



Gene gun immunization with clinically relevant allergens aggravates allergen induced pathology and is contraindicated for allergen immunotherapy

Sandra Scheibelhofer^a, Angelika Stoecklinger^a, Christina Gruber^a, Cornelia Hauser-Kronberger^b, Beate Alinger^b, Peter Hammerl^a, Josef Thalhamer^{a,*}, Richard Weiss^a

^a Christian Doppler Laboratory for Allergy Diagnosis and Therapy, Department of Molecular Biology, University of Salzburg, Hellbrunnerstrasse 34, 5020 Salzburg, Austria

^b Department of Pathology, General Hospital and Paracelsus University Salzburg, Müllner Hauptstrasse 48, 5020 Salzburg, Austria

Received 22 August 2006; accepted 29 September 2006

Abstract

Gene gun immunization has been associated with the induction of a heterologous type of immune response characterized by a T_H1 -like immune reaction on the cellular level, i.e. generation of IFN- γ secreting CD8⁺ T-cells, yet a T_H2 biased serology as indicated by high IgG1:IgG2a ratios and induction of IgE. Nevertheless, gene gun immunization using the model molecule β -galactosidase has been argued to prevent IgE induction and to promote T_H1 cells with respect to allergy DNA immunization.

In our current study, we evaluated the potential of gene gun immunization to prevent type I allergic reactions comparing β -galactosidase with two clinically relevant allergens, and further investigated the effect of gene gun immunization on relevant lung parameters.

BALB/c mice were immunized with plasmids encoding the birch pollen allergen Bet v 1, the grass pollen allergen Phl p 5, or the model molecule β -galactosidase, either by gene gun or intradermal injection followed by sensitization and intranasal provocation with the respective allergen. IgG1 and IgG2a antibody titers were determined by ELISA. IgE levels were evaluated in a rat basophil release assay. The severity of eosinophilia was determined in bronchoalveolar lavages, and the overall infiltrate was analyzed by histology on lung paraffin sections.

Gene gun immunization induced a T_H2 -biased immune reaction, which did not prevent from production of IgE after subsequent sensitization. This T_H2 effect was influenced by the nature of the antigen, with a more pronounced T_H2 -bias for the allergens Bet v 1 and Phl p 5 compared to β -galactosidase. Gene gun immunization with all three antigens promoted eosinophil influx into the lung and did not alleviate lung pathology after intranasal provocation.

In contrast to needle injection of plasmid DNA, which triggers a clearly T_H1 -biased and allergy-preventing immune response, gene gun application fails to induce anti-allergic reactions with all tested antigens and is therefore contraindicated for allergen-specific immunotherapy.

© 2006 Elsevier Ltd. All rights reserved.

Keywords: Type I allergy; DNA-based vaccine; Genetic immunization; Gene gun; Immunoglobulin E; Bet v 1a; Phl p 5; β -Galactosidase

1. Introduction

Inoculation of small animals with allergen-encoding plasmids has been used in a variety of studies. We and others have demonstrated that, by stimulating T_H1 -biased immune reactions, injection of DNA in saline solution into muscle or skin effectively prevents from type I allergic reactions and exhibits therapeutic potential (reviewed in Weiss et al., 2006a). Yet, the relatively high amount of plasmid DNA used represents the major disadvantage of this method. The dose necessary to elicit an adequate immune response in humans would have to be in the

Abbreviations: i.d., intradermal; β -Gal, β -galactosidase; pCMV- β -Gal, DNA plasmid expressing β -galactosidase; pCMV-Bet, DNA plasmid expressing Bet v 1a; pCMV-P5, DNA plasmid expressing Phl p5; pCMV, DNA plasmid without insert; SIT, specific immunotherapy; BAL, bronchoalveolar lavage; alum, Al(OH)₃; H&E, hematoxylin/eosin; PAS, periodic acid Schiff

* Corresponding author. Tel.: +43 662 8044 5737; fax: +43 662 8044 5751.

E-mail address: Josef.Thalhamer@sbg.ac.at (J. Thalhamer).

milligram range (Wang et al., 1998). Genetic immunization with gene gun, which propels plasmid-coated gold particles of about 1 μm size into the uppermost layers of the skin, offered an alternative method to induce immunogenicity with strikingly reduced vaccine doses. The gene gun proved as an excellent tool to induce IFN- γ -secreting CD8 $^{+}$ cells (Yoshida et al., 2000), indicating at least partly a T $_{\text{H}}$ 1 milieu, which tempts to employ this technology against allergic diseases. However, several studies including our own have reported that gene gun immunization favors the production of IgG1 and IgE antibodies, and T $_{\text{H}}$ 2 cytokines, rendering this method questionable for allergen immunotherapy (Feltquate et al., 1997; Oran and Robinson, 2003; Pertmer et al., 1996; Scheiblhofer et al., 2003; Weiss et al., 2000). In accordance with these data, Toda et al. described differential outcomes using intramuscular injection or gene gun immunization for plasmids encoding the Japanese cedar pollen allergen Cry j 1. Intramuscular inoculation of mice induced predominantly IgG2a antibodies and IFN- γ but not IL-4 secretion and suppressed IgE and IgG1 antibody responses upon sensitization with recombinant allergen in Al(OH) $_3$ (alum). In contrast, gene gun immunization induced the synthesis of mainly IgG1, enhanced production of IgE to subsequent sensitization, and led to in vitro secretion of both, IL-4 and IFN- γ (Toda et al., 2000). In clear contrast to these results, Ludwig-Portugall et al. (2004) proposed that gene gun-mediated immunization with the model molecule β -galactosidase (β -Gal) prevented long-term IgE antibody production. They also found a reduction of IgG1, an increase in IgG2a antibodies, a mixed T $_{\text{H}}$ 1/T $_{\text{H}}$ 2 response on the cytokine level, and IFN- γ -producing CD8 $^{+}$ effector T cells. The authors speculated that the discrepancy between their work and that of Toda et al. might be explained by different experimental settings.

