

Mice Vaccinated with Allergen-Pulsed Myeloid Dendritic Cells Are Not Protected from Developing Allergen-Induced Th2 Responses

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Key Words

Atopic asthma · Dendritic cells · Th2 immune responses · Vaccination

Abstract

Background: Dendritic cells (DC) play a decisive role in the induction of allergen-induced Th1 and Th2 responses. Since the induction of allergen-specific Th1 responses has shown to inhibit allergen-induced Th2-type inflammation, in this study we investigated whether manipulated myeloid-derived DC pulsed with the specific allergen would predominantly induce allergen-specific Th1 responses thereby reducing the development of Th2 responses. **Methods:** Murine bone marrow (BM)-DC were generated and pulsed with ovalbumin (OVA) and CpG oligodeoxynucleotides (CpG-ODN). Langerhans cells (LC) were also isolated and pulsed in vitro with OVA. Subsequently, mice were vaccinated intravenous-

ly with either CpG/OVA-pulsed BM-DC or OVA-pulsed LC, and the protocol to induce OVA-specific Th2 responses using OVA/alum sensitization was initiated. Airway inflammation and OVA-specific serum antibody levels were evaluated 6 days after the intranasal challenge with OVA. **Results:** The application of CpG/OVA-pulsed BM-DC was unable to reduce airway eosinophilia and inflammation in OVA/alum-immunized mice. OVA-specific IgG1 or IgE serum levels were also not reduced. The experiments using LC pulsed with OVA yielded similar results. However, mice vaccinated with CpG/OVA-pulsed BM-DC had greatly enhanced levels of serum OVA-specific IgG2a, suggesting the induction of allergen-specific Th1 responses in vivo. Moreover, allergen-induced mast cell degranulation was decreased using this approach. **Conclusions:** Taken together, our results demonstrated that the vaccination with OVA-pulsed BM-DC matured with CpG-ODN or OVA-pulsed LC did not result in a reduction in allergen-specific Th2 responses in a murine model of severe atopic asthma. Other DC-based vaccination strategies should be evaluated in order to prevent the development of allergic disorders.

Supported by the 'Bundesministerium für Bildung und Forschung', the 'Bayerisches Staatsministerium für Wissenschaft, Forschung und Kunst' and the 'Bayerische Forschungsförderung-Forimmun'.

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Introduction

Atopic asthma is a chronic inflammatory disorder in which allergen-sensitized individuals develop reversible airway obstruction and airway hyperreactivity. Its incidence has steadily increased in the last decades, reaching epidemic proportions. The disease is strongly associated with the development of allergen-specific CD4⁺ Th2 cells which are known to influence a variety of processes such as the IgE synthesis by B lymphocytes, the growth and differentiation of eosinophils and the development of bronchial hyperreactivity and goblet cell metaplasia through the production of IL-4, IL-5, IL-9 and IL-13 [1].

However, to recognize the allergen peptides and become differentiated into Th2 lymphocytes, CD4⁺ T cells need the help of antigen-presenting cells. Although dendritic cells (DC) as well as B cells and macrophages are all professional antigen-presenting cells, only DC have the capacity to activate naïve T lymphocytes. In the respiratory tract, an extensive network of DC is located in the mucosa of the nose and the large conducting airways of the lung. In these places, DC have the capacity to capture the allergen, and then they become mature and migrate to the draining lymph nodes where the presentation of the allergen to naïve CD4⁺ T cells (in the context of MHC class II molecules) is initiated. Here, DC provide T cells with essential costimulatory molecules and cytokines which finally determine (in the presence of IL-4) the induction of allergen-specific Th2 responses [2].

The critical contribution of DC in the pathogenesis of atopic asthma was suggested by the increased presence of these cells in the airways of atopic asthmatics and allergen-exposed animals [2]. Later, it was demonstrated that the depletion of airway DC during secondary exposure to the inhaled allergen resulted in the complete disappearance of allergen-induced eosinophilic airway inflammation and goblet cell metaplasia [3]. Furthermore, the application of allergen-pulsed myeloid DCs into the airways was also able to induce airway allergic inflammation after challenge with the specific allergen [4–6].

Based on these findings, it is predicted that by altering the properties of DC priming naïve allergen-specific CD4⁺ T cells it may be possible to prevent the development of asthma. Animals vaccinated with DC pulsed with specific antigens have been shown to be protected against infectious diseases or cancer [7]. More recently, bone marrow-derived DC (BM-DC) matured with CpG-ODN in the presence of *Leishmania major* lysate were shown to be able to reduce the footpad swelling and par-

asitic load in susceptible mice infected with *L. major*. The protection was mediated by the induction of specific Th1 responses [8].

Since the induction of allergen-specific Th1 responses was shown to prevent allergic asthma in several animal models [9], we investigated if DC pulsed with allergen and intravenously applied into mice induce allergen-specific Th1 responses thereby suppressing the development of allergen-specific Th2 responses in a prophylactic setting. Here we showed that in both BALB/c and C57BL/6 mice the application of murine bone marrow (BM)-DC pulsed with CpG/OVA was unable to reduce OVA/alum-induced allergic Th2 responses. Similar results were observed in experiments using Langerhans cells (LC) pulsed with OVA. Other DC-based vaccination strategies should be evaluated in order to prevent the development of severe atopic asthma.

