

# **The Influence of CpG Motifs on a Protein or DNA-Based Th2-Type Immune Response against Major Pollen Allergens Bet v 1a, Phl p 2 and *Escherichia coli*-Derived $\beta$ -Galactosidase**

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## **Key Words**

DNA immunization · DNA immunotherapeutics · CpG motifs · BALB/c · Bet v 1 · Phl p 2 ·  $\beta$ -Galactosidase

## **Abstract**

**Background:** DNA immunization and protein immunization with CpG motifs as adjuvants represent promising approaches in allergen-specific immunotherapy. **Objective:** We investigated the effect of coinjection or prepriming with CpG-ODN on Th2-type responses induced by gene gun and protein immunization. **Methods:** BALB/c mice were immunized with the gene gun using plasmid DNA containing the cDNAs coding for the genes of Bet v 1a, Phl p 2 and  $\beta$ -galactosidase or with the purified Al(OH)<sub>3</sub>-adsorbed proteins. In addition, CpG-ODN were applied by coinjection or by prepriming treatment. Antibody and cytokine responses were measured by ELISA, proliferative and cytotoxic responses were determined by standard labeling procedures. Furthermore, the allergenic activity of sera was measured by passive cutaneous anaphylaxis. **Results:** Gene gun immunization and protein immunization induced a clear Th2-type re-

sponse for all antigens. The Th1-promoting effect of CpG-ODN coinjection together with gene gun immunization was restricted to  $\beta$ -galactosidase as indicated by the increase of IgG2a and a marked expression of IFN- $\gamma$ . CpG motifs also increased the specific cytotoxic response against  $\beta$ -galactosidase. Prepriming with CpG-ODN and gene gun or protein immunization with Bet v 1a exhibited no significant difference to the non-CpG control group. However, sera from mice preprimed with CpG-ODN induced no anaphylaxis with gene gun immunization, but with protein immunization. **Conclusions:** The effect of CpG motifs in vivo depends on a variety of parameters like the nature of the antigen and the immunization modality. Furthermore, our studies indicate that a combination of CpG + DNA immunization may be more effective in antagonizing Th2 responses than the combination of CpG + protein immunization.

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## Introduction

In the past few years genetic immunization has been demonstrated as a new and powerful method inducing protective immunity against a variety of pathogens like viruses, bacteria and parasites [1]. Beyond these applications gene vaccination has also been successfully used to develop strategies against tumors [2] and even allergic responses [3, 4].

The latter gained increasing importance by the fact that intramuscular or intradermal injection of plasmid DNA encoding antigens caused a strong T helper cell type 1 (Th1) response in contrast to the Th2-type response after conventional immunization with protein [5]. A number of recent studies indicated that the major reason for this Th1-type induction are immunostimulatory sequences present on the plasmid DNA of the vectors used for immunization. These bacterial sequences, also designated as CpG motifs, contain unmethylated CpG dinucleotides flanked by two 5' purines and two 3' pyrimidines and were shown to stimulate the innate immune system to produce Th1-inducing cytokines [6, 7]. In fact, also CpG-containing oligodeoxynucleotides applied together with protein were able to potentiate the development of Th1 cells [8].

In the present work we investigated the Th1-promoting effect of CpG motifs on Th2-type responses induced by DNA immunization with gene gun or conventional protein immunization. In contrast to intramuscular or intradermal needle injection of DNA in saline, the injection of plasmid DNA coated on gold particles with a biolistic device causes a Th2-type response with high titers of IgG1 and IgE [9]. Therefore, the question was to what extent was CpG-ODN given as coinjection or by prepriming able to influence protein or DNA-based Th2-type responses.

## Materials and Methods

### *DNA Immunization Vectors and Oligodeoxynucleotides*

Expression plasmids pCMV-Bet and pCMV- $\beta$ gal were constructed, tested and purified as previously described [4, 10]. Phl p 2 was subcloned via PCR from the prokaryotic expression vector pMW172 using *Xho*I and *Xba*I. ODN were purchased from MWG-Biotech (Ebersberg, Germany). The sequence of the CpG-ODN was 5'-TCCATGACGTTCTGACGTT-3' (CpG motifs are underlined). The synthetic ODN were dissolved in LPS-free H<sub>2</sub>O; the LPS content was < 1 ng LPS/mg DNA.

### *Immunization and Experimental Groups*

Female BALB/c mice, aged between 5 and 6 weeks (n = 6 for each group), were maintained at the central animal care unit at the University of Salzburg according to the local guidelines for animal care.