To clarify whether gene gun inoculation represents a suitable method for anti-allergic intervention we compared the outcomes of gene gun-mediated administration of plasmids encoding the model antigen β -Gal and two clinically relevant allergens, Bet v 1 and Phl p 5, to our standard method intradermal (i.d.) needle injection.

Our study demonstrates that, in contrast to i.d. injection, gene gun immunization induces T $_{\text{H}}$ 2-biased immune responses characterized by decreased IgG2a:IgG1 ratios and high levels of specific IgE antibodies with anaphylactic potential, and shows for the first time that gene gun immunization promotes lung eosinophilia after allergen provocation via the airways.

2. Materials and methods

2.1. Plasmids

The CMV promoter-based β -Gal plasmid (pCMV- β -Gal, previously designated pCI- β Gal) and vector pCMV-Bet containing the coding sequence of Bet v 1a have been described (Brtko et al., 1999; Hartl et al., 1999). The DNA sequence encoding Phl p 5 was subcloned from λ gt11-cDNA (Vrtala et al., 1993) into the mammalian expression vector pCI (Promega, Mannheim, Germany) using standard molecular methods, and the resulting vector was designated pCMV-P5. All plasmids were prepared

using Endofree Plasmid Gigakits (Qiagen, Hilden, Germany). Endotoxin levels were <0.1 EU per microgram of DNA as determined by Limulus amoebocyte assay (Pyroquant, Walldorf, Germany).

2.2. Mice and immunizations

Female BALB/c mice ($n=3$; 6–8 weeks of age from Charles River, Sulzfeld, Germany) were immunized 3 times at 2-week-intervals either by particle bombardment onto the shaved abdomen using a Helios gene gun (Bio-Rad, Munich, Germany) or by i.d. needle injection with pCMV- β -Gal, pCMV-Bet, pCMV-P5, or empty mock vector pCMV. For gene gun immunizations, plasmids were coated onto 1.6 μm gold particles and a helium discharge pressure of 400 psi was used to deliver a total of 4 μg /immunization by two shots. I.d. immunizations were performed with 100 μg plasmid in a volume of 200 μl PBS distributed to several spots on the shaved back. Control animals were left untreated until sensitization. Two weeks after the last immunization mice were sensitized by 5 intraperitoneal injections of 1 μg recombinant β -Gal (Sigma-Aldrich, Deisenhofen, Germany), Bet v 1 (Biomay, Vienna, Austria), or Phl p 5 (Biomay) adsorbed to 100 μl alum (Serva, Heidelberg, Germany) in a total volume of 200 μl PBS at two-week-intervals. Two weeks after the last sensitization pulmonary provocation was performed by intranasal application of 5 μg β -Gal, Bet v 1, or Phl p 5 in 50 μl PBS on three consecutive days. Additional groups of mice were challenged via the airways two weeks after the third gene gun immunization without prior sensitization to assess whether gene gun alone would facilitate the induction of airway pathology. All experiments using gene gun or protein sensitization without pre-immunization were repeated once with similar results and data from both sets of experiments were pooled ($n=6$).

All animal experiments were conducted according to local guidelines approved by the Austrian Ministry of Science.

2.3. Serology

IgG1 and IgG2a antibody levels in sera taken at regular intervals were determined by a luminescence-based ELISA as described (Hartl et al., 2004). As a functional read-out for IgE-mediated degranulation, a β -hexosaminidase release assay was performed using the rat basophil leukemia cell line RBL-2H3 as described (Hochreiter et al., 2003). Sera were serially diluted and endpoint titers were defined as the dilution that resulted in a specific release of 5%.

2.4. Analysis of bronchoalveolar lavages

Bronchoalveolar lavages (BALs) were carried out with 2×1 ml fresh, ice-cold PBS using a hypodermic needle. Samples were centrifuged through a 40 μm cell strainer (BD-Biosciences, Schwenchat, Austria) and supernatants were stored for further analysis of cytokines. Erythrocytes were lysed for 10 min at room temperature with 500 μl FACS-Lyse (BD-Biosciences). Cells were labelled with anti-CD45 FITC/anti-

Gr1 PE (BD-Biosciences, 1:400) on ice for at least 10 min, and analyzed on a FACSCaliburTM flow cytometer. Eosinophils were distinguished from other leukocyte populations by their CD45^{med} Gr1^{low} side-scatter^{high} phenotype and their relative amount compared to total CD45⁺ cells was calculated.

Cytokine levels in BAL fluids were quantitated by sandwich ELISA (BD Biosciences, R&D Systems, Minneapolis, MN) according to the manufacturers' instructions.

2.5. Lung histology

Lungs were fixed in 4% formalin at 4 °C overnight and embedded in paraffin. Tissue sections of 2 µm were stained with haematoxylin/eosin dye (H&E) or periodic acid Schiff (PAS) using standard protocols. All sections were analyzed by bright field microscopy by two independent investigators who were blind to the treatments. Severity of infiltrates on H&E sections and goblet cell metaplasia on PAS sections was scored from 1 (no or mild infiltrate/metaplasia) to 4 (severe infiltrate/metaplasia).

2.6. Statistical analysis

Statistical differences between DNA-immunized and the respective mock vector-immunized groups were assessed via Student's *t*-test. Groups that failed normality test and scored H&E- as well as PAS-sections were analyzed by Mann–Whitney rank sum test.

3. Results

3.1. Needle injection and gene gun inoculation induce different types of serological immune reactions, additionally influenced by the nature of the respective antigen

The effect of sensitization with recombinant protein in alum on animals pre-vaccinated with DNA vaccines either by i.d. needle injection or gene gun was tested with three different antigens, the *E. coli*-derived model molecule β-Gal, the birch pollen allergen Bet v 1 and the timothy grass pollen allergen Phl p 5.

Sensitization of naïve mice with recombinant Bet v 1 and Phl p 5 resulted in strongly polarized serological T_H2 type reactions indicated by an almost complete lack of antigen-specific IgG2a and high levels of IgG1 with IgG2a:IgG1 ratios of 12×10^{-3} and 5×10^{-3} , respectively. β-Gal harbored a subtle inherent T_H1 adjuvant effect that resulted in higher levels of IgG2a with an IgG2a:IgG1 ratio of 74×10^{-3} (Fig. 1A).