Methods

Mice

Female BALB/c or C57BL/6 mice were purchased from Charles River (Sulzfeld, Germany) and maintained under conventional conditions in an isolation facility. 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.

Generation and Analysis of BM-DC

BM-DC were generated from BM of BALB/c or C57BL/6 mice in the presence of high doses of GM-CSF as previously described [10]. Briefly, freshly prepared BM cells were cultured in Click RPMI 1640 medium (Biochrom, Berlin, Germany) supplemented with 10% heat-inactivated FCS (PAA Laboratories, Linz, Austria), 2 mM *L*-glutamine, 10 mM HEPES buffer, 60 µg/ml penicillin and 20 µg/ml gentamicin in the presence of 200 U/ml GM-CSF (Pepro-Tech, London, UK). Cultures were fed with GM-CSF on days 3, 6 and 8. After 10 days, nonadherent cells were counted, resuspended at 1×10^6 cells/ml and pulsed on 24-well plates (Nunc, Roskilde, Denmark) for 18 h with either 25 µg/ml CpG (CpG-ODN 1668, 5'-TCCATGACGTTCTGATGCT-3' obtained from Qiagen Operon, Cologne, Germany) or 1 µg/ml lipopolysaccharide (LPS; Sigma, St. Louis, Mo., USA). BM-DC which were not treated with either CpG or LPS and resuspended only with supplemented RPMI medium were used as controls. The culture supernatants were harvested and tested for the presence of cytokines by ELISA. The expression levels of CD40, CD86, CD80 and MHC class II on the surface of pulsed DC were determined by FACS staining using the following mAbs (Pharmingen, San Diego, Calif., USA): FITC anti-mouse CD40 (HM40-3), PE anti-mouse CD86 (GL1), FITC anti-mouse CD80 (16-10A1) and PE anti-mouse I-Ab (AF6-120.1). Isotype-matched mAbs were used to control unspecific staining. Fluorescence intensity was measured using a FACSCalibur flow cytometer (Beckton Dickinson, San Jose, Calif., USA). RNA from pulsed BM-DC was obtained using the Rneasy Mini Kit from Qia-

gen (Hilden, Germany). mRNA from cytokines and chemokine receptors was detected by RNase Protection Assay (Pharmingen) following the manufacturer's instructions.

Treatment of Mice and Induction of OVA-Specific Th2 Responses

For all our experimental protocols, BM-DC (5×10^5) pulsed with 25 $\mu\text{g/ml}$ CpG and/or 100 $\mu\text{g/ml}$ OVA (Sigma) were injected intravenously into the tail vein of naïve mice on day -7. First, we evaluated the intrinsic ability of BM-DC to induce allergic airway inflammation in mice by applying unpulsed BM-DC (cultured without OVA and CpG) and OVA- or CpG/OVA-pulsed BM-DC. On days 0 and 1, mice were anesthetized by an intraperitoneal injection of ketamine/xylazine (Sigma) and challenged intranasally with 50 μl PBS containing 100 μg OVA. Analyses were performed 6 days after the last intranasal challenge. In another set of experiments, DC-vaccinated mice were treated twice with a mixture of 2 μg OVA i.p. (Sigma) in 200 μl alum adjuvant (Serva, Heidelberg, Germany) on days 0 and 14 in order to induce severe allergic responses. The intranasal challenge with OVA in PBS was applied on day 24 and analyses were performed on day 30.

Bronchoalveolar Lavage

At the indicated time points, mice were sacrificed, blood samples collected, the trachea cannulated and a bronchoalveolar lavage (BAL) performed by flushing the lung and airways four times with 1 ml PBS containing 10% heat-inactivated FCS. BAL cells were counted, spun onto glass slides using a cytospin (Shandon Southern Products, Asmoor, UK) and stained with Diff-Quick (Dade Behring, Marburg, Germany) according to the manufacturer's instructions. Numbers of eosinophils, neutrophils, lymphocytes and macrophages present in the BAL were determined microscopically using standard histological criteria.

Culture Condition of Cells

Single-cell suspensions from the mediastinal lymph nodes (MLN) of the different groups of mice were prepared by teasing the MLN through a steel mesh and discarding the cell debris. Total MLN cells were counted and resuspended at 2×10^6 cells/ml in RPMI medium (Sigma) supplemented with sodium bicarbonate (3.024 g/l), 10 $\mu\text{g/ml}$ streptomycin, 10 U/ml penicillin, 50 μM 2-mercaptoethanol and 10% heat-inactivated FCS. The cell preparations were added to cell culture plates (2×10^6 cells/ml) and left in medium alone, stimulated with 40 $\mu\text{g/ml}$ OVA or with a plate-bound mAb to CD3 (145-2C11, 25 $\mu\text{g/ml}$) together with 200 U/ml recombinant human 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.

Detection of Immunoglobulins and Cytokines by ELISA

For the detection of OVA-specific immunoglobulin levels or cytokines in the BAL fluid and cell culture supernatants, sandwich ELISAs were performed as previously described [11]. Serum titers were expressed as the reciprocal value of the serum dilutions, which were 2-fold over the background optical density of serum from age-matched noninfected mice measured at 1/10 dilution (optical density <0.1). For quantification of the cytokines, titration was performed using murine recombinant cytokines (Pharmingen).

