Gene Therapy (2002) 9, 147–156
© 2002 Nature Publishing Group All rights reserved 0969-7128/02 $25.00
www.nature.com/gt

RESEARCH ARTICLE

Efficacy of recombinant adenovirus as vector for allergen gene therapy in a mouse model of type I allergy

S Sudowe, E Montermann, J Steitz, T Tüting, J Knop and AB Reske-Kunz

Clinical Research Unit Allergology, Department of Dermatology, Johannes Gutenberg-University, Mainz, Germany

DNA-based immunization represents an attractive alternative approach to the current treatment of allergic diseases by specific immunotherapy with allergen extracts. In this study, we used a replication-deficient adenovirus vector (AdCMV), to examine the in vivo efficacy of preventive and therapeutic genetic immunization in a mouse model of type I allergy. Primary immunization with a recombinant adenovirus expressing the model antigen β-galactosidase (AdCMV-βgal) induced a Th1 immune response (predominance of IgG2a antibodies, high frequency of IFN-γ producing T cells) and large numbers of cytotoxic T lymphocytes. Prophylactic vaccination with AdCMV-βgal abolished the production of specific IgE following subsequent immunization with βgal-protein, and skewed the Th2-biased immune response to a Th1-orientated response. In contrast, therapeutic administration of AdCMV-βgal after priming with βgal-protein neither significantly inhibited ongoing IgE production nor modulated a manifest Th2 immune response. Thus, allergen gene transfer via recombinant adenovirus represents an effective method to establish protection against the development of allergic disorders, but does not qualify as a therapeutic tool to interfere with ongoing high IgE production.
Gene Therapy (2002) **9,** 147–156. DOI: 10.1038/sj/gt/3302625

Keywords: *allergen gene transfer; DNA immunization; recombinant adenovirus; β-galactosidase; IgE; type I allergy*

Introduction

Type I allergy represents a major health problem in industrialized countries with almost 20% of the population suffering from clinical disorders, such as allergic bronchial asthma or allergic rhino-conjunctivitis.[1] It is well established that the crucial event in the pathogenesis of allergic diseases is the increased and sustained production of allergen-specific immunoglobulin E antibodies (IgE Ab), which are bound by high-affinity Fcϵ receptors on mast cells and basophils. On cross-linking with allergen, they trigger the release of preformed molecules like histamine and of other proinflammatory mediators, which lead to the typical symptoms of type I hypersensitivities, including systemic reactions known as anaphylaxis. The only treatment of allergy that not only is antisymptomatic, but also affects the underlying processes of allergic diseases, is specific immunotherapy (SIT), ie the subcutaneous injection of gradually increasing doses of standardized allergen extracts into sensitized subjects, in order to achieve a state of tolerance to subsequent natural allergen exposure. Although SIT has been demonstrated to be effective in allergic reactions to some allergens like hymenoptera venom, house dust mite antigens and certain grass and tree pollens,[2–4] the application of SIT in hypersensitivities against a number of other allergens (eg latex, food allergens) is limited due to their high potential to induce side-effects like severe anaphylactic reactions.[5,6] The inconvenience of frequent dosing and the duration of therapy of several years are additional factors that restrict the use of SIT. With regard to safety and efficacy, research focuses on new approaches for the treatment of type I allergy.

DNA vaccination represents an attractive alternative to the current protein-based desensitization protocols. In different animal models, allergen genes have been transferred into the host using various routes, eg as 'naked' plasmid DNA by intramuscular or intradermal injection[7–15] or by ballistic transfection with the gene gun,[12,16] as plasmid DNA–polymer complexes by oral delivery,[17] or by means of recombinant mycobacteria as gene vector.[18] Prophylactic DNA immunization lead to inhibition of IgE Ab production[7,8,12–16] and anaphylactic hypersensitivity,[13,16] as well as decreased airway hyperresponsiveness.[8,14] Induction of $CD8^+$ T cells, as well as a shift towards a Th1 immune response contributed to this effect.[7,8,13,14,19,20] However, successful curative allergen gene transfer for the suppression of already established IgE Ab immune responses was reported only by three groups,[7,14,15] rendering it likely to improve therapeutic DNA vaccination by gene transfer with more efficient vehicles.

