Mycobacterium bovis Bacillus Calmette-Guérin Killed by Extended Freeze-Drying Reduces Colitis in Mice

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BACKGROUND & AIMS: *Mycobacterium bovis* Bacillus Calmette-Guérin (BCG), killed by extended freeze-drying (EFD), induces secretion of interleukin-10 and reduces lung inflammation in a mouse model of asthma. We investigated the effects of EFD BCG in mouse models of inflammatory bowel disease. **METHODS:** EFD BCG was administered subcutaneously to mice with colitis induced by dextran sodium sulfate (DSS), oxazolone, or adoptive transfer of $CD4^+CD45RB^{high}Foxp3^-$ T cells from C57Bl/6 Foxp3GFP mice to $RAG2^{-/-}$ mice. **RESULTS:** EFD BCG, administered either before induction of DSS and oxazolone colitis or after development of acute or chronic DSS-induced colitis, reduced symptom scores, loss of body weight, and inflammation. Although transfer of $CD4^+CD45RB^{high}Foxp3^-$ cells induced colitis in $RAG2^{-/-}$ mice, administration of EFD BCG at the time of the transfer converted $Foxp3^-$ T cells to $Foxp3^+$ T cells and the mice did not develop colitis. EFD BCG protected mice from colitis via a mechanism that required expansion of T regulatory cells and production of interleukin-10 and transforming growth factor β. EFD BCG activated the retinoid X receptor (RXR)-α–peroxisome proliferator-activated receptor (PPAR)-γ heterodimer, blocked translocation of nuclear factor κB to the nucleus, and reduced colonic inflammation; it did not increase the number of colon tumors that formed in mice with chronic DSS-induced colitis. **CONCLUSIONS:** EFD BCG controls severe colitis in mice by expanding T regulatory cell populations and PPAR-γ and might be developed to treat patients with inflammatory bowel disease.

Keywords: Killed BCG; IL-10; Therapeutic Vaccine; Immune Regulation.

BASIC AND TRANSLATIONAL AT

Inflammatory bowel diseases (IBDs) are chronic disorders of the gastrointestinal tract, including Crohn's disease and ulcerative colitis. The pathogenesis of IBD has not been completely elucidated, but there is increasing evidence that inflammatory cytokines and chemokines produced in the lesions with local neutrophil accumulation seem to play a major role in acute flares of the disease.[1–3] The pathogenesis of IBD involves gut epithelial barrier defects, environmental, microbial, and genetic factors.[1,4,5] IBD has been linked to deficiencies in the regulation of the immune response, resulting in an excess of inflammatory stimuli and mediators.[6]

Analyses of immune responses in animal models have improved our understanding of the physiopathology of IBD.[7,8] However, the treatment of patients with IBD remains a major challenge in clinical practice. Recently developed therapeutic approaches such as inhibitors of proinflammatory cytokines, blockade of T cells or of lymphocyte migration, or peroxisome proliferator-activated receptor (PPAR)-γ agonists may be associated with side effects, and treatment failure may occur over time.[9–11] PPAR-γ expression is impaired in the epithelial cells of the colon in patients with ulcerative colitis, suggesting that PPAR-γ that inhibits nuclear factor κB (NF-κB) p65 translocation may have a protective effect in IBD.[12,13] Accumulating data from clinical and experimental studies have also highlighted the important role of T regulatory cells (Tregs) producing transforming growth factor (TGF)-β or interleukin (IL)-10 in the control of IBD.[14,15] We have shown that *Mycobacterium bovis* Bacillus Calmette-Guérin (BCG) killed by extended freeze-drying (EFD) reverses established inflammation in a murine model of asthma by increasing the number of IL-10-producing Tregs and by enhancing PPAR-γ expression.[16,17] Therefore, we analyzed the effect of EFD BCG in 3 experimental models of IBD: (1) dextran sodium sulfate (DSS)-induced colitis, which is a useful murine model of IBD for studies of the cellular basis of disease and the validation of therapeutic agents for human IBD[18,19]; (2) oxazolone-induced colitis[20]; and (3) spontaneous colitis developing after the adoptive

Abbreviations used in this paper: **BCG, Bacillus Calmette–Guérin; DSS, dextran sodium sulfate; EFD, extended freeze-dried; IFN, interferon; IL, interleukin; MLN, mesenteric lymph node; MPO, myeloperoxidase; NF-κB, nuclear factor κB; PPAR, peroxisome proliferator-activated receptor; RXR, retinoid X receptor; TGF, transforming growth factor; Treg, T regulatory cell.**

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transfer of naïve T cells ($CD4^+CD45RB^{high}$) into $RAG2^{-/-}$ mice.[21]

We found that EFD BCG prevented DSS- and oxazolone-induced colitis and cured mice with established acute and chronic DSS-induced colitis, as shown by normal colon length as well as decreased proinflammatory cytokines and myeloperoxidase (MPO) activity. EFD BCG enhanced PPAR-γ expression and inhibited nuclear NF-κB translocation. Furthermore, it converted naïve T cells into Tregs expressing Foxp3 at the local (gut) and systemic (spleen) levels in the model of adoptive transfer to $RAG2^{-/-}$ mice. Blockades of anti-inflammatory cytokines (IL-10, TGF-β) and Treg depletion cancelled the protective effect of EFD BCG in DSS-induced colitis.

