

Vaccinations with T-helper type 1 directing adjuvants have different suppressive effects on the development of allergen-induced T-helper type 2 responses

C. M. Trujillo-Vargas*, K. D. Mayer†, T. Bickert*, A. Palmethofer‡, S. Grunewald§, J. R. Ramirez-Pineda¶, T. Polte||, G. Hansen||, G. Wohlleben* and K. J. Erb*

*Center for Infectious Diseases, University of Würzburg, Würzburg, Germany, †Trudeau Institute Inc., New York, NY, USA, ‡Institute of Pathology, University of Würzburg, Würzburg, Germany, §Department of Dermatology, University of Würzburg, Würzburg, Germany, ¶Programa de Estudio y Control en Enfermedades Tropicales-PECET, Universidad de Antioquia, Medellin, Colombia and ||Martin-Luther-University Halle-Wittenberg, Halle, Germany

Summary

Background Allergen-induced T-helper type 2 (Th2) responses can be inhibited with Th1 directing vaccines. However, studies comparing the efficacy of the different adjuvants have not been performed in detail.

Objective For this reason we compare the effects of live (BCG), heat-killed (hk)-BCG, CpG-ODN (oligodeoxynucleotide) or PPD on the development of allergen-induced Th2 responses in mice.

Methods Ovalbumin (OVA)-specific allergic responses were induced in C57BL/6 mice by two intraperitoneally (i.p.) applications of OVA/alum followed by the intranasal challenge with OVA. The different Th1-inducing adjuvants were applied to the mice together with OVA/alum i.p. during the OVA-sensitization period and, subsequently, different parameters of allergic immune responses were evaluated.

Results All the adjuvants were effective in inhibiting the development of allergen-induced airway eosinophilia, mucous production and, with the exception of PPD, also airway hyper-reactivity, when they were applied together with OVA/alum. However, allergen-specific IgG1 and IgE serum levels were only reduced in live BCG- and PPD-treated mice. Suppression of airway eosinophilia was not observed in IFN- γ - or IL-12-deficient mice (hk-BCG, CpG-ODN and PPD). Interestingly, live BCG was still able to suppress allergen-induced Th2 responses in the absence of either IFN- γ or IL-12. When mice vaccinated with the different adjuvants together with OVA/alum were subjected to a second period of OVA/alum immunization, only live and hk-BCG were able to efficiently suppress the development of airway inflammation. This effect could be adoptively transferred by splenic CD4⁺ T cells.

Conclusions Taken together our data suggest that live BCG > hk-BCG > CpG-ODN > PPD are effective in suppressing allergen-induced Th2 responses. The degree of suppression and the component of the Th2 response affected (airway inflammation vs. the production of allergen-specific IgE and IgG1) were dependent upon the adjuvant used and how it was applied. Our results contribute to the design of novel vaccines protecting humans from developing allergic disorders.

Keywords adjuvants, allergy, BCG, Th1/Th2 cells, vaccination

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Introduction

Allergic asthma is a disorder characterized by chronic airway inflammation, goblet cell metaplasia and airway hyper-reactivity (AHR). The most important pre-requisites for the development of this disease are the exposure to allergens, the production of IL-4 and the subsequent generation of allergen-specific CD4⁺ T-helper type 2 (Th2) cells in the lymphoid organs after coming in contact with dendritic cells (DC)

presenting allergen-derived peptides bound to MHC class II molecules. Effector Th2 cells secrete IL-4, IL-5, IL-9 and IL-13 leading to the induction of asthma through the production of allergen-specific IgE by B cells (mediating mast cell degranulation), the development and recruitment of eosinophils into the lung, smooth muscle contraction and mucous production in the airways [1].

Although some of the most important immunological mechanisms leading to the development of allergic asthma have been identified, the incidence, severity and mortality rates all have steadily increased over the past decades despite the use of corticosteroids and other drugs. The reason for it remains unknown but has been speculated to be because of environmental factors. One factor believed to have a strong

Correspondence: Claudia M. Trujillo-Vargas, Center for Infectious Diseases, University of Würzburg, Röntgenring 11, 97070 Würzburg, Germany.

E-mail: claudia.trujillo@mail.uni-wuerzburg.de

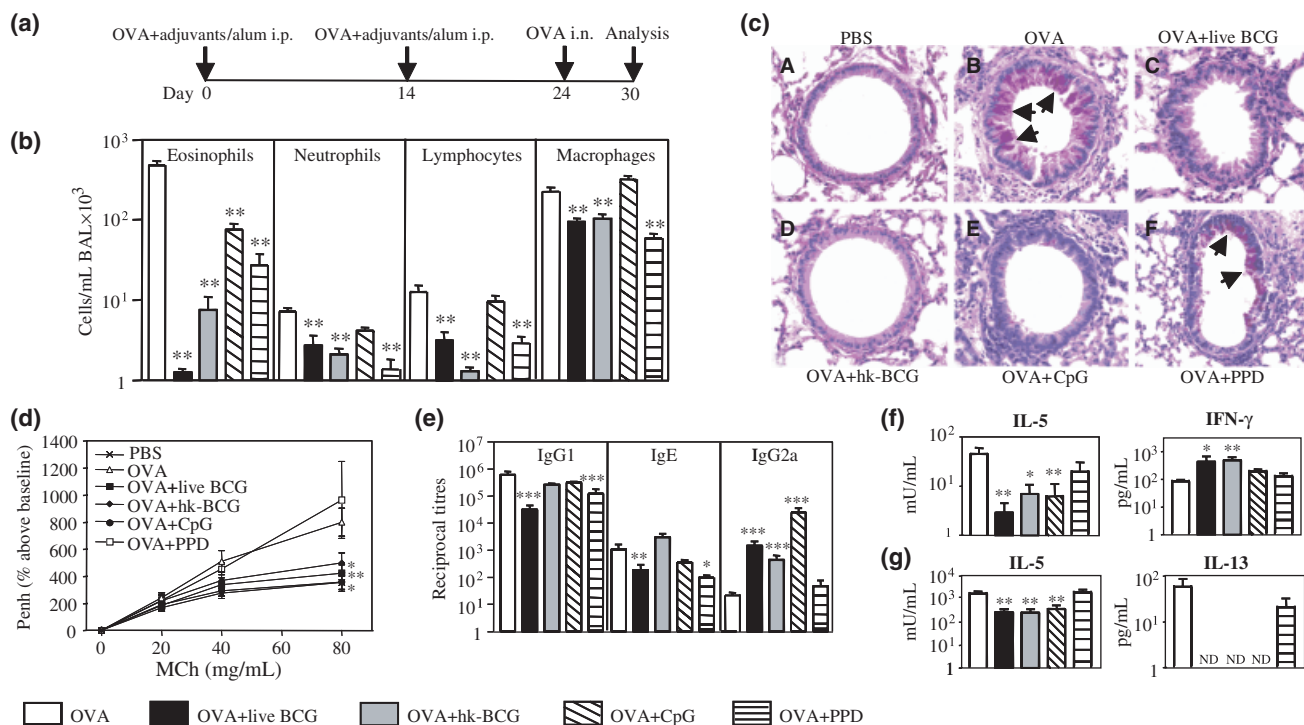


Fig. 1. Vaccination with live *Bacillus-Calmette-Guerin* (BCG), heat-killed -BCG, CpG-oligodeoxynucleotide or PPD together with ovalbumin (OVA)/alum. Mice were vaccinated with the different adjuvants during the OVA sensitization period (a). The numbers of the different cell types present in the bronchoalveolar lavage (BAL) (b), the mucous production in the lung determined by staining lung sections with periodic acid-Schiff (representative examples of 6 mice/group are shown, $\times 200$ magnification) (c), the airway hyper-reactivity in response to increasing concentrations of methacholine (d), and the serum OVA-specific antibody levels (e) are shown. The cytokine levels detected in the BAL fluid (f) and in the mediastinal lymph nodes cell cultures stimulated with OVA (g) are also depicted. Mean values of 7–12 mice/group $\pm$ SEM are shown. The experiments were repeated once (with the exception of D) with similar results. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ compared with the values obtained from mice immunized only with OVA. Arrows indicate areas with goblet cell metaplasia. ND, not detectable.

