of the various BCG preparations on the p38 MAPK pathway and I- κ B α phosphorylation. After OVA challenge, EFD BCG, but not PBS or live or HK BCG treatments, blocked p38 MAPK and I- κ B α phosphorylation in lung extracts (Fig. 7A).

Because PPAR- γ expression was enhanced after EFD BCG treatment whereas NF- κ B p65 translocation was blocked (Figs. 4C, 5C), we next sought to determine whether PPAR- γ is enhanced after live and HK BCG treatments. The expression of PPAR- γ was significantly

Table II. Number of Tregs (CD4+CD25⁺) in inguinal DLNs, spleens, and lungs of BP2 mice depleted or not depleted with anti-CD25 Ab

No. Tregs $\times 10^5$	Naive	PBS	EFD	EFD + Isotype	EFD + Anti-CD25	(%) ^a
24 h						
Inguinal DLNs	3.03 $\pm$ 0.3	3.10 $\pm$ 0.19	4.94 $\pm$ 0.13	4.76 $\pm$ 0.24	0.86 $\pm$ 0.04	82.0
Spleen	32.01 $\pm$ 1.02	31.84 $\pm$ 0.70	30.90 $\pm$ 1.40	32.39 $\pm$ 0.35	10.27 $\pm$ 0.60	68.3
Lungs	1.43 $\pm$ 0.09	1.18 $\pm$ 0.06	1.29 $\pm$ 0.03	1.26 $\pm$ 0.11	0.40 $\pm$ 0.01	68.3
96 h						
Inguinal DLNs	3.00 $\pm$ 0.18	3.06 $\pm$ 0.17	25.69 $\pm$ 0.85	26.44 $\pm$ 0.59	2.10 $\pm$ 0.18	92.1
Spleen	29.11 $\pm$ 1.00	31.79 $\pm$ 3.48	41.23 $\pm$ 2.24	39.97 $\pm$ 0.38	9.96 $\pm$ 0.52	75.1
Lungs	1.51 $\pm$ 0.10	1.38 $\pm$ 0.04	1.32 $\pm$ 0.04	1.31 $\pm$ 0.11	0.39 $\pm$ 0.01	49.0
28 d ^b						
Inguinal DLNs	3.29 $\pm$ 0.24	3.00 $\pm$ 0.16	3.43 $\pm$ 0.19	3.23 $\pm$ 0.14	2.76 $\pm$ 0.67	14.6
Spleen	29.24 $\pm$ 1.69	25.71 $\pm$ 0.44	82.39 $\pm$ 2.18	83.43 $\pm$ 3.37	28.83 $\pm$ 0.84	65.5
Lungs	1.38 $\pm$ 0.07	1.49 $\pm$ 0.14	4.12 $\pm$ 0.32	4.09 $\pm$ 0.19	1.94 $\pm$ 0.17	52.6

BP2 mice were OVA-sensitized and PBS- or EFD BCG-treated as described in *Materials and Methods*. One group received either isotype or anti-CD25 Abs at the time of EFD BCG treatment. The number of Tregs (CD4+CD25⁺) was detected after flow cytometry analysis of DLN, spleen, and lung cells recovered 24, 48 h, and 28 d after the treatment.

^aThe percentage of Treg depletion after anti-CD25 treatment was calculated versus the EFD BCG, isotype-receiving group.

^bTwenty-eight days after the treatments and 24 h after the OVA challenge.

