

Original article

Inhibition of type I allergic responses with nanogram doses of replicon-based DNA vaccines

Background: Allergic diseases have become a major public health problem in developed countries; yet, no reliable, safe and consistently effective treatment is available. DNA immunization has been shown to prevent and balance established allergic responses, however, the high dose of conventional DNA vaccines necessary for the induction of anti-allergic reactions and their poor immunogenicity in primates require the development of new allergy DNA vaccines. We evaluated protective and therapeutic effects of a Semliki-Forest Virus replicase-based vs a conventional DNA vaccine in BALB/c mice using the model allergen β -galactosidase.

Methods: Immunoglobulin (Ig)E suppression was determined by a basophil release assay as an *in vitro* correlate for allergen-specific crosslinking capacity of IgE reflecting the *in vivo* situation in an allergic individual. Th1 memory responses were measured by cytokine detection via enzyme-linked immunosorbent assay (ELISA) and enzyme-linked immunospot assay (ELISPOT).

Results: Nanogram amounts of a replicase-based vector triggered a Th1 response comparable with that achieved with the injection of 20 000-times more copies of a conventional DNA plasmid, and induced IgE suppression in both a protective and a therapeutic setting.

Conclusions: Replicase-based DNA vaccines fulfill the stringent criteria for an allergy DNA vaccine, i.e. low dose, strong Th1 immunogenicity and memory, lack of 'therapy-induced' IgE production and anaphylactic side effects. Moreover, by triggering apoptosis in transfected cells, their unique 'immunize and disappear' feature minimizes the hypothetical risks of genomic integration or induction of autoimmunity.

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At present, the only curative clinical approach against type I allergy is specific immunotherapy (SIT), which is performed by repeated injections of escalating doses of allergen extracts over months and even years. However, the immunological mechanisms underlying this therapy are still controversially discussed (1).

In recent years, DNA-based immunization emerged as an alternative approach yielding promising results against allergic immune responses in animal models. The anti-allergic properties of DNA vaccines can be clearly attributed to the induction of a predominant Th1 type immune response (reviewed in Refs 2–4).

Despite the encouraging data from numerous animal studies, results from preclinical as well as from clinical trials imply that DNA vaccines might be less immunogenic in primates and humans than anticipated (5–7). Furthermore, with respect to allergy treatment, the high amount of plasmid DNA necessary for intramuscular

(i.m.) or intradermal (i.d.) needle injection still remains the major shortcoming of DNA vaccines.

Recently, alphavirus replicase-based DNA vaccines have been designed, which proved to elicit cellular as well as humoral immune responses even at nanogram doses (8–10).

The 'anti-viral' response type characterized by low antibody production and strong Th1/CD8⁺ cell activation induced by these vectors render them an attractive tool for anti-allergic vaccination. In the present study, we used the β -galactosidase (β Gal) model system to demonstrate for the first time that replicase-based DNA vaccines at nanogram doses induce Th1-biased immune reactions that protect from Th2 responses as well as alleviate established allergic reactions.

Materials and methods

Plasmids

Semliki forest virus (SFV) based DNA replicons encoding β Gal (pSFV- β previously designated pRep-CMV-*LacZ*) (10) and the

Abbreviations: β Gal, β -galactosidase; i.d., intradermal; i.m., intramuscular; s.c., subcutaneous; SFV, Semliki forest virus; SIT, specific immunotherapy.

CMV promoter-based β Gal plasmid (pCMV- β , previously designated pCI- β Gal) (11) have been described. Plasmids were prepared using Endofree Plasmid Gigakits (Qiagen, Hilden, Germany). Endotoxin levels were <0.1 EU/ μ g of DNA as determined by Limulus amoebocyte assay.

In vitro transfection and analysis of antigen expression

Baby hamster kidney (BHK)-21 cells seeded at 1.6×10^4 cells/microplate well were cultured for 5 h and transfected with equimolar amounts of pCMV- β and pSFV- β at a total amount of 200, 150, 100, 50, or 25 ng DNA/well using LipofectAMINE PLUS (Invitrogen, Lofer, Austria). 64 h after transfection, cells were stained with 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-gal; Life Technologies Inc., Karlsruhe, Germany) at 37°C for 30 min according to standard protocols. Transfection efficacy was determined by counting cells positively stained for β Gal. Total amount of expressed β Gal in lysed cells was measured with a Galactostar kit (Tropix, Bedford, MA, USA).