impact on the development of allergic asthma is the reduction in the incidence of infectious diseases early during infancy [2, 3]. The rationale behind this hypothesis is that children infected with viruses or bacteria early in life do not develop asthma because these infections establish a Th1-biased immunity leading to the inhibition of allergen-specific Th2 cell priming or Th2 effector function. However, recent data clearly indicate that T regulatory (Tr) responses associated with infections also contribute to the suppression of allergen-specific Th2 responses [4, 5]. Although it remains to be elucidated if the lack of Th1 or Tr responses contribute to the rise in allergic disorders in humans, it is well established that IFN- γ can suppress the development of Th2 cells both *in vitro* and *in vivo* [6]. Based on these findings numerous studies have addressed the question if it is possible to protect mice from developing allergic Th2 responses by immunizing them with vaccines that induce Th1 responses [2, 7–9]. Live and dead bacteria, bacterial components such as purified protein derivative from *Mycobacterium tuberculosis* (PPD), CpG-oligodeoxynucleotides (CpG-ODN) and plasmid DNA have been used successfully [2, 10–15]. However, it is not clear which approach is the most efficient, as studies comparing the suppressive effects of the different approaches have not been performed in detail.

The aim of this study was to compare the efficacy of several known Th1 directing adjuvants including live (BCG), heat-killed (hk) BCG, CpG-ODN or PPD as components of vaccines aimed at protecting mice from developing allergen-induced airway inflammation and allergen-specific IgE and

IgG1. Our data show that the degree of suppression and the component of the Th2 response affected (airway inflammation vs. the production of allergen-specific IgE and IgG1) were dependent upon the adjuvant used and how it was applied. However, live BCG was more efficient in inhibiting the development of allergen-specific Th2 responses than hk-BCG, CpG-ODN or PPD.

Methods

Mice

Female C57BL/6 mice were purchased from Charles River (Sulzfeld, Germany) and were maintained under conventional conditions in an isolation facility. IFN- γ -, IL-12p35- and IL-12p35/p40-deficient mice (all on a C57BL/6 genetic background) were generously provided by O. Liesenfeld (Berlin, Germany). IL-10-deficient mice (on a C57BL/6 genetic background) were a gift of W. Müller (Cologne, Germany). At the onset of the experiments, all animals were between 6 and 8 weeks of age. All experiments were performed according to the guidelines of the local and government authorities for the care and use of experimental animals.

Induction of ovalbumin-specific T-helper type 2 responses

In order to induce ovalbumin (OVA)-specific Th2 responses, mice were treated twice intraperitoneally (i.p.) (days 0 and 14) with a mixture of 2 μ g OVA (Sigma Chemical Co., St Louis,

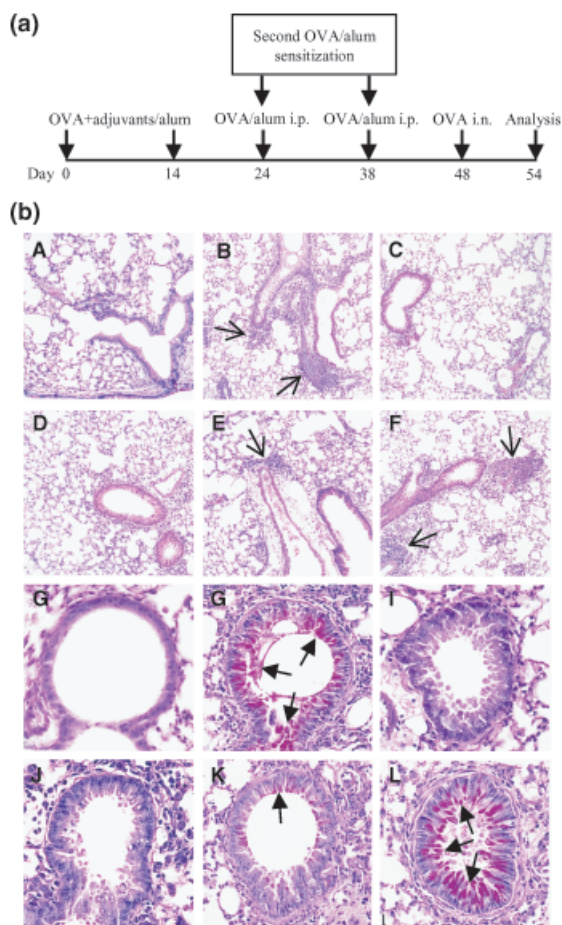


Fig. 2. Effect of the different adjuvants on the development of airway inflammation and goblet cell metaplasia after a second round of ovalbumin (OVA) sensitization. The ability of the different adjuvants applied together with OVA/alum intraperitoneally to inhibit OVA-specific T-helper type 2 responses was also analysed after a second period of OVA sensitization (a). The sections of the lungs subjected to haematoxylin and eosin (A–F) or periodic acid-Schiff (G–L) staining from untreated mice (A, G), OVA-immunized mice (B, H), live *Bacillus-Calmette-Guerin* (BCG) (C, I), heat-killed-BCG (D, J), CpG-oligodeoxynucleotide (E, K) or PPD-treated mice (F, L) are shown. Representative examples of 6 mice/group are shown (b) (A–F $\times 20$ and G–L $\times 200$ magnification). Open arrows indicate areas with peribronchial or perivascular inflammation. Closed arrows indicate areas with goblet cell metaplasia.

MO, USA) in 200 μ L alum adjuvant (Serva, Heidelberg, Germany). Subsequently, mice were anaesthetized by an i.p. injection of Ketamine/Xylazine (Sigma Chemical Co.) and challenged intranasal (i.n.) (day 24) with 50 μ L PBS containing 100 μ g OVA. Analyses were performed 6 days after the i.n. challenge unless otherwise indicated.

Adjuvants BCG (Immunocyst Connaught, Ithringen, Germany) was hk by incubating the mycobacteria twice for 1 h at 80 °C in a water bath. The lack of viability of hk-BCG was monitored by plating serial dilutions onto plates containing Middlebrook 7H10 agar (Difco, Augsburg, Germany) supplemented with 10% v/v Middlebrook ADC enrichment (Becton Dickinson, Franklin Lakes, NJ, USA). Colonies were counted after 21 days of incubation at 37 °C in 9% CO₂. No colony forming units (CFU) could be detected in the hk-BCG stock solution. Live BCG was used as positive control for viability and to verify the amounts of BCG being used for the

immunizations. Phosphorothioate CpG-ODN 1826 (5'-TCC ATG ACG TTC CTG ACG TT-3') was synthesized by Sigma Chemical Co. PPD was purchased from Serum Statens Institute (Copenhagen, Denmark). No endotoxin was detected in any of the adjuvants using the Limulus assay. Live BCG or hk-BCG (2×10^6 CFU/mouse), CpG (30 μ g/mouse) or PPD (40 μ g/mouse) were applied to the mice together with OVA/alum as shown in Figs 1, 2, 4 and 5.

Bronchoalveolar lavage

At the indicated time-points mice were killed, blood samples collected, the trachea cannulated and a bronchoalveolar lavage (BAL) performed by flushing lung and airways four times with 1 mL PBS containing 10% heat-inactivated fetal calf serum (PAA laboratories, Linz, Austria).

Culture condition of cells

Single-cell suspensions from the mediastinal lymph nodes (MLN) of the different groups of mice were prepared as described previously [9]. The cell preparations were added to cell culture plates (2×10^6 cells/mL) and left in medium alone, stimulated with 40 μ g/mL OVA or with a plate-bound mAb to CD3 ϵ (145-2C11, 25 μ g/mL) together with 200 U/mL recombinant IL-2 (Novartis, Basel, Switzerland) at 37 °C in a humidified atmosphere containing 5% CO₂. After 48 h, the culture supernatants were harvested and tested for the presence of cytokines by ELISA.

