IL-18 does not increase allergic airway disease in mice when produced by BCG

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Abstract
Whilst BCG inhibits allergic airway responses in murine models, IL-18 has adversary effects depending on its environment. We therefore constructed a BCG strain producing murine IL-18 (BCG-IL-18) and evaluated its efficiency to prevent an asthma-like reaction in mice.

BALB/cByJ mice were sensitized (day (D) 1 and D10) by intraperitoneal injection of ovalbumin (OVA)-alum and primary (D20-22) and secondary (D62, 63) challenged with OVA aerosols. BCG or BCG-IL-18 were intraperitoneal administered 1hr before each immunization (D1 and D10). BCG-IL-18 and BCG were shown to similarly inhibit the development of AHR, mucus production, eosinophil influx and local Th2 cytokine production in BAL, both after the primary and secondary challenge.

These data show that IL-18 did not increase allergic airway responses in the context of the mycobacterial infection, and suggest that BCG-IL-18 and BCG are able to prevent the development of local Th2 responses and therefore inhibit allergen-induced airway responses even after restimulation.

Key words: Asthma, mouse model, BCG, IL-18, airway hyperresponsiveness

Abbreviations: AHR = Airway hyperresponsiveness, BAL: Bronchoalveolar lavage, BCG: Mycobacterium bovis Bacillus Calmette-Guerin
Introduction

Airway inflammation and hyperresponsiveness, increased Th2 cytokine production in bronchoalveolar lavage and enhanced IgE secretion in serum are the hallmarks of allergic asthma. Increased prevalence of asthma has been related to decreased microbial infection or immunization during early infancy. In particular, *Mycobacterium bovis* Bacillus Calmette-Guerin (BCG) vaccination have been suggested to decrease the risk of asthma in a population with family history of rhinitis or eczema [1, 2]. Studies in mouse models have shown that BCG immunization inhibits the development of asthma features in ovalbumin (OVA)-sensitized mice [3-8]. Several authors reported a switch toward a Th1 pattern of response [3, 5, 9], with little evidence of increased regulatory cell activity, since production of the regulatory cytokine IL-10 as well as production of IL-4 was suppressed [3, 9].

Originally named IFN-γ-inducing factor, IL-18 is important for the generation of protective immunity to mycobacteria. IL-18-deficient mice infected with *Mycobacterium tuberculosis* and *Mycobacterium bovis* BCG developed increased granulomatous lesions while IFN-γ production decreased [10]. IL-18 role on allergic inflammation is complex, as it can promote both Th1 and Th2 responses depending on its environment, the time of its administration and/or the response assessment. IL-18 deficiency selectively enhances OVA-induced eosinophilia in mice, whereas IL-18 gene transfer by adenovirus into the respiratory tract at the time of OVA airway challenge prevents the development of AHR and decreases allergen-specific IL-4 production, airway eosinophilia, mucus production and increases IFN-γ production [11, 12]. In contrast, high doses of IL-18 administered alone or concomitantly with the antigen have been shown to increase allergic sensitization including serum IgE, Th2 cytokines and airway eosinophilia, especially 3 weeks after IL-18 injection [13]. Intraperitoneal coadministration of IL-18 and IL-12 or IL-18 and IL-2 during the airway challenge period inhibits airway hyperresponsiveness (AHR), eosinophilia and serum IgE.
levels [14, 15], whilst intranasal of IL-18 plus IL-2 induced allergic airway disease in naïve mice [16].

A BCG producing IL-18 was shown to exhibit enhanced antimicrobial immunity compared to non recombinant BCG due to increased production of IFN-γ [17]. Therefore we constructed a recombinant BCG producing IL-18 (BCG-IL-18) in order to increase the efficiency of intraperitoneal injection of BCG in preventing allergic reactions. This strain produces low doses of IL-18 and increases Th1 response [18]. We previously showed that i.p. BCG-IL-18 immunization at the time of OVA–sensitization decreases bronchoalveolar lavage (BAL) eosinophilia, IL-5 production by lymph node cells, but increases IFN-γ production by lymph node cells [19]. The aim of the present work was firstly to evaluate the effect of non-recombinant BCG and BCG-IL-18 immunization on AHR, and secondly to analyze BCG and BCG-IL-18 effects in the long term, i.e. after a secondary challenge occurring 6 weeks after the primary OVA challenge. We showed that both BCG and BCG-IL-18 were able to similarly prevent AHR and airway inflammation (BAL eosinophilia, Th2 cytokine production in BAL and mucus production) after primary and secondary challenges.
Materials and Methods