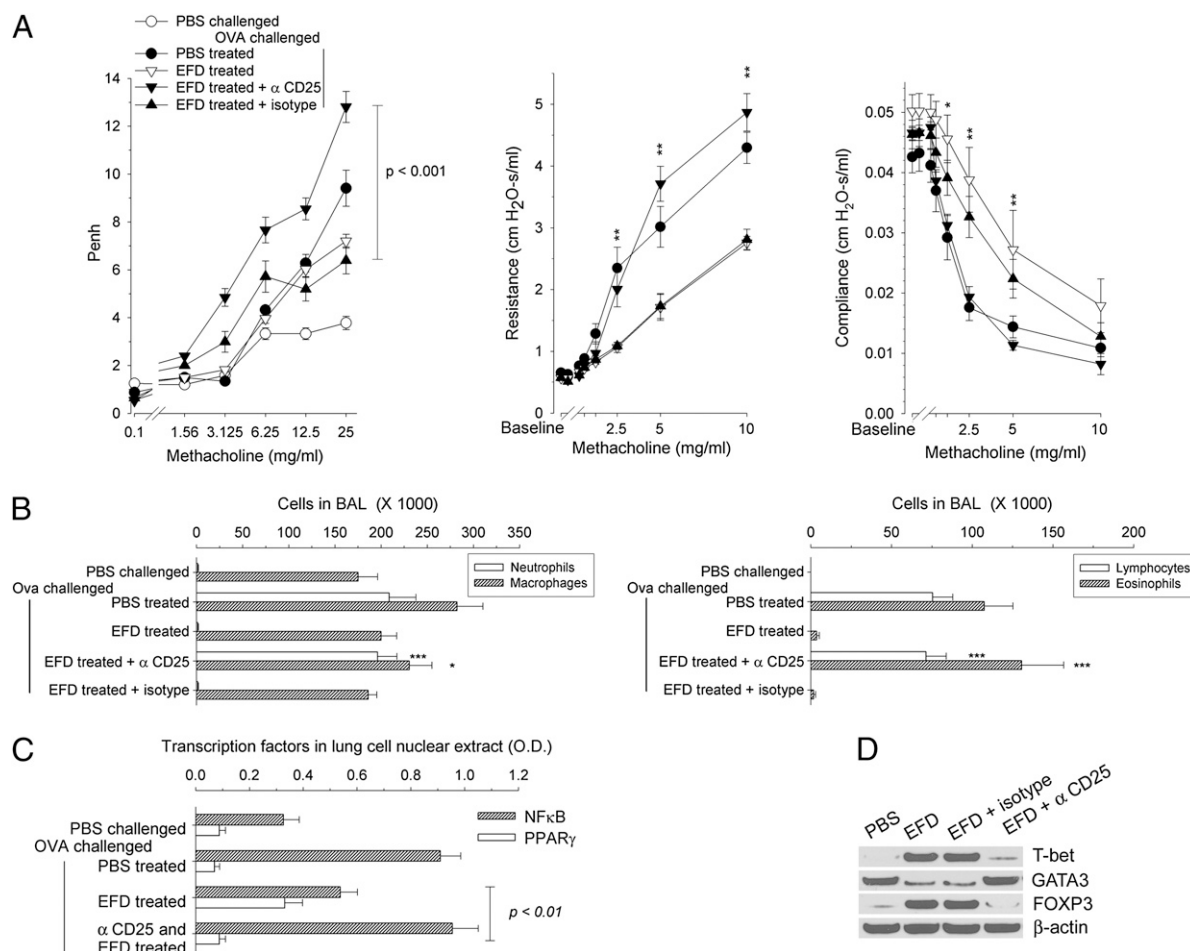


FIGURE 5. CD25 depletion abrogates the protective effect of EFD BCG. OVA-sensitized and EFD BCG-treated mice, CD25-depleted or not depleted at the times of the treatment (six mice per group), were PBS- or OVA-challenged 28 d later. (A) Twenty-four hours after the challenge, Penh values and resistance-compliance were recorded after increasing doses of methacholine. (B) Inflammatory cell numbers in the BAL fluid. (C) NF-κB p65 translocation and PPAR-γ expression were measured in nuclear lung cell extracts. (D) T-bet, GATA-3, and FOXP3 transcription factors expression in lung cell extracts from OVA-challenged mice. $*p < 0.05$; $***p < 0.001$; Student *t* test.

lower in lung cell extracts of PBS- and live and HK BCG-treated mice as compared with those of EFD BCG-treated (Fig. 7B). COX-2 is enhanced in asthma (26), and ligands of RXRα and PPAR-γ decrease COX-2 expression (27). In correlation with high PPAR-γ expression, only EFD BCG treatment blocked the phosphorylation of RXRα and reduced COX-2 expression (Fig. 7C, 7D).