Enzyme-linked immunosorbent assay for the detection of immunoglobulins and cytokines

For the detection of serum OVA-specific Ig levels or cytokines in the BAL fluid and cell culture supernatants, sandwich ELISAs were performed as previously described [9].

Adoptive transfer of cells

Mice were vaccinated as indicated in Fig. 2a. Six days after the OVA i.n. challenge mice were killed and single-cell suspensions from spleens from each group of mice were prepared. In a separate experiment CD4⁺ T cells were purified from the spleens of the different groups of mice using Cederlane's mouse CD4 cell recovery column kit following the manufacturer's instructions (Cederlane Labs, ON, Canada). This procedure allowed the enrichment of mouse CD4⁺ T cells leading to 85–95% purity as determined by FACS staining. 2×10^7 total spleen cells or 1×10^7 purified CD4⁺ T cells (in 100 μ L PBS) from the different groups of mice were i.v. injected into untreated mice. One day later all the animals were subjected to the protocol that induces OVA-specific Th2 responses. As a control, a separate group of mice injected with no cells were also subjected to the Th2-inducing protocol.

Histological analysis

Lungs were fixed in 10% phosphate-buffered formalin for 24 h and embedded in paraffin wax. Sections (2–3 μ m) were cut and stained using standard histological protocols with haematoxylin and eosin (H&E) or periodic acid-Schiff (PAS) reagent. The stained sections were visualized by light

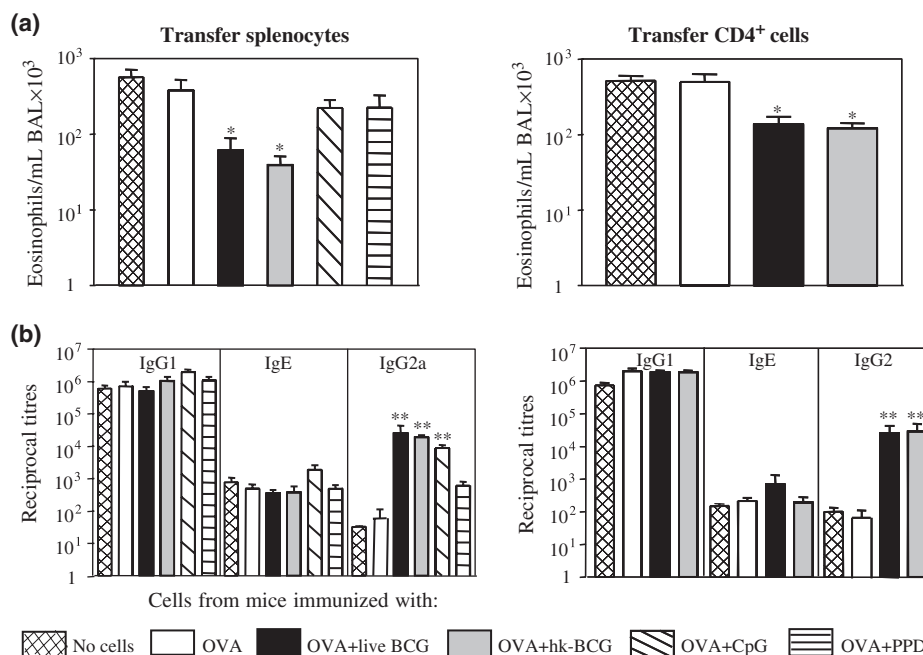


Fig. 3. Adoptive transfer of total splenic cells or CD4⁺ T cells from mice vaccinated with the different T-helper type 1 (Th1)-inducing adjuvants. Suspensions of total splenocytes or purified CD4⁺ T cells were prepared from mice vaccinated as described in Fig. 2a. Naïve C57BL/6 mice were then injected with these cells and 1 day later subjected to the protocol which induces ovalbumin (OVA)-specific Th2 responses as described in 'Methods'. The absolute number of eosinophils present in the bronchoalveolar lavage (a) and the serum titres of OVA-specific antibodies (b) in the different groups of mice (mean of 7–9 mice/group $\pm$ SEM) are shown. * $P < 0.05$ and ** $P < 0.01$ compared with the values obtained in mice adoptively transferred with cells from mice immunized only with OVA.

microscopy. The intensity of peribronchial and perivascular inflammation as well as the degree of goblet cell metaplasia was scored by two independent observers (0 = no inflammation or goblet cell metaplasia, 1 = slight inflammation or goblet cell metaplasia, 2 = moderate inflammation or goblet cell metaplasia, 3 = strong inflammation or goblet cell metaplasia).

Cutaneous anaphylaxis

Active cutaneous anaphylaxis was tested in the different groups of mice 6 days after the OVA intranasal challenge as described previously, by intradermal injection of 50 μ L PBS containing 10, 1 or 0.1 μ g/mL OVA [16]. The degree of bluing as indicator for the intensity of mast cell degranulation was scored by two independent observers.

Airway hyper-reactivity measurements

AHR was assessed by methacholine-induced airflow obstruction, using a whole-body plethysmograph (model PLT UNR MS, EMKA Technologies, Paris, France) as previously described [17]. In Fig. 1d the AHR measurements were performed on day 34 after OVA was applied intranasally on days 24, 25, 26 and 31, 32, 33. No changes in Penh baseline were observed in any of the animals used in this study.

Stimulation of bone marrow-derived dendritic cells

Bone marrow-derived dendritic cell (BM-DC) were generated from bone marrow of C57BL/6 mice in the presence of high doses of GM-CSF (200 U/mL; PeproTech Inc., London, UK) as previously described [18]. After 10 days, cells were counted, resuspended at 2×10^6 cells/mL and stimulated in 24-well

plates (NUNC, Neerijse, Belgium) for 24 h with live BCG or hk-BCG (at 1×10^6 or 1×10^5 CFU/mL), CpG-ODN (30 μ g/mL) or PPD (20 μ g/mL). BM-DC resuspended only with supplemented RPMI medium were used as controls. The culture supernatants were harvested and tested for the presence of cytokines by ELISA 24 h later. In parallel, the expression levels of CD86, CD80 and MHC class II on the surface of the BM-DC were determined by FACS staining. Total RNA was also extracted 24 h after stimulation using the Rneasy Mini Kit from Qiagen, (Hilden, Germany). To determine the mRNA abundancies of the various chemokine receptors RNase protection analyses were performed (kit used was purchased from Pharmingen, San Diego, CA, USA).

Statistical analysis

Results were evaluated using one-way ANOVA.

Results

The application of live BCG, heat-killed-BCG, CpG-oligodeoxynucleotide or PPD together with ovalbumin/alum reduces the development of allergen-induced T-helper type 2 responses

Previous results have clearly shown that the application of allergen-specific Th1 cells or Th1-inducing adjuvants reduces the development of allergic responses [2]. In this study, we wanted to compare the capacity of different Th1-inducing adjuvants *in vivo* to suppress allergen-specific Th2 responses. For this purpose we applied live or hk-BCG, CpG-ODN or PPD together with OVA/alum i.p. and