Replication-defective adenovirus (Ad) vectors have become a popular tool for gene transfer into mammalian cells and gained widespread application in a variety of preclinical disease models[21] and clinical gene therapy trials.[22] In previous reports,[23,24] it was shown in mice that

Correspondence: AB Reske-Kunz, Clinical Research Unit Allergology, Department of Dermatology, Johannes Gutenberg-University, Obere Zahlbacher Str 63, D-55131 Mainz, Germany
Received 17 July 2001; accepted 29 November 2001

the primary immune response after intraperitoneal administration of antigen-expressing Ad revealed two features that are known to play prominent roles in the control and counter-regulation of IgE Ab production: the induction of potent cell-mediated immunity in the form of $CD8^+$ cytotoxic T lymphocytes (CTL)[25] and the activation of $CD4^+$ T cells with Th1-polarized phenotype.[26] These characteristics lead us to speculate that recombinant Ad might represent an efficient vehicle for allergen gene transfer. We tested this notion in a mouse model of type I allergy where we took advantage of the fact that repeated immunization with low doses of protein antigen induces considerable IgE Ab formation[27] and thus resembles the main hallmark of type I allergy. In this setting, we evaluated the efficacy of preventive or therapeutic vaccination with replication-defective recombinant Ad expressing the model antigen β-galactosidase (βgal) under the control of the CMV promoter (AdCMV-βgal) to modulate the immune response following immunization with βgal-protein.

Results

Humoral immune response after primary immunization with AdCMV-βgal or βgal-protein

To analyze the primary immune response after administration of βgal-expressing recombinant Ad, BALB/c mice were immunized by a single intraperitoneal injection of 5×10^8 p.f.u AdCMV-βgal, a viral load, which was shown to be effectively immunogenic.[28,29] After 35 days, production of specific antibodies was characterized by a predominance of immunoglobulins of the IgG2a isotype over the IgG1 subclass (Figure 1a), indicating a Th1-orientated immune response. This notion was further confirmed by the finding that production of IgE Ab could not be demonstrated (Figure 1d). After application of recombinant control Ad encoding 'enhanced green fluorescent protein' EGFP (AdCMV-EGFP), no βgal-specific humoral immune response was detected (Figure 1b and d). In contrast, repeated intraperitoneal immunization of BALB/c mice with βgal-protein, adsorbed to the adjuvant aluminiumhydroxide (alum), elicited a strong Th2 immune response with considerably increased IgE Ab titers (Figure 1d). The specific humoral immune response after three consecutive injections of βgal-protein was dominated by IgG1 Ab which were produced in more than 30-fold excess over IgG2a Ab (Figure 1c), confirming the notion of a Th2-polarized immune response.

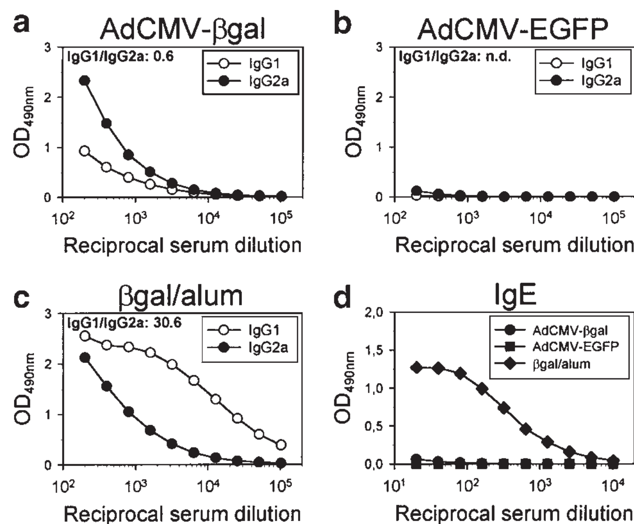

Figure 1 Production of βgal-specific antibodies after primary immunization. BALB/c mice (n = 4) were immunized by a single injection (day 0) of 5×10^8 p.f.u. recombinant adenovirus AdCMV-βgal (a, d) and AdCMV-EGFP (b, d), respectively, or after repeated immunization (days 0, 14 and 28) with 1 μg βgal-protein adsorbed to alum (c, d). On day 35 sera were recovered and βgal-specific IgG1 and IgG2a Ab (a–c), as well as IgE Ab (d) titers were determined by ELISA.

