

Extended freeze-dried *Mycobacterium bovis* Bacillus Calmette–Guérin induces the release of interleukin-12 but not tumour necrosis factor- α by alveolar macrophages, both *in vitro* and *in vivo*

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Summary

Background Airway hyper-responsiveness (AHR), chronic airway inflammation and predominance of the T helper type-2 (Th2; IL-4, IL-5, IL-13) over the Th1 (IL-2, IFN- γ) immune response are hallmarks of asthma. Alveolar macrophages (AM) are the most numerous cells in the airway lumen, where they represent the first immune cell population encountered by inhaled antigens. AM act as antigen-presenting cells (APC) and they release various soluble mediators and enzymes. AM thus play a prominent role in the modulation of the local immunity in airways. In allergic airways, AM have been implicated in the pathogenesis of inflammation by promoting the Th2 versus the Th1 cytokine patterns.

Objectives Infections with attenuated bacteria or challenges with bacterial products may involve AM. Such stimuli have been shown to potentially restore the Th1/Th2 balance in asthmatic airways, but they also induce the release of inflammatory mediators. We investigated the response of AM when stimulated by two preparations of non-proliferating Bacillus Calmette–Guérin (BCG).

Methods We evaluated the cytokine production by AM from BP2 and C57BL/6 mice when cultured with heat-killed (HK) and extended freeze-dried (EFD) BCG. We then investigated *in vivo* the release of soluble factors in the airway lumen of mice after instillation of these BCG preparations. Finally, we studied the profile of cytokine transcripts in the lung of mice pre-treated with BCG and then challenged with ovalbumin (OVA).

Results HK BCG induced the production of both TNF- α and IL-12, and did not prevent high levels of Th2 cytokine transcripts. In contrast, EFD BCG induced a response dominated by the production of IL-12, with no later over-expression of Th2 cytokine transcripts.

Conclusion Our results show that EFD BCG induce the release of the Th1-promoting cytokine IL-12 by AM, without the deleterious effects of HK BCG. These data suggest that EFD BCG may be considered as a potential novel treatment to restore the Th1/Th2 imbalance in asthma.

Keywords asthma, BCG, IL-12, macrophage, TNF- α

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Introduction

Chronic inflammation and airway hyper-responsiveness (AHR) are hallmarks of asthma, an allergic disorder associated with an increased number of CD4⁺ lymphocytes showing a T helper type-2 (Th2) profile [1]. During the past decade, the prevalence of asthma has increased significantly in countries sharing a 'western lifestyle' [2]. The hypothesis has emerged that reduced exposure to bacterial infections, vaccination and use of antibiotics early in life would favour a predominant Th2 (IL-4, IL-5,

IL-13) immune response in susceptible individuals, whereas early bacterial infections or other immunological modifiers could promote a Th1 (IL-2, IFN- γ) profile, thus explaining the lower incidence of asthma in other countries [3, 4].

Alveolar macrophages (AM) represent the most abundant immune cell population in the airway lumen [5]. Thus they constitute the first line of defense of the respiratory tract, notably by producing inflammatory and regulatory mediators in response to exogenous stimuli [6]. Previous studies have shown that AM from immunized guinea pigs, as those from allergic patients, are less responsive to the inhibitory effects of prostaglandin E2 and β -adrenergic agonists [7], and may contribute to the development of an allergic reaction [8, 9]. Indeed, the number of activated macrophages mainly producing inflammatory mediators is increased in asthmatic airways [10], and during asthma attacks both the early and late phases of acute inflammation involve AM [11]. Besides, AM have been

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shown to stimulate the Th2 cytokine unbalance in asthma [12], while activated macrophages in asthmatic airways over-express CD80 (B7.1) and CD86 (B7.2) molecules, which are required to support the production of Th2 cytokines by T cells [13]. Thus, AM may be involved in the pathogenesis of asthma, especially by stimulating the Th2 versus the Th1 cytokine pattern in allergic airways [14, 15].

Recent reports have highlighted the potential of bacterial infections or bacterial components to restore this Th1/Th2 balance in asthmatic airways. The production of various soluble factors by macrophages and dendritic cells has been described, including the inflammatory cytokines IL-1 β , IL-6 and TNF- α [16], or modulatory cytokines such as IL-10, IL-12 and IL-18 [15]. In particular, Erb and co-workers have demonstrated that the intranasal treatment with *Mycobacterium bovis* Bacillus Calmette–Guérin (BCG) suppresses several pathologic features in a mouse model of asthma [17].

However, one of the potential drawbacks for the use of live BCG in the treatment of asthma is their ability to multiply and, accordingly, to promote a sustained local inflammation. As a consequence, the protection against allergen-induced eosinophilic inflammation and AHR is masked for at least 2 months, the anti-allergic activity being recovered only when inflammation is over [18]. In order to eliminate the presence of living bacteria into the lung, with their potential risk of uncontrolled infection in immunocompromised host, we prepared and compared two different preparations of killed BCG.