Sensitization and airway challenge for acute and chronic inflammatory reaction

10-12 week old female BALB/c mice (H-2d) (Iffa Credo, l’Arbresle, France) were sensitized by intraperitoneal (i.p.) injection of 20 μg ovalbumin (OVA) (Grade V, Sigma Chemical Co., St. Louis, MO) emulsified in 2.25 mg aluminum hydroxide (AlumImject: Pierce, Rockford, IL) in a total volume of 100 μl on days (D)1 and 10. Mice were challenged (20 min) via the airways with OVA (1% in saline) for 3 days (D20, 21 and 22) using ultrasonic nebulization. Mice were divided into 2 groups: the groups of OVA-sensitized mice were i.p. injected with OVA and received OVA challenges aerosols. The groups of non-sensitized mice only received OVA-challenges by aerosols.

In the primary challenge protocol, 48 hours after the last OVA challenge (D24), AHR and tissues were assessed. For the secondary challenge protocol, 6 weeks after the first challenge, mice were exposed to OVA challenges (1% in saline on D62, 5% in saline on D63), and airway reactivity and tissues were assessed on D64. Non-sensitized control groups only received OVA-challenges by aerosols.

Immunization with BCG or IL-18-producing recombinant BCG

Non-sensitized and sensitized mice were or were not treated with BCG or BCG-IL-18. Mycobacterium bovis BCG (Pasteur strain 1173P2; WHO Stockholm, Sweden) and the recombinant BCG producing IL-18 (BCG-IL-18) [18] were grown, using stationary flasks, at 37°C in Sauton medium containing 10 μg/ml HgCl₂ when required and frozen until use. Non-recombinant BCG or rBCG producing IL-18 were administered intraperitoneally on D0 and D10 (5 X 10⁶ CFU in a final volume of 100 μl).
**Airway Hyperresponsiveness**

Airway responsiveness was assessed using single-chamber whole body plethysmography (Buxco Electronics, Sharon, CT), as described previously [20]. Enhanced pause (Penh) was used as the measure of airway responsiveness in this study. In the plethysmography, mice were exposed for 2 min to nebulized PBS and followed by increasing concentrations of nebulized methacholine (MCh) (1.5–12 mg/ml in PBS) (Sigma-Aldrich) using an AeroSonic ultrasonic nebulizer (Systam). After each nebulization, recordings were taken for 3 min. The Penh values measured during each 3-min sequence were averaged and were expressed for each MCh concentration as the percentage of baseline Penh values measured after PBS exposure. There were no significant differences in any of the treatment groups for baseline (PBS) Penh.

**Bronchoalveolar Lavage**

Immediately after assessment of AHR, lungs were lavaged via the tracheal tube with PBS (1x 1ml). Total leukocyte numbers were counted with a hemocytometer. Differential cell counts were performed on bronchoalveolar lavages by counting at least 200 cells on cytocentrifuged preparations, stained with Diff Quick (Dade Behring, Deerfield, IL) and differentiated by standard hematological procedures.

**Histochemistry**

Lungs were fixed by inflation (1 ml) and immersion in Immunohistofix® (Aphase, Gosselies, Belgium) and embedded in Immunohistowax® (Aphase). For detection of mucus containing cells in fixed airway tissue, 5µm sections were stained with Periodic Acid Schiff (PAS) (Sigma Aldrich, Missouri, USA) and hematoxylin (Labonord, Templemars, France) as previously described [21].
Measurement of cytokines

IFN-γ, IL-5, IL-12 and IL-13 levels in the bronchoalveolar lavage fluid (BALF) were measured by ELISA according to the manufacturer direction (Pharmedium, San Diego, CA, for IFNγ, IL-5, IL-12; R&D systems, Mineapolis, MN, for IL-13). The limits of detection were 4 pg/ml for IL-5, 10 pg/ml for IL-12 and IFN-γ, and 1.5 pg/ml for IL-13.

