

## Effects of *Mycobacterium bovis* BCG on the development of allergic inflammation and bronchial hyperresponsiveness in hyper-IgE BP2 mice vaccinated as newborns

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### Abstract

Asthma may result from excessive Th-2 response in children not previously exposed to Th-1-inducing infections. We tested the hypothesis that BCG vaccination in Th-2-susceptible newborn BP2 mice blocks allergic inflammation and bronchial hyperreactivity (BHR). Ten day-old BP2 mice received  $10^5$  CFU of BCG 1173P2 intranasally (IN), and 6, 10 or 14 weeks thereafter were sensitized with 100  $\mu$ g ovalbumin (OVA) in aluminium hydroxide twice subcutaneously (SC) at 1 week interval, and challenged 1 week after the second sensitization with 10  $\mu$ g OVA IN. Compared to OVA-challenged unvaccinated mice, those that received BCG 8 weeks before challenge developed intense bronchial inflammation, BHR, and high IgE titers. Inflammation involved T cells, macrophages, dendritic cells and was accompanied by increased levels of Interleukin-5 (IL-5) in the bronchoalveolar lavages (BAL). However, animals challenged 16 weeks after BCG vaccination did not develop BHR nor bronchial hypereosinophilia, and showed reduced IgE levels. Bronchial infiltration by immunocompetent cells was also significantly reduced. Increased levels of gamma-interferon (IFN- $\gamma$ ) after in vitro stimulation of tracheo-bronchial lymph node cells accompanied this blockage, but levels of IL-5 remained high. We demonstrate that 16 weeks after vaccination with BCG in newborn BP2 mice which have a high Th-2 background, allergic inflammation and BHR were blocked, even though a clear Th-1 shift was not achieved. © 2001 Elsevier Science Ltd. All rights reserved.

**Keywords:** Newborn BP2 mice; BCG; BHR

### 1. Introduction

Chronic inflammation of the respiratory mucosa plays a fundamental role in the pathogenesis of asthma, a disease that affects more individuals in industrialized than in developing countries [1]. This discrepancy may result from the genetic background, from higher levels of industrial pollution [2] or from the decline of infectious diseases in the developed world [3,4]. Allergic asthma is characterized by bronchial hyperreactivity

(BHR) associated to eosinophilic airway inflammation [5,6] and high levels of serum immunoglobulin E (IgE) [7].

Th-2 cells secreting interleukin-4 (IL-4) and interleukin-5 (IL-5) play a central role in initiating and sustaining the asthmatic response by regulating the production of IgE and the growth, differentiation and recruitment of basophils and eosinophils, respectively [8]. While Th-2 cells promote airway inflammation in asthma, it has been proposed that Th-1 cells, through secretion of interferon (IFN)- $\gamma$ , protect against allergic disease by dampening the activity of Th-2 cells. Immunotherapy for allergic diseases and asthma reduces IL-4 production [9] and increases IFN- $\gamma$  production by allergen-specific Th-1 cells [10]. Infectious diseases such

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as measles, influenza and tuberculosis, by inducing Th-1 type responses, may hamper the development of allergen-specific Th-2 cells and prevent allergy [11].

Recent studies demonstrated that *Mycobacterium bovis* BCG infection suppresses allergen-induced airway eosinophilia in C57Bl/6 mice [12] and airway hyperresponsiveness in BALB/c mice [13]. However, mice used in these studies are prone to Th-1-type immune responses upon mycobacterial infection. In addition, BCG immunization in C57Bl/6 mice may more easily skew the cytokine production toward proliferation of Th-1-related cells [14], thus protecting against the development of Th-2-dependent allergic disease.

We have previously developed a model of allergic asthma in which ovalbumin (OVA)-sensitized BP2 mice (high antibody-responders), a strain with a high Th-2 background, develop, after intranasal OVA challenge, higher BHR and IgE titers than BALB/c mice [15]. Gheorghiu et al. [16] have demonstrated that BP2 mice vaccinated with BCG were protected against virulent challenge with *Mycobacterium tuberculosis*. However, the protective effect of BCG is stronger in low antibody-producing mice, which might result from higher levels of living BCG found in these mice at the time of challenge [16]. We now investigated whether BCG vaccination in newborn and adult BP2 mice prevents the development of allergic asthma-like responses in the lungs and the possible mechanisms involved. Further, since a potential anti-allergic vaccine should address preferably to infants, the murine model was adapted to BCG inoculations in newborn animals.

## 2. Materials and methods

### 2.1. Animals

Male newborn (10 days) and adult (6 weeks) BP2 mice were obtained from 'Centre d'Elevage R. Janvier', France.

### 2.2. Microorganisms

The *M. bovis* BCG Pasteur strain 1173P2 and the attenuated mutant BCG *erp::aph* strain [17] were grown as dispersed bacilli in Beck–Proskauer medium [18] and the vaccine suspensions were stored at  $-70^{\circ}\text{C}$  until use. The number of colony forming units (CFU) per ml was determined by plating suitable saline dilutions on Middlebrook 7H10 agar medium.

### 2.3. Immunizations

In preliminary experiments, adult BP2 mice were anesthetized and IN immunized with  $10^2$ ,  $10^6$  or  $10^8$

CFU of BCG 1173P2 in 10  $\mu\text{l}$  to determine dose-effect. Thereafter, two groups of adult BP2 mice were IN immunized with  $10^6$  CFU of either BCG 1173P2 or mutant BCG *erp::aph*. In a second phase of the study, newborn BP2 mice were IN immunized with  $10^5$  CFU of BCG 1173P2.

### 2.4. Ovalbumin sensitization and challenge

At various times post-BCG 1173P2 or mutant BCG *erp::aph* immunizations (6–24 weeks), adult BP2 mice were SC sensitized twice at a 1-week interval with 0.4 ml 0.9% NaCl containing 100  $\mu\text{g}$  OVA (Immunobiologicals, Lisle, IL, USA) and 1.6 mg aluminium hydroxide (Merck, Darmstadt, Germany). One week after the second sensitization, mice were anesthetized and challenged IN with 10  $\mu\text{g}$  OVA in 50  $\mu\text{l}$  alum-free 0.9% NaCl solution or with 0.9% NaCl solution as control. BP2 mice immunized as newborns with BCG 1173P2, were sensitized with OVA 6, 10 and 14 weeks after and challenged using the same protocol used for adult mice. Each study group included four to six mice and the animals were used 24 h after OVA challenge. Controls were littermates that were submitted to the same protocol of OVA sensitization and challenge but did not receive BCG immunization.

### 2.5. Bronchial hyperreactivity

BHR was evaluated on conscious mice as described previously [19]. Mice were placed in a barometric plethysmographic chamber (Buxco Electronics, Sharon, CT, USA) and respiratory parameters were measured before and after an aerosol of methacholine (Sigma–Aldrich, Stenheim, Germany) delivered for 20 s at 60 mM in the aerosolator. The bronchopulmonary resistance was expressed as enhanced pause (Penh), calculated as:  $(\text{expiratory time (tE)}/40\% \text{ of relaxation time (trel)} - 1) \times \text{peak expiratory flow (PEF)}/\text{peak inspiratory flow (PIF)} \times 0.67$ , according to the manufacturer's recommendations. Every 20 s an average value of Penh was recorded. For the graphic representation, each value was expressed for every minute.