Prophylactic vaccination with AdCMV-βgal abrogates the production of IgE Ab following subsequent immunization with βgal-protein

To investigate whether βgal-specific IgE Ab production is modulated by preventive treatment with recombinant Ad, BALB/c mice were immunized with AdCMV-βgal or with the irrelevant vector AdCMV-EGFP. Six weeks later each group was divided and IgE Ab production was induced in one half, as well as in untreated control mice by immunization with βgal-protein in alum, while the other half received PBS in alum (Figure 2a). Prophylactic vaccination with AdCMV-βgal abrogated specific IgE Ab production induced by subsequent immunization with

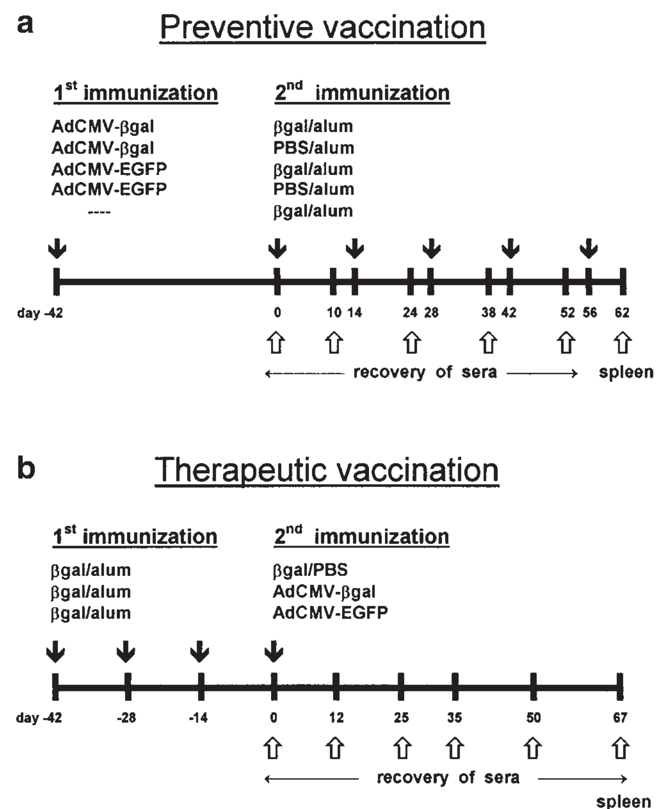

Figure 2 Experimental setting for preventive and therapeutic vaccination. Black arrows indicate time-points at which mice were vaccinated with recombinant adenovirus or immunized with βgal-protein or PBS adsorbed to alum. White arrows indicate time-points at which sera were recovered for determination of specific antibody titers. Spleen cells were prepared for further analysis 62 days after the beginning of immunization with βgal/alum in the preventive approach and 67 days after secondary immunization in the therapeutic approach.

βgal-protein (Figure 3). The amount of IgE Ab in the sera of these mice on day 52 of the immunization period was reduced to less than 1% in comparison with the IgE Ab content in immune sera from mice that had not been vaccinated with recombinant Ad ($P < 0.05$). The inhibition was antigen-specific, since application of the control vector AdCMV-EGFP before immunization with βgal-protein in fact enhanced IgE Ab secretion ($P < 0.05$). The almost complete absence of specific IgE Ab producing plasma cells in animals vaccinated with AdCMV-βgal was confirmed at the cellular level by enumeration of antibody secreting cells among splenocytes *ex vivo* with an antigen-specific ELISPOT assay (Table 1). The frequency of IgE Ab secreting cells was decreased to approximately 8% by preimmunization with AdCMV-βgal ($P < 0.05$) and was in the range of results obtained with animals which had been vaccinated with recombinant Ad, but were not challenged with βgal-protein.