Preparation and Antigen Pulsing of LC

Epidermal LC suspensions were prepared from mouse ear skin by trypsinization procedures as previously described [12]. Briefly, the ventral, thick ear halves were incubated in a solution of 1% trypsin in PBS for 90 min, and the dorsal, thin ear halves were treated with 0.6% trypsin in PBS for 45 min. These preparations contained ~3–5% of LC that constitutively express MHC class II as well as MHC class II-negative keratinocytes, a source of GM-CSF that is essential for LC differentiation. The proportion of LC was determined by fluorescence labeling of MHC class II-positive cells. The preparations were completely devoid of macrophages, as documented by the lack of staining with the fluorescence-labeled mAb F4/80. For antigen pulsing, epidermal cell suspensions were cultured for 24 h in Click RPMI 1640 medium (Biochrom), supplemented with 10% heat-inactivated FCS, 2 mM L-glutamine, 10 mM HEPES buffer, 60 $\mu\text{g/ml}$ penicillin and 20 $\mu\text{g/ml}$ gentamicin, in the presence of 25 $\mu\text{g/ml}$ OVA. Control cultures were set up in the absence of OVA (unpulsed LC). Thereafter, the cells were washed, resuspended in PBS and injected intravenously into the tail of naïve mice (3×10^5 cells/mouse). In these mice, the protocol to induce OVA-specific Th2 responses was initiated 7 days later.

Active Cutaneous Anaphylaxis

Active cutaneous anaphylaxis was tested in the different groups of mice 6 days after the intranasal OVA challenge as described previously [13]. Briefly, 200 μl of Evan's blue were intravenously applied to the mice followed by subcutaneous injection of 50 μl PBS containing 10, 1 or 0.1 $\mu\text{g/ml}$ OVA. Mice were sacrificed 10 min later, and the degree of bluing as an indicator of the intensity of mast cell degranulation was scored by two independent observers (0 = no bluing, 1 = slight bluing, 2 = moderate bluing and 3 = strong bluing).

Statistical Analysis

Results were evaluated using Student's *t* test in order to establish differences between groups.

Results

CpG Induces Maturation and Production of Cytokines by BM-DC in vitro

CpG-ODNs have previously been described as potent maturation factors for DC through the ligation with a Toll-like receptor (TLR-9) [14]. In order to determine if the CpG-ODN (CpG 1668) we selected for our studies had the desired effects on DC, we generated BM-DC and pulsed them with CpG 1668, and then analyzed the maturation and the cytokine production by these BM-DC. As previously reported, CpG was able to upregulate the expression levels of the costimulatory molecules CD40, CD86 and CD80 and induce an increase in the levels of MHC class II on the surface of BM-DC to levels comparable to those induced by LPS (fig. 1A). BM-DC pulsed with CpG also resulted in increased levels of IL-10, IL-6, IL-1 β and IL-12p70 in comparison to unpulsed BM-DC