### 2.6. Bronchoalveolar lavage

After evaluating bronchoconstriction as described above, mice were anesthetized with urethane (15 mg/10 g body weight i.p.), and the abdominal cavity was opened. Blood samples for serum were collected from the vena cava. The trachea was cannulated, and bronchoalveolar lavage (BAL) was performed by lavages with 1.5 ml PBS supplemented with 10  $\mu\text{g}/\text{ml}$  of gentamycin (Roche Diagnostics, Meylan, France). Total cell numbers were determined and cytopins were prepared for each sample by centrifugation of 50  $\mu\text{l}$  BAL

fluid. After fixation, cytopspins were stained with Diff Quik (Baxter Dade AG, Duedingen, Switzerland) and differential cell counts were performed. Cell-free lavage fluids were used to analyze cytokine production and the cells were resuspended in 1 ml PBS and plated on Middlebrook 7H11 medium, the number of BCG CFU were counted after 21 days incubation at 37°C.

## 2.7. Immunohistochemical studies of the lung

Mice were exsanguinated via the abdominal aorta and the contents of the thoracic cavity resected 'en bloc'. After bronchoalveolar lavage, the lungs were inflated with 1 ml of 50% Optimum Cutting Temperature (OCT; Sakura Finetek, Torrance, CA, USA) in saline solution. The left lobe was placed in an Eppendorf tube and immediately frozen in liquid nitrogen for cryostat sectioning. The frozen blocks were kept at  $-80^{\circ}\text{C}$  prior to use. Sections ( $5\text{ }\mu\text{m}$ ) were cut in a cryostat, kept at  $-21^{\circ}\text{C}$  and collected on glass slides previously coated with poly-L-lysine (Sigma), fixed in chloroform-acetone v/v (Merck) for 10 min, wrapped in a plastic film and kept at  $-20^{\circ}\text{C}$  prior to use. Representative sections of each block were also stained with hematoxylin-eosin (Rhône-Poulenc, Viliers-Saint Paul, France) for conventional histology. Consecutive sections of each block were stained for cyanide-resistant eosinophil peroxidase (EPO) activity, employing potassium cyanide (Merck), diaminobenzidine (Sigma) and hydrogen peroxide (Merck) to count eosinophils. Consecutive sections of each block were stained with the following purified monoclonal antibodies (all from Pharmingen, Grenoble, France): CD3 (clone 17A2; T-cell receptor), CD4 (clone H129.19; T helper cells), CD8 (clone Ly-2; T suppressor-cytotoxic cells), Mac-3 (clone M3/84; mononuclear phagocytes). All the others were biotinylated antibodies: I-Aq (clone KH116; Major Histocompatibility Complex Class II), CD80 (clone 16.10A1; co-stimulatory molecule), CD86 (clone GL1; co-stimulatory molecule), CD40 (clone 3/23), CD11c (integrin  $\alpha_x$  chain, highly expressed by mouse dendritic cells). Alkaline phosphatase-anti-alkaline phosphatase (APAAP) staining procedure was performed using rabbit immunoglobulin (Ig) to rat Ig (D455, Dako A/S, Glostrup, Denmark), followed by rat APAAP (D488, Dako) or by alkaline phosphatase-conjugated streptavidin AP(D0396, Dako) for the biotinylated primary antibodies. The reaction was developed with the substrates Fast Red TR (Sigma) and naphthol AS MX phosphate (Sigma), and light hematoxylin counterstaining. Sections were stained, coded and read in a 'blind' fashion. Positive cells were enumerated in two compartments: peribronchial (between the basal lamina and the beginning of the alveolar area) and perivascular (between the endothelium and the begin-

ning of the alveolar area) by means of an eyepiece graticule comprising 100 squares of known area. The area of the compartments and the number of positive cells were determined on each microscope field, and at least ten fields were analyzed. The results of each stained slide were expressed as the number of positive cells per unit area ( $6.25 \times 10^4\text{ }\mu\text{m}^2$ , the total area of the graticule). Results were calculated for each experimental group and expressed as mean  $\pm$  S.E.M. of the indicated number of experiments. Unpaired Student's *t*-test was used to assess comparability between means. A value of  $P < 0.05$  was considered significant.

## 2.8. Th-1 and Th-2 cytokine assay

Cytokine productions were measured *ex vivo* in bronchoalveolar lavages and *in vitro* after stimulation with OVA. Briefly, cell suspensions from tracheo-bronchial lymph nodes were cultured in RPMI 1640 (Seromed, Munich, Germany) supplemented with 10% fetal calf serum, 2 mM L-glutamine (Seromed),  $5 \times 10^{-5}\text{ M}$  2-mercaptoethanol, and antibiotics. A total of  $4 \times 10^6$  cells were incubated with medium alone or with 2  $\mu\text{g}$  of OVA per 1 ml in flat-bottom 24-well plates (Nunc, Roskilde, Denmark). Supernatants were harvested at 72 h, and the levels of different cytokines were determined by ELISA.

IL-4 was measured with a commercial ELISA (Endogen, Woburn, MA, USA). Briefly, 96-well plates were coated with 2  $\mu\text{g}/\text{ml}$  of rat anti-mouse IL-4 (BVD4-1D11), the plates were next incubated with supernatants or dilutions of recombinant IL-4 (rIL-4) standard, then a biotinylated rat anti-IL-4 antibody (BVD6-24G2) at 0.5  $\text{mg}/\text{ml}$  was added. The reaction was revealed with *o*-phenylenediamine dihydrochloride substrate (Sigma) in phosphate buffer and read at 490 nm. IL-5 was measured using the immunometric assay as described previously [20]. Briefly, 96-well plates were coated with 10  $\mu\text{g}/\text{ml}$  of rat anti-mouse IL-5 (TRFK-4) supernatants or dilutions of rIL-5 standard were added followed by an acetylcholinesterase-labelled rat anti-mouse IL-5 antibody (TRFK-5) at 10 Ellman units/ml. Absorbance was read at 405 nm. IFN- $\gamma$  was measured with a commercial ELISA (Biosource, Nivelles, Belgium). Briefly, 96-well plates were coated with rat anti mouse IFN- $\gamma$  (R4-6A2), the plates were incubated with supernatants and dilutions of rIFN- $\gamma$  standard. A biotinylated anti-IFN- $\gamma$  antibody (XMG2.2) was added and the reaction was revealed with *o*-phenylenediamine dihydrochloride substrate and read at 490 nm.

## 2.9. Immediate and delayed type hypersensitivity

Immediate and delayed-type hypersensitivity (DTH) responses of newborn BP2 mice were tested 16 weeks

after BCG immunization and sensitization with OVA. Briefly, 50  $\mu$ l of OVA (10  $\mu$ g) or purified protein derivative (PPD; Ministry of Agriculture, Weybridge, UK; 4  $\mu$ g) were injected into the footpad of each group of mice (immunized or not with BCG). The immediate and delayed type hypersensitivity were quantified 1, 3, 4, 6 and 24 h later by measuring the difference in millimeters with a dial gauge caliper (sensitivity 0.05 mm) between the footpad thicknesses before and after OVA or PPD injections.

### 2.10. Statistical analysis

Results are expressed as mean  $\pm$  S.E.M. ( $n = 3-8$ ). One-way ANOVA was used to determine significance among the groups. If a significance variance was found, an unpaired Student's *t*-test was used to assess comparability between means. A value of  $P \leq 0.05$  was considered significant. For histological studies statistical analysis were performed with two-way ANOVA.