Mice and immunization

Female WT BALB/c mice or *IFN- γ ^{-/-}* and *IL-12p40^{-/-}* BALB/c mice (6–8 weeks of age from Charles River, Sulzfeld, Germany) were immunized i.d. twice at an 8-week (experiment 1) or a 1-week (experiment 2) interval with plasmid DNA doses from 0.01 to 100 μ g of pCMV- β , pSFV- β , or empty mock vectors (pCMV, pSFV) as described (12) (Table 1). Control animals were left untreated until sensitization. Four weeks after the second immunization mice were challenged by four rounds of sensitization via subcutaneous (s.c.) injection of 5 μ g purified *Escherichia coli* β Gal protein (Sigma, Vienna, Austria) in sterile PBS with 100 μ l Al(OH)₃ (Serva, Heidelberg, Germany) in a total volume of 200 μ l into two spots on the back. The first two sensitization rounds were applied at a 1-week interval, after 2 weeks two more sensitizations at a 1-week interval were performed. Additional groups of five mice (pCMV- β 100 and pSFV- β 1 μ g) were sacrificed 2 weeks after the 2nd immunization to assess the prechallenge cytokine secretion status of splenocytes.

For evaluation of the therapeutic efficacy of pCMV- β and pSFV- β mice were presensitized by s.c. injection of recombinant β Gal/alum as described above on days 0, 14, and 21. Four weeks after the last sensitization mice received four therapeutic injections at weekly intervals of either 100 μ g pCMV- β (pCMV- β 100) or 1 μ g pSFV- β (pSFV- β 1) or empty mock vectors (pCMV 100, pSFV 1), or were left untreated (control) (Table 1). To assess the therapeutic effect, this procedure was followed by another sensitization and

later a second round of treatment and sensitization as shown in Fig. 5A.

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

Serology

Immunoglobulin G1 and IgG2a serum antibody levels were determined by a luminescence-based enzyme-linked immunosorbent assay (ELISA) as described (12).

Lymphocyte cultures

Culture of splenocytes was performed as described (13) (except that 1% mouse serum instead of 5% calf serum was used and cells were plated at a density of 2×10^5 cells per well). Cells were stimulated with recombinant β Gal at a concentration of 20 μ g/ml for 72 h. IL-5, IL-10, IFN- γ and TNF- α were measured in culture supernatants by Fluorescent Bead Immunoassay (Bender MedSystems, Vienna, Austria) on a Luminex 100 LabMap System (Luminex, Austin, TX, USA).

ELISPOT assay

Lymphocytes prepared as above were cultured in anti-IFN- γ or IL-5 (clones AN-18.17.24 and TRFK5, 4 μ g/ml) coated ELISPOT plates (Millipore, Vienna, Austria) with either recombinant β Gal (20 μ g/ml) or with 10 μ g/ml of the H-2L^d restricted peptide TPHPARIGL for 24 h as described for proliferation cultures. Cytokines were detected with biotinylated mAbs (2 μ g/ml, clones R4-6A2 and TRFK4) followed by streptavidin-HRP (1 : 1000, Becton Dickinson Pharmingen, Schwenchat, Austria). The assay was developed using 3-amino-9-ethyl-carbazole substrate (Acros, Geel, Belgium).

β -hexosaminidase release from rat basophil leukemia cells

As a functional read-out for immunoglobulin (Ig)E-mediated degranulation, a β -hexosaminidase release assay was performed using RBL-2H3 cells as described (13).