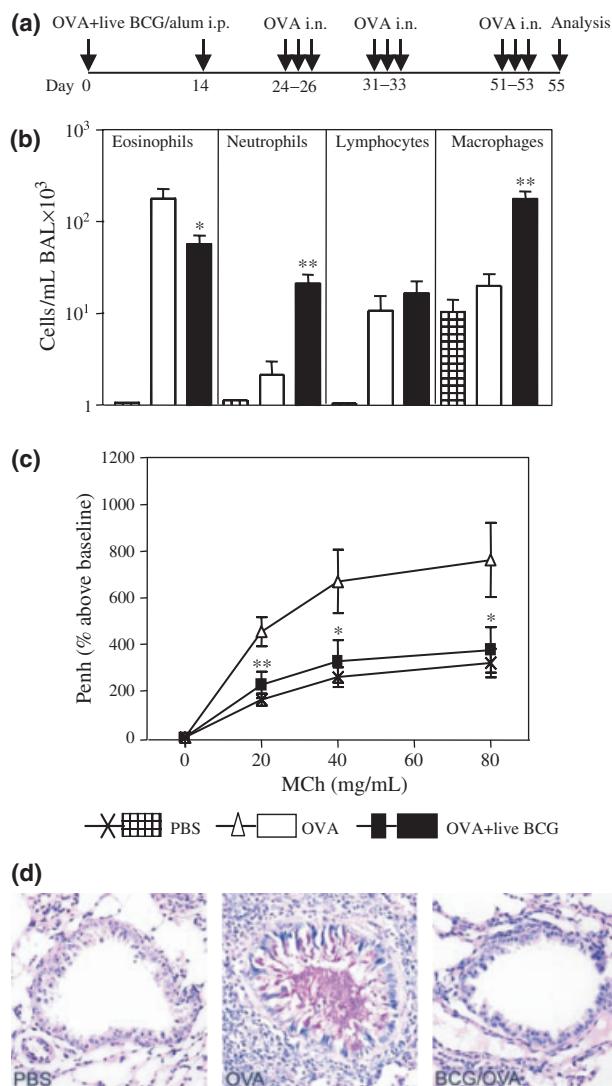


Fig. 4. Effect of multiple ovalbumin (OVA) intranasal (i.n.) challenges in mice vaccinated with OVA + live *Bacillus-Calmette-Guérin* (BCG)/alum. Mice were injected with OVA + live BCG/alum and then subjected to multiple OVA i.n. challenges (a). The absolute numbers of the different cell types present in the bronchoalveolar lavage (b) and the airway hyper-reactivity in response to increasing concentrations of methacholine (c) (mean $\pm$ SEM from 8–10 mice/group) are shown. * $P < 0.05$ and ** $P < 0.01$ compared with the mice injected only with OVA/alum. Goblet cell metaplasia and mucous production in the lung were also evaluated (d). Representative examples of the lungs from 6–8 mice/group ($\times 200$ magnification) are shown.

analysed if allergen-specific Th1 responses were initiated and allergen-specific Th2 responses were suppressed (Fig. 1a). Figure 1b shows that the application of the different adjuvants resulted in a strong decrease in airway eosinophilia in comparison with mice sensitized only with OVA. The strongest effect was observed using live BCG, which induced a 400-fold reduction in the number of eosinophils in the BAL. Hk-BCG, CpG-ODN and PPD also induced a significant but slighter reduction in airway eosinophilia (64-, 6- and 17-fold reduction compared with OVA only treated mice, respectively).

Goblet cell metaplasia in the airways were virtually absent in mice treated with live BCG, hk-BCG and CpG-ODN (Fig. 1c).

The use of PPD also reduced mucous production and goblet cell metaplasia, but to a lower degree than live BCG, hk-BCG and CpG-ODN (mean scores of goblet cell metaplasia in the different groups: OVA/alum: 2, OVA + live BCG/alum: 0, OVA + hk-BCG/alum: 0, OVA + CpG/alum: 0, OVA + PPD/alum: 1).

The reduction of airway eosinophilia also correlated with significantly reduced AHR and decreased levels of IL-5 detected in the BAL fluid when the mice were treated with live BCG, hk-BCG or CpG-ODN (Figs 1d and f). Detectable IFN- γ levels were only increased in the airways when live or hk-BCG was applied. No IL-4 or IL-13 could be detected in the BAL fluid. The application of live BCG, hk-BCG or CpG-ODN but not PPD also resulted in a significant decrease in the levels of IL-5 and IL-13 produced by MLN cells after stimulation with OVA *in vitro* (Fig. 1g). No significant differences in the levels of IL-10 or IFN- γ were found in the supernatants of the MLN cells from the different groups of mice stimulated with OVA (data not shown). Similar results were obtained when the cells were restimulated with anti-CD3/IL-2 and no cytokines were detected when the cells were cultured in medium only. IL-4 could not be detected in any of the MLN cell cultures (data not shown).

Surprisingly, although all the compounds tested suppressed Th2 responses in the lung, only the application of live BCG and PPD resulted in reduced OVA-specific IgG1 as well as OVA-specific IgE levels in the serum. In contrast, application of live BCG, hk-BCG and CpG-ODN but not PPD led to increased levels of OVA-specific IgG2a in the serum (Fig. 1e). Although all the adjuvants modulated the allergen-specific B cell response, only the application of live BCG resulted in reduced cutaneous mast cell reactivity after the intradermal application of OVA (data not shown).

Adjuvant-induced suppression of allergic T-helper type 2 responses is dependent upon the presence of interleukin-12 or interferon- γ

The reduction of type 2 responses by the application of live BCG, hk-BCG or CpG-ODN was associated with increased Th1 responses. This suggests that Th1 cells secreting IFN- γ were responsible for this effect. However, recently it was also reported that mycobacteria-induced suppression of Th2 responses may also be because of Tr and not Th1 responses [4]. Furthermore, Kline et al. [19] showed that the application of CpG-ODN could still inhibit the development of allergen-specific Th2 responses in the absence of IL-12 or IFN- γ . To confirm the hypothesis that our observed effects were because of Th1 responses we repeated the experiments described in Fig. 1a in either IL-12 p35- or IFN- γ -deficient mice. Table 1 shows that the suppressive effect of hk-BCG, CpG-ODN or PPD on allergen-induced eosinophilia was totally dependent on the presence of IL-12 or IFN- γ . In contrast, in the presence of live BCG, eosinophil numbers were significantly reduced indicating that this effect was only partially dependent on either one of these two cytokines.

To assess whether live BCG mediates the suppressive effect on Th2 responses by generating IL-10-producing Tr cells or alternatively by inducing IL-23 (a heterodimer composed of the IL-12p40 subunit and the IL-12p35-related molecule p19

which also acts on Th1 effector/memory CD4⁺ T cells [20]), the experiments described in Fig. 1a were repeated in IL-10- and IL-12p35/p40-deficient mice. Interestingly, live BCG-mediated suppressive effect on allergen-induced airway eosinophilia was still observed in both IL-10- and IL-12p35/p40-deficient mice (results in IL-10-deficient mice: OVA/alum: 52.2 ± 21.9 vs. OVA + live BCG/alum: $1.5 \pm 0.5^*$ eosinophils/mL BAL $\times 10^3$; results in IL-12p35/p40-deficient mice: OVA/alum: 333.7 ± 137.2 vs. OVA + live BCG/alum: $0.7 \pm 0.2^{**}$ eosinophils/mL BAL $\times 10^3$; mean of 4–7 mice/group $\pm$ SEM; $^*P < 0.05$ and $^{**}P < 0.01$).

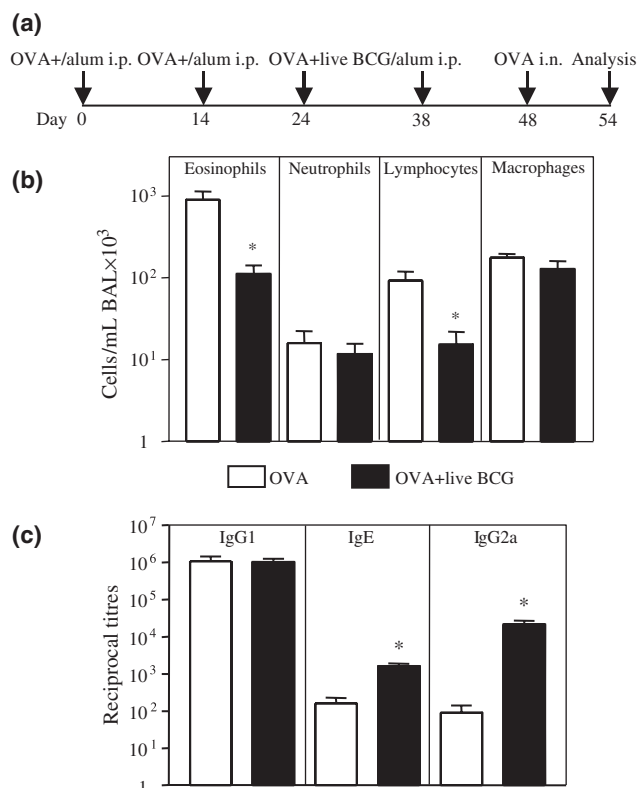


Fig. 5. Effect of live *Bacillus-Calmette-Guerin* (BCG) on the allergic responses using a therapeutic protocol. Mice were first sensitized with ovalbumin (OVA)/alum intraperitoneally (i.p.) and 10 days later, subjected to the vaccination with OVA + live BCG/alum i.p. Subsequently, OVA intranasal challenge was applied and the allergic responses were evaluated 6 days later (a). The absolute numbers of the different cell types present in the bronchoalveolar lavage (b) and the serum OVA-specific antibody levels (c) either in untreated (OVA) or treated mice (OVA + live BCG) (mean values of eight mice/group $\pm$ SEM from two separate experiments) are shown. $^*P < 0.05$ compared with the mice injected only with OVA/alum.