Next, we investigated the type of serological immune response induced by the three antigens when applied as DNA vaccines either by gene gun or by i.d. needle injection. Gene gun immunization induced clearly T_H2-polarized serological reactions for all molecules with low IgG2a:IgG1 ratios similar to protein immunization, again with a higher proportion of IgG2a in the β-Gal group (IgG2a:IgG1 ratio of 325×10^{-3} – Fig. 1B). In contrast, i.d. needle immunization elicited a significantly elevated IgG2a:IgG1 ratio compared with gene gun for all three antigens (Fig. 1C; β-Gal: $P < 0.001$; Bet v 1 and Phl p 5: $P < 0.024$, rank sum test).

As we know from previous experiments, T_H1 cells induced by pre-vaccination can be re-activated and further expanded by a subsequent protein sensitization and thereby counterbalance the sensitization-induced T_H2 type reaction (Bauer et al., 2006; Scheibelhofer et al., 2006). Therefore, we sensitized the pre-vaccinated animals with the respective proteins in alum and analyzed the resulting serological profile. In animals pre-vaccinated with the β-Gal plasmid DNA via gene gun, sensitization significantly increased the IgG2a:IgG1 ratio ($P < 0.005$) compared with mock immunized animals. However, the clinically relevant allergens Bet v 1 or Phl p 5 displayed a clear serological T_H2 response that did not differ from mock immunized or non-immunized control animals, and obviously was not influenced by pre-vaccination with gene gun (Fig. 1D). In contrast, sensitization of mice pre-vaccinated by i.d. needle injection (Fig. 1E) elicited a marked proportion of IgG2a (β-Gal: $P < 0.01$; Bet v 1: $P = 0.1$, rank sum test; Phl p 5 $P < 0.001$ compared with mock immunized animals) indicating a T_H1 memory response that resulted from needle pre-vaccination.

3.2. Gene gun immunization does not protect against IgE induction upon sensitization with recombinant allergen

Sensitization with protein/alum induced biologically active antigen-specific IgE as determined by RBL release assay (Fig. 2A). Gene gun immunization alone induced considerable amounts of IgE (Fig. 2B, open bars) that were boosted by subsequent sensitization with protein (Fig. 2B, hatched bars). No significant reduction of IgE in gene gun pre-vaccinated animals could be found when compared with mock-immunized or non-immunized animals in any group (Fig. 2B).

In contrast, pre-vaccination by i.d. needle injection induced only marginal amounts of IgE and successfully suppressed IgE upon sensitization as compared with non-vaccinated groups (Fig. 2C, hatched bars; rank sum test: $P < 0.05$). Differences between pre-immunized and mock immunized animals were only significant for the Bet v 1 group (β-Gal: $P = 0.052$; Bet v 1a: $P < 0.001$; Phl p 5: $P = 0.07$), indicating a slight mock vector effect possibly induced by CpG motifs (Jilek et al., 2001).

3.3. Gene gun immunization does not alleviate lung pathology, but even induces airway eosinophilia

Subsequently, we provoked the mice by intranasal application of recombinant antigen to induce airway inflammation. Animals pre-vaccinated via gene-gun displayed a high percentage of eosinophils in BALs with no differences compared with mock immunized animals. In contrast, mice pre-vaccinated by i.d. needle immunization showed a reduction of eosinophils in BALs. However, significant differences compared with mock immunized animals could only be seen in the β-Gal group ($P < 0.001$) (Fig. 3A and B). Interestingly, gene gun immunization alone was able to induce marked eosinophilia in BALs in the Phl p 5 group (Fig. 3C). All three antigens induced BAL eosinophilia when given intranasally to non-immunized but sensitized animals, with Phl p 5 being more allergenic than β-Gal or Bet v 1 (Fig. 3D).