Statistical analysis

Statistical differences between means of individual sample groups were assessed using Sigma Plot 3.1. Unless otherwise stated, sample

Table 1. Experimental groups

Prevention					
Experiment 1		Experiment 2		Therapy	
Group and dosage (μ g)	Number of animals	Group and dosage (μ g)	Number of animals	Group and dosage (μ g)	Number of animals
pCMV- β 100	3	pCMV- β 1	4	pCMV- β 100	6
pCMV- β 10	3	pSFV- β 1	4	pSFV- β 1	6
pCMV- β 1	3	pSFV- β 0,1	4	pCMV 100	6
pSFV- β 100	3	pSFV- β 0,01	4	pSFV 1	6
pSFV- β 10	3	pCMV 100	3	Control	6
pSFV- β 1	3	pCMV 1	3		
Control	3	pSFV 1	3		
		Control	4		

groups passed normality and equal variance test and were analyzed via *t*-test. *P*-values of serially diluted serum samples from different groups that passed normality test were calculated by ANOVA.

Results

In vitro expression of β Gal

To assess the amount of translated protein, BHK-21 cells were transfected with either the conventional (pCMV- β) or the replicase-based (pSFV- β) DNA vaccine. Using equimolar amounts of the different constructs at various total DNA amounts, the average amount of β Gal expression per cell was calculated (Fig. 1). β Gal expression from pSFV- β was about 1.75-fold less compared with pCMV- β ($P < 0.001$).

Replicase-based DNA vaccines induce immune responses with a Th1-biased antibody profile at doses as low as 10 ng

We tested whether i.d. injection of low doses of replicase-based DNA vaccines encoding the model allergen β Gal still stimulates an antigen-specific Th1-biased immune response, a prerequisite for any DNA vaccine intended for allergy treatment. Furthermore, we investigated whether recruitment of Th1 cells by replicon-based vaccines has any influence on a subsequent Th2-sensitization with the allergen. For this purpose, conventional and replicase-based DNA vaccines were injected at different doses, and sera of individual animals obtained before and after sensitization were analyzed for specific IgG1 and IgG2a antibodies.

Both, the conventional and the replicase-based DNA vaccines induced IgG1 and IgG2a antibodies with

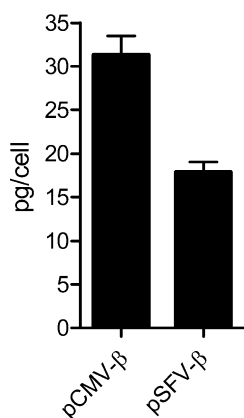


Figure 1. *In vitro* expression of β -galactosidase (β Gal). BHK-21 cells were transfected with equimolar amounts of pCMV- β or pSFV- β plasmids encoding β Gal. Total amount of expressed β Gal per transfected cell was measured 64 h after transfection. Data are shown as picogram per transfected cell. Results are expressed as a mean $\pm$ SEM of five transfections using different amounts of DNA. Results are representative of two independent experiments.