Prophylactic vaccination with AdCMV-βgal shifts the immune response induced by immunization with βgal-protein from a Th2 phenotype to a Th1-like phenotype and generates large numbers of IFN-γ producing CD8⁺ effector T cells

On day 10 after immunization with βgal-protein production of specific IgG1 Ab, as well as IgG2a Ab was considerably stimulated in mice that had received an initial vaccination with AdCMV-βgal (Figure 4a and b; $P < 0.05$). At later time-points of the immunization period, the amount of IgG1 Ab in sera of these mice was significantly reduced while the content of IgG2a Ab was strongly enhanced as compared with sera of control mice that had not received preventive vaccination or had been immunized with the irrelevant vector AdCMV-EGFP ($P < 0.05$). As a consequence, the ratio of βgal-specific IgG1 *versus* IgG2a in AdCMV-βgal-vaccinated mice was strongly decreased compared with control mice: IgG1 and IgG2a Ab titers in immune sera of AdCMV-βgal vaccinated mice were almost equal, while in sera of control animals a preponderance of IgG1 Ab was found (Figure 4c). This observation was verified on the cellular basis by data obtained by enumeration of IgG1 and IgG2a Ab secreting B cells. In control mice without prevaccination or vaccinated with AdCMV-EGFP the number of IgG1 Ab producing plasma cells exceeded the number of IgG2a Ab forming plasma cells by a factor of 26 and 12, respectively, while in the experimental group of mice that had been vaccinated with AdCMV-βgal IgG1 and IgG2a Ab secreting B cells were detected in nearly the same frequency (Table 1). Thus the development of a Th2-biased antibody isotype profile as the result of immunization with βgal-protein in alum was prevented by protective application of AdCMV-βgal. Instead a humoral response with Th1-like phenotype was induced.

In accordance with these data the profile of cytokines produced by splenocytes after *in vitro* restimulation with βgal-protein, reflecting activation of MHC class II-restricted $CD4^+$ T cells, indicated a more Th1-orientated immune response in the experimental group of prevaccinated mice: the frequency of IL-4-producing T cells was markedly reduced to 32% of the level in control mice that had not received an immunization with recombinant adenovirus ($P < 0.05$) and which showed a clear-cut Th2 cytokine pattern, characterized by a preponderance of IL-4-secreting T cells (Figure 5) and documented by a ratio of IL-4 *versus* IFN-γ producing T cells of 8.9. Moreover, the release of the Th2 cytokines IL-5 and IL-10 into culture supernatants after *in vitro* stimulation of spleen cells with βgal for 3 days was significantly decreased to 20% and 50%, respectively, in cultures of splenocytes from AdCMV-βgal-vaccinated mice *versus* nonvaccinated mice (data not shown). In contrast, the expansion of IFN-γ producing T cells was slightly enhanced ($P = 0.21$), so that in consequence the ratio of IL-4 *versus* IFN-γ producing T cells was decreased to 1.7 for mice prevaccinated with AdCMV-βgal. However, the frequency of IFN-γ producing T cells was increased to a lower extent than was observed with spleen cell cultures of mice solely immunized with βgal-encoding recombinant Ad (Figure 5; $P < 0.05$). The cytokine profile in the latter group was characterized by a 10-fold predominance of IFN-γ over IL-4 producing T cells (ratio IL-4 *versus* IFN-γ: 0.1), supporting the notion of a Th1-biased immune response which had already been postulated on the basis of the IgG2a dominated antibody isotype pattern.