The application of live BCG or heat-killed-BCG but not CpG-oligodeoxynucleotide or PPD reduces allergen-induced airway inflammation after secondary sensitization with allergen

Next we examined the inhibitory effect of live or hk-BCG, CpG-ODN or PPD applied together with OVA/alum on the development of allergic Th2 responses induced by a second period of OVA sensitization (Fig. 2a). Table 2 shows that using this protocol the application of live BCG was able to induce a 320-fold reduction in the generation of OVA-induced eosinophilia in the airways. The application of hk-BCG also reduced airway eosinophilia significantly but to a lesser extent compared with its effect using the protocol shown in Fig. 1 (sevenfold reduction in comparison with 64-fold reduction using the protocol shown in Fig. 1a). Most importantly, the initial suppressive effects of CpG-ODN or PPD on airway eosinophilia were no longer present after a second sensitization with OVA. No significant reduction in the levels of allergen-specific IgG1 and IgE serum levels were found with any of the adjuvants used. Similar to the results shown in Fig. 1e we again found that the application of live or hk-BCG or CpG-ODN resulted in increased levels of OVA-specific IgG2a in the serum. Only the reduction in the airway eosinophilia induced by live BCG was associated with decreased levels of IL-5 in the BAL. Although we found increased levels of IFN- γ in the BAL of mice treated with the different adjuvants, the amounts were not significantly higher than in the OVA only treated group. Also, no significant changes in the levels of IL-5, IL-10 or IFN- γ in OVA-stimulated MLN cell cultures from the different groups of mice could be observed (data not shown), and no IL-4 was detected in the BAL fluid or in any of the culture supernatants.

The examination of lung sections revealed that the application of live or hk-BCG again strongly reduced pulmonary inflammation, goblet cell metaplasia and mucous production in mice sensitized with OVA. In contrast, the application of CpG-ODN or PPD only led to a slight reduction in OVA-induced airway inflammation (Fig. 2b, A–F). Interestingly, mucous production and goblet cell metaplasia were reduced in CpG-ODN but not PPD-treated mice in comparison with OVA only sensitized animals (Fig. 2b, K, L) (mean scores of pulmonary inflammation/goblet cell metaplasia in the different groups: OVA/alum: 3/3, OVA + live BCG/alum: 1/0, OVA + hk-BCG/alum: 1/0, OVA + CpG/alum: 2/1, OVA + PPD/alum: 2/3).

Mice were also vaccinated s.c. with the different adjuvants together with OVA/alum to investigate if similar effects were observed in comparison with applying the vaccine i.p. We found that live BCG was the only adjuvant

Table 1. Suppression of airway eosinophilia after the application of the different adjuvants in the absence of either IL-12p35 or IFN- γ

		OVA	OVA/live BCG	OVA/hk-BCG	OVA/CpG	OVA/PPD
IL-12p35 ^{-/-} mice	Eos/mL BAL $\times 10^3$	207.0 $\pm$ 98.2	21.4 $\pm$ 13.3*	120.9 $\pm$ 48.2	123.6 $\pm$ 31.2	65.0 $\pm$ 26.8
IFN- γ ^{-/-} mice	Eos/mL BAL $\times 10^3$	277.7 $\pm$ 90.4	11.6 $\pm$ 8.9**	245.7 $\pm$ 86.2	533.8 $\pm$ 141.8	124.4 $\pm$ 39.2

IL-12p35- and IFN- γ -deficient mice (both on a C57BL/6 background) were treated as described in Fig. 1a. The absolute number of eosinophils present in the BAL fluid in the different groups in either IL-12p35^{-/-} or IFN- γ ^{-/-} mice are shown (mean $\pm$ SEM from 5–7 mice/group).

OVA, ovalbumin; hk, heat-killed; BAL, bronchoalveolar lavage.

* $P < 0.05$.

** $P < 0.01$ compared with values in mice only immunized with OVA.

Table 2. Allergen-induced immune responses in mice vaccinated with the different adjuvants and OVA and then subjected to a secondary sensitization with OVA/alum

	OVA	OVA/live BCG	OVA/hk-BCG	OVA/CpG	OVA/PPD
Eosinophils/mL BAL $\times 10^3$	449.3 $\pm$ 84.4	1.4 $\pm$ 0.3**	60.7 $\pm$ 15.4**	185.6 $\pm$ 43.4	274.7 $\pm$ 71.3
OVA-specific IgG1 ($\times 10^3$)	977.3 $\pm$ 246.5	402.8 $\pm$ 178.4	1046.0 $\pm$ 261.0	2845.0 $\pm$ 935.9	1275.0 $\pm$ 276.8
Antibodies (reciprocal titres)					
IgE	220.9 $\pm$ 39.2	317.0 $\pm$ 98.7	581.1 $\pm$ 114.1*	1566.0 $\pm$ 355.0**	325.8 $\pm$ 96.4
IgG2a	107.5 $\pm$ 41.8	10500.0 $\pm$ 6586.0**	4858.0 $\pm$ 1965.0**	10480.0 $\pm$ 4649.0**	152.3 $\pm$ 52.8
Cytokines BAL					
IL-5 (mU/mL)	239.5 $\pm$ 28.4	141.6 $\pm$ 13.2*	206.7 $\pm$ 19.1	227.1 $\pm$ 22.1	206.5 $\pm$ 22.7
IFN- γ (pg/mL)	264.7 $\pm$ 37.7	391.0 $\pm$ 89.8	435.8 $\pm$ 95.1	482.4 $\pm$ 111.8	328.1 $\pm$ 64.4

Mice were treated as described in Fig. 2a. Six days after the intranasal challenge with OVA mice were analysed as described in 'Methods'. Shown are the mean values of 18–23 mice/group $\pm$ SEM from three separate experiments.

OVA, ovalbumin; hk, heat-killed; BAL, bronchoalveolar lavage.