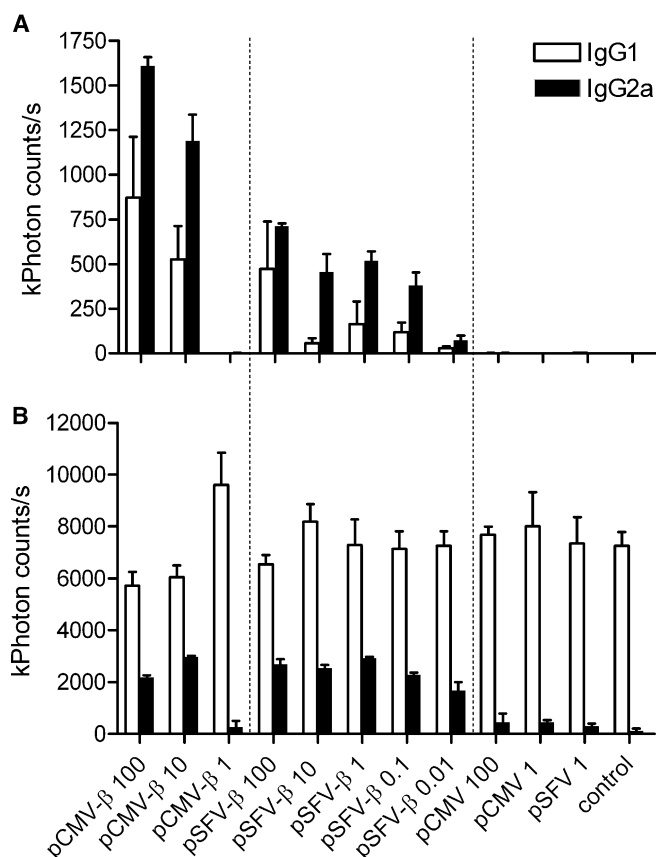


Figure 2. β -galactosidase (β Gal)-specific IgG subclass distribution after immunization with conventional and replicase-based DNA vaccines. (A), IgG1 and IgG2a levels after two immunizations with DNA vaccine. (B), Antibody levels after four subsequent sensitization rounds with β Gal protein. Data are shown as mean $\pm$ SEM of representative groups from prevention experiments 1 and 2 ($n = 3-4$; see Table 1 for details).

prevalence for the latter (Fig. 2A). While at doses of 10 and 100 μ g the conventional DNA vaccine (pCMV- β) induced higher antibody levels compared with all regimens of the replicase-based DNA vaccine (pSFV- β), 1 μ g of pCMV- β failed to induce a detectable antibody response. Mock vector groups (pCMV 100, pCMV 1, pSFV 1, i.e. empty vector at 100 or 1 μ g/mouse) showed no β Gal-specific antibodies. In contrast, as little as 10 ng pSFV- β induced detectable antibodies (IgG2a: $P < 0.05$; Mann-Whitney). The IgG2a titre was dose-independent in the range between 100 μ g and 100 ng of the replicon vaccine. Sensitization with recombinant protein in alum induced a marked increase of IgG1 antibodies in all groups, regardless of the prevaccination protocol. Prevaccination with the pSFV- β construct at any dose or with high doses of pCMV- β (10 and 100 μ g) resulted in a boost of IgG2a after sensitization. Prevaccination with 1–100 μ g of the empty vectors as well as 1 μ g of the conventional vaccine induced no increase of IgG2a during sensitization excluding unspecific CpG mediated effects.

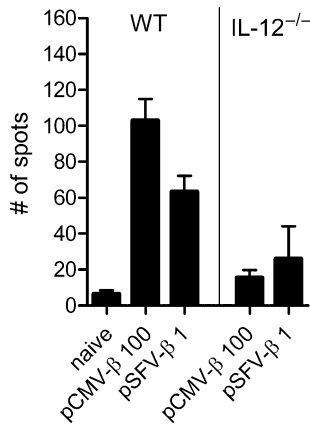


Figure 3. Interferon- γ ELISPOTs from splenocytes re-stimulated with β -galactosidase (β Gal) protein after immunization with conventional and replicase-based DNA vaccines. Data are shown as mean $\pm$ SEM of individual animals (WT: $n = 5$; KO: $n = 4$).

Replicase-based DNA vaccines stimulate the production of IFN- γ

For evaluation of the immune status prior to sensitization mice were immunized twice (1-week interval) with either 1 μ g pSFV- β or 100 μ g pCMV- β . Re-stimulation of splenocytes with protein yielded significant proliferation (pSFV- β 1: $P < 0.001$; pCMV- β 100: $P < 0.05$) compared with naïve controls with a higher stimulation index for the conventional DNA vaccine (pSFV- β 1: 2 ± 0.088 , pCMV- β 100: 3 ± 0.42). Both vaccines induced significant numbers of IFN- γ producing cells (pSFV- β 1: $P < 0.05$, Mann-Whitney; pCMV- β 100: $P < 0.005$), with higher numbers in the pCMV- β 100 group compared with the pSFV- β 1 group ($P < 0.05$) as determined by ELISPOT (Fig. 3). Analysis of culture supernatants by ELISA revealed corresponding levels of IFN- γ (pSFV- β 1: 0.71 ± 0.20 ng/ml, pCMV- β 100: 1.53 ± 0.60 ng/ml) whereas no significant production of IL-5, IL-10, or TNF- α was detectable in any of the groups. IFN- α production was partially independent from IL-12 for both vaccines as significant numbers of IFN- γ secreting cells could be detected in *IL-12p40*^{-/-} mice (Fig. 3). While IFN- γ knockout mice produced mainly IgG1 and only marginal IgG2a antibodies, IL-12-deficient mice responded with a Th1-specific serological profile (data not shown). The production of IFN- γ in *IL-12*^{-/-} mice has already been demonstrated by others and IL-18 has been proposed to substitute IL-12 in these animals (14).