In the present report, the early effects of these two preparations of killed BCG: 'classical' heat-killed (HK) BCG and BCG killed by extended freeze-drying were compared. HK and extended freeze-dried (EFD) BCG were assayed on cultured AM, in order to mimic *in vitro* the early reaction of the innate immunity after instillation of non-proliferating bacteria. HK BCG were strong inducers of the TNF- α and IL-12 release by AM. EFD BCG also induced an important production of IL-12 in AM but they failed to generate TNF- α . This dissociation in the production of TNF- α and IL-12 was confirmed by comparing *in vivo* the instillation of HK and EFD BCG into the mouse airways. Finally, we investigated whether such a treatment with BCG preparations in newborn mice could modulate the lung expression of cytokines when mice were ovalbumin-sensitized and submitted to an allergic challenge thereafter. Whereas mice pre-treated with HK BCG showed high levels of both Th1 and Th2 cytokine transcripts, mice pre-treated with EFD BCG showed transcript levels similar to normal controls, suggesting that the pre-immunization with EFD BCG might restore the Th1/Th2 balance.

Materials and methods

Animals, ovalbumin sensitization and challenge

Male BP2 and C57BL/6 mice (7–10 weeks), and newborn BP2 mice (10 days) were obtained from the 'Centre d'Elevage R. Janvier' (Le Genest St Isle, France). Data were all obtained from adult animals, either naive or ovalbumin (OVA)-sensitized. Sensitization to OVA (weeks 8 and 9) consisted of two subcutaneous injections (one injection/week) with 0.4 mL 0.9% NaCl containing 1 μ g OVA (Immunobiologicals, Lisle, IL, USA) and 1.6 mg aluminium hydroxide (Merck, Darmstadt,

Germany). When indicated, OVA-sensitized adult mice were also challenged by the intranasal injection of 50 μ L 0.9% NaCl containing OVA (1 mg/mL), at least 1 week after sensitization (week 10).

Preparation of killed BCG

The *M. bovis* BCG Pasteur strain 1173P2 has been grown on Sauton medium according to conditions used for vaccine production. The bacteria collected after 14 days of culture were extensively washed with distilled water onto a fiberglass filter (GF/C). Starting from this 'cake' two different procedures were used to kill the bacteria, a classical heat-killing procedure and a killing by an extensive freeze-drying procedure.

Heat killing was performed by autoclaving during 15 min at 120 °C a suspension of 10⁸ bacteria/mL in a borate buffer at pH 8.

Killing of the bacteria by extensive freeze-drying was performed on a frozen mixture of bacteria in water (1/1), left in the freeze-dryer during 4 days at a pressure of 80 μ Bar. The remaining water was then in the range of 0.5%. The bacterial corpses were kept in air at room temperature. The eventual persisting viability was checked on 1 mg of bacterial corpses (around 10⁹ bacteria), dispersed in saline (10 mL) and plated on Middlebrook 7H10 agar medium (Difco, Detroit, MI, USA).

Treatment of newborn and adult mice with BCG preparations

Eleven-day-old newborn BP2 mice were treated by the intranasal instillation of 10 μ L 0.9% NaCl containing 10⁵ bacterial corpses of HK or EFD *M. bovis* BCG Pasteur strain 1173P2, while OVA-sensitization was performed between weeks 7 and 10. The immune response in these HK or EFD pre-treated mice thus was explored exclusively in adult mice, at least 6 weeks after OVA-sensitization.

For *in vivo* experiments, BP2 mice were anaesthetized with an intramuscular injection of 100 μ L 0.9% NaCl containing 10% ketamine (Merial, Lyon, France) and 10% xylazine (Bayer Pharma, Puteaux, France). A catheter was placed in the trachea and 50 μ L 0.9% NaCl, either alone or containing 10⁷ bacterial corpses of HK or EFD BCG, were injected. Mice awoke 1 h later, and did not show any evidence of sickness during the following days.

Bronchoalveolar lavage, cell culture and stimulating reagents

Animals were anaesthetized by the intraperitoneal injection of a lethal dose of urethane (Sigma-Aldrich, St Quentin Fallavier, France, 15 mg/10 g body weight). Then the trachea was denuded and cannulated. For cytokine determination, lungs were rinsed with a total volume of 2.5 mL 0.9% NaCl containing EDTA (1 mM, Sigma-Aldrich) and leupeptin (10 mM, Sigma-Aldrich), and bronchoalveolar lavage (BAL) were centrifuged (400 g, 20 min). Then supernatants were aliquoted and stored at –20 °C until analysis.

For cell culture, AM were collected by rinsing the lungs with a total volume of 5 mL Hank's balanced salt solution (HBSS) containing 2% fetal calf serum (FCS; Boehringer Mannheim, Mannheim, Germany). Microscopic examination of BAL revealed that in mice sensitized (but not challenged) with OVA