Gene gun immunization was performed with 2  $\mu$ g DNA on day 0, 27, 56 and 90 for pCMV-Bet and pCMV-Phl, and on day 0, 28 and 65 for pCMV- $\beta$ gal. The CpG-ODN concentration was 50  $\mu$ g in 100  $\mu$ l PBS for both coinjection and prepriming. Prepriming was done on days -9 and -3 followed by one DNA immunization on day 0 and two protein injections on days 0 and 7 with 5  $\mu$ g purified Al(OH)<sub>3</sub>-adsorbed protein.

IgG, IgG1, IgG2a, IgE antibodies and the cytokines IFN- $\gamma$  and IL-4 from supernatants of proliferation cultures [4] were measured with a luminometric ELISA as previously described [11].

### *Cytotoxicity Assay*

Cell-mediated cytotoxicity was examined in the pCMV- $\beta$ gal group by a standard 4-hour <sup>51</sup>Cr release assay, as described [12], aa 96–103 of  $\beta$ -galactosidase served as target CTL epitope.

### *Passive Cutaneous Anaphylaxis*

Anaphylactic activity of sera obtained from immunized mice was determined by passive cutaneous anaphylaxis (PCA) reactions in naive BALB/c mice as described [13]. Briefly, pooled sera were diluted 1:10 in sterile PBS and 30  $\mu$ l were injected intradermally into the shaved back. After 2 h the animals were challenged intravenously with 100  $\mu$ l of 0.5% Evan's blue solution containing 250  $\mu$ g of birch pollen protein extract.

### *Statistical Analysis*

Data are expressed as means  $\pm$  SEM. Statistical significance was assessed using an unpaired Student t test.

## Results

Gene gun immunization was performed with expression plasmids encoding for the major allergen of birch pollen, Bet v 1a [14], the grass pollen Phl p 2 [15] and the enzyme  $\beta$ -galactosidase from *Escherichia coli* (termed pCMV-Bet, pCMV-Phl and pCMV- $\beta$ gal). All constructs elicited 100% seroconversion after three injections of 2  $\mu$ g DNA in each group (fig. 1). For all three antigens the antibody subclass distribution revealed a predominant expression of IgG1 and a significant increase ( $p < 0.05$ ) of the IgE antibody isotype (fig. 1). Coinjection of 50  $\mu$ g CpG-ODN did not significantly influence the IgG1 production but induced elevated IgG2a titers with pCMV-Bet and pCMV- $\beta$ gal ( $p < 0.05$ ), but not with pCMV-Phl. Surprisingly, the expression of IgE antibodies was even increased by the coadministration of CpG motifs in the pCMV-Bet group ( $p < 0.05$ ). For both the pCMV- $\beta$ gal and pCMV-Phl groups no significant change of IgE production could be detected after CpG coinjection (fig. 1). Only in the  $\beta$ -galactosidase group did CpG motifs cause a significantly increased expression of IFN- $\gamma$  ( $p < 0.05$ , table 1). Gene gun immunization led to an antigen-specific cytotoxic response against  $\beta$ -galactosidase which was increased by coinjection of CpG-ODN ( $p < 0.01$ ). The per-