The numbers of βgal-specific $CD8^+$ effector T cells in mice of the different experimental groups were determined *ex vivo* using a modified ELISPOT assay, in which spleen cells or isolated $CD8^+$ T cells were stimulated by a $H\text{-}2L^d$-restricted nonapeptide of βgal to produce IFN-γ. The low frequency of specific class I-restricted T cells

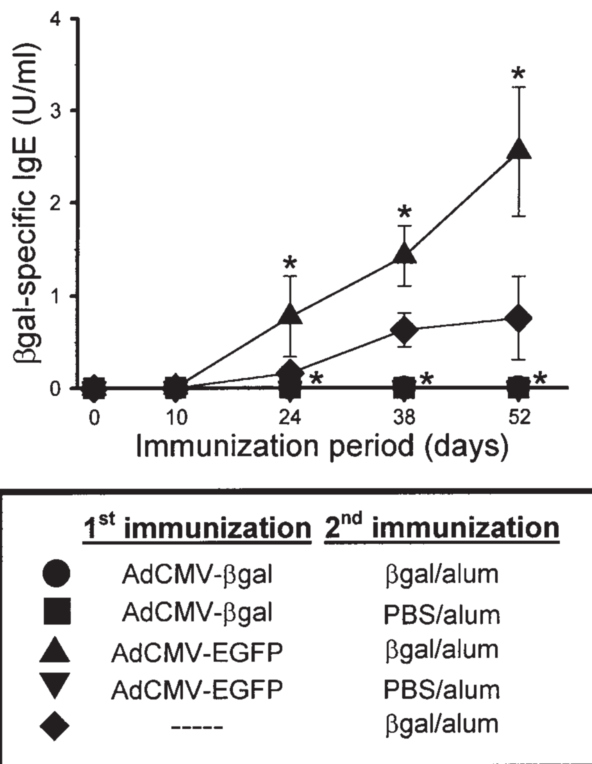

Figure 3 *Production of βgal-specific IgE Ab after preventive vaccination. BALB/c mice were vaccinated with 5×10^8 p.f.u. recombinant adenovirus AdCMV-βgal or AdCMV-EGFP and subsequently immunized with 1 μg βgal-protein adsorbed to alum or mock-injected with PBS in alum (for details see Figure 2a). On various days of the immunization period as indicated, sera were recovered and the content of βgal-specific IgE Ab was determined by ELISA. Data are presented as mean ± s.e.m. (n = 5). Statistically significant differences between βgal-immunized control mice and mice prevaccinated with recombinant adenovirus are indicated (*$P < 0.05$).*

Table 1 Enumeration of βgal-specific antibody producing plasma cells *ex vivo*

First immunization	*Second immunization*	*ASC per 10^7 spleen cells*			*Ratio IgG1/IgG2a*
		IgE	*IgG1*	*IgG2a*	
AdCMV-βgal	βgal/alum	5 ± 5[a,*]	639 ± 116*	725 ± 78*	0.9
AdCMV-βgal	PBS/alum	5 ± 5	59 ± 12	105 ± 15	0.6
AdCMV-EGFP	βgal/alum	117 ± 5*	812 ± 81*	88 ± 35	11.9
AdCMV-EGFP	PBS/alum	<5	<10	<5	—
—	βgal/alum	61 ± 14	3.099 ± 605	120 ± 24	25.8

Results were obtained 62 days after beginning of immunization with βgal-protein.
[a]Data are presented as mean of quadruplicates ± s.e.m.
*Indicates significant differences ($P < 0.05$) between βgal-immunized control mice and mice prevaccinated with recombinant adenovirus.
ASC, antibody secreting cells.