* $P < 0.05$,

** $P < 0.01$ compared with OVA only treated mice.

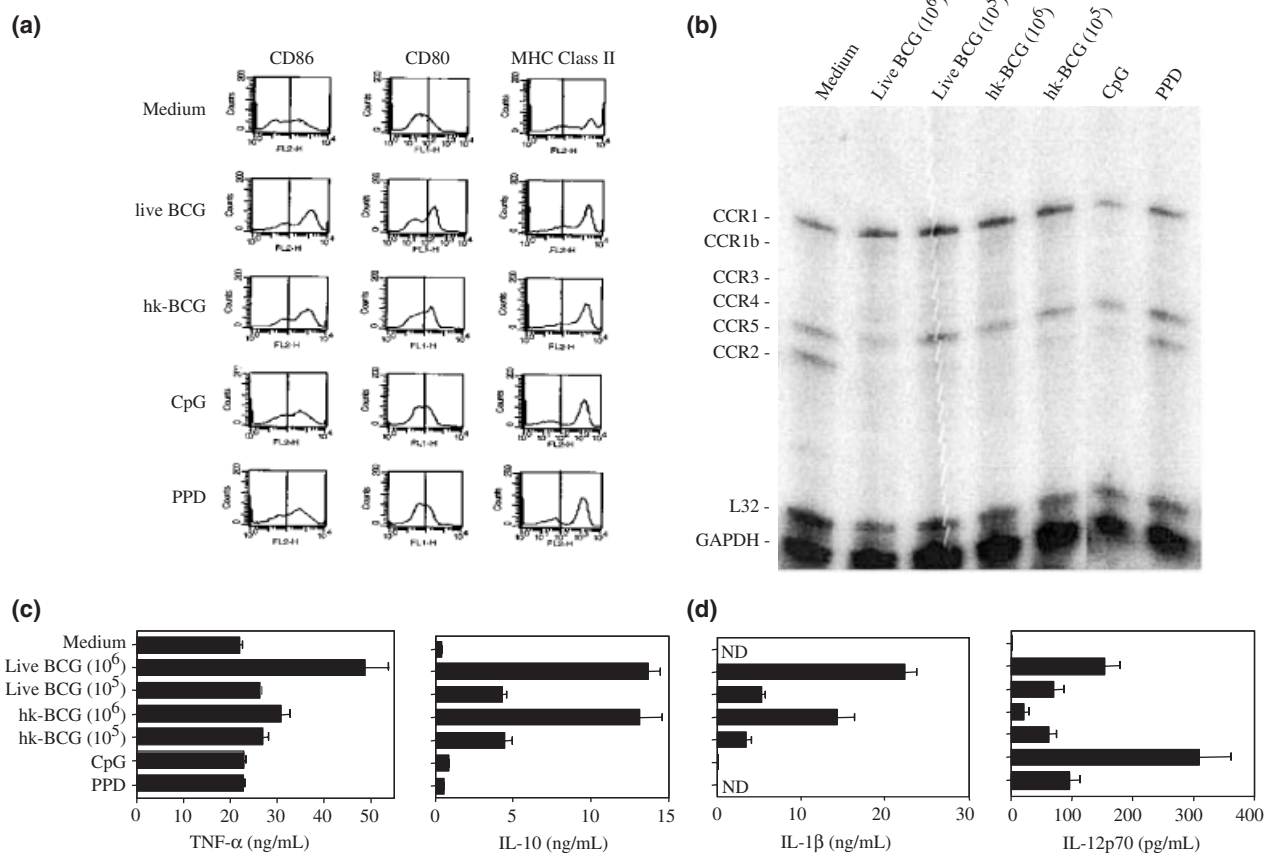


Fig. 6. Activation of bone marrow-derived dendritic cell (BM-DC) by live *Bacillus-Calmette-Guerin* (BCG), heat-killed-BCG, CpG-oligodeoxynucleotide and PPD. BM-DC were prepared and stimulated with the different adjuvants as described in 'Methods'. The surface expression levels of CD86, CD80 and MHC Class II (a), the RNA transcripts for different chemokine receptors (b) and the cytokine levels in the supernatants of BM-DC cultures after 24 h stimulation (c) are shown. The results are representative for two separate experiments.

able to significantly reduce airway eosinophilia when applied s.c. (OVA/alum: 501.6 $\pm$ 72.4; OVA + live BCG/alum: 208.0 $\pm$ 61.4*; OVA + hk-BCG/alum: 280.7 $\pm$ 41.4; OVA + CpG/alum: 654.4 $\pm$ 143.8; OVA + PPD/alum: 449.0 $\pm$ 77.2 eosinophils/mL BAL $\times 10^3$; mean of 9–15 mice/group from two separate experiments $\pm$ SEM; * $P < 0.05$ between OVA/alum and OVA + live BCG/alum-treated groups).

The adoptive transfer of total spleen cells or splenic CD4⁺ T cells from mice immunized with the allergen and live or heat-killed-BCG reduces allergen-induced pulmonary eosinophilia

Next we investigated whether the suppressive effects induced by the different adjuvants on the development of allergic Th2 responses were mediated by lymphocytes. For this purpose

single-cell suspensions were prepared from spleens of mice, which had been vaccinated with the different adjuvants or OVA/alum alone and subjected to a second round of OVA/alum sensitization (Fig. 2a). The isolated spleen cells were transferred i.v. into naïve mice as described in 'Methods'. One day after cell transfer, the mice were subjected to the OVA immunization protocol. As controls, naïve mice transferred with no cells were also included. Figure 3 shows that the adoptive transfer of spleen cells from mice injected either with live or hk-BCG but not CpG-ODN or PPD (together with OVA/alum) resulted in a significant reduction in the numbers of eosinophils in the BAL compared with the control mice transferred with spleen cells from OVA/alum only treated mice or mice transferred with no cells. OVA-specific IgG1 and IgE levels in the serum were not affected by the transfer of spleen cells from any of the different groups of mice. In contrast, the transfer of spleen cells from live BCG, hk-BCG or CpG-ODN but not PPD-immunized mice resulted in strongly enhanced OVA-specific IgG2a serum levels.

We repeated the experiment using purified CD4⁺ T cells from the spleen of live and hk-BCG-treated mice. Figure 3 shows that the results were very similar to the ones found when using total spleen cells suggesting that the application of live and hk-BCG results in the generation of CD4⁺ T cells which protect from the development of airway eosinophilia but not allergen-specific IgE. The greatly enhanced OVA-specific IgG2a levels detected in the protected mice indicate that the transferred cells contained allergen-specific Th1 cells.

Mice immunized with live BCG together with ovalbumin show increased numbers of neutrophils and macrophages in the airways after repetitive ovalbumin intranasal challenges

None of our experiments have provided any evidence that Th1-mediated inflammation occurred in the lungs of any of the mice vaccinated with the different Th1-inducing adjuvants. However, it is possible that after repetitive exposures to the allergen, allergen-specific Th1 cells may increase in numbers and lead to inflammation and tissue destruction in the airways. To test this hypothesis we immunized mice with OVA + live BCG/alum (the strongest Th2 response inhibitor) and subjected the animals to nine repetitive OVA airway challenges (Fig. 4a) and analysed if Th1 cell-mediated inflammation occurs. Figure 4b shows that the numbers of macrophages and neutrophils but not eosinophils increased in the BAL fluid of these mice. However, in contrast to the OVA only treated mice, these animals did not develop AHR or goblet cell metaplasia (Figs 4c and d) (mean scores of goblet cell metaplasia: PBS: 0, OVA/alum: 3, OVA + live BCG/alum: 0). Moreover, perivascular and peribronchial inflammation was still decreased when OVA/live BCG/alum was applied before repetitive i.n. challenges (mean scores of pulmonary inflammation: PBS: 0, OVA/alum: 3, OVA + live BCG/alum: 2). BCG needed to be alive to inhibit the development of AHR, as using hk-BCG instead of live BCG did not result in the suppression of AHR (data not shown).

Live BCG reduces airway inflammation but induces increasing levels of allergen-specific immunoglobulin E in animals previously sensitized with ovalbumin/alum.

We also evaluated the effects of live BCG on allergic responses using a therapeutic instead of a prophylactic protocol. For this purpose, mice were sensitized with OVA/alum and 10 days later, the application of live BCG together with OVA/alum i.p. was performed. Subsequently, the mice were subjected to the OVA intranasal challenge and allergic responses were evaluated 6 days later (Fig. 5a). Using this therapeutic protocol, live BCG was still able to reduce the eosinophilia and the inflammatory response in the airways (Fig. 5b). No variation in the levels of OVA-specific IgG1 in the serum were observed in comparison with mice injected only with OVA/alum, but interestingly, when live BCG was applied to previously OVA-sensitized mice, increased serum levels of OVA-specific IgE were detected (Fig. 5c). Similar to the results obtained using a prophylactic protocol (see Table 2), live BCG was still effective in increasing the serum levels of OVA-specific IgG2a using this therapeutic protocol (Fig. 5c).