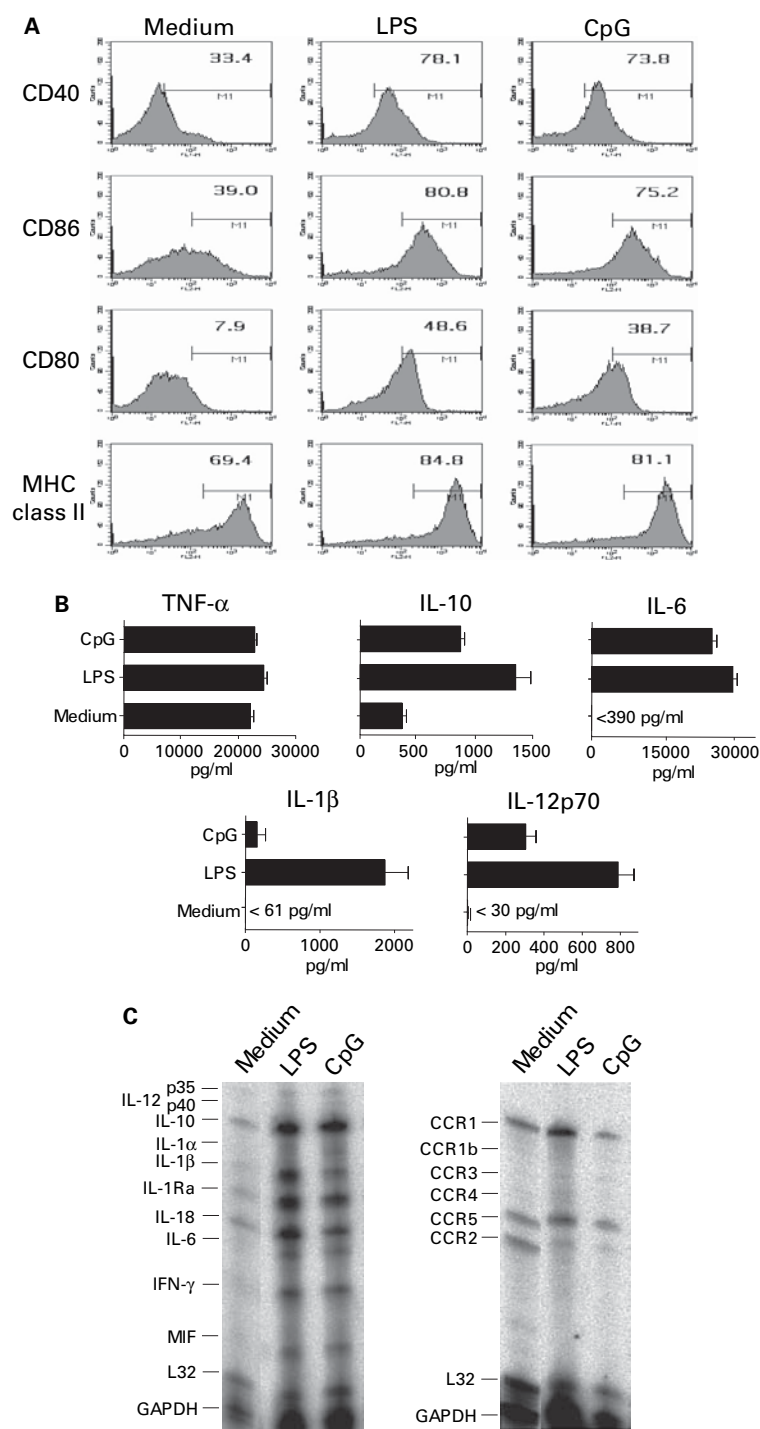
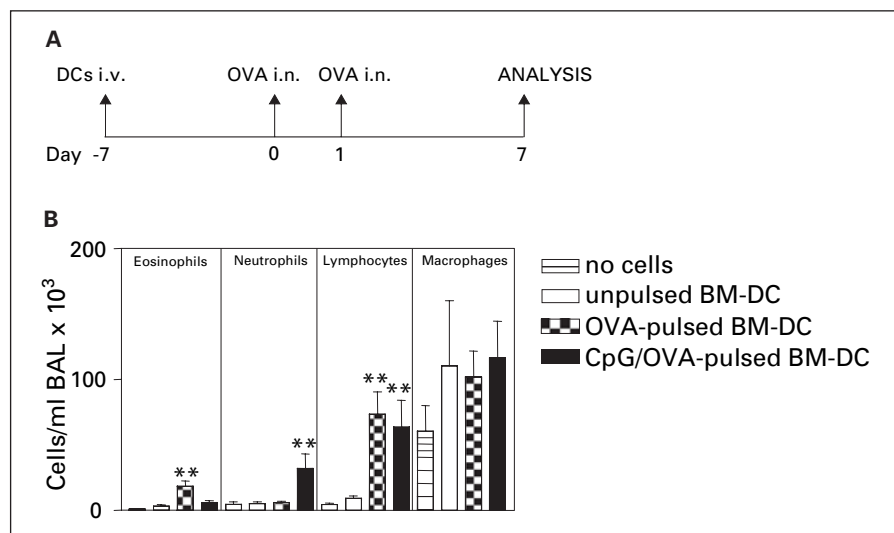


Fig. 1. Activation of BM-DC by CpG-ODN induces maturation and cytokine production. BM-DC were prepared and stimulated with either LPS or CpG as described. The surface expression levels of CD40, CD86, CD80 and MHC class II (**A**), the cytokine levels in the supernatants of BM-DC after 18-hour stimulation (**B**) and the RNA transcripts of different cytokines and chemokine receptors (**C**) of BM-DC pulsed with CpG in comparison with those in either LPS-pulsed BM-DC or BM-DC cultured in the absence of either CpG or LPS (Medium) are shown. The results are representative of two separate experiments.

Fig. 2. Priming with CpG/OVA-pulsed BM-DC is not able to induce eosinophilia but increases instead the influx of neutrophils in the airways upon allergen rechallenge. Unpulsed-BM-DC (cultured in the absence of CpG and OVA), OVA-pulsed or CpG/OVA-pulsed BM-DC were intravenously given to BALB/c mice 1 week before the intranasal OVA challenge (A). The numbers of the different cell types present in the BAL fluid (B) 6 days after the last intranasal application of OVA are indicated. Mean values of 6 mice/group $\pm$ SEM are given. ** $p < 0.01$ compared to the values obtained from mice immunized with either no cells or unpulsed BM-DC.



(fig. 1B). These results were confirmed by RNase protection assays as shown in figure 1C. We also analyzed if CpG-ODNs were able to downmodulate the mRNA expression levels of the chemokine receptors CCR5 and CCR2. Figure 1C shows that CpG-ODN as well as LPS induced the downregulation of these two chemokine receptors, indicative of BM-DC maturation. Thus, the CpG-ODN used in our experiments has the expected and desired effects on immature BM-DC in vitro.