## 3. Results

### 3.1. BCG dose effect on allergy prevention in adult BP2 mice

Adult BP2 mice were immunized IN with  $10^2$ ,  $10^6$  and  $10^8$  CFU of BCG 1173P2 strain to determine the dose-dependent effects on the different end-points. The three doses of BCG reduced significantly, as compared to non-immunized control mice, the eosinophilia in BAL (Fig. 1(A)), however, only the dose of  $10^6$  CFU reduced significantly the OVA-specific IgE in the sera (Fig. 1(B)), moreover, this dose was the only one able to suppress BHR (Fig. 1(C)). Accordingly, the doses of  $10^6$  CFU for adult mice and  $10^5$  CFU for newborn mice were selected to study long-term effect of BCG immunization to prevent allergy.

### 3.2. Long-term effect of BCG immunization on bronchial hyperreactivity and eosinophilia in adult BP2 mice

Adult BP2 mice that were IN immunized with  $10^6$  CFU of BCG 1173P2 or of the attenuated mutant *erp::aph*, then sensitized and challenged with OVA at various time points as described above, showed, at 8 and 12 weeks post BCG 1173P2 or BCG mutant *erp::aph* immunization, a decreased BHR in response to aerosolized methacholine 24 h after the ovalbumin challenge (Fig. 2A, B). At 8 weeks, the sum of the Penh (values calculated for 10 min) of BCG 1173P2 or BCG mutant-immunized groups was non-significant as compared to control group challenged with saline ( $P > 0.05$ ) and significant as compared to control group challenged with OVA ( $P < 0.05$ ). At 12 weeks post-immunization, the sum of the Penh was significant as compared to control groups challenged with saline or with OVA and at 26 weeks post-immunization, the sum of the Penh was significantly decreased in BCG-immunized group as compared to control group challenged with OVA ( $P < 0.05$ ) and non-significantly different from control group challenged with saline ( $P > 0.05$ ). In contrast, the sum of the Penh was significantly increased in BCG *erp::aph* group as compared to control groups challenged with saline or with OVA ( $P < 0.05$ ).

BCG immunization before OVA sensitization and challenge had a suppressive effect on the recruitment of eosinophils into the lungs (Fig. 3). At 8, 12 and 26 weeks post-BCG immunization, a lower percentage of eosinophils, respectively, 10, 7 and 6%, was found in mice immunized with wild type BCG as compared to 34, 41 and 24% found in control mice, however, the eosinophilia was significantly reduced only at 12 weeks ( $P < 0.05$ ). Attenuated mutant *erp::aph* reduced slightly and not significantly ( $P > 0.05$ ) the eosinophil numbers in the BAL.

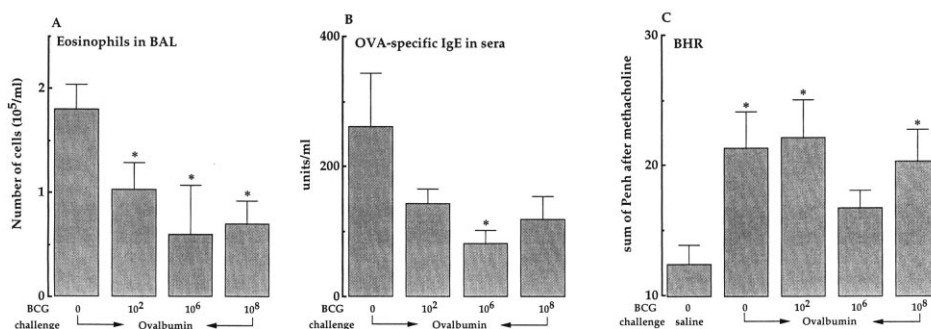


Fig. 1. BCG dose effect on eosinophilia in the BAL (A), on OVA-specific IgE in the sera (B) and on BHR (C) in adult BP2 mice. (A) and (B) groups of mice immunized intranasally with  $10^2$ ,  $10^6$  or  $10^8$  viable units of BCG were compared to a non-immunized group. \*  $P < 0.05$ , the three doses reduced significantly eosinophilia in the BAL,  $10^6$  viable units of BCG reduced significantly the OVA-specific IgE in the sera. (C) BCG-immunized groups OVA-challenged were compared to non-immunized group saline-challenged, \*  $P < 0.05$ , the sum of the Penh in the groups immunized with  $10^2$  or  $10^8$  viable units of BCG is significantly different from the saline group.

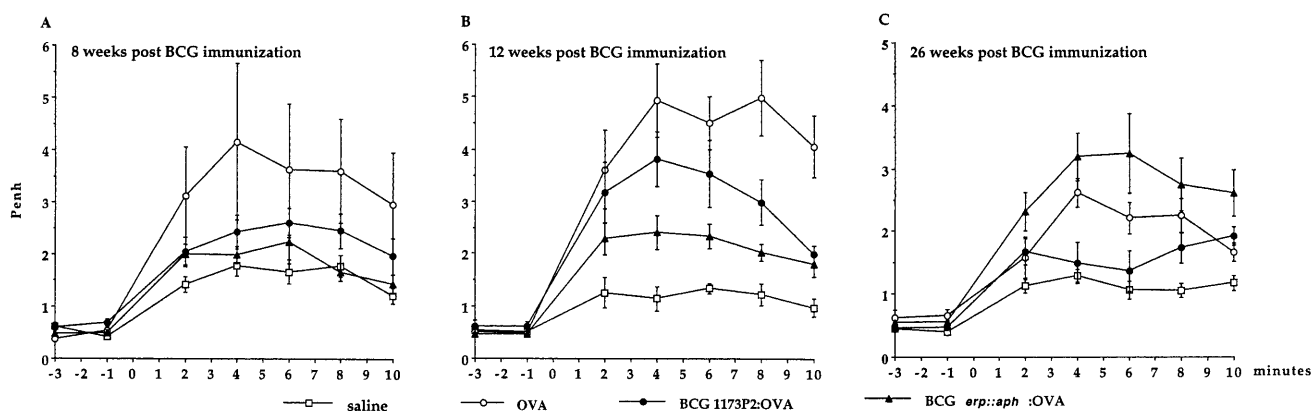


Fig. 2. Long term effect of BCG immunization on BHR in adult BP2 mice. BCG 1173P2 (—●—) and BCG *erp::aph* (—▲—) immunized and non-immunized mice (—○—) were sensitized and challenged with OVA at various times after BCG immunization, age-matched control mice were challenged with saline (—□—). BHR was monitored 24 h after the challenge. The sum of the Penh (for 10 min) of BCG1173P2 and BCG *erp::aph* groups were compared to the sum of the Penh of OVA and saline groups. At 8 weeks, the immunized groups were significantly different from OVA group ( $P < 0.05$ ) and non-significantly different from saline group ( $P > 0.05$ ). At 12 weeks, the immunized groups were significantly different from the OVA and saline groups ( $P < 0.05$ ). At 26 weeks, the sum of the Penh, as compared to the OVA group, is significantly increased in the group BCG *erp::aph* immunized and significantly decreased in the group BCG 1173P2 immunized ( $P < 0.05$ ), the latter group is not significantly different from the saline group ( $P > 0.05$ ).

### 3.3. BCG enumeration in BAL of adult BP2 mice

The CFU of BCG 1173P2 or mutant *erp::aph* were counted in the BAL, tracheo-bronchial lymph nodes and spleens of adult OVA-sensitized BP2 mice, 24 h after the challenge at 8, 12 and 26 weeks after BCG immunizations. Wild type BCG was recovered from the BAL until the end of the experiment, whereas CFU of the attenuated mutant *erp::aph* were never found (Table 1), this in accordance with previous results demonstrating the rapid clearance in the lungs of the attenuated mutant *erp::aph* [17]. Whatever the BCG used, wild type or attenuated mutant, no detectable CFU were found in tracheo-bronchial lymph nodes and spleen (data not shown).