Measurement of total and OVA-specific serum IgE

Serum levels of total IgE, and OVA-specific IgE were measured by ELISA as previously described [19]. Briefly, 96-well plates (Immulon 2, Dynatech, Chantilly, VA) were coated with either OVA (5 µg/ml) or purified anti-IgE (clone 02111D, Pharmingen). After addition of serum samples, a biotinylated anti-IgE antibody (clone 02122D, Pharmingen) was used as detecting antibody, and the reaction amplified with avidin-horseradish peroxidase (Sigma). The OVA-specific antibody titers of the samples were related to pooled standards that were generated in the laboratory and expressed as ELISA units per ml (EU/ml). Total IgE levels were calculated by comparison with known mouse IgE standards (Pharmingen). The limit of detection was 100 pg/ml for total IgE.

Statistical analysis

Values for all measurements were expressed as the mean ± standard error of the mean (SEM). Statistical analyses were done using STATVIEW 5 software. Differences within all groups were first evaluated for each parameter using the Kruskal Wallis test. When statistical significance was observed, differences were subsequently analyzed by using the Mann Whitney test. p values for significance were set to 0.05.
Results

BCG and BCG-IL-18 prevented airway hyperresponsiveness following primary and secondary challenge

OVA-sensitized mice developed airway hyperresponsiveness compared to the non-sensitized mice after primary (figure 1A) and secondary (figure 1B) challenge. Mouse treatment by intraperitoneal injections with BCG or IL-18-producing-BCG (BCG-IL-18) before and during sensitization significantly inhibited airway hyperresponsiveness (figure 1A). In order to evaluate the long term effect of BCG and BCG-IL-18 on AHR, we waited 6 weeks after the primary challenge and then rechallenged the animals. It has previously been shown that at this time point, the inflammatory reaction and AHR developing after primary challenge are resolved but that a secondary airway challenge induces a strong inflammatory reaction with concomitant development of AHR [22]. Under these conditions, BCG and BCG-IL-18 treatment inhibited the increase in airway reactivity (Figure 1B). Baseline airway reactivity was not affected by OVA or BCG or BCG-IL-18 treatment (data not shown).

BCG and BCG-IL-18 decreased BAL inflammation and goblet cell hyperplasia following primary and secondary challenge

In OVA-sensitized mice, inflammatory cell recruitment into the airways was increased after primary as well as secondary airway challenge (Figure 2). Increased total cell numbers in BAL were largely due to increased numbers of eosinophils. There was also a small but significant (p=0.0005) increase in the number of lymphocytes compared to the non-sensitized mice (Figure 2). Administration of BCG or BCG-IL-18, led to a significant (p<0.05) decrease in total cell numbers, macrophage, lymphocyte and eosinophil numbers in the primary challenge protocol compared to OVA-sensitized mice (Figure 2A). In the secondary challenge...
protocol, administration of BCG or BCG-IL-18 significantly decreased eosinophil numbers only (Figure 2B).

Lung tissue was obtained and processed 48 hr after allergen provocation. To assess the degree of goblet cell hyperplasia, tissue sections were stained with Periodic Acid Schiff (PAS). After primary (Figure 3A-D) and secondary (Figure 3E-H) challenge, non-sensitized mice showed no PAS-positive cells, whereas non-treated OVA-sensitized mice showed many PAS-positive cells (92% and 81% of PAS positive cells/mm basement membrane for the primary and secondary challenge protocol respectively). In contrast, sensitized and BCG or BCG-IL-18 treated mice showed only scattered PAS-positive cells (71% and 66% of PAS positive cells/mm basement membrane for BCG treated mice in the primary and secondary challenge protocol respectively and 48% and 40% of PAS positive cells/mm basement membrane for BCG-IL-18 treated mice in the primary and secondary challenge protocol respectively), with a stronger inhibition for BCG-IL-18 treatment after the secondary challenge (Figure 3H).