Effects of the different adjuvants on the maturation and cytokine production of bone marrow-derived dendritic cell in vitro

Figure 6a shows that live BCG and hk-BCG were more effective than CpG-ODN or PPD in increasing the surface expression of the maturation markers CD80 and CD86 on BM-DC generated *in vitro*. Figure 6b shows that all the adjuvants except PPD induced the down-regulation of the mRNA expression levels of CCR5 and CCR2, indicative of BM-DC maturation [21]. However, live BCG was the only stimulus able to induce the secretion of high levels of TNF- α , IL-1 β and IL-12p70 by these cells. Live and hk-BCG but not CpG-ODN or PPD were also able to induce the production of IL-10 by BM-DC. CpG-ODN induced the secretion of large amounts of IL-12p70 (Fig. 6c).

Discussion

Numerous studies have shown that the induction of allergen-specific or unspecific Th1 responses is associated with the inhibition of allergic Th2 responses in the airways [1, 2, 7–15, 22, 23]. Several epidemiological studies indicate that this may also be true in humans [2, 10]. In order to induce Th1 responses different approaches have been used. The most effective ones include live or dead bacteria, bacterial products such as LPS, monophosphoryl lipid A, PPD or CpG-ODN and DNA vaccines [2, 7–15]. However, it is not clear which approach is the most efficient. The aim of our study was to compare the inhibitory effects on Th2 cell development of several known Th1 directing adjuvants as components of anti-allergy vaccines.

We found that all the different adjuvants tested (live BCG, hk-BCG, CpG and PPD) reduced the development of allergic inflammation in the airways including eosinophilia and mucous production when they were applied together with the allergen during the i.p. sensitization. The strongest effects were induced by live BCG and hk-BCG. The finding that live BCG also

reduced the development of allergen-specific IgG1 and IgE serum levels could be expected as this application was the most efficient in reducing Th2 responses in the lung. However, the result that the application of hk-BCG or CpG-ODN did not reduce the amounts of OVA-specific IgG1 or IgE in the serum was surprising, especially as in both cases increased OVA-specific IgG2a levels were found. Another unexpected finding was that the application of PPD decreased OVA-specific IgE levels in the serum. Currently we have no explanation how the addition of PPD to OVA/alum mediates the decrease in IgG1 and IgE production without affecting OVA-specific IgG2a levels in the serum. However, our observation is supported by Hattori et al. [15] who also found that the application of PPD reduced the induction of *Schistosoma mansoni* egg antigen-specific IgG1 and IgE titres in mice.

In addition, our findings indicate that the inhibitory effects on Th2 responses were dependent on the induction of Th1 responses. This hypothesis was confirmed by repeating the experiments in IFN- γ and IL-12 p35-deficient mice. Taken together, the strong reduction in airway eosinophilia observed in mice immunized with OVA together with live BCG, hk-BCG or CpG-ODN may be explained by a reduction in Th2 cell development induced by Th1 cells. Interestingly, live BCG was still able to reduce Th2 responses in the lung independent of IFN- γ or IL-12, albeit to a lower degree than observed in wild-type mice. In addition, we also found that the suppressive effect of live BCG on allergen-induced airway eosinophilia was still observed in IL-12p35/IL-12p40-double deficient and IL-10-deficient mice. These data indicate that IFN- γ , IL-12, IL-23 and IL-10 are not solely responsible for live BCG-induced suppression of Th2 responses. Taking into account the proposed role of type I interferons in down-modulating allergen-induced Th2 responses [24] and considering that mycobacteria or mycobacterial components have been shown to induce this type of interferons [25, 26], it could be argued that the suppressive effect of live BCG on the development of OVA-induced Th2 responses we observed in our experiments might be because of the induction of IFN- α/β by these bacteria. However, we could not detect IFN- α either in peritoneal lavages after 4 or 16 h of the application of OVA + live BCG/alum i.p. or in the BAL or MLN-cell cultures after vaccination with OVA + live BCG/alum and challenge with OVA (data not shown). These findings indicate that IFN- α may not play a role in the BCG-mediated inhibition of allergen-specific Th2 responses. We are currently further investigating the mechanism of the Th1-independent suppression of allergic responses induced by live BCG.

Our results shown above suggest that inducing allergen-specific Th1 responses by the application of the different adjuvants may be useful for the development of an anti-asthma vaccine. However, an efficient vaccine should not only protect from Th2 cell development during the application of the vaccine but most importantly also after vaccination when the individual is re-exposed to the allergen. We found that only the application of live or hk-BCG but not CpG-ODN or PPD was able to reduce allergen-induced Th2 responses in the lung after a second round of OVA/alum sensitization. This effect was very long lasting, as airway eosinophilia was still significantly reduced when the second round of OVA sensitization started 12 weeks after the vaccination (data not shown). Furthermore, suppression of

airway eosinophilia could be transferred to naïve mice by CD4⁺ T cells purified from the spleen of immunized mice, clearly demonstrating that the anti-allergic effect of live BCG and hk-BCG was induced by this subpopulation of cells. We recently reported that infection with influenza A virus reduces the development of airway eosinophilia by interfering with the recruitment of Th2 cells into the airways and this effect was associated with increased levels of IFN- γ and IFN- γ producing cells in the airways [9]. It is tempting to speculate that the suppression of allergen-induced airway eosinophilia and mucous production observed in live and hk-BCG vaccinated mice may be because of the presence of Th1 cells secreting IFN- γ in the lung, which results in the inefficient recruitment of allergen-specific Th2 cells into the airways. Supporting this view was our finding that vaccination with OVA + live BCG/alum prior to the application of *in vitro* generated OVA-specific Th2 cells i.v. reduced the recruitment of these cells into the airways in comparison with OVA/alum only immunized mice (data not shown).

The results of our experiments show that mice are efficiently protected from developing airway inflammation and AHR when live BCG is used as the vaccine adjuvant. This suggests that an anti-asthma vaccine using live BCG may also be effective in humans. However, our data also indicate that the protective effects are associated and most likely mediated to a large extent by Th1 cells. As Th1 cells are powerful mediators of inflammation, it is important to rule out that a vaccine aimed at protecting humans from asthma utilizing allergen-specific Th1 responses does not lead to pulmonary Th1 cell-mediated inflammation and tissue destruction. None of our experiments have provided any evidence that this occurs in mice vaccinated with different Th1 inducing adjuvants and i.n. challenged once with OVA. However, when mice were vaccinated with OVA + live BCG/alum and then repetitively exposed to OVA i.n. increased amounts of macrophages and neutrophils were detected in the airways. This indicates that a vaccine inducing Th1 responses may lead to allergen-induced Th1 inflammation in the airways. Supporting this view are our own results demonstrating that live BCG induce the secretion of increasing amounts of TNF- α and IL-1 β , two important proinflammatory cytokines, by BM-DC *in vitro*. In addition, we have also evaluated a therapeutic protocol using live BCG where sensitization with OVA/alum i.p. is followed by two i.p. injections of OVA + live BCG/alum and the intranasal OVA challenge. Interestingly, by using this approach, live BCG was still able to significantly decrease the airway eosinophilia in comparison with the application of only OVA/alum, but a significant increase in the serum levels of OVA-specific IgE was observed. Although our interest within this work was more directed to prophylactic approaches, these data demonstrate that the effects of BCG on the allergic Th2 responses depend on the protocol used.

On the other hand, the finding that live and hk-BCG were much more effective than CpG-ODN in inhibiting allergen-induced Th2 responses as demonstrated here was surprising. However, we used the same CpG-ODN that has previously shown to be efficient in inhibiting allergen-induced Th2 responses [12, 27]. Furthermore, the CpG-ODN used in our studies induced the maturation of BM-DC and the secretion of IL-12 by these cells *in vitro*. Based on these observations it is very unlikely that our results are due to a lack of function

on the part of the used CpG-ODN. The enhanced OVA-specific IgG2a serum levels detected in the CpG-ODN-treated mice in comparison with the live BCG and hk-BCG-treated mice support this view. It is possible that using higher amounts of CpG-ODN or applying them more frequently may yield different results. However, the frequent application of CpG-ODN may lead to unwanted side-effects, as recently reported by Heikenwalder et al. [28]. These authors observed immunotoxic and hepatotoxic effects of the daily treatment with CpG-ODN in mice.