Application of OVA-Pulsed BM-DC Leads to Airway Eosinophilia whereas CpG/OVA-Pulsed BM-DC Induce Airway Neutrophilia upon Allergen Rechallenge

The main goal of our study was to design a DC-based vaccination strategy leading to the induction of allergen-specific Th1 cells which then in turn protect mice from developing allergen-specific Th2 responses. However, several authors have reported that the application of allergen-loaded DC is sufficient to induce eosinophilic airway inflammation upon allergen rechallenge [4–6]. To evaluate the effects of our BM-DC in vivo, we injected mice with either unpulsed BM-DC, or OVA-pulsed or CpG/OVA-pulsed BM-DC and then we applied OVA intranasally as shown in figure 2A. Six days after the last intranasal OVA challenge, BALs were performed and the absolute number of the different cell types was determined. The application of unpulsed BM-DC induced no changes in the cellular composition of the BAL in comparison to the application of no cells (fig. 2B). However, when mice were intravenously injected with OVA-pulsed

BM-DC, a significant increase in the number of eosinophils in the airways upon intranasal challenge was observed. Interestingly, the application of CpG/OVA-pulsed BM-DC did not lead to eosinophilic inflammation but induced instead a significant influx of neutrophils in the airways. Both treatments, i.e. the application of OVA- or CpG/OVA-pulsed BM-DC, also induced a significant increase in the number of lymphocytes in the airways in comparison to the application of either no cells or unpulsed BM-DC (fig. 2B). These results demonstrate that our BM-DC are also functional in vivo and confirm previous reports showing that allergen-pulsed DC are able to induce airway eosinophilia after intranasal allergen rechallenge [4–6]. In addition, the induction of airway neutrophilia by using CpG/OVA-pulsed BM-DC indicates that upon allergen rechallenge, this treatment modulates DC function leading to the induction of proinflammatory responses in the airways.

Vaccination with BM-DC Pulsed with CpG/OVA Does Not Lead to the Suppression of OVA-Induced Th2 Responses in vivo

The above-shown results clearly demonstrate that CpG/OVA-pulsed BM-DC do not induce airway eosinophilia. Furthermore, BM-DC activated by CpG DNA ex vivo in the presence of the specific antigen have recently shown to be potent inducers of antigen-specific Th1 responses conferring protection from infection with *L. major* [8]. For these reasons we analyzed whether the vaccination of mice with CpG/OVA-pulsed BM-DC protects from allergen-specific Th2 responses after sensitization

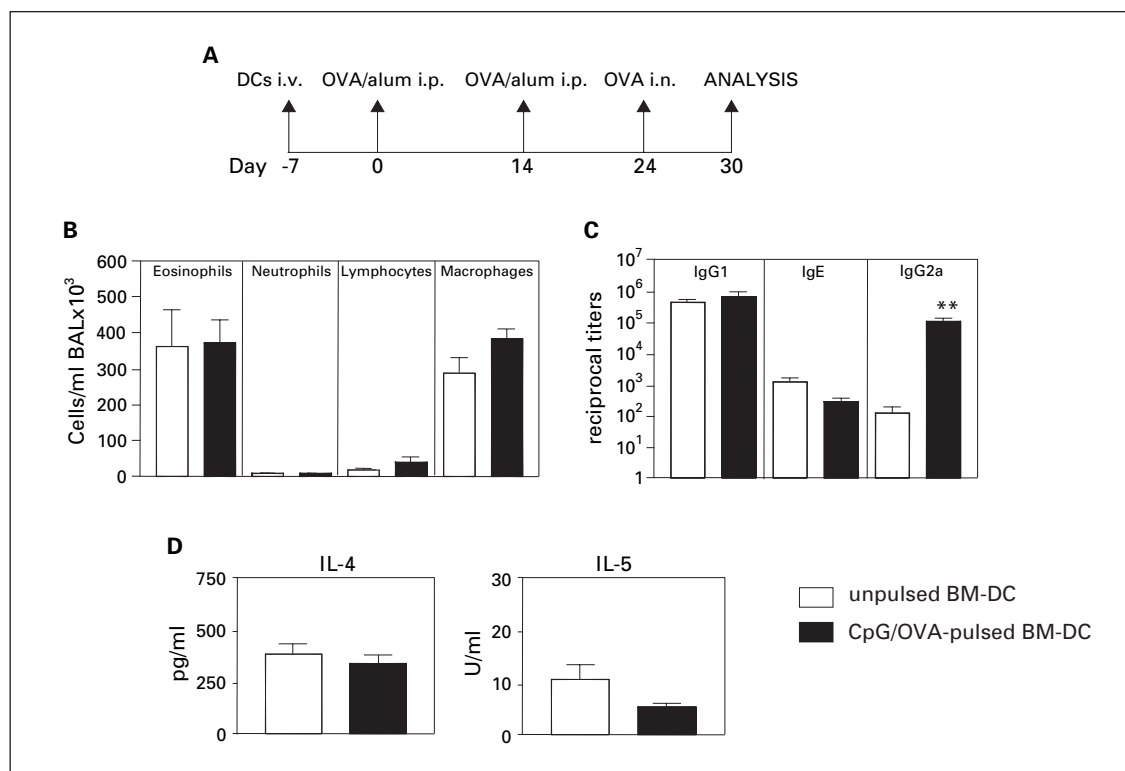


Fig. 3. CpG/OVA-pulsed BM-DC do not decrease OVA-induced Th2 responses in vivo. BALB/c mice were intravenously vaccinated with BM-DC pulsed with CpG/OVA 1 week before the initiation of the OVA/alum sensitization period (**A**). The numbers of the different cell types present in the BAL fluid (**B**), the serum OVA-specific antibody levels (**C**) and the IL-4 and IL-5 levels detected in the supernatants of MLN cell cultures stimulated with anti-CD3/IL-2 (**D**) from mice injected with CpG/OVA-pulsed BM-DC were compared with those in mice injected with unpulsed BM-DC. Mean values of 5–7 mice/group $\pm$ SEM are shown. The experiments were repeated once with similar results. ** $p < 0.01$ compared to the values obtained from mice immunized with unpulsed BM-DC.