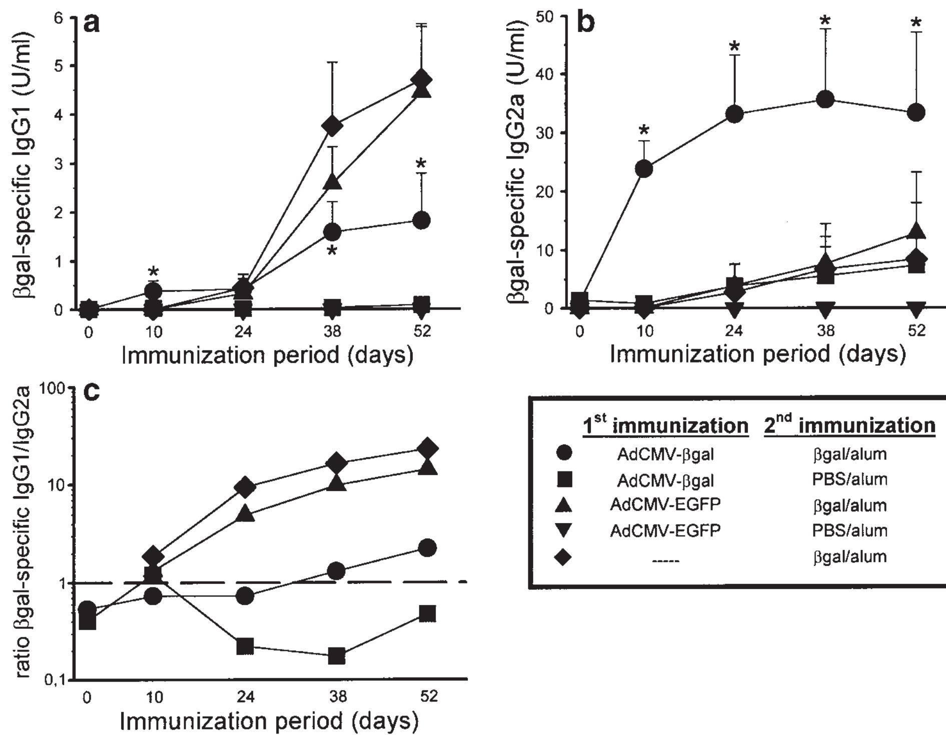

Figure 4 Production of βgal-specific IgG1 and IgG2a Ab after preventive vaccination. BALB/c mice were vaccinated with 5×10^8 p.f.u. recombinant adenovirus AdCMV-βgal or AdCMV-EGFP and subsequently immunized with 1 μg βgal-protein adsorbed to alum or mock-injected with PBS in alum (for details see Figure 2a). On various days of the immunization period as indicated, sera were recovered and the content of βgal-specific IgG1 Ab (a) and IgG2a Ab (b) was determined by ELISA, normalized to a standard reference serum and denoted as U/ml. Data are presented as mean ± s.e.m. (n = 5). Statistically significant differences between βgal-immunized control mice and mice prevaccinated with recombinant adenovirus are indicated ($P < 0.05$). The ratio of βgal-specific IgG1 versus IgG2a (c) is presented as the quotient of IgG1 Ab and IgG2a Ab titers determined by linear regression analysis in a parallel ELISA.*

in unvaccinated control mice after immunization with βgal-protein was increased by a factor of 9.3 (for spleen cells) to 8.9 (for $CD8^+$ T cells) in mice that had received a prophylactic vaccination with AdCMV-βgal (Table 2; $P < 0.05$). The induction of IFN-γ producing $CD8^+$ effector T cells in mice initially vaccinated with AdCMV-βgal was in a comparable range, irrespective of subsequent immunization with βgal-protein (Table 2).

Therapeutic vaccination with AdCMV-βgal does not suppress established IgE Ab immune responses induced by priming with βgal-protein

To investigate whether already established IgE Ab responses are affected by gene transfer via recombinant Ad, production of βgal-specific IgE in BALB/c mice was at first induced by three consecutive immunizations with protein in alum (Figure 2b). Subsequently, animals were injected either with βgal in PBS for exogenous antigen delivery, with AdCMV-βgal to induce endogenous production of βgal or with AdCMV-EGFP as control vector. Thereafter the content of specific antibodies in the immune sera was measured for a period of 67 days.

Inhibition of manifest IgE Ab production was not achieved by curative application of AdCMV-βgal. After an initial rise in IgE Ab formation immediately after gene transfer, titers of IgE Ab decreased over the immunization period to the same extent as in nonvaccinated animals or mice vaccinated with the control vector AdCMV-