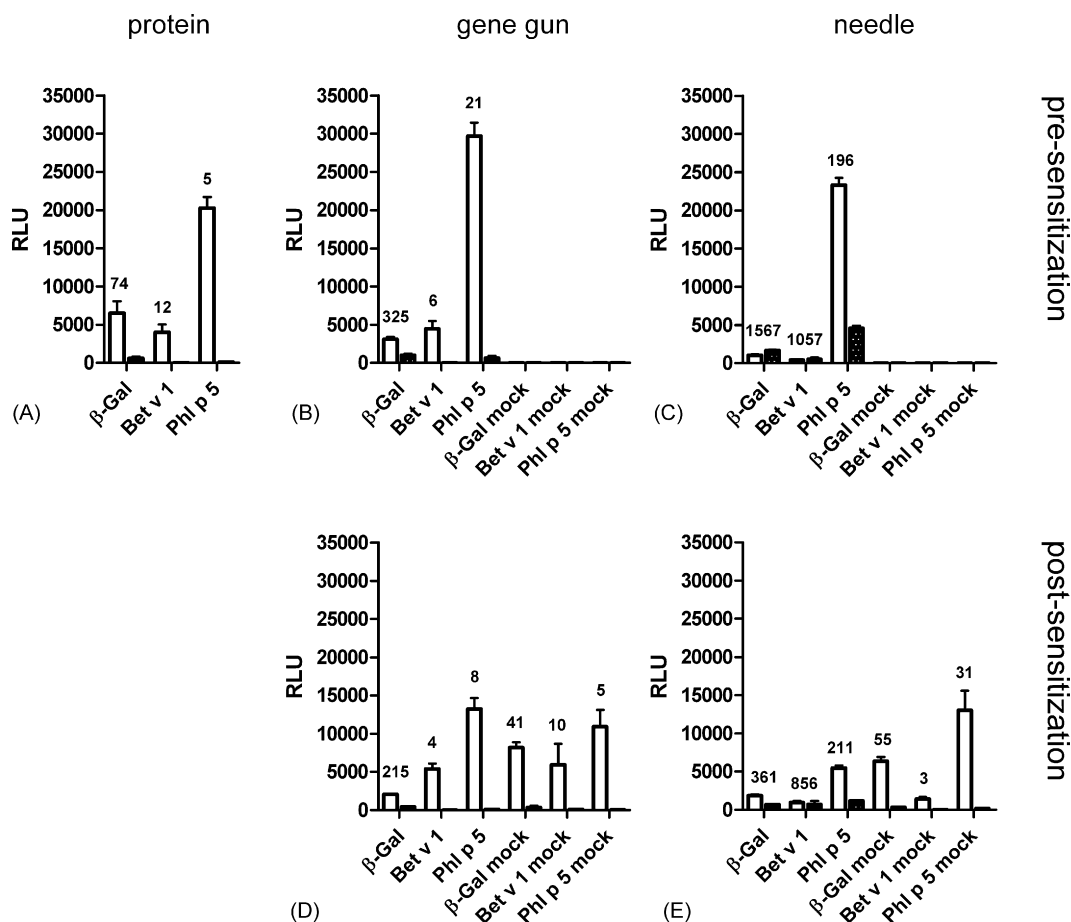


Fig. 1. Antigen-specific IgG1 (open bars) and IgG2a (hatched bars) levels from animals sensitized with recombinant protein (A) or pre-vaccinated with the respective DNA vaccines (β -Gal: pCMV- β -Gal; Bet v 1: pCMV-Bet; Phl p 5: pCMV-P5) by gene gun (B – before sensitization, D – after sensitization) and i.d. needle injection (C – before sensitization, E – after sensitization). Mock immunized animals (i.e. mice that had received empty vector pCMV either via gene gun or i.d. injection) served as controls. Pre-sensitization sera were diluted 1:5000 (panels B and C), sera taken after sensitization 1:50,000 (panels A, D and E). Data are presented as mean $\pm$ S.E.M. IgG2a:IgG1 ratios are displayed as numbers $\times 10^{-3}$ above the respective groups; RLU: relative light units.

The reduction of eosinophils in BALs of i.d. immunized mice was only partly reflected by the levels of cytokines found in BAL fluids. Although no significant differences could be observed when compared with mock immunized animals, there was a trend towards lower IL-5 values in BALs of i.d. immunized ani-

mals (Fig. 4B) in the β -Gal and Phl p 5 groups compared with gene gun immunized mice (Fig. 4A and B), thus paralleling eosinophil counts of the same mice. In contrast to gene gun, i.d. immunization induced IFN- γ in the BAL fluids, indicating recruitment of vaccine-induced T_H1 lymphocytes into the lung.

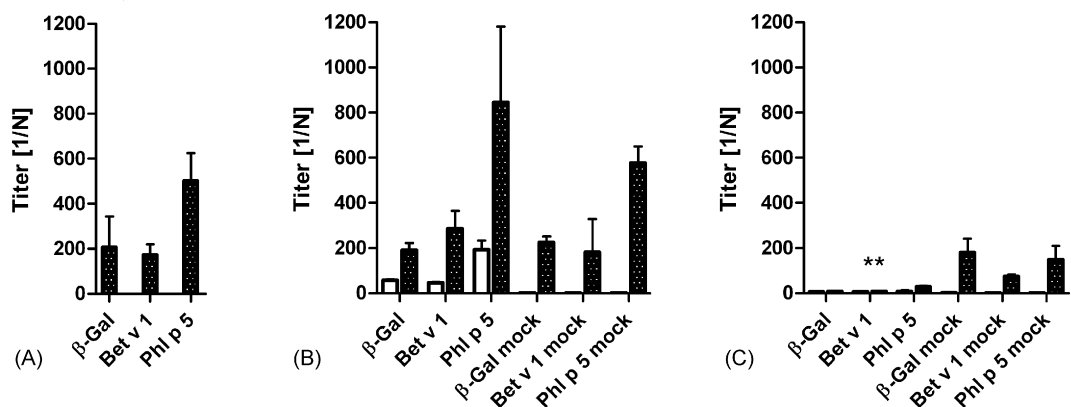


Fig. 2. Antigen-specific IgE measured by RBL assay. IgE titers after sensitization without pre-treatment (A). IgE titers after pre-vaccination via gene gun (B) or i.d. needle immunization (C) with plasmids encoding the respective antigens. IgE values induced by pre-vaccination (before sensitization) are shown as open bars; IgE levels after sensitization are shown as hatched bars. Data are presented as mean $\pm$ S.E.M. *P*-values compared with the respective mock groups: (**) < 0.01.