with OVA/alum. Surprisingly, the application of BM-DC pulsed ex vivo with CpG/OVA 1 week before the initiation of the protocol to induce OVA-specific allergic Th2 responses in BALB/c mice was unable to reduce the eosinophilia and the inflammatory response in the airways in comparison to the application of either unpulsed BM-DC (fig. 3A, B), or OVA-only pulsed or CpG-only pulsed BM-DC (data not shown). The levels of OVA-specific IgG1 and IgE in the serum were also not modified by the application of CpG/OVA-pulsed BM-DC (fig. 3C). However, using this approach, the production of OVA-specific IgG2a was greatly increased (fig. 3C). Since the production of IgG2a is dependent on IFN- γ , this result suggested that the applied BM-DC were functional in vivo and induced OVA-specific Th1 responses. Furthermore, we also found that the active cutaneous anaphylaxis after the application of 1 and 0.1 μ g of OVA was reduced in

mice vaccinated with CpG/OVA-pulsed BM-DC in comparison with the control group [means $\pm$ SEM of the bluing intensity in control vs. CpG/OVA-pulsed BM-DC-vaccinated group (4–5 mice/group): at 10 μ g/ml OVA, 2.66 ± 0.33 vs. 2.7 ± 0.25 ; at 1 μ g/ml OVA, 2.66 ± 0.33 vs. 1.50 ± 0.28 ($p < 0.05$), and at 0.1 μ g/ml OVA, 1.0 ± 0.00 vs. 0.25 ± 0.25].

To further evaluate the effect of the application of BM-DC pulsed with CpG/OVA in the generation of Th2 responses in comparison to the application of unpulsed BM-DC in BALB/c mice, we measured the production of IL-4 and IL-5 in the supernatants of MLN cultures after stimulation with anti-CD3+IL-2, OVA or medium alone. The application of CpG/OVA-pulsed BM-DC did not result in a significant decrease in the levels of IL-4 and IL-5 produced by MLN cells after stimulation with anti-CD3/IL-2 in vitro (fig. 3D). No cytokines were detected

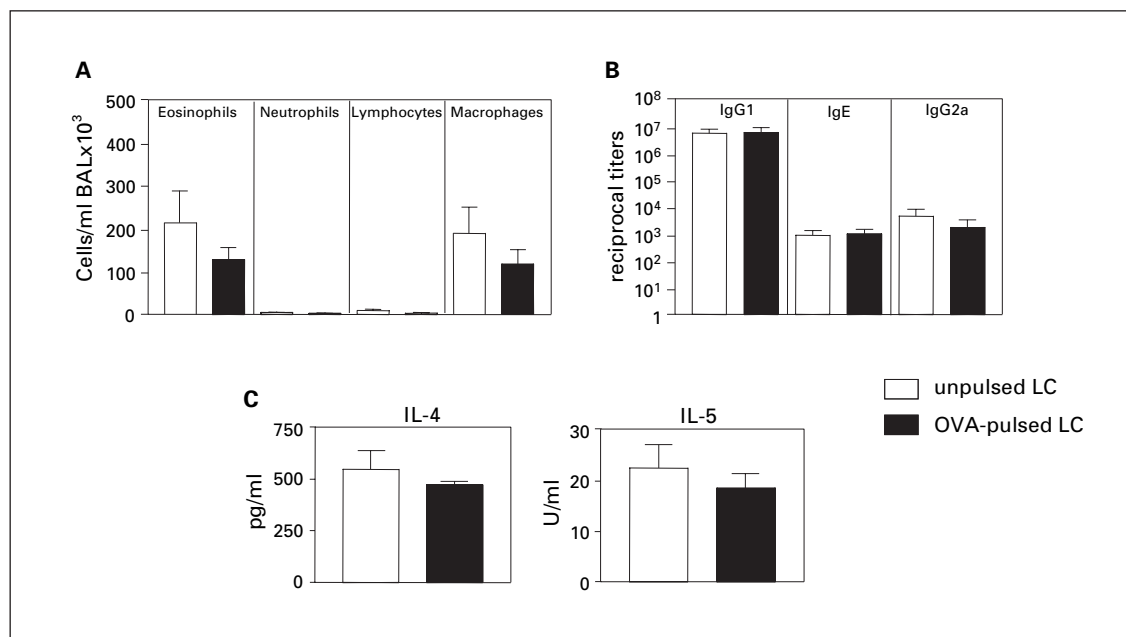


Fig. 4. LC loaded with the allergen ex vivo and intravenously given to mice also could not reduce allergic Th2-mediated inflammation in BALB/c mice. Mice were injected with OVA-pulsed LC and then subjected to the OVA immunization protocol as shown in figure 2A. The absolute numbers of the different cell types present in the BAL fluid (**A**), the serum OVA-specific antibody levels (**B**) and the production of IL-4 and IL-5 in supernatants of MLN cultures after stimulation with anti-CD3/IL-2 (**C**) from mice subjected to the intravenous administration of either unpulsed or OVA-pulsed LC (mean $\pm$ SEM of 5–7 mice per group) are given. The experiment was repeated once with similar results.

when the cells were cultured with OVA or in medium only.