**Effect of BCG and BCG-IL-18 on cytokine production following primary and secondary challenges**

BAL fluid was obtained in order to assess Th1 (IFN-γ), pro-Th1 (IL-12), and Th2 (IL-5, IL-13) cytokine levels, 48 or 24 hours after primary or secondary allergen challenge, respectively. Th1 (IFN-γ) and pro-Th1 (IL-12) cytokines were decreased in OVA-sensitized mice compared to non-sensitized mice, significantly after the primary challenge, and non-significantly after the secondary challenge (Figure 4). IL-5 and IL-13 production was significantly increased in OVA-sensitized mice after the primary and the secondary challenge. Treatment of OVA-sensitized mice with BCG or BCG-IL-18 did not significantly affect IFN-γ, IL-12 (Figure 4) or IL-10 (data not shown) production, but significantly inhibited IL-5 and IL-13 production both in primary and secondary challenge protocols.
**BCG and BCG-IL-18 treatment did not modify total IgE production but inhibited OVA-specific IgE**

Serum from OVA-sensitized mice showed elevated total IgE levels and OVA-specific IgE antibodies compared to non-sensitized mice following the primary and the secondary challenge protocol (Figure 5). Treatment with BCG and BCG-IL-18 did not affect total IgE measured after the primary and the secondary challenge (Figures 5A, 5B). OVA-specific IgE measured after the primary or the secondary challenge were not modified after BCG treatment (Figures 5C, 5D). In contrast, treatment with BCG-IL-18 significantly increased levels of OVA-specific IgE after the primary and decreased them after the secondary challenge compared to OVA-sensitized mice (Figures 5C, 5D).
**Discussion**

In this study we showed that BCG and BCG-IL-18 given at the time of OVA-sensitization were efficient to prevent AHR even after a secondary challenge performed 6 weeks after the initial allergen challenge. This effect is related to the decrease in the local Th2 immune response, as seen with the decreased BAL IL-5 and IL-13 production. In human as well as in mouse models, Th2 cells are indeed considered as the central cells involved in the development of asthma [23, 24]. Others have shown that BCG was effective in attenuating allergic airway inflammation and associated changes in pulmonary function in animal models. Administered before OVA sensitization, BCG decreases BAL eosinophilia and IL-5 production in the draining lymph nodes in an IFN-γ dependent manner [4]. In our hands, in a previous study, similar effects were found both with intraperitoneal and intranasal administration at the dose of \(5 \times 10^6\) CFU, when BCG was given at the time of OVA-sensitization [19]. Systemically injected (by intravenous injection) 14 days before the challenge, BCG also suppressed OVA-induced airway responses [9]. In the present study, BCG was injected i.p. at the time of sensitization, and inhibition of airway responses was still observed 10 weeks after the first injection. Only few studies investigated BCG long-term effects: similar results to ours were obtained although BCG was always given long before OVA-sensitization. Given from 4 to 16 weeks before OVA-sensitization by i.n. immunization, BCG prevented airway allergic inflammation [7, 8].

After primary and secondary challenge, BCG and BCG-IL-18 treatment locally decreased the production of IL-5 and IL-13, in parallel to decreased mucus hyperplasia, arguing for a role, in this process, of IL-13, the main cytokine involved in mucus production [25, 26]. However, BCG treatment did not modify IFN-γ and IL-12 production in BAL, in contrast to decreased IFN-γ production seen in the spleen [9] or the draining lymph nodes [19]. This absence of effect on IFN-γ production in BAL may be due to the way of injection (i.p. instead i.n. route.
of administration) and the analyzed tissue. Another hypothesis, pointed out by Trujillo and colleagues [27] suggests that the treatment before sensitization may inhibit the onset and consequently the recruitment of Th2 cells thus preventing from allergic reaction. Our previous studies are in favor of this hypothesis as we showed that i.p. administration of BCG or BCG-IL-18 increased in vitro production of IFN-γ by splenocytes or lymph node cells, whilst decreasing IL-5 production [18, 19].

In the present work, total IgE production was unchanged after BCG treatment either following primary or secondary challenge, suggesting that BCG was not able to totally prevent Th2 responses. Although Mycobacterium tuberculosis has been shown to induce IgE production [28], BCG was shown to prevent OVA-specific IgE production after systemic (intravenous), but not local (i.n.) administration [4, 7, 9]. In our study, BCG does not seem to induce total IgE either as non-sensitized mice treated with BCG-IL-18 did not develop total IgE response, either in the primary or the secondary challenge protocol. However BCG and BCG-IL-18 treatment decreased OVA-specific IgE after the secondary challenge, as well as OVA-specific IgG1 (data not shown). Only BCG-IL-18 treatment effect was significant suggesting a potentiating effect of IL-18 produced by BCG and a long term effect of BCG-IL-18.