as well as in controls, 96–100% of the recovered cells were macrophages. A few granulocytes were scarcely found, while no lymphocytes could be identified. The number of AM was evaluated by means of an electronic counter (Coulter Electronics, Luton, UK). After centrifugation (400 g, 20 min), cell pellets were resuspended in RPMI-1640 medium (Life Technologies, Grand Island, NY, USA) supplemented with 3% FCS, 2 mM glutamine, 100 U/mL penicillin and 100 µg/mL streptomycin (ICN Biochemicals, Aurora, OH, USA). AM were dispensed at a concentration of 10^5 cells/well in 96-well tissue culture plates (TPP, ATGC Technologies, Noisy-le-Grand, France), and allowed to adhere at least 2 h at 37 °C in 5% CO₂ humidified atmosphere. After 2 h of adherence, only adherent macrophages were identified in wells. We assumed that other cell types as well as non-adherent/dead macrophages (estimated less than 10%) were then removed by washing with RPMI-1640 medium. Isolated macrophages thus obtained were incubated for up to 96 h at 37 °C in 5% CO₂ humidified atmosphere with RPMI-1640 (supplemented with FCS, glutamine and antibiotics), containing stimulating reagents as follows: lipopolysaccharide (LPS; *Escherichia coli* 055:B5, Difco Laboratories) 1 µg/mL, HK or EFD BCG 5:1 (bacterial corpses : macrophage ratio). Non-adherent cells were absent of wells during the following days of cell culture. Aliquots of supernatant were collected at 2, 4, 6, 8, 12, 24, 48, 72 and 96 h of cell culture, and stored at –20 °C until analysis.

Cytokine and protein assays

Cytokine and proteins were measured either by enzyme-linked immunosorbent assay [ELISA; fibronectin (FN), IFN-γ, IL-12p40 and IL-13] or SPI-EIA (IL-10, TNF-α), in 96-well microtitre plates (MaxiSorb, Nunc, Roskilde, Denmark). INF-α and IL-12p40 secretions were measured with commercial kits from Biosource (Nivelles, Belgium), while IL-13 was measured with commercial kits from R&D (Minneapolis, MN, USA), according to the manufacturers' instructions. IL-10 and TNF-α assays were performed using pure or acetyl cholinesterase-conjugated rat anti-murine monoclonal antibodies (JES-25 A and MP6XT22/MP6XT3, respectively), as previously described [19, 20]. FN amounts were measured by an indirect competitive ELISA method described elsewhere [21]. For ELISA, the minimal detectable dose indicated by the manufacturers was 7 pg/mL, while the SPI-EIA methods developed in our laboratory allowed the detection of minimal doses of 10 pg/mL.

RNAs extraction, reverse-transcription, and multiplex polymerase chain reaction

BP2 mice pretreated with HK or EFD BCG as newborns were sensitized and challenged with OVA as adults. They were anaesthetized by the lethal injection of urethane (Sigma-Aldrich, 15 mg/10 g body weight). The right cardiac ventricle was perfused with 10 mL 0.9% NaCl to eliminate blood from the pulmonary vessels, and lungs were then resected, immediately frozen in liquid nitrogen and stored at –80 °C until RNA extraction.

Total RNAs were isolated with RNEasy columns (Quiagen, Courtaboeuf, France) according to manufacturer's instructions. The purity of extracted mRNAs was estimated by the ratio between readings taken at 260 nm and 280 nm (A_{260}/A_{280}

in water). Then, the integrity of mRNAs was checked by denaturing-agarose gel electrophoresis and ethidium bromide staining.

The cDNAs were produced by incubating 5 µg of total RNAs with 0.5-µg hexamers as random primers (Amersham Pharmacia Biotech, Buckinghamshire, UK), 0.5 µg RNAGuard (Amersham), 200 U M-MTLV reverse transcriptase RNase H[–], 5 µL enzyme buffer (Promega, Charbonnières, France) and 1.2 µL dNTP (100 mM, Eurobio, Les Ulis, France), in a total volume of 20 µL (H₂O ppi, qsp), for 1 h at 42 °C, followed by 2 min at 95 °C.

For the detection of IFN-γ, IL-2, IL-4, IL-5, IL-10, IL-12p40 and IL-13 cDNAs, multiplex PCR was performed using CytoXpress kits from Biosource. Variations in RNAs extraction, reverse transcription and PCR were normalized using GAPDH expression. The amplification was performed on a Peltier type 200 thermal cycler apparatus (MJ Research, Watertown, MA, USA). The optimal cDNAs dilution (1/10) and choice of the DNA polymerase (Biotaq, QBiogene, Illkirch, France) were determined by preliminary experiments. For a 45-µL reaction, 5 µL of diluted cDNAs and 2.5 U DNA polymerase were used with primers, dNTP and buffer provided by the manufacturer. The thermocycling profile was as follows: 96 °C 1 min, then 96 °C 1 min and 56 °C 4 min (two cycles), then 94 °C 1 min and 56 °C 2.5 min (40 cycles). The reaction was achieved by incubation at 70 °C for 10 min.

PCR products were resolved on a 2% agarose gel (MetaPhor, FMC Bioproducts, Rockland, ME, USA) containing 0.5 µg/mL ethidium bromide (Sigma-Aldrich). Images of gels were recorded after amplification with an Ultra-Lum system (Ultra-Lum, Serlabo, Bonneuil-Marne, France) under ultraviolet light, and semiquantification was achieved using ImageQuant on a Storm (Molecular Dynamics, Sunnyvale, CA, USA).