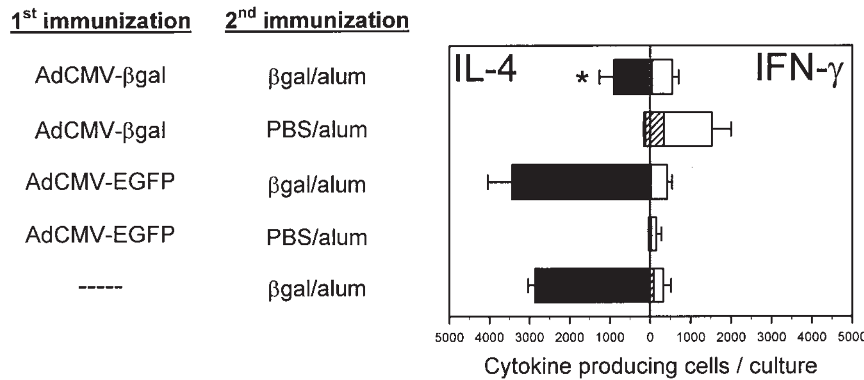

Figure 5 *Frequency of IL-4 and IFN-γ producing cells after preventive vaccination. BALB/c mice were vaccinated with 5 × 10⁸ p.f.u. recombinant adenovirus AdCMV-βgal or AdCMV-EGFP and subsequently immunized with 1 μg βgal-protein adsorbed to alum or mock-injected with PBS in alum (for details see Figure 2a). Sixty-two days after the beginning of immunization with βgal-protein, IL-4 (solid bars) and IFN-γ (open bars) producing cells among splenocytes, restimulated with recombinant βgal, were enumerated by ELISPOT assay. Numbers of unspecific IL-4 and IFN-γ secreting cells, determined from unstimulated cultures, are depicted as hatched bars. Data are presented as mean of triplicates ± s.e.m. Statistically significant differences between βgal-immunized control mice and mice prevaccinated with recombinant adenovirus are indicated (*P < 0.05).*

Table 2 Enumeration of βgal-specific IFN-γ producing CD8⁺ effector T cells

First immunization	*Second immunization*	*IFN-γ spots per 10^6 spleen cells*		*IFN-γ spots per 10^5 CD8⁺ T cells*	
		Medium	*+ $\beta gal_{876-884}$*	*Medium*	*+ $\beta gal_{876-884}$*
AdCMV-βgal	βgal/alum	11 ± 2[a]	234 ± 20*	15 ± 7	157 ± 11*
AdCMV-βgal	PBS/alum	49 ± 24	197 ± 21	23 ± 15	198 ± 12
AdCMV-EGFP	βgal/alum	3 ± 1	46 ± 5	ND	ND
AdCMV-EGFP	PBS/alum	1 ± 1	1 ± 1	ND	ND
—	βgal/alum	12 ± 4	36 ± 3	4 ± 2	20 ± 7

Results were obtained 62 days after beginning of immunization with βgal-protein.
[a]Data are presented as mean of triplicates ± s.e.m.
*Indicates significant differences ($P < 0.05$) between βgal-immunized control mice and mice prevaccinated with recombinant adenovirus.
ND, not determined.

EGFP (Figure 6a). Likewise, the distribution of IgG isotypes in the immune sera did not change significantly for 67 days: all three experimental groups showed a marked Th2 determined pattern, as shown by the predominant production of IgG1 (Figure 6b). The cytokine profile of splenocytes stimulated with βgal-protein *in vitro*, reflecting activation of MHC class II-restricted CD4⁺ T cells, also revealed no fundamental differences between control animals and mice vaccinated with AdCMV-βgal. The predominant expansion of IL-4 producing cells again indicated a Th2-biased immune response in all groups (Table 3). However, one major characteristic in mice vaccinated with AdCMV-βgal was noted: frequencies of IFN-γ producing CD8⁺ effector T cells, activated following incubation with the MHC class I-restricted βgal-derived peptide, were considerably raised by a factor of 28 as compared with mice which had not been vaccinated with recombinant Ad (Table 3; $P < 0.05$).