BCG-IL-18 did not demonstrate a stronger ability to prevent airway inflammation and hyperresponsiveness than BCG alone, in contrast to what we previously showed [19]. The protocol used to sensitize mice in this first study was much weaker (sensitizing doses were twice lesser and OVA aerosol concentration were 0.1% OVA instead of 1% in the present study) and did not allow the measurement of significant AHR. The absence of a differential effect between BCG and BCG-IL-18 may therefore be related to the strength of the sensitization protocol and the already maximal effect of the non-recombinant BCG on AHR. We previously showed that BCG and BCG-IL-18 were still viable and detected after 94 days. Moreover, although undetectable, IL-18 was efficiently produced in vivo by the recombinant
BGC, as suggested by its potentiating effect over non recombinant BCG [18, 19]. In the present study, BCG-IL-18 significantly modified OVA-specific IgE production in contrast to non recombinant BCG, suggesting that IL-18 is really produced in vivo, probably in low concentration.

Finally, after the primary and secondary OVA-challenge, BCG or BCG-IL-18 treatment inhibits BAL eosinophilia. BCG was already known to inhibit this feature of asthma when given before sensitization [7]. Interestingly BCG-IL-18 increased the number of BAL lymphocytes compared to BCG-treated mice and non-treated mice. This was not accompanied with decreased or increased airway responses, in contrast to the increased Th2 response induced by IL-18 as previously shown [13]. The only parameter increased after BCG-IL-18 treatment compared to BCG treatment or no treatment was the OVA-specific IgE after the primary challenge. These data suggest a systemic transient effect of IL-18 produced by BCG. IL-18 has been shown previously to increase Th2 responses and more particularly IgE production [13, 29, 30]. The modulation of the production of Th1/Th2 cytokines by IL-18 was suggested to be time dependent [31], which might relate to our observation that OVA-specific IgE transiently increased due to IL-18 produced by i.p. injected BCG. This increase in IgE production was not accompanied by effect on airway inflammation and function. Dissociation of serum IgE and airway inflammation and hyperresponsiveness was previously demonstrated in the same model [32]. Conflicting data have been reported on the role of IL-18 in airway inflammation. In the presence of IL-12, IL-18 administered i.p. before the allergen challenge has been reported to attenuate eosinophil accumulation [14]. OVA-sensitized IL-18 deficient mice exhibit greater BAL eosinophilia than wild-type mice [11]. In contrast, local instillation of recombinant IL-18 was reported to favor the eosinophil recruitment through the release of eotaxin from bronchial epithelial cells without any effect on AHR [33]. Moreover i.p. injection of large quantities of IL-18, in the absence of IL-12, also increases the recruitment of
eosinophils in the airways [34]. Finally, lower doses of IL-18 regularly administered intraperitoneally increase eosinophil recruitment in the airways 3 weeks after the last injection [13]. BCG-IL-18 administered in our study produces only low doses of IL-18 as previously demonstrated [18] and no IL-18 could be detected in BAL (data not shown). Moreover, BCG has been shown to favor IL-12 production by macrophages [6]. The low dose of IL-18 released and the cytokine environment may explain the absence of an increased AHR and eosinophilia in BCG-IL-18 treated mice even after OVA restimulation.

In conclusion, we showed that BCG as well as BCG producing IL-18 administered at the time of sensitization prevents airway responses after primary as well as secondary OVA-challenge, suggesting their effect after systemic injection, which had not been demonstrated yet. Moreover, our study suggests that, thank to the environment induced by BCG, low doses of IL-18 do not increase Th2 responses, even after secondary allergen challenge.
Figure legends

Figure 1: BCG and BCG-IL-18 prevent the development of AHR

OVA-sensitized mice were OVA-sensitized and challenged, whereas non-sensitized mice were challenged only. At the time of each OVA sensitization, treated mice were injected either with BCG or BCG-IL-18. Airway reactivity was evaluated after inhalation of increasing doses of metacholine by whole body plethysmography and penh (enhanced pause) measurement after primary (A) or secondary (B) challenge. Results are expressed as mean ± sem of penh percentage increase above PBS inhalation. Statistical analysis were performed and showed p<0.04 for differences between OVA-challenged and OVA-sensitized and challenged mice (+), between OVA-sensitized and challenged mice and OVA-sensitized and challenged mice + BCG treatment (#), and between OVA-sensitized and challenged mice and OVA-sensitized and challenged mice + BCG-IL-18 treatment (&).