Nanogram doses of replicase-based DNA vaccines protect from induction of IgE antibodies following sensitization with recombinant protein

Sensitization with recombinant protein in alum triggered a strong Th2 type response with high IgG1 titres and only marginal IgG2a (Fig. 2B). IgE antibodies were deter-

mined by a RBL cell release assay as an *in vitro* correlate for allergic responses. RBL cells bind mouse IgE via their Fc ϵ -receptors, and cross-linking with antigen causes β -hexosaminidase release. In addition to the highly sensitive detection of IgE, this method also provides information about the release-triggering capacity of the detected IgE antibodies.

Prevaccination with 100 and 10 μ g of the conventional DNA vaccine prevented IgE production ($P < 0.001$; Fig. 4A). Protection was completely lost with the 1 μ g dose ($P = 0.251$). In contrast, the replicase-based plasmid still provided 100% protection at the 1 μ g dose ($P < 0.001$). The protective efficacy decreased in the 100 and 10 ng dose group ($P < 0.05$), but even in the latter, IgE production was completely inhibited in one animal and reduced in two further animals ($n = 4$). Despite their reduced capacity to mount IFN- γ responses, *IL-12*^{-/-} mice could be protected from IgE induction

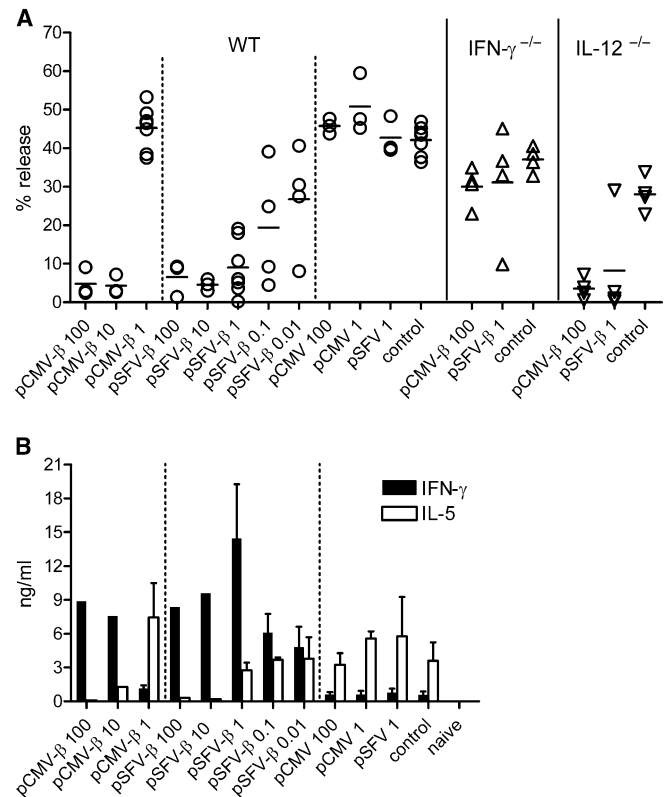


Figure 4. (A) β -galactosidase-specific IgE levels in sera of conventional and replicase-based DNA vaccinated mice after four rounds of sensitization with protein/alum. Data are shown as percentage specific release as determined by RBL assay. Values represent individual samples from prevention experiments 1 and 2 ($n = 3$ –8; see Table 1 for details). (B) Interferon- γ (IFN- γ) and IL-5 levels in supernatants of splenocytes of the respective animals re-stimulated with β Gal protein for 3 days. Data show representative groups from prevention experiments 1 and 2. Data from experiment 1 was obtained from pooled samples, individual samples from experiment 2 are shown as mean $\pm$ SEM ($n = 3$ –4; see Table 1 for details).

(pSFV- β 1: $P < 0.05$; pCMV- β 100: $P < 0.001$) while a total lack of IFN- γ abrogates the protective effect of both vaccine types.

Prevaccination with empty vectors was not protective, clearly excluding a significant role of antigen-independent processes induced by CpG motifs within the vaccine constructs.