### 3.4. Long-term effect of BCG on bronchial hyperreactivity in BP2 mice immunized as newborns

Newborn BP2 mice that were IN immunized with  $10^5$  CFU of BCG 1173P2 and were sensitized and challenged with OVA at various time points as described above. At 8 weeks, when we compared the sum of the Penh of the BCG-immunized group, no difference with the control group challenged with OVA ( $P > 0.05$ ) and a significant difference with control group challenged with saline ( $P < 0.05$ ) were observed (Fig. 4A). At 12 weeks, mice immunized as newborns developed a lower BHR than controls, the sum of the Penh being significantly different from those of control groups challenged with OVA or saline ( $P < 0.05$ ; Fig. 4B) and at 16 weeks post-BCG immunization a significant suppression of BHR was observed, the sum of the Penh in the BCG-immunized group was significantly lower as compared

to the control group challenged with OVA ( $P < 0.05$ ) and not significant as compared to the control group challenged with saline ( $P > 0.05$ ; Fig. 4C).

### 3.5. Long term effect of BCG on cytokine production in BAL and tracheo-bronchial lymph nodes of BP2 mice immunized as newborns

BP2 mice that were sensitized twice with OVA 6, 10 or 14 weeks after immunization with BCG as newborns, had a non-significant reduction of IL-4 produc-

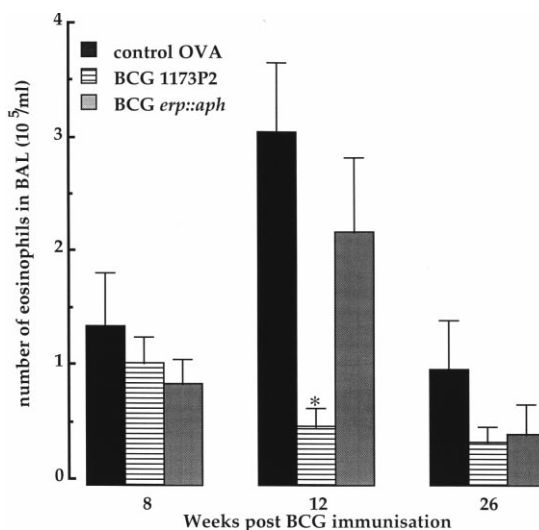


Fig. 3. Effect of BCG immunization on the number of eosinophils recovered in the BAL of adult BP2 mice. At various times after BCG 1173P2 and BCG *erp::aph* immunizations, adult BP2 mice were OVA-sensitized and -challenged, and the number of eosinophils in the BAL was compared to that of non-immunized control group, \*  $P < 0.05$ .

Table 1

Number of BCG 1173P2 or BCG *erp::aph* (CFU) in BAL of adult BP2 mice at various times after intranasal administration of  $10^6$  CFU at day 0<sup>a</sup>

| Weeks | BCG 1173P2     | BCG <i>erp::aph</i> |
|-------|----------------|---------------------|
| 8     | 1395 $\pm$ 275 | <10                 |
| 12    | 407 $\pm$ 229  | <10                 |
| 26    | 65 $\pm$ 53    | <10                 |

<sup>a</sup> BAL were collected from adult BP2 mice at various times after administration of BCG 1173P2 or BCG *erp::aph* ( $10^6$  CFU). The lavage fluids were centrifuged and the cells were resuspended in 1 ml PBS and plated on Middlebrook 7H11 medium, the number of BCG CFU (mean  $\pm$  S.E.M.) were counted after 21 days incubation at 37°C.

tion in BAL after OVA challenge at 8, 12 or 16 weeks ( $P > 0.05$ ). IL-5 production was enhanced in BAL of BCG immunized mice at 8 weeks and slightly reduced at 12 and 16 weeks without significant differences among the groups ( $P > 0.05$ ). Compared to the non-immunized control group, IFN- $\gamma$  production was significantly enhanced at 8 weeks ( $P < 0.05$ ) and at 16 weeks IFN- $\gamma$  production was decreased in the BCG immunized group (Fig. 5).

Since the effect of BCG seemed not to clearly influence the cytokine production in the BAL, we investigate whether BCG administered IN might decrease the Th-2 response in regional lymph nodes, we evaluated the cytokine production after OVA-specific stimulation in vitro by tracheo-bronchial lymph node cells. In BP2 mice immunized as newborns, the OVA-specific secretion of IL-5 cytokine by tracheo-bronchial lymph node cells was not suppressed 16 weeks after the BCG immunization, but higher secretion of IFN- $\gamma$  after in vitro stimulation was found at 8 and 16 weeks (Fig. 6). No

IL-4 production could be detected after in vitro stimulation of tracheo-bronchial lymph node cells with OVA or with mycobacterial antigens (data not shown).

### 3.6. Effect of BCG on OVA-induced eosinophilia and IgE OVA-specific serum immunoglobulin in BP2 mice immunized as newborns

The suppressive effect of BCG administered to newborn BP2 mice on the eosinophilia of the adult animals, as compared to age-matched non-immunized mice, was observed only at the later time point of the experiment ( $P < 0.05$ ; Fig. 7(A)). The specific anti-OVA IgE levels were reduced in BCG-immunized mice as compared to control mice at 12 and 16 weeks after immunization without significant differences ( $P > 0.05$ ; Fig. 7(B)).

### 3.7. Effect of BCG immunization on immediate and delayed type hypersensitivity

No significant difference was observed after 1 h in the intensity of edema in response to OVA injected into the footpad when mice immunized 16 weeks before with BCG were compared to control mice ( $P > 0.05$ ). Three and 4 h after OVA injection, BCG-immunized mice showed a footpad diameter significantly lower than control mice ( $P < 0.05$ ). Twenty-four hours after PPD injection, only animals from the BCG-immunized group developed a significant increase of the footpad diameter ( $P < 0.05$ ). BP2 mice, BCG immunized as newborns and sensitized with OVA showed 16 weeks after immunization a reduced immediate hypersensitivity to OVA as compared to control mice and, on the contrary, develop a significant delayed type hypersensitivity to mycobacterial antigens (PPD; Fig. 8).