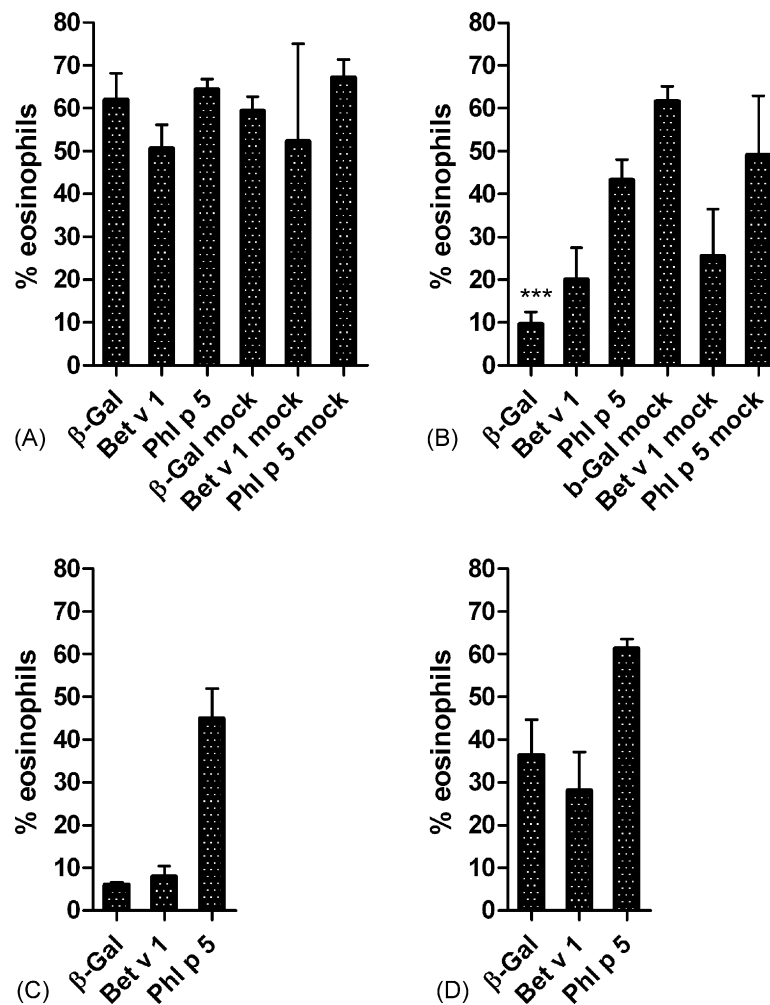


Fig. 3. Percentage of BAL eosinophils after intranasal provocation with the respective allergens. Percentage of eosinophils in BALs was determined in mice pre-vaccinated with gene gun (A) or via i.d. needle injection (B) after sensitization. Panel C shows gene gun immunized animals without subsequent sensitization, panel D shows sensitized animals that had not been pre-vaccinated. Data are presented as mean $\pm$ S.E.M. *P*-values compared with the respective mock groups: (***) < 0.001 .

Again, the T_H1 -prone immunogenic nature of the β -Gal antigen becomes obvious in this experiment (Fig. 4C and D).

To investigate whether these differences in BALs would be reflected by the overall leukocyte infiltrate in the lung, H&E-stained paraffin sections were scored from 0 to 4 according to severity of the infiltrate. Phl p 5 again proved to be the most aggressive allergen of the tested antigens with the highest infiltration scores (Fig. 5E). β -Gal showed slightly less severe infiltration scores (Fig. 5A), while Bet v 1 induced comparatively mild infiltration (Fig. 5C), supporting the results from the other read outs that indicate that the used protocol was suboptimal in inducing an allergic phenotype. No significant effect on the total amount of infiltrate could be observed by pre-vaccination, which is in agreement with previous data from our lab showing that DNA pre-vaccination changes the quality of the infiltrate (Fig. 3) rather than the overall quantity (Gabler et al., 2006).

Additionally, goblet cell metaplasia was scored from 0 to 4 on PAS stained paraffin sections (Fig. 5B, D and F). In contrast to gene gun immunization, i.d. pre-immunization with β -Gal (Fig. 5B) and Bet v 1 (Fig. 5D) showed a trend towards a reduction of goblet cell metaplasia. However, no significant dif-

ferences in pre-immunized mice compared to mock immunized mice could be observed.

4. Discussion

Several theories have evolved to explain the difference in immune response types elicited by gene gun and needle administration of plasmid DNA. Unmethylated immunostimulatory sequences – designated CpG motifs – in bacterial DNA have been shown to directly or indirectly trigger the production of the T_H1 -promoting cytokines IL-12, IL-6, IFN- α , β , γ , and TNF- α by macrophages, dendritic cells, and natural killer cells, and also to activate B and T cells (Krieg, 2002). Therefore, injection of large amounts of CpG-containing bacterial plasmids has been proposed to deliver a T_H1 -biased adjuvant effect. The 10–100-fold lower plasmid dose inoculated by gene gun has been estimated to be insufficient for T_H1 adjuvanticity (Barry and Johnston, 1997). However, recent studies demonstrated that Toll-like receptor 9 knock out mice adequately respond to DNA-based immunization although they lack the receptor specific for recognition of CpG DNA (Babiuk et al., 2004; Spies et al.,

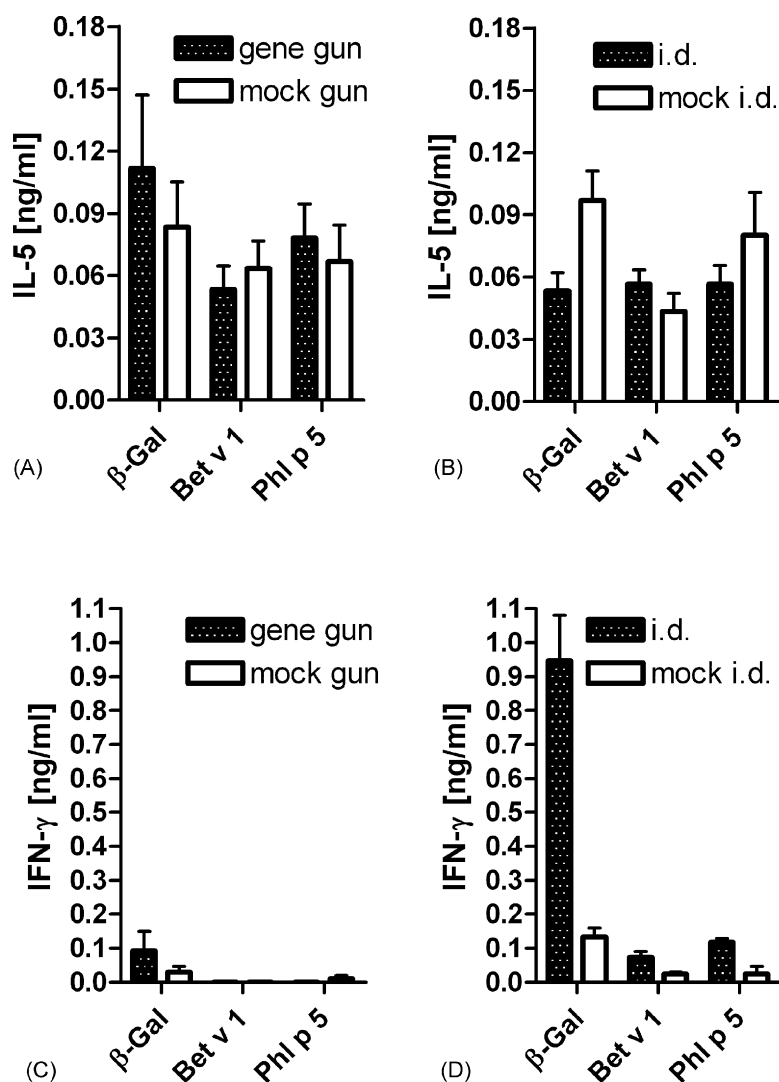


Fig. 4. Levels of IL-5 (A and B) and IFN- γ (C and D) in BAL fluids of mice pre-immunized with DNA vaccines encoding the respective antigens (hatched bars), or with empty mock vector (open bars) via gene gun (A and C), or i.d. needle injection (B and D) after sensitization and subsequent provocation via the airways. Data are shown as mean $\pm$ S.E.M.