Nanogram doses of replicase-based DNA vaccines induce a robust and antigen-specific Th1 type response with a sustained Th1 cytokine milieu

The DNA immunization with both, conventional and replicon-based vaccines, induced IgG2a antibodies (Fig. 2A), which were further boosted by a subsequent Th2-stimulating sensitization procedure (Fig. 2B). This indicates that Th1 cells recruited by the DNA vaccine have been further expanded by the sensitization. We tested this assumption by analyzing the key cytokines IFN- γ and IL-5 in culture supernatants of spleen cells from all groups (Fig. 4B). Sensitization without prevaccination induced significant production of IL-5 ($P < 0.05$; Mann–Whitney), comparable with the mock vector groups. Prevaccination with 100 and 10 μ g of the conventional DNA vaccine elicited strong IL-5 suppression as well as marked IFN- γ enhancement. However, in the 1 μ g DNA vaccine group IFN- γ was not significantly increased and IL-5 was not significantly reduced. These data correlate with the ability of the vaccine to protect from IgE production (Fig. 4A).

In contrast, prevaccination with DNA replicons induced strong IFN- γ production at all vaccine doses tested ($P < 0.05$; Mann–Whitney). IL-5 suppression was achieved with as little as 1 μ g of the vaccine. No significant production of IL-10 or TNF- α could be detected in any of the groups. Comparing the vaccination groups, the expression of IFN- γ appeared to strongly correlate ($r_s = 0.688$, $P < 0.001$, Spearman rank order correlation) with protection from a subsequent Th2 response regarding IgE production (see DNA replicon doses 10 ng to 10 μ g, Fig. 4A, B). The IFN- γ secretion pattern was confirmed by ELISPOT analysis (data not shown).

Replicase-based DNA vaccination down-regulates an established allergic immune response

A therapeutic experimental approach was employed to evaluate whether replicase-based DNA vaccines can affect an ongoing allergic response. Animals were alternately sensitized with recombinant protein and treated with DNA vaccines (Fig. 5A). This regimen reflects a stringent therapeutic situation in the presence of repeated allergen exposure. The 100 μ g dose of pCMV- β was compared with the 1 μ g dose of pSFV- β . Functional IgE production was measured by RBL-release assay after the 1st (data not shown) and 2nd re-challenge. Treatment

with both vaccines revealed significant suppression of the IgE response (Fig. 5C) at both time points ($P < 0.01$ and $P < 0.05$, ANOVA), while empty vector was ineffective ($P > 0.35$ and $P > 0.11$, ANOVA). Treatment also resulted in significant reduction of IgG1 levels at both time points (Fig. 5B) (P values ranging from <0.0001 to 0.014, ANOVA). IgG2a levels of the treated groups did not significantly differ from those of untreated mice (Fig. 5B). Two weeks after the last sensitization round cytokine secretion of protein or peptide stimulated splenocytes was analyzed. Although treatment with either DNA vaccine induced high levels of IFN- γ secreting cells (Fig. 5D), the amount of IL-5 secreting cells remained unaffected (Fig. 5E). In all groups IL-10 levels between 1.8 and 3.1 ng/ml could be detected in supernatants of splenocytes re-stimulated with β Gal protein. Increased IL-10 levels are a well-described effect of repeated s.c. protein application as seen in conventional SIT. No TNF- α was detected (data not shown).

Discussion

The DNA vaccines have emerged as a promising approach for prevention and treatment of type I allergy in the mouse model (4, 15–17). Induction of Th1 memory cells can inhibit the development of Th2 type responses associated with the production of IgE molecules as well as mitigate an established allergic response (18). The efficacy of DNA vaccines with respect to allergy treatment is independent of a humoral response (13) and cannot be attributed to ‘blocking’ antibodies as hypothesized for SIT (19). Also in contrast to SIT (20), published data (13) and the present results provide no indication for tolerance involved in the anti-allergic mechanisms of DNA vaccines.

However, several points regarding the use of DNA vaccines in humans have to be considered: (i) Studies in primates and humans revealed that milligram amounts of DNA are necessary to elicit strong immune responses (5, 7, 21). Although there is no evidence for adverse effects of DNA vaccination (22, 23), there are concerns regarding generation of autoimmunity or the potential for genomic integration when injecting large doses of plasmid DNA.