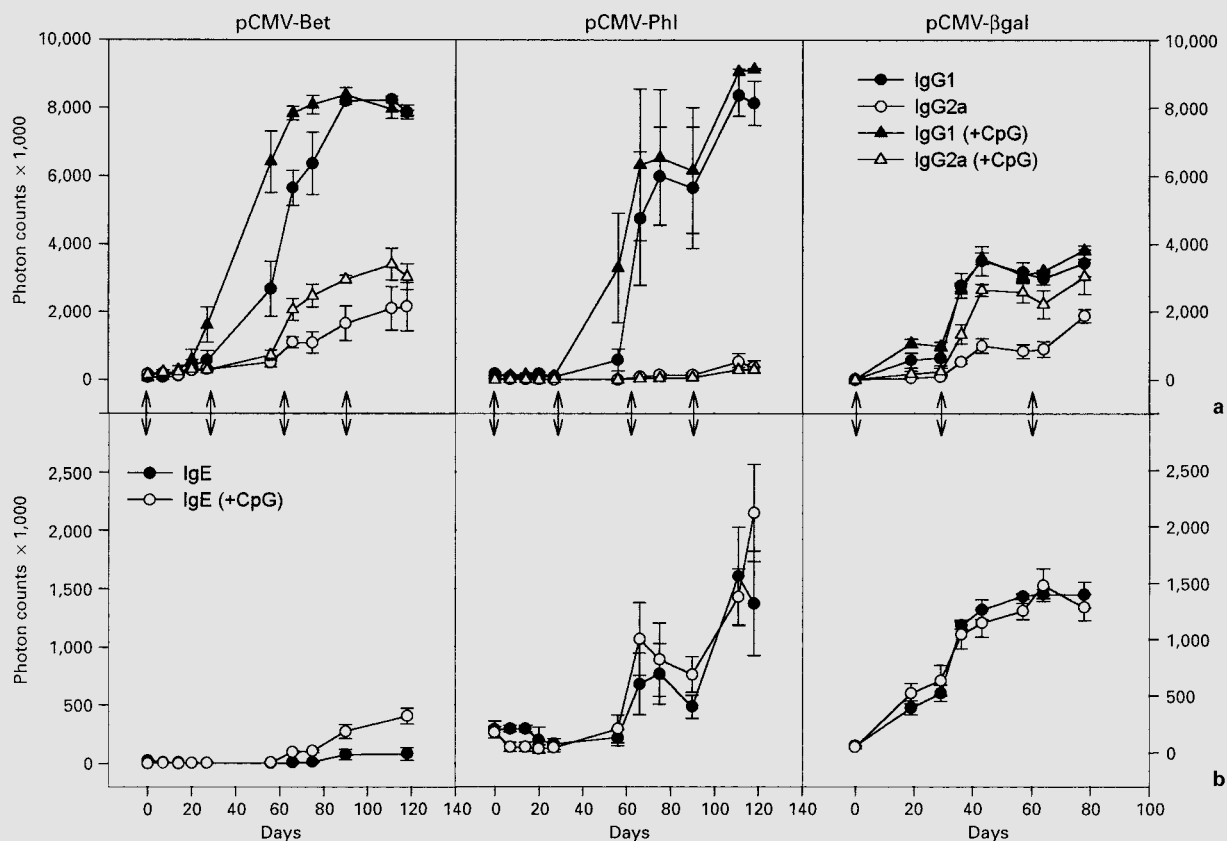


**Fig. 1.** ELISA of IgG1, IgG2a (a) and IgE (b) immune responses after gene gun immunization with 2 µg plasmid DNA encoding for Bet v 1a (pCMV-Bet), Phl p 2 (pCMV-Phl) and β-galactosidase (pCMV-βgal). Additionally, one group each received 50 µg CpG-ODN. Serum dilutions were 1:100 except for Bet v 1a and Phl p 2 IgE detection (1:20) and β-galactosidase IgG1 and IgG2a measurement (1:2,000). Immunizations are indicated by arrows, data are expressed as mean ± SEM of 6 mice.

**Table 1.** Lymphoproliferation assay and cytokine measurement of proliferation supernatants of spleen cells from mice immunized with plasmid DNA applied by the gene gun

| Construct       | Stimulation index | IFN-γ, pg/ml      | IL-4, pg/ml |
|-----------------|-------------------|-------------------|-------------|
| pCMV-Bet        | 4.18 ± 0.38       | 2,874.8 ± 704.3   | 64.9 ± 1.4  |
| pCMV-Bet + CpG  | 3.18 ± 0.27       | 3,016.0 ± 1,973.8 | 78.9 ± 26.4 |
| pCMV-Phl        | 2.16 ± 0.34       | 76.9 ± 1.7        | 10.7 ± 0.5  |
| pCMV-Phl + CpG  | 1.78 ± 0.25       | 72.1 ± 9.2        | 9.2 ± 0.3   |
| pCMV-βgal       | 1.69 ± 0.22       | 4,741.3 ± 203.6   | 27.9 ± 3.1  |
| pCMV-βgal + CpG | 2.57 ± 0.36       | 9,957.7 ± 1,623.4 | 37.0 ± 2.6  |
| Control         | 1.18 ± 0.08       | 94.2 ± 17.6       | 9.0 ± 0.6   |

Stimulation was performed with the respective recombinant antigen. The proliferation data represent the mean stimulation index of 4 mice (five wells per animal).