2003). Additionally, we could show that the gene gun shot itself delivers a strong T_H2 -promoting effect, as propelling empty gold particles into the skin induced T_H2 responses even against plasmid-encoded antigens applied via i.d. injection immediately after bombardment (Weiss et al., 2002).

Gun and needle application also differ with respect to the mode and efficacy of transfection. Following i.d. or intramuscular injection, the transfection rate is relatively low and plasmids have to be taken up from extracellular spaces. In contrast, gene gun immunization directly transfects cells by depositing DNA-coated beads within the cytoplasm, leading to a high transfection rate. One could speculate that the difference in protein expression levels between the two methods would lead to differentiation of distinct $CD4^+$ T cells, but to our knowledge no such mechanism has been described so far.

However, while gene gun immunization mainly deposits the plasmid DNA in the epidermal layers of the skin, i.d. injection targets the dermis; hence different classes of professional antigen presenting cells will process and present the encoded antigen.

In case of gene gun immunization, predominantly Langerhans cells will be targeted. Strid et al. propose that Langerhans cells naturally favor the induction of T_H2 -type immunity when exposed to protein antigen in the absence of adjuvants. The immunological microenvironment at the surface of the skin is naturally prone to promote tolerance and/or to dampen T_H1 responses without affecting T_H2 responses when exposed to antigen (Kissenpfennig and Malissen, 2006; Strid et al., 2004; Strid and Strobel, 2005). This could explain why empty gold particles propelled into the skin of mice, and hence targeting Langerhans cells, induce a T_H2 -driving regulatory signal (Weiss et al., 2002). On the other hand, T_H1 -type immunity may be initiated when antigen invades further into the body as a result of injury caused by i.d. injection of DNA (Strid and Strobel, 2005). A similar effect can be achieved by particle bombardment at increased helium discharge pressure. Targeting gold particles to deeper layers of the skin enhanced the T_H1 polarization of the elicited immune response against β -Gal (unpublished observations). In accordance with this we could recently demonstrate

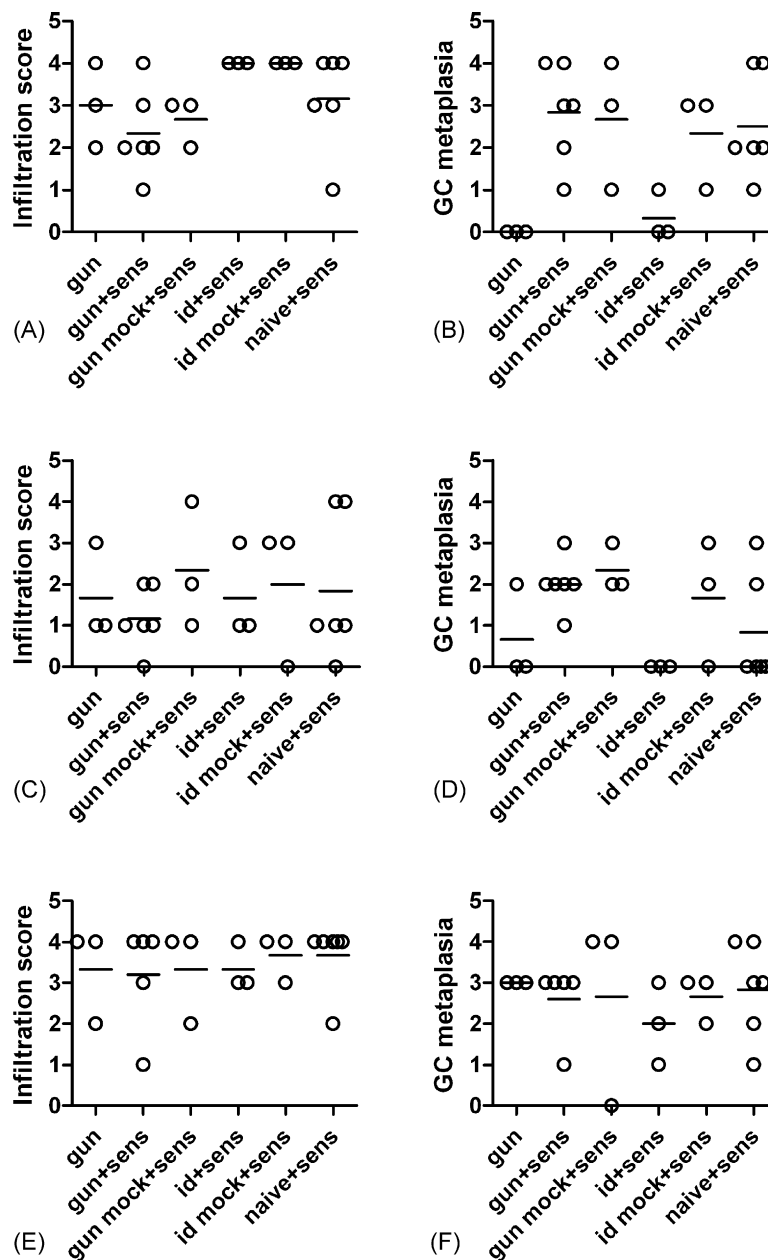


Fig. 5. Infiltration scores and goblet cell (GC) metaplasia scores of paraffin sections of lungs from mice pre-immunized with the different DNA vaccines or empty mock vector (mock) by either gene gun (gun) or i.d. needle injection (id). Indicated groups were subsequently sensitized (sens), and all groups were provoked by intranasal application of the respective antigen. Panels A and B: β -Gal; Panels C and D: Bet v 1; Panels E and F: Phl p 5. Data are shown as individual scores for each animal.

that targeting *Leishmania* antigen to Langerhans cells via gene gun bombardment can modulate a curative T_H1 type in naturally resistant C57/B6 mice into a T_H2 -biased immune response resulting in an aggravated course of disease similar to the phenotype usually observed in BALB/c mice (Weiss et al., 2006).