In conclusion, our data show that all the tested Th1-inducing adjuvants had suppressive effects on the development of allergen-induced Th2 responses although live BCG was the product with the most potent anti-allergy properties. Moreover, live BCG was the only adjuvant able to suppress OVA-induced airway eosinophilia when applied s.c. Interestingly, clinical therapeutic trials have shown that the percutaneous application of live BCG could improve symptoms in patients with moderate-to-severe asthma [29]. These positive effects were not observed when adults with allergic asthma were treated with hk-BCG on which, conversely, excessive local reactions were present [30]. However, a prophylactic approach using these products has still not been evaluated in humans [31]. Controlled studies are necessary in order to definitely elucidate whether live BCG could be a promising adjuvant to prevent allergic responses in humans and whether strong proinflammatory side-effects because of the application of this product could be controlled or ruled out. In this context, the findings of our study contribute to the design of efficient and safe vaccines protecting humans from developing allergic disorders in the future.

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References

- Maddox L, Schwartz DA. The pathophysiology of asthma. *Annu Rev Med* 2002; 53:477–98.
- Erb KJ, Wohlleben G. Novel vaccines protecting against the development of allergic disorders: a double-edged sword? *Curr Opin Immunol* 2002; 14:633–43.
- Yazdanbakhsh M, Kremsner PG, van Ree R. Allergy, parasites, and the hygiene hypothesis. *Science* 2002; 296:490–4.
- Zuany-Amorim C, Sawicka E, Manlius C et al. Suppression of airway eosinophilia by killed *Mycobacterium vaccae*-induced allergen-specific regulatory T-cells. *Nat Med* 2002; 8:625–9.
- Umetsu DT, Akbari O, DeKruyff RH. Regulatory T cells control the development of allergic disease and asthma. *J Allergy Clin Immunol* 2003; 112:480–7.
- O'Garra A. Cytokines induce the development of functionally heterogeneous T helper cell subsets. *Immunity* 1998; 8:275–83.
- Erb KJ, Holloway JW, Soback A, Moll H, Le Gros G. Infection of mice with *Mycobacterium bovis*-Bacillus Calmette-Guerin (BCG) suppresses allergen-induced airway eosinophilia. *J Exp Med* 1998; 187:561–9.
- Hansen G, Yeung VP, Berry G, Umetsu DT, DeKruyff RH. Vaccination with heat-killed *Listeria* as adjuvant reverses established allergen-induced airway hyperreactivity and inflammation: role of CD8+ T cells and IL-18. *J Immunol* 2000; 164:223–30.
- Wohlleben G, Muller J, Tatsch U et al. Influenza A virus infection inhibits the efficient recruitment of Th2 cells into the airways and the development of airway eosinophilia. *J Immunol* 2003; 170:4601–11.
- Trujillo C, Erb KJ. Inhibition of allergic disorders by infection with bacteria or the exposure to bacterial products. *Int J Med Microbiol* 2003; 293:123–31.
- Wohlleben G, Erb KJ. Atopic disorders: a vaccine around the corner? *Trends Immunol* 2001; 22:618–26.
- Jain VV, Kitagaki K, Businga T et al. CpG-oligodeoxynucleotides inhibit airway remodeling in a murine model of chronic asthma. *J Allergy Clin Immunol* 2002; 110:867–72.
- Smit JJ, Loveren HV, Hoekstra MO, Schijf MA, Folkerts G, Nijkamp FP. *Mycobacterium vaccae* administration during allergen sensitization or challenge suppresses asthmatic features. *Clin Exp Allergy* 2003; 33:1083–9.
- Li XM, Srivastava K, Huleatt JW, Bottomly K, Burks AW, Sampson HA. Engineered recombinant peanut protein and heat-killed *Listeria monocytogenes* coadministration protects against peanut-induced anaphylaxis in a murine model. *J Immunol* 2003; 170:3289–95.
- Hattori H, Okano M, Yamamoto T et al. Intranasal application of purified protein derivative suppresses the initiation but not the exacerbation of allergic rhinitis in mice. *Clin Exp Allergy* 2002; 32:951–9.
- Grunewald SM, Hahn C, Wohlleben G et al. Infection with influenza A virus leads to flu antigen-induced cutaneous anaphylaxis in mice. *J Invest Dermatol* 2002; 118:645–51.
- Hamelmann E, Schwarze J, Takeda K et al. Noninvasive measurement of airway responsiveness in allergic mice using barometric plethysmography. *Am J Respir Crit Care Med* 1997; 156:766–75.
- Lutz MB, Kukutsch N, Ogilvie ALJ et al. An advanced culture method for generating large quantities of highly pure dendritic cells from mouse bone marrow. *J Immunol Methods* 1999; 223:77–92.
- Kline JN, Krieg AM, Waldschmidt TJ, Ballas ZK, Jain V, Businga TR. CpG oligodeoxynucleotides do not require TH1 cytokines to prevent eosinophilic airway inflammation in a murine model of asthma. *J Allergy Clin Immunol* 1999; 104:1258–64.
- Brombacher F, Kastelein RA, Alber G. Novel IL-12 family members shed light on the orchestration of Th1 responses. *Trends Immunol* 2003; 24:207–12.
- Caux C, Ait-Yahia S, Chemin K et al. Dendritic cell biology and regulation of dendritic cell trafficking by chemokines. *Springer Semin Immunopathol* 2000; 22:345–69.
- Cohn L, Homer RJ, Niu N, Bottomly K. T helper 1 cells and interferon gamma regulate allergic airway inflammation and mucus production. *J Exp Med* 1999; 190:1309–18.
- Huang TJ, MacAry PA, Eynott P et al. Allergen-specific Th1 cells counteract efferent Th2 cell-dependent bronchial hyperresponsiveness and eosinophilic inflammation partly via IFN-gamma. *J Immunol* 2001; 166:207–17.
- Tilg H, Kaser A. Type I interferons and their therapeutic role in Th2-regulated inflammatory disorders. *Expert Opin Biol Ther* 2004; 4:469–81.
- Nakane A, Minigawa T. Induction of alpha and beta interferons during the hyporeactive state of gamma interferon by *Mycobacterium bovis* BCG cell wall fraction in *Mycobacterium bovis* BCG-sensitized mice. *Infect Immun* 1982; 36:966–70.
- Adams VC, Hunt JR, Martinelli R, Palmer R, Rook GA, Brunet LR. *Mycobacterium vaccae* induces a population of pulmonary CD11+ cells with regulatory potential in allergic mice. *Eur J Immunol* 2004; 34:631–8.

- 27 Jain VV, Businga TR, Kitagaki K, George CL, O'Shaughnessy PT, Kline JN. Mucosal immunotherapy with CpG oligodeoxynucleotides reverses a murine model of chronic asthma induced by repeated antigen exposure. *Am J Physiol Lung Cell Mol Physiol* 2003; 285:L1137–46.
- 28 Heikenwalder M, Polymenidou M, Junt T et al. Lymphoid follicle destruction and immunosuppression after repeated CpG oligodeoxynucleotide administration. *Nat Med* 2004; 10: 187–92.
- 29 Choi IS, Koh YI. Therapeutic effects of BCG vaccination in adult asthmatic patients: a randomized, controlled trial. *Ann Allergy Asthma Immunol* 2002; 88:584–91.
- 30 Shirtcliffe PM, Easthope SE, Weatherall M, Beasley R. Effect of repeated intradermal injections of heat-inactivated *Mycobacterium bovis* Bacillus Calmette–Guérin in adult asthma. *Clin Exp Allergy* 2004; 34:207–12.
- 31 Renz H. Usefulness of mycobacteria in redirecting the immune response in atopic disease. *Clin Exp Allergy* 2004; 34:167–69.