Replicase-based vaccines obviously solve the dose problem observed with many conventional DNA vaccines. In mice, approximately 1–10 μ g DNA injected into the muscle or skin seems to represent a ‘threshold’ for successful immunization with conventional DNA vaccines (24). However, even at 10 ng (which equals 20 000-fold less copies of pSFV- β compared with the standard dose of 100 μ g pCMV- β), the replicase-based DNA vaccine is still capable of recruiting Th1 cells, as demonstrated by the induction of Th1-memory (IFN- γ suppressing the generation of allergen-induced IgE antibodies and IL-5). The present data clearly indicate that IL-5 suppression is not necessary for full protection against

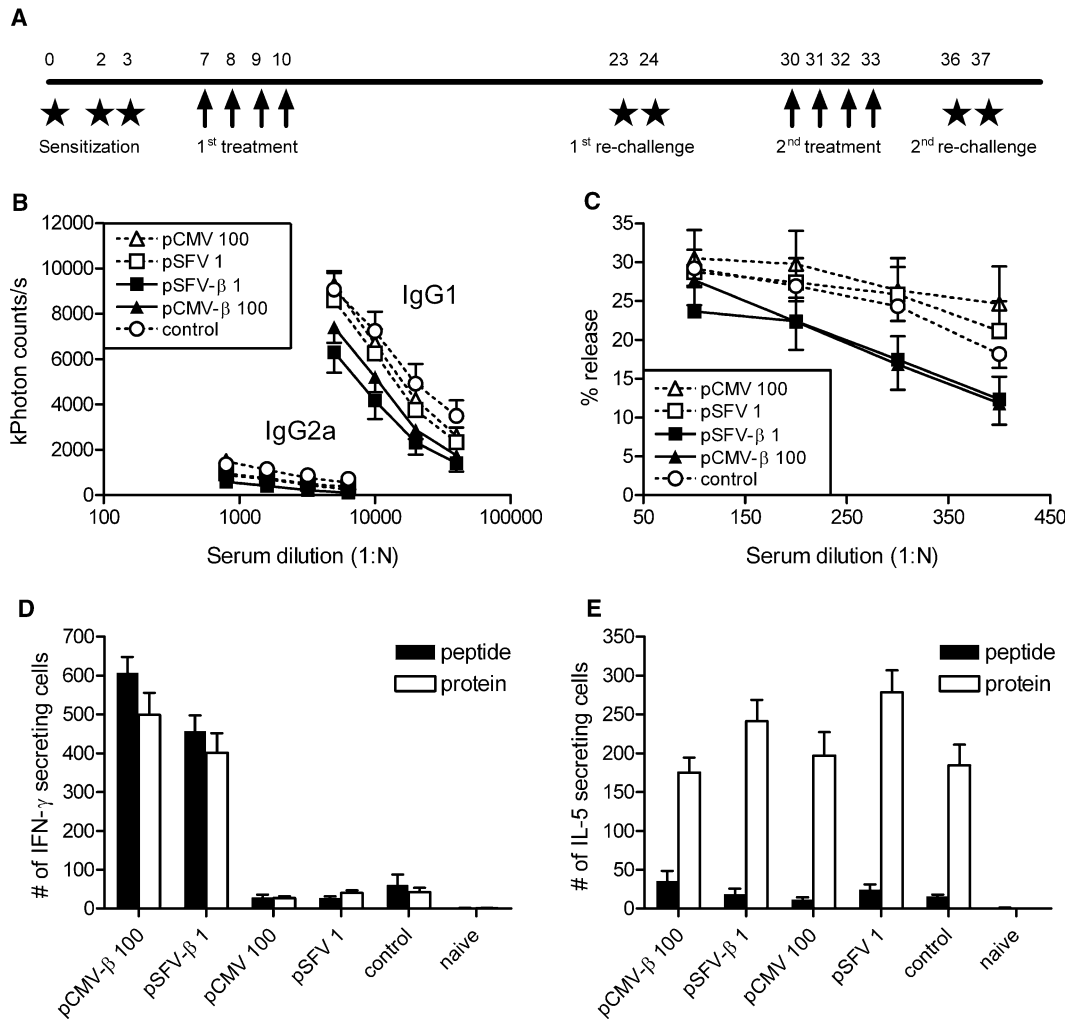


Figure 5. Treatment of sensitized animals with conventional and replicase-based DNA vaccines. (A) Treatment regimen (weeks): Injections with DNA vaccines are displayed as arrows, injections with recombinant protein/alum are displayed as stars. Effect of treatment on serum levels of β -galactosidase (β Gal)-specific IgG1 (B) and IgE (C) after the 2nd re-challenge. IgG2a levels (B) were not significantly different and not all individual data points are visible. Data are shown as mean $\pm$ SEM ($n = 6$). The number of IFN- γ (D) and IL-5 (E) secreting cells after treatment was determined following 24 h of *in vitro* re-stimulation with either β Gal protein or a class I peptide. Data are shown as mean $\pm$ SEM ($n = 6$).

Th2 sensitization since protection correlates with the increase in IFN- γ production.

(ii) Conventional DNA vaccines that displayed high immunogenicity in mice or other animals yielded disappointing results in primates and humans (5–7). One reason for this so-called ‘primate barrier’ may be functional differences or differences in the distribution of the CpG receptor TLR-9 between dendritic cell (DC) subsets in mice and humans (25), resulting in reduced primary danger signals of DNA vaccines in the latter. Thus, replicase-based DNA vaccines may not only offer an attractive solution for the dose problem of DNA vaccines but may also overcome the primate-specific impairment of immunogenicity, as they display additional danger signals different from CpG motifs – probably