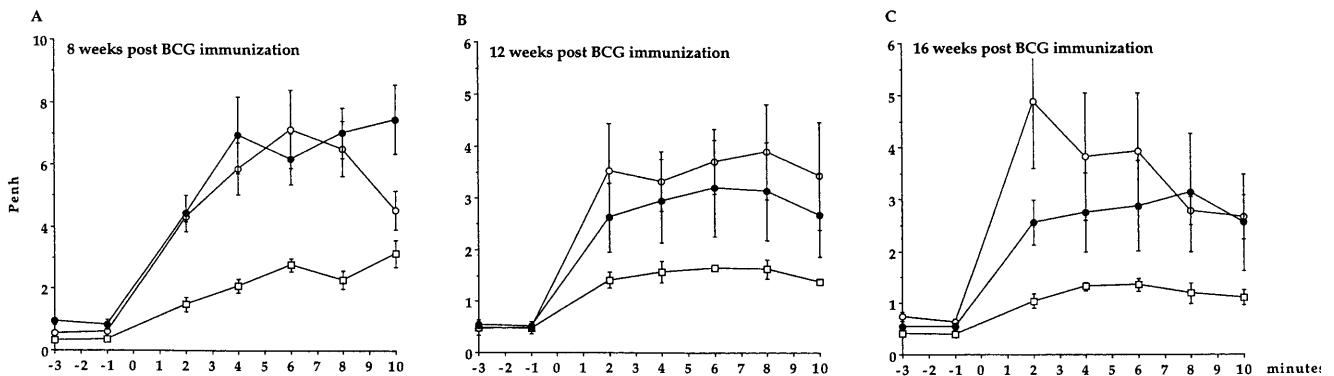


Fig. 4. Long term effect of BCG on BHR in BP2 mice immunized as newborns. BCG 1173P2 immunized (—●—) and non-immunized (—○—) mice were sensitized and challenged with OVA at various times, age-matched control mice (—□—) were challenged with saline. BHR was monitored 24 h after the challenge. The sum of the Penh (for 10 min) of BCG immunized group, was compared to the sum of the Penh of OVA and saline groups. At 8 weeks, the sum of the Penh in the BCG immunized group is not significantly different from the OVA group ( $P > 0.05$ ) and significantly different from the saline group ( $P < 0.05$ ). At 12 weeks, the sum of the Penh in the BCG immunized group is significantly different from OVA and saline groups ( $P < 0.05$ ). At 16 weeks, the sum of the Penh in the BCG immunized group is significantly different from the OVA group ( $P < 0.05$ ) and non-significantly different from the saline group ( $P > 0.05$ ).

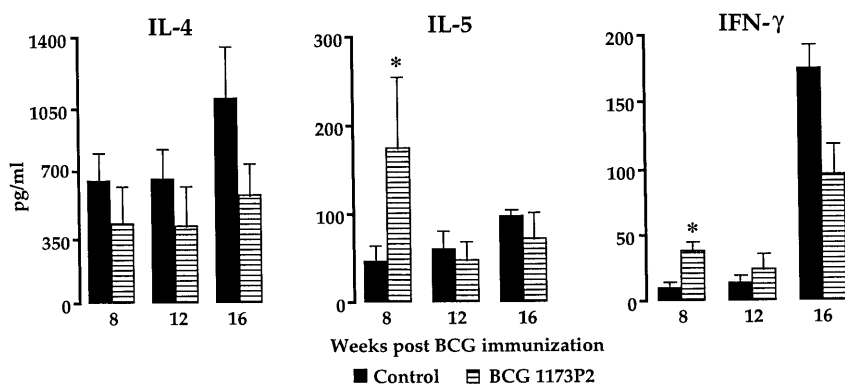


Fig. 5. Long term effect of BCG on cytokine production in the BAL of BP2 mice immunized as newborns. BCG 1173P2 immunized and age-matched control mice were sensitized and challenged with OVA at various times. BAL were recovered 24 h after the challenge and IL-4, IL-5 and IFN- $\gamma$  productions were analyzed using sandwich ELISA. The cytokine levels are expressed in pg/ml, \* $P < 0.05$ .

### 3.8. Phenotype of lung cells

Cell counts at the peribronchial (Table 2) and perivascular (data not shown) compartments of the lungs demonstrated a marked increase in the eosinophilic inflammation in BP2 mice that were sensitized twice with OVA 6 weeks after sham or BCG-immunization as newborns and challenged at 8 weeks ( $P < 0.05$ ). The inflammatory response at this earlier time point was very intense and involved T-lymphocytes, MHC class II molecules, CD11c surface receptor and costimulatory molecules CD86 and particularly CD40. At the later time point, the eosinophil infiltration in the lungs was significantly reduced when compared with allergic animals that did not receive BCG as newborns, and this was paralleled by the reduction in numbers of T-lymphocytes, macrophages, dendritic cells and co-stimulatory molecules. Compared to naive, newborn BCG-immunized mice showed 16 h after significant increases in MHC classII, CD11c, CD40, CD80 and CD86 markers. Mice BCG-immunized as newborns and not sensitized with OVA showed at the later time point studied (8 weeks), as compared to naive mice, significant increases in all markers, except eosinophil (EPO), CD3 and CD8 markers (Table 3). Figs. 9 and 10 illustrate the dampening of the lung inflammation in BCG immunized and OVA-sensitized mice, as compared with non-vaccinated animals.

## 4. Discussion

This study demonstrates that intranasal immunization with wild type BCG 1173P2 prevents in the long term the development of BHR and lung inflammation in adult BP2 mice. The persistence in the lungs of wild type BCG 1173P2 stimulated probably a Th-1 type response in situ and suppressed OVA-induced eosinophilia and BHR after immunization more effi-

ciently than attenuated mutant in the long term. In newborn BP2 mice, compared with OVA-challenged non-vaccinated mice, those that received BCG 8 weeks before challenge were unable to block BHR. This was accompanied by intense bronchial inflammation involving T-cells, macrophages, dendritic cells, increased levels of IFN- $\gamma$  and IL-5 in BAL. However, animals challenged with OVA 12, and particularly 16 weeks after BCG vaccination, did not develop BHR nor bronchial eosinophilia and had reduced levels of IgE. Bronchial infiltration by immunocompetent cells was also significantly reduced. Decreased levels of IL-4 in the BAL and increased production of IFN- $\gamma$  by tracheo-bronchial lymph node cells, accompanied the blockage of BHR. However, IL-5 levels remained elevated in BAL, suggesting that BCG vaccination in newborn BP2 mice with a high Th-2 background might block allergic inflammation and BHR, even though a clear Th-1 shift was not achieved.

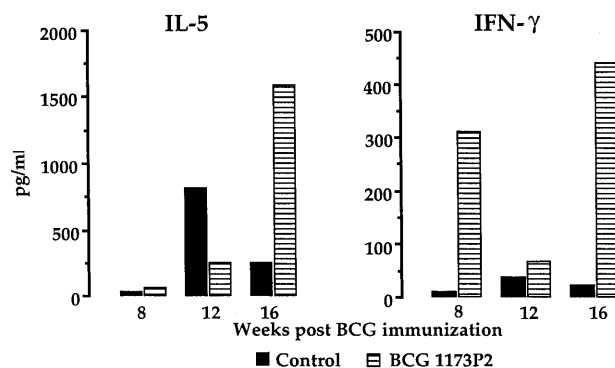


Fig. 6. Cytokine production in tracheo-bronchial lymph node cells of BP2 mice BCG immunized as newborns. BCG 1173P2 immunized and age-matched control mice were sensitized and challenged with OVA at various times as indicated. Tracheo-bronchial lymph node were removed from five mice in each group and cells were pooled and cultured for 72 h in the presence of OVA (2  $\mu$ g/ml), then the supernatants were analyzed for IL-5 and IFN- $\gamma$  production using sandwich ELISA. The cytokine levels are expressed in pg/ml and are representative of two different experiments.

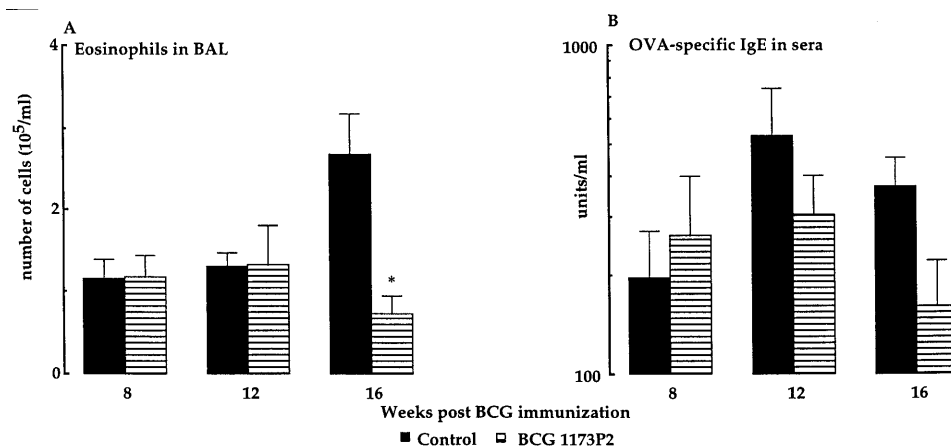


Fig. 7. Effect of BCG on the number of eosinophils recovered in the BAL and on OVA-specific IgE in the sera of BP2 mice immunized as newborns. At various times after BCG immunization, mice were sensitized and challenged with OVA, the number of eosinophils in the BAL (A) and OVA-specific IgE in the sera (B) were compared to age-matched control group, \*  $P < 0.05$ .