Statistical analysis

For Figs 1–3 results were expressed as means ± SEM for at least three independent experiments. Variations were tested with one-way analysis of variance (ANOVA) and Student's *t*-test when necessary. Differences were considered statistically significant when *P* < 0.05.

Results

Release of TNF-α and IL-12 by alveolar macrophages stimulated with LPS from *E. coli*

In order to define the pattern of cytokines released by AM in contact with bacteria-derived products, we investigated the presence of IFN-γ, IL-10, IL-12, IL-13 and TNF-α in supernatants collected from cultured AM after stimulation with LPS from *E. coli*. While non-stimulated AM failed to produce any detectable amounts of those cytokines (not shown), AM stimulated with LPS only secreted TNF-α and IL-12 (Fig. 1).

AM isolated from BP2 and C57BL/6 mouse strains showed a peak release of TNF-α at 48 h and 72 h, respectively (Fig. 1a). The pattern of TNF-α secretion for both strains was similar, but after the 48 h peak the secretion decreased more rapidly for AM from BP2 mice. Thus AM from C57BL/6 mice showed a higher secretion of TNF-α at 72 h and 96 h (*P* < 0.05 and *P* < 0.01, respectively).

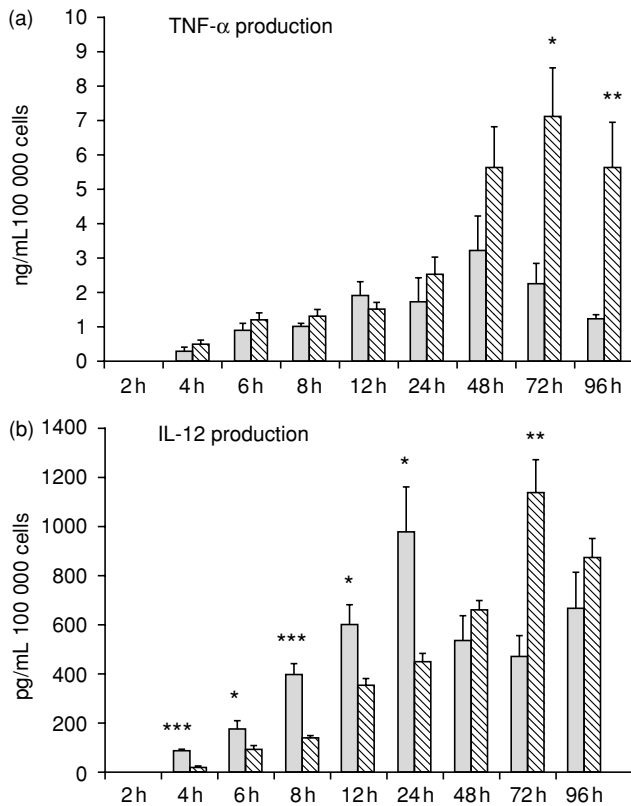


Fig. 1. Effects of the stimulation with LPS on TNF- α and IL-12 production by alveolar macrophages (AM). AM obtained from BAL of BP2 (grey bars) and C57BL/6 (diagonal bars) mice were cultured in 96-well tissue plates (10^5 cells/well) with LPS at $1 \mu\text{g/mL}$ for 4 days. Supernatant samples were collected at each time point and then analysed by SPI-EIA (TNF- α) or ELISA (IL-12). Analyses of differences between BP2 and C57BL/6 mouse strains were reported on the figure as *** $P < 0.005$; ** $P < 0.01$; * $P < 0.05$ (Student's *t*-test).

The release of IL-12 showed a slightly different pattern for AM isolated from either BP2 or C57BL/6 mice (Fig. 1b). The secretion of IL-12 peaked at 24 h for AM from BP2 mice, and at 72 h for AM from C57BL/6 mice. The earlier response, judged on the secretion of IL-12, was higher for BP2 mice during the first day ($P < 0.05$), while the secretion of IL-12 was higher for C57BL/6 mice at 72 h ($P < 0.005$).

Thus, AM from BP2 and C57BL/6 mice both reacted to the stimulation with Gram-negative bacteria LPS by secreting large quantities of TNF- α and IL-12 for at least 96 h.

Release of TNF- α and/or IL-12 by alveolar macrophages when stimulated with HK and EFD BCG

As observed with LPS, AM failed to produce any detectable amounts of IFN- γ , IL-10 and IL-13 when stimulated with either HK or EFD BCG.

First, HK and EFD BCG were tested as inducers of the TNF- α secretion by AM from BP2 and C57BL/6 mice (Figs 2a and b, respectively). In both strains, only HK BCG induced the sustained release of TNF- α by AM, while levels released with EFD BCG remained undetectable. Thus, AM from both BP2 and C57BL/6 mice reacted to stimulation with HK BCG by secreting TNF- α , but EFD BCG failed to do so.

We then investigated the release of IL-12 by AM when stimulated by HK or EFD BCG. BCG preparations induced distinct patterns of IL-12 secretion by AM from BP2 and C57BL/6 mice. AM from BP2 mice produced large amounts of IL-12 when stimulated either by HK or EFD BCG (Fig. 2c). In contrast, stimulated AM from C57BL/6 mice secreted low levels of IL-12 (Fig. 2d).