The results of the present study, performed with the model molecule β -Gal and two clinically highly relevant allergens, again confirmed the general T_H2 -bias of genetic immunization with gene gun (Alvarez et al., 2005; Feltquate et al., 1997; Oran and Robinson, 2003; Weiss et al., 2002) and also confirmed previous data showing that i.d. needle immunization induces effective T_H1 -biased immune responses that can modulate a sub-

sequent sensitization with all antigens tested so far (Bauer et al., 2003; Bauer et al., 2006; Gabler et al., 2006; Scheiblhofer et al., 2006).

In contradiction to previously published data (Ludwig-Portugall et al., 2004), gene gun application did not prevent from IgE expression, but even contributes to IgE production, possibly because of its strong humoral T_H2 -bias. Gene gun immunization itself can even promote lung eosinophilia when applying plasmids encoding a molecule with high allergenic potential, and this method rather deteriorates than alleviates lung pathology following sensitization. The T_H1 nature of the β -Gal molecule itself which, even with gene gun immunization, shifts the balance of

the immune response towards T_H1 misled to the impression of an anti-allergic potential of gene gun immunization (Ludwig-Portugall et al., 2004), which was clearly refutable with the real allergens Bet v 1 and Phl p 5.

The observed differences between the allergens and β -Gal indicate a possibly important role of the immunogenic/antigenic/allergenic nature of the molecule itself. Our current study shows that the nature of the antigen can have a substantial effect on the type of immune reaction elicited. β -Gal is a very strong antigen, unique in its ability to induce a T_H1-biased serological immune profile even after gene gun immunization. The inherent T_H1 capacity of β -Gal is also sufficient to suppress the induction of IgG1 after subsequent sensitization, but this effect does not extend to the suppression of IgE or to lung parameters. Bet v 1a represents an allergen with overall weak immunogenicity, whereas Phl p 5 is an exceptionally strong immunogen. However, both induced clearly T_H2-biased immune reactions when applied via gene gun, indicating that the inherent T_H1/T_H2 bias of an antigen is not directly linked to its immunogenicity, but to yet unknown factors.

5. Conclusions

In contrast to DNA-based desensitization with needle injection of allergen-encoding plasmids, which triggers protective T_H1 type immune reactions, gene gun immunization induces detrimental T_H2-biased immune responses. Our data clearly indicate that the use of the gene gun is contraindicated for protective or therapeutic approaches to treat allergic diseases.

Acknowledgements

This study was supported by the Austrian Science Foundation, project # S8813, L21-B13, and T133-B08.