Vaccination in early childhood is an effective mean of protecting infants from infectious diseases and may also be extended to allergic asthma. Accordingly, the purpose of our study was to determine whether BCG immunization in adult and more so in newborn BP2 mice could prevent in the long-run the allergic disease and, if so, whether counterbalancing Th-2-type immune response in favour of Th-1-type might be the underlying mechanism. In preliminary experiments, we showed that intranasal immunization of adult BP2 mice with BCG 1173P2 suppressed bronchial hyperreactivity and decreased eosinophilia in the lungs. The attenuated mutant BCG *erp::aph*, which was rapidly cleared from the lungs [17] suppressed effectively BHR only at the beginning of the experiment, if the immunization occurred 8 weeks before the OVA sensitization. The long term persistence of viable BCG in the BAL seemed to play a role in inducing the suppression of BHR and reduction of eosinophilia, because BCG *erp::aph* strain, whose viable units were not recovered in the BAL, was unable to suppress BHR and eosinophilia at the later time point. At the end of our study (26 weeks), few viable bacteria remained in the BAL, but they were sufficient to promote a Th-1 background. Nevertheless, it would be hazardous to recommend multiple BCG immunizations by intranasal route with the purpose of maintaining viable mycobacteria in the lungs. The majority of the population infected with a few bacilli of *M. tuberculosis* never develop the disease and is long-term

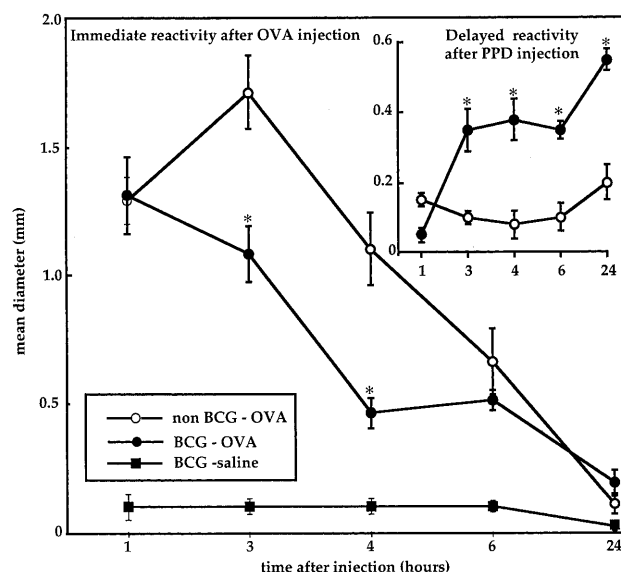


Fig. 8. Effect of BCG on immediate hypersensitivity, after OVA injection (10  $\mu\text{g}$ ), and delayed type hypersensitivity after PPD injection (4  $\mu\text{g}$ ) in the footpad of BP2 mice 16 weeks after immunization. Mice BCG immunized as newborns and age-matched control mice were sensitized or not with OVA before testing. BCG-immunized group was compared to non-immunized group, \*  $P < 0.05$ .

protected against a virulent reinfection. In our mice model it seemed that vaccination with BCG 1173P2 strain could long-term decrease BHR, however, these preliminary results are far from permitting clinical applicability.

Fig. 9. Immunohistochemical study in airways of BP2 mice BCG immunized as newborns 16 weeks before. Cyanide-resistant eosinophil peroxidase staining (positive cells in brown) in BCG-immunized, OVA-sensitized and challenged mice (A) or in non-immunized, OVA-sensitized and challenged mice (B). CD4<sup>+</sup> T-cells (positive cells in red) in BCG-immunized, OVA-sensitized and challenged mice (C) or in non-immunized, OVA-sensitized and challenged mice (D). Original magnification  $\times 400$ .

Fig. 10. Immunohistochemical study in airways of BP2 mice, BCG immunized as newborns 16 weeks before. CD11c dendritic cells (positive cells in red) in BCG-immunized, OVA-sensitized and challenged mice (A) or in non-immunized, OVA-sensitized and challenged mice (B). Cells expressing CD80 co-stimulatory molecules (positive cells in red) in BCG immunized, OVA-sensitized and challenged mice (C) or in non-immunized, OVA-sensitized and challenged mice (D). Original magnification  $\times 400$ .