dsRNA-mediated virus-like danger signals – involved in the initiation of Th1 immune responses by replicase-based vaccines. Additionally, transfection with replicase-based DNA vaccines results in apoptosis of transfected cells (9, 10, 26). Apoptotic cells can serve as activating targets for local DCs (27, 28). Moreover, pro-apoptotic signals delivered by alphaviral replicons may directly activate DCs (29). dsRNA intermediates generated during vaccine replication are probably not recognized in the cytosol of the transfected cells, but TLR3 recognition most likely takes place in CD8 α^+ DCs, that internalize apoptotic cells (reviewed in Ref. 30). Indeed, it has been shown that dsRNA can mature DCs that in turn secrete IL-12 and can expand allogeneic T cells into highly polarized Th1 cells with 70–90% of the cells secreting high amounts of

IFN- γ (31), and that this dsRNA induced secretion of IL-12 is MyD88 dependent, a key element in the TLR-3 receptor signaling pathway (32).

(iii) The third aspect regarding the use of anti-allergy DNA vaccines in humans is their safety profile. Anaphylactic reactions because of crosslinking of existing IgE antibodies on mast cells caused by injected protein represents the most severe side effect of SIT at the beginning of therapy. SIT itself can trigger 'therapy-induced' IgE molecules responsible for anaphylactic adverse effects during the course of immunotherapy.

Like conventional DNA vaccines, replicase-based DNA vaccines do not induce IgE, even after repeated injections (15, 18). In addition, they do not trigger anaphylactic reactions based on pre-existing IgE molecules crosslinked with the vaccine-derived allergen because of the slow release of minute amounts of protein. Despite the amplification of the antigen-encoding RNA, pSFV- β expressed even lower amounts of protein than equimolar levels of pCMV- β (Fig. 1) indicating that not the amount of antigen, but rather replicase-specific danger signals may be responsible for the high immunogenicity at low vaccine doses. Repli-

case-based plasmids also induce apoptotic elimination of transfected host cells, which may be the reason why, despite their excellent immunogenicity with respect to stimulation of CD4⁺ and CD8⁺ cells, replicase-based DNA vaccines stimulate weaker, or in some cases no antibody responses. This presents an additional safety feature as persistence of the vaccine (33–35) and long-term protein expression is sometimes considered a problem, particularly for allergy DNA vaccines, since translated antigen (allergen) represents a source for anaphylactic side effects.

In conclusion, replicase-based DNA vaccines for allergy treatment meet the stringent requirements for optimized allergy DNA vaccine approaches and production of 'next generation' DNA vaccines for future clinical application.

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