These results clearly demonstrate that BM-DC pulsed with CpG/OVA induce Th1-type B-cell responses but do not suppress the development of OVA-specific Th2 responses when applied intravenously into BALB/c mice before the initiation of the OVA/alum sensitization.

Intravenous Injection of LC Pulsed with OVA Also Does Not Reduce the Development of OVA-Induced Th2 Responses in Mice

As demonstrated above, BM-DC generated in the presence of high doses of GM-CSF were not able to protect BALB/c mice from the development of allergic Th2 responses after their maturation ex vivo in the presence of the allergen. Since these cells clearly show a myeloid phenotype (CD11c+, CD8 α –, data not shown) we next wanted to confirm our results using LC, a type of skin DC also derived from myeloid precursors that constitutively express high levels of MHC class II molecules and are highly specialized in antigen presentation [12]. Further-

more, we have recently shown that a similar approach using LC pulsed with defined antigen protected mice from developing lethal Th2 responses after infection with *L. major* [12]. Therefore, LC prepared from the ears of mice and pulsed with OVA were administered intravenously to naïve mice using the same protocol as for CpG/OVA-pulsed BM-DC (fig. 3A). Figure 4A, B shows that the administration of the OVA-pulsed LC also did not significantly suppress the airway eosinophilia or the levels of OVA-specific IgG1 or IgE in the serum in comparison to the administration of unpulsed LC. In addition, the levels of IL-4 and IL-5 in cultures of MLN after stimulation with anti-CD3/IL-2 were also not reduced (fig. 4C). Thus, our previous results using BM-DC matured with CpG/OVA were confirmed using LC loaded ex vivo with OVA. However, the administration of LC pulsed with OVA did not lead to enhanced OVA-specific IgG2a levels.

Table 1. Allergen-induced immune responses in C57BL/6 mice vaccinated intravenously with either CpG/OVA-pulsed BM-DC or OVA-pulsed LC and then subjected to the immunization with OVA/alum

	BM-DC		LC	
	unpulsed	CpG/OVA pulsed	unpulsed	OVA pulsed
Cells/ml BAL $\times 10^3$				
Eosinophils	227.8 $\pm$ 87.9	147.3 $\pm$ 27.2	299.7 $\pm$ 117.9	190.8 $\pm$ 71.4
Neutrophils	4.8 $\pm$ 1.4	2.5 $\pm$ 0.5	5.0 $\pm$ 1.4	2.8 $\pm$ 0.9
Lymphocytes	5.2 $\pm$ 1.3	2.5 $\pm$ 0.5	16.0 $\pm$ 4.3	11.0 $\pm$ 5.9
Macrophages	94.5 $\pm$ 28.3	108.7 $\pm$ 21.6	161.0 $\pm$ 21.6	88.8 $\pm$ 21.5
OVA-specific antibodies, reciprocal titers				
IgG1, $\times 10^3$	291.9 $\pm$ 101.6	478.1 $\pm$ 67.4	468.6 $\pm$ 131.9	656.9 $\pm$ 277.8
IgE	232.3 $\pm$ 93.5	341.0 $\pm$ 174.8	898.2 $\pm$ 391.6	923.8 $\pm$ 280.7
IgG2a	26.2 $\pm$ 6.9	14,650.0 $\pm$ 6,213.0**	25.1 $\pm$ 8.2	9.2 $\pm$ 4.0
Cytokines MLN				
IL-4, pg/ml	596.4 $\pm$ 45.8	514.2 $\pm$ 26.3	≤ 14	≤ 14
IL-5, U/ml	26.4 $\pm$ 1.2	12.3 $\pm$ 2.7*	28.9 $\pm$ 3.3	18.9 $\pm$ 2.5*

Mice were treated as described in figure 3A. Six days after the intranasal OVA challenge, mice were analyzed as described in the legend to figure 3. Mean values of 5–7 mice/group $\pm$ SEM. * $p < 0.05$, ** $p < 0.01$, compared to mice injected with either unpulsed BM-DC or LC, respectively.

Effect of the Administration of Myeloid DC Loaded ex vivo with OVA in the Induction of OVA-Specific Th2 Responses in C57BL/6 Mice

Since the development of allergen-specific Th2-type responses in mice could be influenced by the genetic background of the strain [15], we also evaluate if the administration of either CpG/OVA-pulsed BM-DC or OVA-pulsed LC decreased the development of OVA-induced Th2 responses in C57BL/6 mice. For this purpose, CpG/OVA-pulsed BM-DC or OVA-pulsed LC were prepared as described above and injected intravenously into C57BL/6 mice using the same protocol as for BALB/c mice (fig. 3A). Table 1 shows that neither the administration of BM-DC pulsed with CpG/OVA nor that of LC pulsed with OVA resulted in a significant reduction in airway eosinophilia after immunization with OVA/alum and the challenge with OVA. Also, the levels of OVA-specific IgG1 and IgE in the serum were not altered by any of the myeloid DC-based vaccines. We again observed an increase in the levels of serum OVA-specific IgG2a in the mice injected intravenously with CpG/OVA-pulsed BM-DC. Using either mature BM-DC or LC loaded ex vivo with OVA intravenously, we observed a significant decrease in the levels of IL-5 (but not IL-4) in the supernatants of MLN cultures from OVA-sensitized C57BL/6 mice after stimulation with anti-CD3/IL-2 (table 1). How-

ever, the decrease in the IL-5 levels in the MLN after un-specific stimulation in vitro did not correlate with a reduction in the absolute numbers of eosinophils in the airways.