Both HK and EFD BCG thus induced the release of IL-12 by AM from BP2 or C57BL/6 mice. These preparations were particularly efficient when applied to AM from BP2 mice.

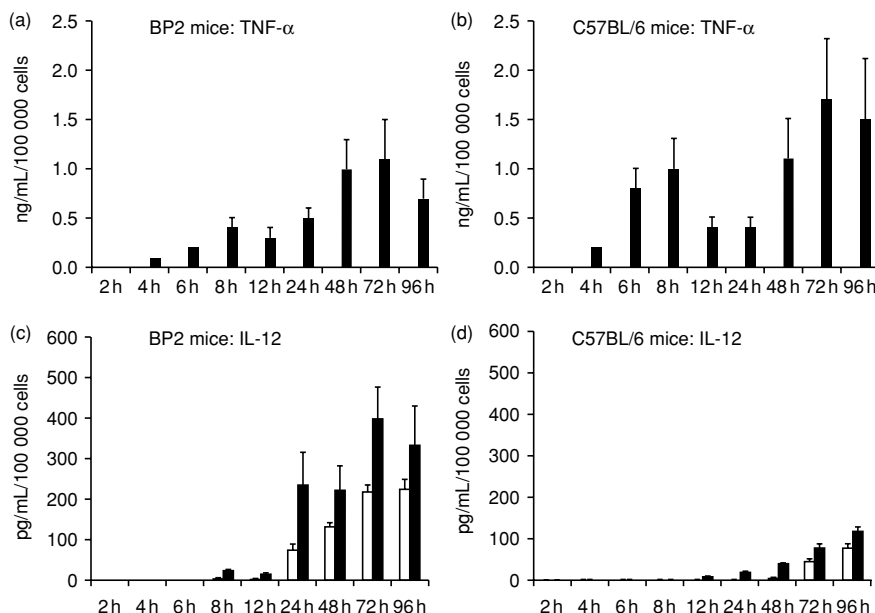


Fig. 2. Effects of the stimulation with EFD BCG (□) and HK BCG (■) on the TNF- α and IL-12 production by AM. AM obtained from BAL of BP2 and C57BL/6 mice were cultured in 96-well tissue plates (10^5 cells/well) with BCG corpses at a ratio of 5:1 for 4 days. Supernatant samples were collected at each time-point and then analysed by SPI-EIA (TNF- α) or ELISA (IL-12). The secretion of TNF- α by AM from BP2 (a) and C57BL/6 mice (b) was detectable only after HK stimulation ($P = 0.002$ and $P = 0.001$, respectively). The secretion of IL-12 by AM from BP2 (c) and for C57BL/6 mice (d) was not statistically different between EFD and HK BCG.

The intratracheal instillation of EFD BCG stimulates the secretion of IL-12 with minor release of TNF- α and FN in the airway lumen of BP2 mice

The secretion of TNF- α by AM stimulated with preparations of BCG may be a drawback for their use *in vivo*. We thus tested whether the production of TNF- α and IL-12 obtained with cultured AM was still observed when preparations of BCG were directly instilled into the trachea of BP2 mice. The amounts of TNF- α and IL-12 were measured on the BAL collected in the hours following instillation. We also investigated the presence of FN as a soluble marker of inflammation in the airway lumen (Fig. 3).

HK BCG induced a significant release of FN in the airway lumen ($P = 0.025$), while the levels observed with EFD BCG were not statistically different from controls instilled with saline alone (Fig. 3a).

HK BCG induced a transient high secretion of TNF- α , 3–24 h after tracheal instillation, while EFD BCG induced only a marginal secretion of TNF- α (Fig. 3b). In contrast, the levels of IL-12 were similar with HK and EFD BCG from as far as 24 h after instillation (Fig. 3c). However, because the production of IL-12 induced with HK BCG continuously increased, the levels were higher at 48 h and 96 h compared to EFD BCG ($P < 0.001$ and $P < 0.005$, respectively).

The intratracheal instillation of BCG preparations thus confirmed that HK BCG induced the release of TNF- α , and IL-12, while EFD BCG only stimulated the latter. The peak of FN in BAL of HK BCG-treated mice confirmed the inflammatory response due to TNF- α secretion.

The preventive treatment with EFD BCG prevents the development of a Th2 unbalance in the lung of OVA-sensitized BP2 mice

The stimulation of cultured AM, as well as the *in vivo* instillation of BCG preparations in mouse airways, revealed that HK and EFD BCG gave rise to distinct responses by the airway innate immunity. We next investigated whether the treatment of newborn mice with HK or EFD BCG could modulate the cytokine balance in the lung of these mice sensitized and challenged with OVA as adults (Fig. 4).

Because the delay of secretion may be variable from one cytokine to another, we performed multiplex RT-PCR to compare at the same time the expression of IFN- γ , IL-2, IL-4, IL-5, IL-10 and IL-12p40 after an OVA challenge. Basal levels in non-treated/non-sensitized mice were given as a reference, while non-treated/OVA-sensitized mice were used as pathologic controls.