Discussion

We have previously described a mouse model in which EFD BCG administered s.c. protected and cured acute lung inflammation, whereas live and HK BCG did not (11). Various mechanisms may

explain the unique immunoregulatory activity of EFD that is absent for live or HK BCG. Surface molecule expression and/or control of APC metabolism may differ according to the method of preparation of each formulation. Functions of macrophages and DC subsets, including cytokine production, could be differentially triggered during the processing of the various BCG preparations used. We showed that EFD BCG expanded or recruited more pDCs than live or HK BCG. The pDCs migrating from the site of injection or locally recruited promote the differentiation of Tregs in the DLNs. These Tregs migrate and home in on various tissues, including spleen and lungs, where they exert an immunoregulatory

FIGURE 6. EFD BCG treatment regulates transcription factors associated with inflammation. (A) IL-17 was measured in the sera of OVA-sensitized, PBS-, EFD- and live and HK BCG-treated and OVA-challenged mice (six mice per group). (B) Transcription factor expression (T-bet, GATA-3, Foxp3, and RORγt) were measured by Western blot in the lung cell extracts from two pools of three mice per group. After Scan-Analyze of the bands, data are expressed as the ratio of target protein to β-actin. Because there were only two Scan-Analyze data points per experiment, statistical studies were not done (#). Data are representative of two independent experiments. $***p < 0.001$; Student *t* test.

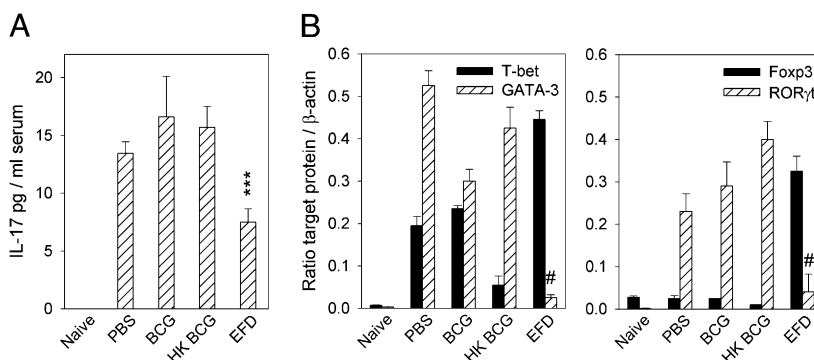
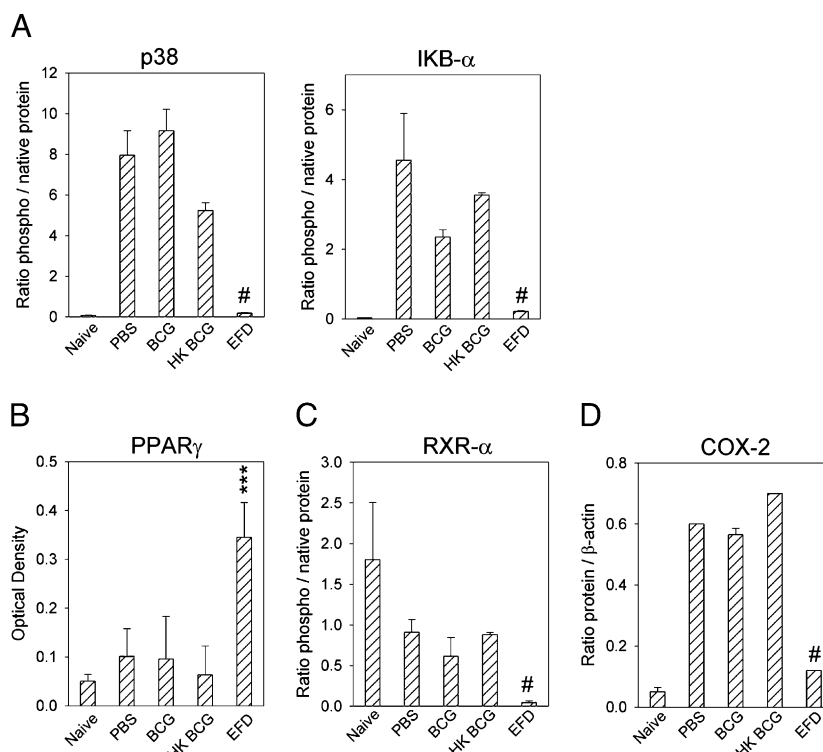