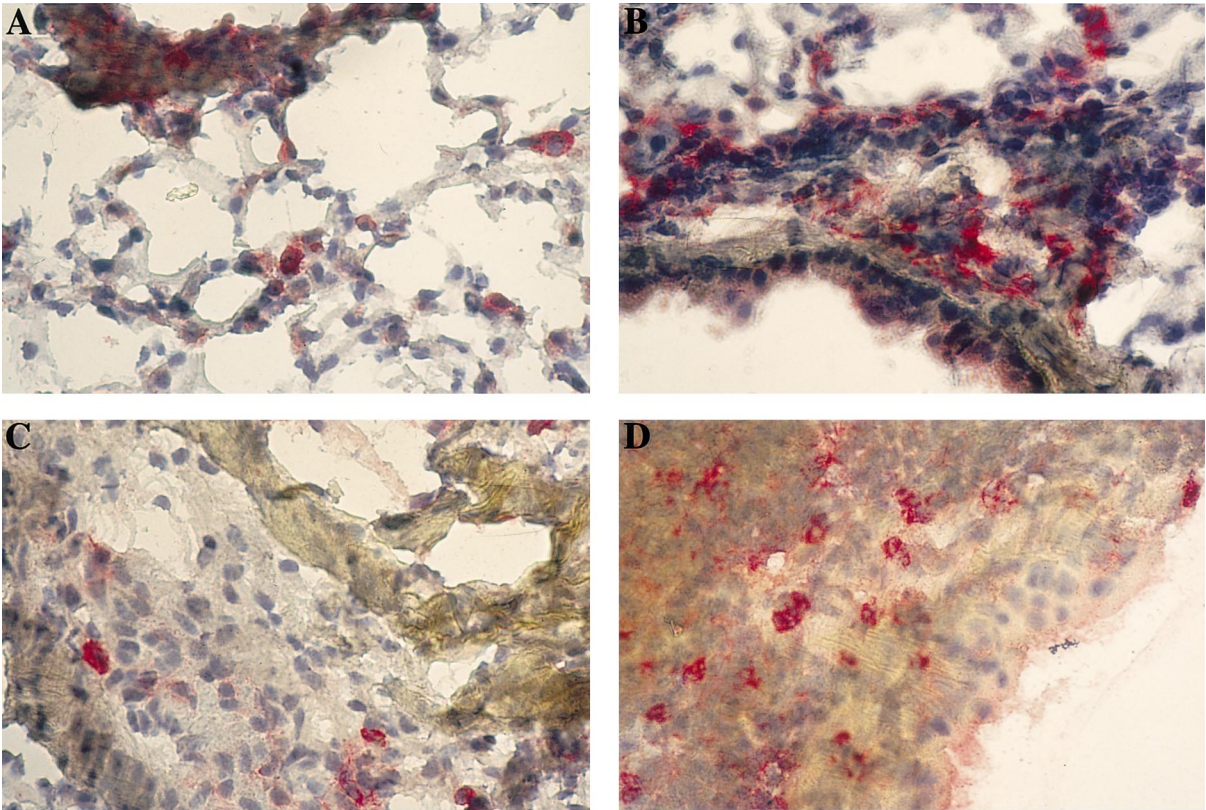


Fig. 9

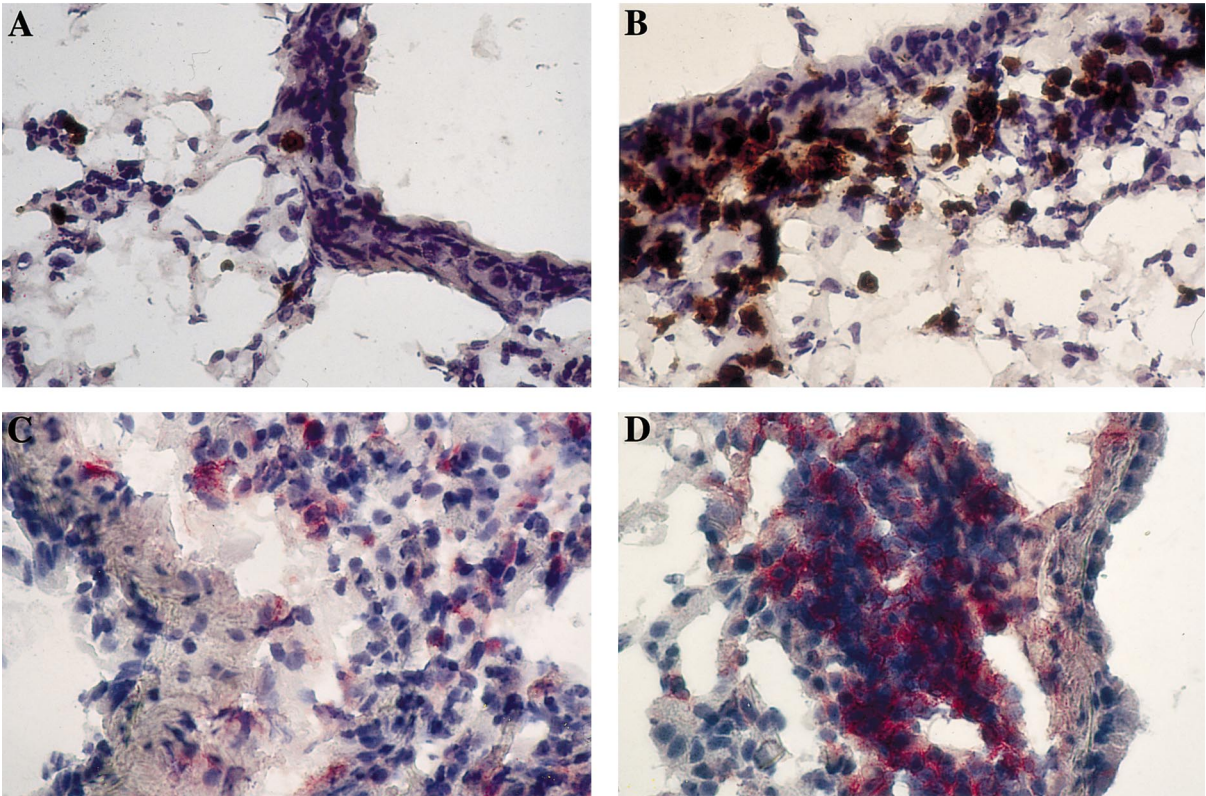


Fig. 10

Table 2

Peribronchial distribution of inflammatory cells in allergic BP2 mice vaccinated or not with BCG as newborns<sup>a</sup>

| Marker       | 8 weeks after BCG |             | 12 weeks after BCG |             | 16 weeks after BCG |             |
|--------------|-------------------|-------------|--------------------|-------------|--------------------|-------------|
|              | OVA only          | BCG:OVA     | OVA only           | BCG:OVA     | OVA only           | BCG:OVA     |
| EPO          | 24.6 ± 9.5        | 50.2 ± 4.9  | 32.1 ± 9.0         | 10.9 ± 4.6  | 51.3 ± 11.0        | 17.9 ± 9.8  |
| CD3          | 25.3 ± 11.2       | 26.4 ± 6.4  | 18.3 ± 4.2         | 5.2 ± 1.8   | 19.3 ± 5.6         | 11.5 ± 7.8  |
| CD4          | 43.8 ± 14.1       | 61.1 ± 17.3 | 44.0 ± 13.4        | 42.8 ± 21.3 | 32.9 ± 7.6         | 18.8 ± 5.5  |
| CD8          | 11.7 ± 4.4        | 23.0 ± 7.3  | 14.6 ± 3.3         | 5.6 ± 0.5   | 19.1 ± 4.3         | 7.2 ± 3.8   |
| Mac3         | 54.7 ± 8.1        | 60.6 ± 20.9 | 42.5 ± 6.4         | 44.6 ± 17.4 | 34.1 ± 7.2         | 25.0 ± 9.3  |
| ClassII (Ia) | 29.0 ± 5.4        | 53.8 ± 11.8 | 24.2 ± 6.5         | 45.7 ± 14.4 | 37.4 ± 4.5         | 43.6 ± 12.0 |
| CD80         | 33.9 ± 9.6        | 18.5 ± 9.3  | 18.2 ± 4.8         | 8.8 ± 1.0   | 16.7 ± 2.8         | 20.7 ± 6.4  |
| CD86         | 25.7 ± 8.4        | 29.1 ± 10.2 | 5.5 ± 0.7          | 9.2 ± 2.8   | 11.3 ± 2.7         | 16.7 ± 6.2  |
| CD11c*       | 30.6 ± 5.3        | 52.3 ± 11.6 | 20.7 ± 3.5         | 19.8 ± 2.3  | 35.0 ± 8.8         | 9.6 ± 1.6   |
| CD40*        | 0.5 ± 0.8         | 19.7 ± 12.2 | 3.1 ± 1.9          | 4.7 ± 2.1   | 2.9 ± 1.3          | 0.7 ± 0.7   |

<sup>a</sup> Lung sections in the peribronchial area (between the basal lamina and the and the beginning of the alveolar area) were stained for cyanide-resistant eosinophil peroxidase (EPO), T-cell receptor (CD3), T-helper cells (CD4), T-suppressor-cytotoxic cells (CD8), mononuclear phagocytes (Mac3), major histocompatibility complex class II (Ia), co-stimulatory molecules (CD80, CD86, and CD40) and dendritic cells (CD11c). The number of positive cells were determined on each microscope field and at least ten fields were analyzed. The results were expressed as the number of positive cells per units area ( $6.25 \times 104 \mu\text{m}^2$ , total area of the graticule). Results were calculated for each experimental group and expressed as mean ± S.E.M.

\* Significant for two-way ANOVA.

BP2 mice were described as high antibody producers which are less susceptible to mycobacterial infection than low antibody producer mice [16]. They display a marked BHR after OVA challenge and show high levels of serum IgE [15]. Despite this strong Th-2 background, adult and newborn BP2 mice could counterbalance long term after BCG immunization the effects of Th-2 type response and suppress airway inflammation and BHR.