The expression of IL-2 and IL-10 mRNAs remained very close to the basal levels in all conditions, except one slight peak (26% of GAPDH level) of IL-10 mRNAs at 1 h, in mice pre-treated with HK BCG (not shown).

The patterns of expression for IL-4 (Fig. 4a) and IL-5 (Fig. 4b) mRNAs were very similar after the OVA challenge. This expression increased with time in controls (max. 60% and 86% of GAPDH level at 6 h, respectively), while mice pre-treated with HK BCG showed an early overexpression of both IL-4 and IL-5 mRNAs (max. 69% and 77% at 1 h, respectively). In contrast, mice pre-treated with EFD BCG showed levels of IL-4 and IL-5 mRNAs similar or lower to basal levels. Only

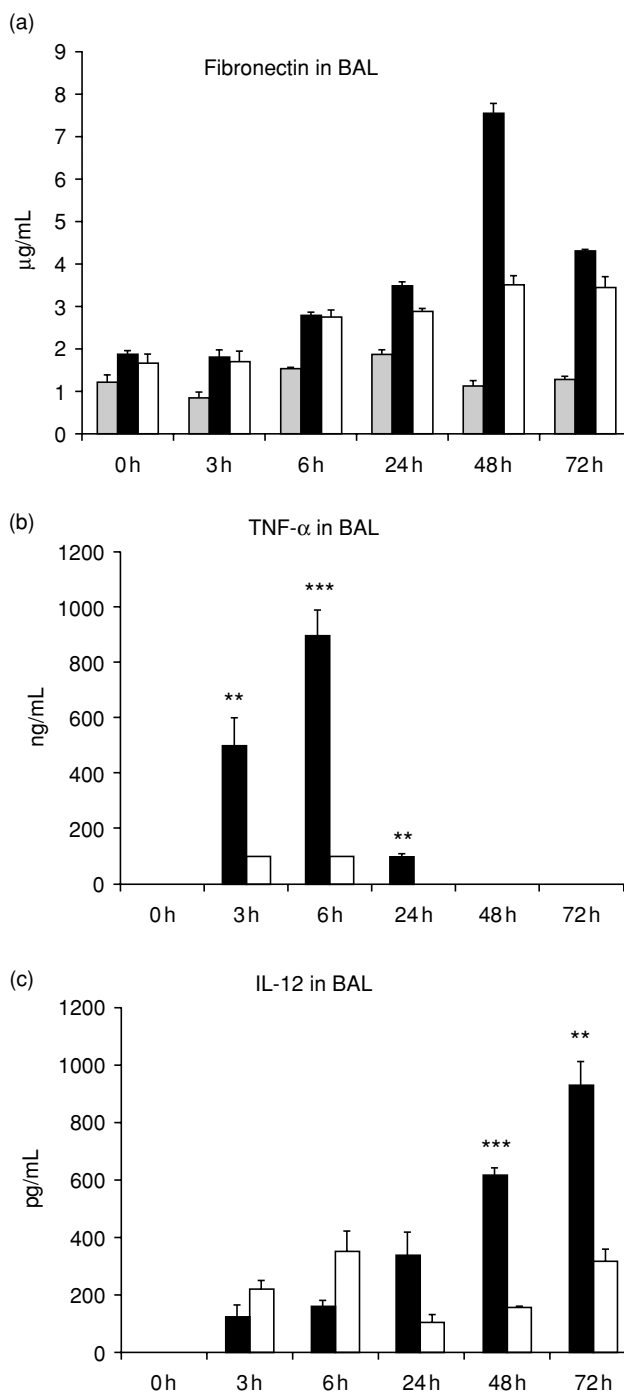


Fig. 3. Effects of the intratracheal instillation with HK or EFD BCG. 10^7 bacterial corpses of HK (■) or EFD BCG (□) in 50 µL of saline were injected in the trachea of adult BP2 mice. Control (grey bars) received saline alone. The concentrations of fibronectin (a), TNF- α (b) and IL-12 (c) were measured in BAL samples collected for 3 days. For the fibronectin content (a), ANOVA was significant ($P = 0.025$) comparing HK BCG to controls but not EFD BCG to controls. Analyses of differences between EFD and HK BCG were reported on the figure as *** $P < 0.001$; ** $P < 0.005$ (Student's *t*-test).

controls showed a late and slight increase of IL-13 mRNAs at 6 h (63% of GAPDH level). Mice pre-treated with HK and EFD BCG expressed levels similar or lower to basal levels (data not shown).