FIGURE 7. EFD BCG treatment upregulates PPAR- γ expression and blocked the phosphorylation of transcription factors associated with inflammation. OVA-sensitized mice (six per group) were PBS-, EFD- and live and HK BCG-treated and OVA-challenged. (A) Phosphorylated and native forms of p38 MAPK and I- κ B α of lung cell extracts (two pools of three mice per group) were measured by Western blot. After Scan-Analyze of the bands, data are expressed as the ratio of phosphoprotein to native protein. (B) PPAR- γ expression (OD) was measured in nuclear lung cell extracts of six mice per group. (C) Phosphorylated and native form of RXR- α in lung cell extracts were measured and expressed as described in A. (D) COX-2 was measured by Western blot in lung cell extracts of two pools of three mice per group. After Scan-Analyze of the bands, data are expressed as the ratio of target protein to β -actin. Because there were only two Scan-Analyze data points per experiment, statistical studies were not done (#). *** $p < 0.001$; Student t test.



function through the production of IL-10. This prevents recruitment or interferes with the functions of allergenic lung DCs. Lung pDCs have been shown to promote an anti-inflammatory response (20, 28). EFD BCG was able to recruit in the inguinal DLNs shortly after injection mature pDCs upregulating MHC class II and DEC205 molecules, previously reported to be expressed only on cDCs (29). The pDCs recruited in the inguinal DLNs after EFD BCG injection expressed the specific marker PDCA-1 but differ from those previously described (18) by expressing very low levels of the Ly6G-Gr1 marker. Further investigation is needed to attribute them to the distinct biological process associated with pDCs, such as IFN- α production in response to TLR-9 stimulation. Our pDCs secreted IL-10 and were involved in the polarization of naive T cells toward cells secreting IL-10, suggesting that these T cells are Tregs rather than tolerogenic Th3 T cells that produce much more TGF- β and develop preferentially in the gut (30). Thus, the selection or differentiation of different types of DCs by various Ags or microbial molecules may greatly influence which Treg subset will develop. Indeed, the pDCs recruited after EFD BCG injection play a key role in activating and expanding immunoregulating Tregs, as demonstrated by transient depletion of pDCs at the time of EFD BCG treatment, which impaired the development of FOXP3-expressing cells and subsequent reduction of AHR. In our model, we never observed an enhancement of the number of pDCs in the lungs of EFD BCG-treated mice in contrast to recent data showing that Flt3L treatment suppressed features of asthma and favored the accumulation of pDCs in the lungs of allergen-challenged mice (31). Our findings emphasize the importance of the pDC subset recruited to the inguinal DLNs, which played an essential role in the local induction of Tregs that migrated to spleen and lungs where they exerted a protective immunoregulatory response. Indeed, depletion of pDCs or CD25⁺ cells at the time of EFD BCG injection, during the initiation of the immune response, abrogated the protective effect of EFD BCG, resulting in a substantial increase in AHR. It has been recently shown that depletion of Tregs during

the initiation of the immune response enhances AHR, whereas, during the effector phase, it fails to induce changes (32).