Figure 2: BCG and BCG-IL-18 effect on BAL composition after primary and secondary challenge.

BAL infiltrate was analyzed after primary (A) or secondary (B) challenge. Results are expressed as mean ± sem of cell number. *: p<0.05.

Figure 3: BCG and BCG-IL-18 effect on mucus hyperplasia after primary and secondary challenge.

Mucus production was detected using Periodic Acid Schiff staining on lung section from non-sensitized mice (A, E), sensitized mice (B, F), sensitized mice treated with BCG (C, G) or BCG-IL-18 (D, H), after primary (A-D) or secondary (E-H) OVA-challenge.
Figure 4: BCG and BCG-IL-18 effect on BAL cytokine production after primary and secondary challenge.

BAL cytokines were measured after primary (A) or secondary (B) challenge. Results are expressed as mean ± sem pg/ml. *: p<0.03.

Figure 5: BCG and BCG-IL-18 decrease OVA-specific IgE, but do not modify serum total IgE.

IgE were measured after primary (A, C) or secondary (B, D) challenge. Results are expressed as mean ± sem ng/ml for total IgE (A, B), and mean ± sem ELISA units/ml (EU/ml) for OVA-specific IgE (C, D). *: p<0.01.
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References


Figure 1

A

- ○ Non-sensitized mice (n=12)
- ● OVA-sensitized mice (n=15)
- □ Non-sensitized BCG-IL-18 treated mice (n=10)
- ■ OVA-sensitized BCG-IL-18 treated mice (n=15)
- ▬ OVA-sensitized BCG treated mice (n=11)

B

- ○ Non-sensitized mice (n=12)
- ● OVA-sensitized mice (n=10)
- □ Non-sensitized BCG-IL-18 treated mice (n=10)
- ■ OVA-sensitized BCG-IL-18 treated mice (n=10)
- ▬ OVA-sensitized BCG treated mice (n=12)
Figure 2

A

- Non-sensitized mice (n=12)
- Non-sensitized BCG-IL-18 treated mice (n=10)
- OVA-sensitized mice (n=15)
- OVA-sensitized BCG-IL-18 treated mice (n=15)
- OVA-sensitized BCG treated mice (n=11)

B

- Non-sensitized mice (n=5)
- Non-sensitized BCG-IL-18 treated mice (n=5)
- OVA-sensitized mice (n=6)
- OVA-sensitized BCG-IL-18 treated mice (n=6)
- OVA-sensitized BCG treated mice (n=6)
Figure 4

A

- IL-5
- IL-13
- IL-12
- IFN-γ

B

- IL-5
- IL-13
- IL-12
- IFN-γ

Legend:
- Non-sensitized mice (n=12)
- Non-sensitized BCG-IL-18 treated mice (n=10)
- OVA-sensitized mice (n=15)
- OVA-sensitized BCG-IL-18 treated mice (n=15)
- OVA-sensitized BCG treated mice (n=11)
- Non-sensitized mice (n=5)
- Non-sensitized BCG-IL-18 treated mice (n=5)
- OVA-sensitized mice (n=6)
- OVA-sensitized BCG-IL-18 treated mice (n=6)
- OVA-sensitized BCG treated mice (n=6)
Figure 5

A: Total IgE (ng/ml)

B: Total IgE (ng/ml)

C: OVA specific IgE (EU/ml)

D: OVA specific IgE (EU/ml)

- Non-sensitized mice (n=11)
- Non-sensitized BCG-IL-18 treated mice (n=10)
- OVA-sensitized mice (n=11)
- OVA-sensitized BCG-IL-18 treated mice (n=10)
- OVA-sensitized BCG treated mice (n=11)

- Non-sensitized mice (n=17)
- Non-sensitized BCG-IL-18 treated mice (n=16)
- OVA-sensitized mice (n=18)
- OVA-sensitized BCG-IL-18 treated mice (n=17)
- OVA-sensitized BCG treated mice (n=19)