A number of studies in mice support the view that the Th-1 environment promoted by infectious diseases, by BCG immunization [12,13], by treatment with CpG-motif oligonucleotides [21] or by killed *M. vaccae* [22] inhibits the development of Th-2 immune response. One possible explanation for these results is that the IFN- $\gamma$  produced during the Th-1 response directly inhibits the development of allergen-specific Th-2 cells. There is mounting evidence that asthma develops predominantly in children that fail to mature their immune system towards a Th-1 pattern [23]. Newborns who have naturally a Th-2 skewed response, and a failure to mature IFN- $\gamma$  responses to inhaled antigens, may prolong the period during which Th-2 responses are predominant [24]. This is the basic reasoning derived from the observations by Shirakawa et al. [4] showing that, in Japan, children vaccinated with BCG were less prone to atopy and allergy. A recent study demonstrated that infants vaccinated with BCG at birth or at 2 months, develop Th-1 and not Th-2 responses directed against BCG antigens [25].

Many studies showed that *M. tuberculosis* and BCG are potent stimulators of DCs [26,27]. Inaba et al. [28] demonstrated that DCs mature after in vitro phagocytosis of BCG and, following their injection into the mice footpads, migrate to lymph nodes and spleen. Here, DCs

might present mycobacterial to T-cells and, accordingly, the activation of mycobacteria-reactive T-cells would enhance cytokine production. In our study, BCG intranasally administered induced the recruitment of antigen-presenting cells (APC), macrophages and probably DCs, expressing CD11c and MHC class II molecules, costimulatory molecules (CD40, CD80 and CD86) in lung peribronchial or perivascular area both at the early (16 h) and the late (8 weeks) stages. The up-regulation of the costimulatory molecule CD86 (B7-2) after BCG immunization and OVA sensitization agrees with an earlier study [29] indicating that CD86 constitutively expressed on monocytes is up-regulated on B cells and

Table 3

Peribronchial distribution of inflammatory cells in BP2 mice vaccinated or not with BCG as newborns<sup>a</sup>

| Marker        | 16 h after BCG |            | 8 weeks after BCG |            |
|---------------|----------------|------------|-------------------|------------|
|               | Control        | BCG only   | Control           | BCG only   |
| EPO           | 0 ± 0          | 0 ± 0      | 4.5 ± 1.6         | 6.3 ± 0.4  |
| CD3           | 4.4 ± 0.8      | 6.8 ± 0.7  | 7.2 ± 1.8         | 3.4 ± 0.9  |
| CD4*          | 7.1 ± 0.7      | 7.4 ± 1.6  | 3.4 ± 0.4         | 12.5 ± 2.2 |
| CD8           | 4.5 ± 0.5      | 5.4 ± 0.9  | 5.4 ± 2.4         | 3.9 ± 0.9  |
| Mac3          | 11.0 ± 2.5     | 14.3 ± 1.7 | 12.9 ± 9.2        | 26.3 ± 0.7 |
| ClassII (Ia)* | 13.5 ± 2.3     | 20.8 ± 4.5 | 4.2 ± 1.6         | 23.0 ± 7.5 |
| CD80*         | 3.3 ± 0.9      | 6.9 ± 1.0  | 2.1 ± 0.4         | 11.0 ± 3.1 |
| CD86*         | 3.1 ± 0.5      | 7.3 ± 3.3  | 1.1 ± 0.6         | 12.8 ± 1.6 |
| CD11c*        | 4.3 ± 1.3      | 14.4 ± 4.6 | 3.6 ± 1.3         | 18.1 ± 0.3 |
| CD40*         | 1.3 ± 1.3      | 2.9 ± 1.1  | 1.0 ± 1.0         | 5.4 ± 0.2  |

<sup>a</sup> Lung sections in the peribronchial area (between the basal lamina and the and the beginning of the alveolar area) were stained with the markers described in Table 2. The results were calculated and expressed as described in Table 2.

\* Significant for two-way ANOVA.

DCs following exposure to OVA. In contrast, BCG immunization before OVA sensitization induced down-regulation of CD80 (B7-1) expression from the early stage of the experiment at 8 weeks and in CD40 expression at 16 weeks. CD40, CD80 and CD86 costimulatory molecules do not deliver similar signals, CD40 and CD80 are not required for T cell IL-4 production, whereas CD86 provides a costimulatory signal for development of Th-2 cytokine patterns [30,31]. The CD80 and CD40 down-regulation, counterbalanced by CD86 up-regulation, might explain the mixed Th-1/Th-2 cytokine production noted in our study.

The recovery of BCG bacilli in BAL supports the view that IFN- $\gamma$  was first produced by local T-cells triggered by mycobacterial antigens presented by DCs or macrophages at the site of BCG infection. Thereafter, the migration of BCG infected APC to draining lymph nodes induced a clonal expansion of T-cells which produced IFN- $\gamma$  at this site, when restimulated in vitro with mycobacterial antigens (data not shown) or OVA. Fenton et al. [32] demonstrated recently that human alveolar macrophages infected in vitro with *M. tuberculosis* produce IFN- $\gamma$ . This production appears to be mediated in part by IL-12 and is blocked by exogenous IL-10. A recent study [33] showed that live and dead BCG induce comparable levels of IFN- $\gamma$  at the site of injection during the first week. In contrast, only live BCG induced IFN- $\gamma$  production in draining lymph nodes long term after immunization. This is in accordance with our results (data not shown) in which BP2 mice immunized with dead BCG and OVA-sensitized were unable to produce, after OVA-challenge, higher levels of IFN- $\gamma$  than non-immunized animals and failed to subsequently block BHR.

IFN- $\gamma$  production may have direct effects on effector cells such as eosinophils. The partial dependence of antigen-induced eosinophilia on IFN- $\gamma$ , in our model, is consistent with a study showing that IFN- $\gamma$  administration can block eosinophilia influx into murine airway [34]. IFN- $\gamma$  and IL-4 generated by antigen-specific lymphocytes modified the IgE synthesis. These cytokines regulate reciprocally the IgE response, and an enhanced IFN- $\gamma$  production would favor suppression of IgE production and decrease the IL-4 production. This hypothesis is supported by the fact that IFN- $\gamma$  levels in BAL, and particularly after stimulation of tracheo-bronchial lymph node cells are high, whereas IL-4 levels are low in BAL and are not detectable in lymph node cells, in correlation with the decreased IgE levels.

The role of IL-5 and eosinophils in the development of BHR in mice is pointed out in many studies. Foster et al. [35] reported that sensitized IL-5<sup>-/-</sup> B6 mice did not develop eosinophilia nor BHR after allergen airway challenge. Hamelmann et al. [36] demonstrated that anti IL-5 antibodies prevents airway hyperresponsiveness in BALB/c mice and we reported similarly for

BP2 mice [15]. In the present study, despite the persistence of IL-5 production in BAL, BCG immunization reduce eosinophil infiltration in the lungs in close association with the suppression of BHR and with lower levels of IgE. The finding by Corry et al. [37] that IL-4 but not IL-5 is required in a murine model of BHR, is in accordance with our results in which BCG immunization decreased IL-4 but not IL-5 production in BAL which was associated with decreasing levels of eosinophilia and BHR.

In summary, we demonstrated that BCG intranasally administered to newborn BP2 mice with a high Th-2 background inhibits the allergen-induced eosinophilia and BHR in the adult mice, under conditions where a clear Th-1 versus Th-2 shift was not achieved.

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