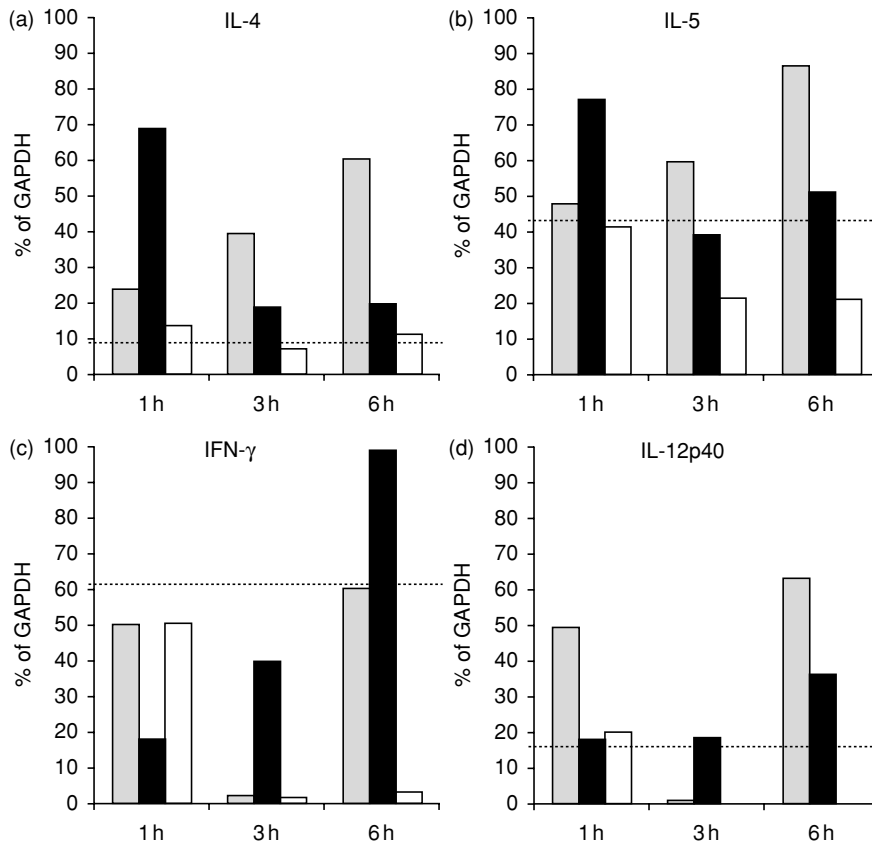


Fig. 4. Expression of cytokine transcripts in the lung of BP2 mice pretreated with HK or EFD BCG and sensitized and challenged with OVA. Groups of five newborn (11 days) BP2 mice were treated by intranasal instillation of 10^5 bacterial corpses of HK or EFD BCG. These mice were then sensitized and challenged with OVA as adults (7–10 weeks). Multiplex RT-PCR was performed on mRNAs extracts from whole lungs for the detection of IL-4 (a), IL-5 (b), IFN- γ (c) and IL-12p40 (d) 1–6 h after OVA challenge. Basal levels from non-treated/non-sensitized mice are given as reference (dot line). Mice sensitized and challenged with OVA are used as controls (grey bars) to compare mice pre-treated as newborns with HK BCG (■) and EFD BCG (□) and then sensitized and challenged with OVA as adults. Results are representative of three separate experiments and expressed as a percentage of GAPDH levels in the whole lung.

The basal expression of IFN- γ mRNAs (Fig. 4c) appeared constitutively high (62% of GAPDH level). As a consequence, the expression of IFN- γ mRNAs observed in controls and EFD BCG pre-treated mice was similar to the basal levels. In mice pre-treated with HK BCG, the expression of IFN- γ mRNAs continuously increased after the OVA challenge, to reach a peak of 99% (GAPDH level) at 6 h. In contrast, the basal expression of IL-12p40 mRNAs was low, with 16% of GAPDH level (Fig. 4d). Interestingly, controls showed a high expression of IL-12p40 mRNAs after OVA challenge, with two peaks at 1 h (49%) and 6 h (63%). Mice pre-treated with HK BCG showed a late and slight increase of IL-12p40 expression at 6 h (36%), while mice pre-treated with EFD BCG remained similar or lower to the basal levels.

The pre-treatment with HK and EFD BCG thus led to highly distinct mRNA expressions in the lung of BP2 mice sensitized and challenged with OVA. After this challenge, control mice expressed high levels of IL-4, IL-5, IL-12p40 and IL-13 mRNAs. Mice pre-treated with HK BCG developed a late expression of IFN- γ mRNAs but also an early expression of mRNAs for IL-4 and IL-5. In contrast, animals pre-treated with EFD BCG continued to show a cytokine transcript expression similar to basal levels, even after the OVA challenge.

Discussion

In the present study, we demonstrated that AM respond *in vitro* to EFD BCG by a significant secretion of the pro-Th1 cytokine

IL-12, along with undetectable levels of TNF- α . These results obtained with macrophages in culture were confirmed *in vivo* by the intratracheal instillation of EFD BCG, showing that this preparation of BCG induces a minimal release of the proinflammatory cytokine TNF- α , along with a rapid and sustained production of IL-12. When newborn mice were pre-treated with EFD BCG, this primary stimulation of the innate immunity appeared to prevent a later Th1/Th2 response in airways of mice sensitized and challenged with OVA. In contrast, the pre-treatment with HK BCG was associated with a strong Th1/Th2 response in the lung of mice sensitized and challenged with OVA.

We and others have previously shown that the instillation of live BCG in mouse airways inhibits several pathologic features of asthma, because it decreases AHR and airway hyper-eosinophilia [18], and it increases the IFN- γ /IL-4 ratio in BAL [22]. Preparations of killed bacteria are known to stimulate the Th1 profile of cytokines [23], suggesting that infection is not a prerequisite for their antiallergic potential [24]. The treatment with mycobacterial products appears able to reduce AHR, hyper-eosinophilia and IL-4 titres in plasma, while augmenting IL-12 titres [25].