References

- Alvarez, D., Harder, G., Fattouh, R., Sun, J., Goncharova, S., Stampfli, M.R., Coyle, A.J., Bramson, J.L., Jordana, M., 2005. Cutaneous antigen priming via gene gun leads to skin-selective Th2 immune-inflammatory responses. *J. Immunol.* 174, 1664–1674.
- Babiuk, S., Mookherjee, N., Pontarollo, R., Griebel, P., van Drunen Littel-van den Hurk, S., Hecker, R., Babiuk, L., 2004. TLR9^{-/-} and TLR9^{+/+} mice display similar immune responses to a DNA vaccine. *Immunology* 113, 114–120.
- Barry, M.A., Johnston, S.A., 1997. Biological features of genetic immunization. *Vaccine* 15, 788–791.
- Bauer, R., Himly, M., Dedic, A., Ferreira, F., Thalhamer, J., Hartl, A., 2003. Optimization of codon usage is required for effective genetic immunization against Art v 1, the major allergen of mugwort pollen. *Allergy* 58, 1003–1010.
- Bauer, R., Scheiblhofer, S., Kern, K., Gruber, C., Stepanoska, T., Thalhamer, T., Hauser-Kronberger, C., Alinger, B., Zoegg, T., Gabler, M., Ferreira, F., Hartl, A., Thalhamer, J., Weiss, R., 2006. Generation of hypoallergenic DNA vaccines by forced ubiquitination: preventive and therapeutic effects in a mouse model of allergy. *J. Allergy Clin. Immunol.* 118, 269–276.
- Brtko, J., Mostböck, S., Scheiblhofer, S., Hartl, A., Thalhamer, J., 1999. DNA immunization is associated with increased activity of type I iodothyronine 5'-deiodinase in mouse liver. *Mol. Cell Endocrinol.* 152, 85–89.
- Feltquate, D.M., Heaney, S., Webster, R.G., Robinson, H.L., 1997. Different T helper cell types and antibody isotypes generated by saline and gene gun DNA immunization. *J. Immunol.* 158, 2278–2284.
- Gabler, M., Scheiblhofer, S., Kern, K., Leitner, W.W., Stoecklinger, A., Hauser-Kronberger, C., Alinger, B., Lechner, B., Prinz, M., Vrtala, S., Valenta, R., Thalhamer, J., Weiss, R., 2006. Immunization with a low-dose replicon DNA vaccine encoding Phl p 5 effectively prevents from allergic sensitization. *J. Allergy Clin. Immunol.* 118, 734–741.
- Hartl, A., Kiesslich, J., Weiss, R., Bernhaupt, A., Mostböck, S., Scheiblhofer, S., Ebner, C., Ferreira, F., Thalhamer, J., 1999. Immune responses after immunization with plasmid DNA encoding Bet v 1, the major allergen of birch pollen. *J. Allergy Clin. Immunol.* 103, 107–113.
- Hartl, A., Weiss, R., Hochreiter, R., Scheiblhofer, S., Thalhamer, J., 2004. DNA vaccines for allergy treatment. *Methods* 32, 328–339.
- Hochreiter, R., Stepanoska, T., Ferreira, F., Valenta, R., Vrtala, S., Thalhamer, J., Hartl, A., 2003. Prevention of allergen-specific IgE production and suppression of an established Th2-type response by immunization with DNA encoding hypoallergenic allergen derivatives of Bet v 1, the major birch-pollen allergen. *Eur. J. Immunol.* 33, 1667–1676.
- Jilek, S., Barbey, C., Spertini, F., Corthesy, B., 2001. Antigen-independent suppression of the allergic immune response to bee venom phospholipase A(2) by DNA vaccination in CBA/J mice. *J. Immunol.* 166, 3612–3621.
- Kissenpfennig, A., Malissen, B., 2006. Langerhans cells-revisiting the paradigm using genetically engineered mice. *Trends Immunol.* 27, 132–139.
- Krieg, A.M., 2002. CpG motifs in bacterial DNA and their immune effects. *Annu. Rev. Immunol.* 20, 709–760.
- Ludwig-Portugall, I., Montermann, E., Kremer, A., Reske-Kunz, A.B., Sudowe, S., 2004. Prevention of long-term IgE antibody production by gene gun-mediated DNA vaccination. *J. Allergy Clin. Immunol.* 114, 951–957.
- Oran, A.E., Robinson, H.L., 2003. DNA vaccines, combining form of antigen and method of delivery to raise a spectrum of IFN-gamma and IL-4-producing CD4⁺ and CD8⁺ T cells. *J. Immunol.* 171, 1999–2005.
- Pertmer, T.M., Roberts, T.R., Haynes, J.R., 1996. Influenza virus nucleoprotein-specific immunoglobulin G subclass and cytokine responses elicited by DNA vaccination are dependent on the route of vector DNA delivery. *J. Virol.* 70, 6119–6125.
- Scheiblhofer, S., Weiss, R., Durnberger, H., Mostböck, S., Breitenbach, M., Livey, I., Thalhamer, J., 2003. A DNA vaccine encoding the outer surface protein C from *Borrelia burgdorferi* is able to induce protective immune responses. *Microbes Infect.* 5, 939–946.
- Scheiblhofer, S., Gabler, M., Leitner, W.W., Bauer, R., Zoegg, T., Ferreira, F., Thalhamer, J., Weiss, R., 2006. Inhibition of type I allergic responses with nanogram doses of replicon-based DNA vaccines. *Allergy* 61, 828–835.
- Spies, B., Hochrein, H., Vabulas, M., Huster, K., Busch, D.H., Schmitz, F., Heit, A., Wagner, H., 2003. Vaccination with plasmid DNA activates dendritic cells via Toll-like receptor 9 (TLR9) but functions in TLR9-deficient mice. *J. Immunol.* 171, 5908–5912.
- Strid, J., Hourihane, J., Kimber, I., Callard, R., Strobel, S., 2004. Disruption of the stratum corneum allows potent epicutaneous immunization with protein antigens resulting in a dominant systemic Th2 response. *Eur. J. Immunol.* 34, 2100–2109.
- Strid, J., Strobel, S., 2005. Skin barrier dysfunction and systemic sensitization to allergens through the skin. *Curr. Drug. Targets Inflamm. Allergy* 4, 531–541.
- Toda, M., Sato, H., Takebe, Y., Taniguchi, Y., Saito, S., Inouye, S., Takemori, T., Sakaguchi, M., 2000. Inhibition of immunoglobulin E response to Japanese cedar pollen allergen (Cry j 1) in mice by DNA immunization: different outcomes dependent on the plasmid DNA inoculation method. *Immunology* 99, 179–186.
- Vrtala, S., Sperr, W.R., Reimitzer, I., van Ree, R., Laffer, S., Muller, W.D., Valent, P., Lechner, K., Rumpold, H., Kraft, D., et al., 1993. cDNA cloning of a major allergen from timothy grass (*Phleum pratense*) pollen; characterization of the recombinant Phl pV allergen. *J. Immunol.* 151, 4773–4781.
- Wang, R., Doolan, D.L., Le, T.P., Hedstrom, R.C., Coonan, K.M., Charoenvit, Y., Jones, T.R., Hobart, P., Margalith, M., Ng, J., Weiss, W.R., Sedegah, M., de Taisne, C., Norman, J.A., Hoffman, S.L., 1998. Induction of antigen-specific

- cytotoxic T lymphocytes in humans by a malaria DNA vaccine. *Science* 282, 476–480.
- Weiss, R., Leitner, W.W., Scheiblhofer, S., Chen, D., Bernhaupt, A., Mostböck, S., Thalhamer, J., Lyon, J.A., 2000. Genetic vaccination against malaria infection by intradermal and epidermal injections of a plasmid containing the gene encoding the *Plasmodium berghei* circumsporozoite protein. *Infect. Immun.* 68, 5914–5919.
- Weiss, R., Scheiblhofer, S., Freund, J., Ferreira, F., Livey, I., Thalhamer, J., 2002. Gene gun bombardment with gold particles displays a particular Th2-promoting signal that overrules the Th1-inducing effect of immunostimulatory CpG motifs in DNA vaccines. *Vaccine* 20, 3148–3154.
- Weiss, R., Scheiblhofer, S., Gabler, M., Ferreira, F., Leitner, W.W., Thalhamer, J., 2006a. Is genetic vaccination against allergy possible? *Int. Arch. Allergy Immunol.* 139, 332–345.
- Weiss, R., Scheiblhofer, S., Thalhamer, J., Bickert, T., Richardt, U., Fleischer, B., Ritter, U., 2006. Epidermal inoculation of *Leishmania* antigen by gold bombardment results in a chronic form of leishmaniasis. *Vaccine*, doi:10.1016/j.vaccine.2006.07.044.
- Yoshida, A., Nagata, T., Uchijima, M., Higashi, T., Koide, Y., 2000. Advantage of gene gun-mediated over intramuscular inoculation of plasmid DNA vaccine in reproducible induction of specific immune responses. *Vaccine* 18, 1725–1729.