**Table 2.** Luminometric ELISA of IgE, IgG1 and IgG2a, and PCA tests of sera from mice preprimed with CpG-ODN followed by immunization with Bet v 1a using the gene gun or by injection of recombinant protein

|                | IgE        | IgG1          | IgG2a         | PCA |
|----------------|------------|---------------|---------------|-----|
| Gene gun       | 216.8 ± 42 | 6,532.8 ± 230 | 132.6 ± 22    | +   |
| Gene gun + CpG | 219.2 ± 21 | 4,439.0 ± 361 | 104.9 ± 24    | –   |
| Protein        | 501.0 ± 27 | 6,952.8 ± 282 | 222.6 ± 27    | ++  |
| Protein + CpG  | 559.9 ± 22 | 7,171.4 ± 618 | 115.6 ± 25    | ++  |
| DNA needle     | 110.9 ± 25 | 3,324.7 ± 502 | 2,978.2 ± 815 | –   |

Plasmid DNA via needle injection served as control for a strong Th1-type response. Anti-sera were collected on day 60, antibody titers are expressed as photon counts × 1,000. For PCA the color intensity and area size were scored from – to ++, the sera of untreated control animals were negative.

cent lysis values at effector/target ratios of 100:1, 50:1 and 25:1 were  $87 \pm 5$ ,  $67 \pm 4$  and  $45 \pm 6$  (plus CpG) and  $52 \pm 7$ ,  $27 \pm 2$  and  $12 \pm 2$  (without CpG). Control values did not exceed 2% lysis. As indicated by a recent publication [8], coadministration of CpG motifs together with the antigen may be a suboptimal procedure. Therefore, we investigated whether prepriming with CpG motifs could overcome the Th2 bias of gene gun immunization or protein immunization. For this purpose mice were treated with intradermal injections of 50 µg CpG-ODN on day –9 and day –3 before immunization which was performed with 2 µg pCMV-Bet (gene gun) or 5 µg recombinant Bet v 1a (s.c.) as described above. In addition, a group of animals was immunized with 100 µg pCMV-Bet plasmid DNA in saline using the needle injection as a control for a strong Th1-type response [4]. Table 2 demonstrates that CpG prepriming decreases the humoral immune response in the gene gun group but does not significantly change the Th2-type response. In contrast, the needle injection clearly induced a strong Th1-type response with high IgG2a and low IgE antibodies. PCA was positive with sera from animals immunized with gene gun and protein with or without CpG-ODN but no anaphylactic reaction was detectable with gene gun and CpG prepriming and with the needle-injected Th1 control group (table 2).

## Discussion

Immunostimulatory sequences like CpG motifs attracted much attention because of their proven ability to induce Th1-related cytokines in vitro and their immune modulating capacity in vivo. Recent publications focused on the concept of using allergens together with CpG motifs as Th1 adjuvants for the treatment of allergy [16].

In the present work this concept was evaluated in vivo using two clinically relevant allergens, the major birch pollen allergen Bet v 1a and the grass pollen allergen Phl p 2 and, in addition, the model molecule β-galactosidase, a highly immunogenic protein from *E. coli*. In agreement with published data genetic immunization with the gene gun induced a Th2-type response against all antigens [9]. A clear Th1-promoting effect of CpG-ODN coinjection was detectable only in the β-galactosidase group. Coinjection of CpG motifs in the Bet v 1a group even elicited a paradox situation. IgG2a in serum and IFN-γ in proliferation supernatants were slightly increased, but surprisingly, also IgE antibodies were elevated. This is in agreement with observations indicating that the nature of the antigen itself plays an important role [17] and that reported CpG effects [8] cannot be generalized for all antigens. In addition, the data clearly point to the questionable role of β-galactosidase as a surrogate model in allergy research. Prepriming, as proposed to optimize the Th1 effect of CpG motifs [8], had no serological effect on the Th2-type response against Bet v 1a, neither caused by the gene gun nor induced by protein immunization.

In order to include a functional parameter reflecting the allergenic activity of serum antibodies PCA assays were performed. Surprisingly, sera from preprimed and gene gun-immunized animals were nonanaphylactic despite exhibiting a clear Th2-type antibody profile. In contrast, sera with nearly identical characteristics from animals immunized with gene gun without CpG prepriming showed strong PCA reactions. Also CpG prepriming had no antianaphylactic effect in the protein immunization group, thus pointing to a possibly overoptimistic expectation concerning the protein-CpG immunotherapeutic concept.

Summing up we can state that CpG effects in vivo may depend on complex parameters like the nature of the antigen and the immunization protocol and modality. The results also clearly indicate that a combination of CpG and DNA immunization may more strongly induce Th1 responses than combinations of CpG and protein-based immunization strategies.

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