Materials and Methods

Mice

Male BALB/c and C57Bl/6 mice were purchased from Charles River France or Centre d'Elevage Janvier (Le Genest, St Isle, France) for the DSS- and oxazolone-induced colitis models. Female Swiss mice from the Centre d'Elevage Janvier were used to study the incidence of adenocarcinomas in DSS-induced long-term colitis.[22] Female $RAG2^{-/-}$ mice (from the central animal house of Institut Pasteur) and C57Bl/6-Foxp3GFP mice (kindly provided by B. Malissen) were used as recipient and donor mice, respectively, in a model of colitis induced by the transfer of naïve T cells. Mice were 6 to 7 weeks old at the start of the study and were kept in specific pathogen-free conditions in accordance with national guidelines for animal welfare.

Acute and Chronic Chemically Induced Colitis

Acute DSS model. DSS (mol wt, 36,000–50,000; MP Biomedicals, Illkirch, France) was added to the drinking water of C57Bl/6 mice (2.5% DSS given for 5 days to mice from Elevage Janvier, and 3% DSS given for 6 days to mice from Charles River France). Mice were treated subcutaneously at the base of the tail with 100 μL phosphate-buffered saline (PBS) or 100 μg EFD BCG prepared as previously described[16]: (1) preventively (21 days before DSS administration), and mice were killed 7 days after the end of DSS treatment or (2) curatively (administration 2 days after the end of the course of DSS treatment), and mice were killed 20 days after EFD BCG treatment.

Chronic DSS model. C57Bl/6 mice were subjected to one cycle of treatment with 1.5% DSS in drinking water for 7 days followed by 10 days of sterile tap water and then to 2 cycles for 5 days of 1.5% DSS followed by 10 days of sterile tap water. Mice were treated subcutaneously at the base of the tail (2 days after the first DSS cycle) with 100 μL PBS or 100 μg EFD BCG. All mice were killed 18 days after the third DSS cycle.

Acute oxazolone model. BALB/c mice were treated subcutaneously, at the base of the tail, with 100 μL PBS or 100 μg EFD BCG. Three weeks later, mice were epicutaneously sensitized with 3% oxazolone (4-ethoxymethylene-2-phenyl-2-oxazolin-5-one; Sigma-Aldrich, St Quentin Fallavier France). Mice were anesthetized 8 days later and treated intrarectally with 1% oxazolone in 50% ethanol as previously described.[23] Control mice received 50% ethanol alone. Mice were killed 4 days after the oxazolone challenge.

Adoptive Transfer of Naïve T Cells

$CD4^+$ spleen cells from female C57Bl/6 Foxp3 GFP naïve mice were purified on an AutoMACS (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's instructions and were then sorted into $CD4^+CD45RB^{high}Foxp3^-$ cells (purity ≥90%) (FACSAria; BD, Le Pont de Claix, France). The purified naïve cells (3×10^5) were intravenously transferred to female $RAG2^{-/-}$ recipient mice receiving concomitant treatment with 100 μg EFD BCG or 100 μL PBS (subcutaneously at the base of the tail).

In Vivo Treatment With Anti–IL-10, Anti–TGF-β, or Anti-CD25 Antibodies

C57Bl/6 mice were treated with EFD BCG 21 days before the induction of acute colitis (3% DSS). Two days after DSS exposure, groups of mice were treated intraperitoneally with (1) 0.5 mg of anti-CD25 antibody (clone PC61, a gift from A. Herbelin), (2) 1 mg of anti–TGF-β antibody (clone 2G7, provided by L. Chatenoud), (3) 0.5 mg of anti–IL-10 antibody (clone JES-2A5), followed by a second injection at the end of DSS treatment (day 6) as previously described,[16] and (4) 0.5 mg of rat immunoglobulin G1 isotypic antibody. All mice were killed 10 days after the start of DSS treatment.

Statistical Analysis

Data are presented as mean ± SD. The Instat package from Graph Pad Software (San Diego, CA) was used to analyze the data, using the *t* test with Welch's correction.

Results

EFD BCG Prevents Acute DSS-Induced Colitis

Acute colitis resulted in substantial body weight loss in PBS-treated mice, whereas mice treated preventively with EFD BCG displayed only slight weight loss (Figure 1*A*). Clinical scores peaked at day 7 with diarrhea and wet blood present on the anus of PBS-treated mice, whereas only one mouse displayed diarrhea and none had wet blood on the anus in the EFD BCG–treated group (Figure 1*A*; $P < .01$). Colon length was significantly shorter in PBS- than in EFD BCG–treated and naïve mice (4.7 ± 0.5 cm, 6.1 ± 0.2 cm, and 6.4 ±0.5 cm, respectively; Figure 1*B*). Inflammatory infiltrate and a loss of normal colonic architecture were observed in the colon of PBS-treated mice, whereas EFD BCG–treated mice displayed only mild infiltration (Figure 1*C*). Five and 10 days after DSS exposure, EFD BCG–treated mice had a higher percentage and absolute number of $CD4^+Foxp3^+$ cells in the spleen, mesenteric lymph nodes (MLNs), and lamina propria than PBS-treated mice (Figure 1*D* and *E* and Supplementary Figure 1). By contrast, total cell and $CD4^+$ T-cell numbers were higher after PBS than after EFD BCG treatment (Supplementary Figure 2).

EFD BCG Cures Acute and Chronic DSS-Induced Colitis

We then assessed the therapeutic effect of EFD BCG administered after the onset (day 6) of acute IBD (2.5% DSS for 5 days), at a time at which clinical scores were high (3.4 ± 0.6) and substantial weight loss was observed (Figure 2*A*). Mouse body weight began to increase 2 days after the EFD BCG treatment, reaching that