Discussion

Our previous work has clearly shown that the administration of Th1-inducing adjuvants such as BCG or mycobacterium-derived products can inhibit the development of allergen-specific Th2 responses [16, 17]. The aim of our present study was to develop a DC-based vaccination strategy also leading to the induction of allergen-specific Th1 cells which then in turn protect mice from developing allergen-specific Th2 responses. We used two different approaches: the intravenous administration of either CpG/allergen-pulsed BM-DC or allergen-pulsed LC. Our results show that both approaches failed to reduce allergic airway inflammation or the production of serum allergen-specific IgG1 or IgE in two different mouse strains (BALB/c and C57BL/6) when the DC cells were applied before the initiation of the OVA/alum sensitization. Surprisingly, using an identical vaccination protocol, we have previously found that *L. major*-induced Th2 responses could be inhibited by vaccination with CpG-

treated BM-DC or LC pulsed with *L. major* lysate [8, 12]. This result suggests that the type of antigen used may be of importance when applying DC-based vaccination protocols. Based on recent reports [18], it can also be postulated that the type of DC is decisive for the efficacy of these approaches. De Heer et al. [18] have shown that different subsets of DCs in the lung govern the induction of tolerance versus immunity to inhaled allergens. Whereas myeloid DC are important for generating T-cell division and priming, plasmacytoid DC (pDC) suppress T-cell effector functions. As the DC subsets used in this study (BM-DC and LC) were all derived from a myeloid lineage, it is possible that they can induce Th1-cell development but that they do not have the ability to efficiently suppress the development of allergen-specific Th2 cells.

In the case of LC, the lack of Th2 suppression may have been due to the lack of effectiveness of the vaccination used, since no enhanced OVA-specific IgG2a levels were detected. However, this cannot explain why the application of BM-DC also failed to reduce Th2-cell development, since this approach led to strongly enhanced levels of OVA-specific IgG2a levels, indicative of Th1 responses. Interestingly, we found that the active cutaneous anaphylaxis after the application of OVA was reduced in mice vaccinated with CpG/OVA-pulsed BM-DC in comparison to the control group. This finding was surprising since OVA-specific IgE and IgG1 (both of which can induce mast cell degranulation in mice) levels were not reduced in these animals. A possible explanation for this finding is that the increased levels of OVA-specific IgG2a interfered with mast cell degranulation after allergen application. This hypothesis is supported by previous findings which demonstrate that mouse IgG2a protects from antibody-mediated mast cell degranulation [19]. A further explanation may be that the vaccination with CpG/OVA-pulsed BM-DC induced non-anaphylactic IgG1. This type of IgG1 was shown to be induced either by IL-12 or Th1-type adjuvants in mice and have lower binding capacity to the mast cell surface preventing its degranulation [20–22]. Although we did not observe an increase in the levels of OVA-specific IgG1 in the DC-vaccinated animals, it is possible that the OVA-specific IgG1 produced after the application of CpG/OVA-pulsed BM-DC comprised mainly non-anaphylactic murine antibodies (since CpG-ODN are potent stimulators of IL-12 production).

The results of our study seem to contradict previously published work. It was reported that the activation of peroxisome proliferator-activated receptor γ (PPAR- γ , an array of receptors that are activated by polyunsaturated

fatty acid derivatives or phospholipids) in allergen-pulsed BM-DC leads to decreased airway eosinophilic inflammation in BALB/c mice treated with these DC [23]. The application of myeloid DC stimulated with LPS together with the allergen has also been reported to suppress airway Th2 responses in mice [24]. Furthermore, a study conducted by Chiang et al. [25] has reported that BM-DC pulsed with the guanosine analogue ribavirin or CpG-ODN (CpG 1826) together with the allergen could downregulate Th2-type immune responses and alleviate airway inflammation in BALB/c mice. A possible reason for the apparent discrepancy between our results and the previously published findings may be that the immunization protocol used by us (sensitization of mice with OVA and alum) induces much stronger allergen-specific Th2 responses than the protocols used by the other investigators (sensitization of mice with OVA without alum). Supporting this view are other studies showing that a protocol used to inhibit allergic asthma in mice produced positive results in a mild but not in a more severe asthma model [26, 27]. Moreover, this assumption is further supported by our observations which indicate that the intravenous administration of CpG/OVA-pulsed BM-DC is not able to induce airway eosinophilia upon intranasal allergen rechallenge in comparison to the application of OVA-pulsed DC. However, when much stronger allergen-specific Th2 responses were induced after OVA/alum sensitization, the application of CpG/OVA-pulsed BM-DC showed no effect on airway inflammation.

Taken together, our study shows that the vaccination with either BM-DC activated with CpG and pulsed with OVA, or alternatively LC pulsed only with OVA 1 week before the sensitization with allergen, did not reduce the development of allergen-specific Th2 responses. However, mast cell degranulation was reduced in our experimental approach suggesting that the vaccination with CpG/OVA-pulsed BM-DC was not totally ineffective in inhibiting allergic responses in mice. Thus, we cannot rule out the possibility that applying CpG/OVA-pulsed BM-DC or OVA-pulsed DC via a different route, more often or in a therapeutic setting may yield more promising results.

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