Here we show that two preparations of inactivated BCG led to contrasting responses by AM. While HK BCG (like LPS) induced the secretion of TNF- α *in vitro* and *in vivo*, EFD BCG failed to do so. It is interesting to note that the sustained production of TNF- α by AM *in vitro* was only transient *in vivo*. It is likely that results obtained from cell cultures magnified levels measured in the airway lumen where AM are scattered. Conversely, cultured AM were not submitted to

downregulating messages as such they received *in vivo*, explaining the biphasic production of TNF- α observed with AM *in vitro*. Macrophages cultured with HK BCG probably showed a self-sustained secretion of TNF- α , while the release of inflammatory mediators *in vivo* is known as a compromise between the elimination of bacteria and the preservation of tissues [26]. The presence of FN in the airway lumen confirmed that HK BCG induces an inflammatory response in the lung, even temporarily, likely linked to the TNF- α secretion. In allergic/asthmatic airways, where inflammation, tissue destruction and abnormal repair are characteristic [27], the use of stimuli triggering the production of inflammatory mediators is obvious to avoid. Moreover, TNF- α induces the production of eotaxin by macrophages and epithelial cells, which may lead to the recruitment of more numerous eosinophils into the asthmatic airways [28]. Finally, a role for TNF- α and its p55 receptor has been recently suggested in the development of AHR [29], although this point is still controversial [30]. Several studies have demonstrated a synergistic effect of TNF- α and IL-12/IL-18 in the induction of the Th1 profile [31, 32], but in actual fact the induction of a strong inflammatory response by AM encountering HK BCG may thereafter promote the development of both the Th1 and Th2 profiles in our mouse model of asthma. This consequence of the pre-treatment of newborn mice with HK BCG was revealed by the overexpression of transcripts for Th1/Th2 cytokines in the lung of these mice sensitized and challenged with OVA as adults. Notably, this treatment with HK BCG promoted a high expression of IL-4, which may be involved in the early development of AHR [33]. Moreover, the continuously increasing secretion of IL-12 (protein and transcripts) observed with HK BCG may be a consequence of the abundant levels of IL-4, as peripheral blood mononuclear cells (PBMC) pre-treated with IL-4 overproduce IL-12 and TNF- α in response to LPS or *Staphylococcus aureus* [34]. Our results with HK BCG and these data obtained from PBMC stimulated with bacteria are in agreement with recent studies demonstrating the production of Th1 and Th2 cytokines in individuals vaccinated with live BCG [35, 36]. Altogether, these observations join the emerging theory that Th1 and Th2 cells co-operate to the development of an eosinophilic airway inflammation, due at least in part to elevated levels of TNF- α [10, 37].

In contrast to HK BCG, the EFD preparation induced no detectable production of TNF- α by AM *in vitro*, and only a mild release of TNF- α *in vivo*. Even though FN titres in the BAL have been used to express exudation [38], they also seem to correlate with tissue injury (Proust and co-workers, unpublished observations). Accordingly, low levels of FN after EFD BCG treatment *in vivo* supported the non-inflammatory properties of this preparation. The pre-treatment of newborn mice with EFD BCG appeared to prevent the overexpression of Th1/Th2 cytokine transcripts in adults sensitized and challenged with OVA. These results obtained on cytokine transcripts are in perfect accord with results in progress showing that pre-treatment with EFD BCG is able to prevent the AHR and other signs of experimental asthma while HK BCG is inactive (manuscript in preparation). This protection may be not directly due to the overproduction of Th1 cytokines at the time of OVA-challenge as Zuany-Amorin and co-workers have shown recently that the protective effect of mycobacteria treatments is IFN- γ -independent, and not due to a basic redirection of a

predominantly Th2-dominated to a Th1-dominated response [39]. In fact, this long-term protection is likely to the proliferation of regulatory T cells keeping the memory of the mycobacteria treatment and preventing the Th2 imbalance in pre-treated OVA-sensitized mice [40].

While the secretion of TNF- α may be deleterious for asthmatic airways, the production of IL-12 by macrophages is a determining factor for the ensuing secretion of IFN- γ by NK cells and T lymphocytes [41], with a positive feedback on other myeloid cells including dendritic cells [42]. Moreover, IL-12 not only stimulates the Th1 profile, but also maintains Th1 cells [43] and promotes the eosinophil apoptosis [44]. The sustained production of IL-12 by AM stimulated with EFD BCG could be the first step to controlling the Th2 inflammation in asthmatic airways. On the contrary, local administration of preparations of inactivated bacteria such as HK BCG, by starting a strong inflammatory response in AM might be more deleterious than beneficial for asthmatic airways.

The low or undetectable levels of TNF- α , the induction of IL-12, as well as the levels of cytokine transcripts similar to basal levels in mice pre-treated early with EFD BCG, altogether suggest that this preparation of inactivated BCG may be considered as a novel tool treatment to restore the Th1/Th2 balance in asthmatic airways.

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