Interactions between pathogens and Tregs are of mutual benefit to the pathogen and host. They allow persistence of infection, maintenance of memory, and resistance to reinfection (33). However, disrupting the balance of Tregs and effector T cells can lead to disease reactivation (34). High *Mycobacterium tuberculosis* loads in humans have been reported to give rise to IL-10-producing T cells that suppress specific immune responses (35). An important finding of the current study is that EFD BCG promotes the generation of IL-10-producing pDCs and Tregs that do not suppress the Th1 immune response, as assessed by upregulation of T-bet expression. To confirm the nonsuppressive effect of EFD BCG, our unpublished data have shown that EFD BCG-treated mice or guinea pigs remained able to control the bacterial load of a virulent *M. tuberculosis* strain and even that the protective capacity of the BCG vaccination against *M. tuberculosis* challenge was not diminished by EFD BCG treatment.

Allergic asthma is considered to be a Th2-dominant inflammatory disease of the lungs; however, Th17 cells may also contribute to the pathogenesis of classically recognized Th2-mediated allergic disorders (36). It was reported that IL-17 increased in BAL and sera from asthmatic patients (37) and IL-17 inhalation led to induction of neutrophilia rather than eosinophilia in the airways of mice and rats (23, 38). In our murine model of asthma, only EFD BCG significantly decreased neutrophils, IL-17, and ROR γ t and enhanced FOXP3 in accordance with recent data showing that FOXP3 directly interacts with ROR γ t (39).

During inflammation, activated monocytes/macrophages, neutrophils, leukocytes, and endothelial cells produce inflammatory markers (TNF- α , IL-6, IL-12, and COX-2), and PPAR- γ negatively regulates proinflammatory gene expression of these markers by interfering with the NF- κ B signaling pathway (40–42). COX-2 has been associated with asthma (26), and in the rat colitis inflammation model, administration of PPAR- γ agonist reduces the expression of COX-2 and NF- κ B p65 proteins (43). In accordance

with these observations, we showed that EFD BCG enhanced PPAR- γ , blocked the NF- κ B pathway, and reduced COX-2 expression. Because of their essential roles in the intracellular signaling network, mitogen-activated protein pathways could be appropriate targets for pharmacological treatment of asthma (44). In our model, live and HK BCG treatments were unable to reduce phosphorylation of p38 MAPK or activation of NF- κ B, whereas EFD BCG blocked both, enhanced PPAR- γ , and therefore decreased AHR, eosinophilia, and GATA-3 expression in accordance with previous results (45, 46). The malfunction of RXR α due to post-translational modification by phosphorylation was associated with colon inflammation or cancer (27, 47). In our model, we found that EFD BCG inhibited RXR α phosphorylation and thus enhanced PPAR- γ expression in the lungs of mice. The precise mechanism by which EFD BCG induces the activation of the RXR α /PPAR- γ heterodimer and negatively regulates lung inflammation remains to be elucidated.

Several new treatments are under development for asthma, but many of them are highly specific and thus might only influence a single aspect of the disease and might have a minor clinical impact (44). In contrast, EFD BCG treatment combines simultaneously: 1) an immunoregulatory effect on Th2 and Th17 cells through a mechanism relying on pDCs and persistent Tregs; 2) a blockade of inflammatory pathways, NF- κ B activation, and p38 phosphorylation; and 3) an increase of PPAR- γ expression. Moreover, EFD BCG does not induce side effects (11) and does not modify ongoing Th1-mediated effector mechanisms. Thus, EFD BCG may become an effective immunotherapeutic preparation against asthma in humans that will not impair capacity to control infections, and it could be used not only in asthma but also in other inflammatory diseases.

Acknowledgments

We thank A. Herbelin and L. Araujo for the gift of anti-CD25 Ab and G. Milon and H. Bercovier for critical reading of the manuscript.

Disclosures

The authors have no financial conflicts of interest.

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