Discussion

In this report we show that vaccination of mice with replication-defective, recombinant AdCMV-βgal prevents the development of specific IgE responses following subsequent immunization with βgal-protein in alum, shifting the immune response from a Th2 to a Th1-like phenotype and generating large numbers of IFN-γ producing CD8⁺ effector T cells. Our finding that the primary immune response after a single administration of AdCMV-βgal was associated with the induction of Th1 lymphocytes confirms data previously obtained following infection of mice with βgal-expressing Ad[24,30] or other viral gene vectors like herpes simplex virus.[31] The Th1 bias was evident from the specific humoral immune response, which was characterized by the predominance of antibodies of the IgG2a isotype over the IgG1 subclass and a lack of IgE Ab production. On the other hand, analysis of the cytokine profile following activation of CD4⁺ T cells by *in vitro* culture with βgal-protein revealed a majority of IFN-γ producing T cells among spleen cells from AdCMV-βgal immunized mice. In addition, induction of large numbers of IFN-γ producing CD8⁺ effector T cells was observed. The question of whether effective priming of naive CD8⁺ T cells with specificity for the encoded protein requires the direct transfection of professional antigen presenting cells (APC) by recombinant Ad and thus the expression of endogenous antigens by APC represents a subject of controversial discussion.[32] Cross-priming by APC, ie the MHC class I-restricted presentation of peptides, derived from exogenously uptaken antigens,[33,34] is an alternative mechanism to explain the induction of CD8⁺ T cells. However, Ad-mediated transfection *ex vivo* of dendritic cells (DC), which are the critical APC for the initiation of cellular immune responses,

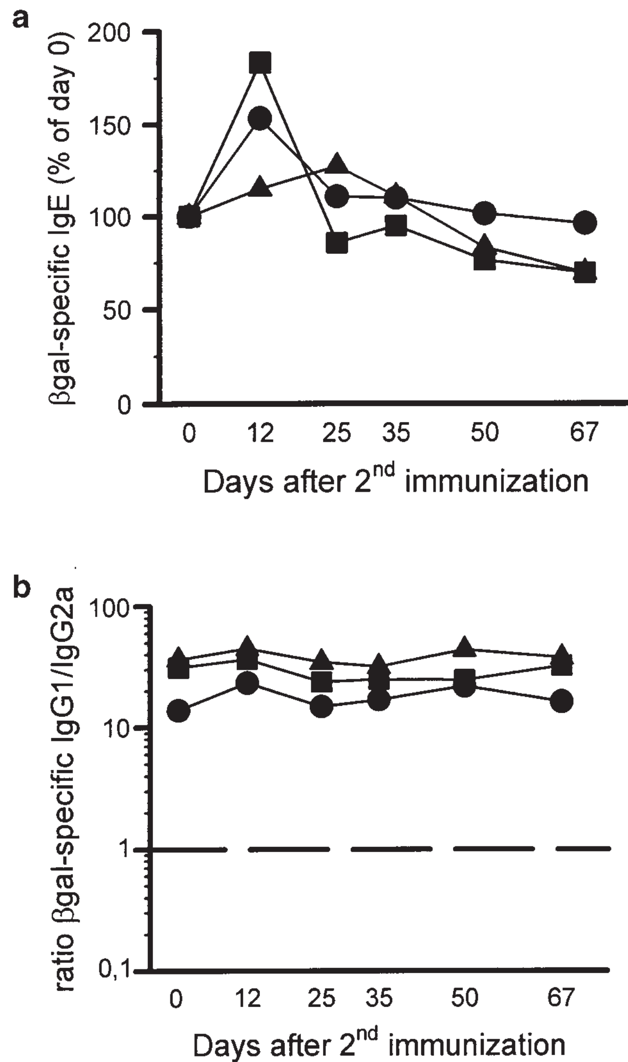

Figure 6 *Production of βgal-specific antibodies after therapeutic vaccination. BALB/c mice were primed with 1 μg βgal-protein adsorbed to alum and subsequently injected with βgal-protein in PBS (●) or vaccinated with 5×10^8 p.f.u. recombinant adenovirus AdCMV-βGal (■) or AdCMV-EGFP (▲) (for details see Figure 2b). On various days after secondary immunization as indicated sera were recovered and βgal-specific IgE, IgG1 and IgG2a Ab titers were measured by ELISA. (a) Mean IgE Ab titers (n = 4) are given as percentage of the mean Ab titer on day 0. (b) The ratio of βgal-specific IgG1 versus IgG2a is presented as the quotient of IgG1 Ab and IgG2a Ab titers determined by linear regression analysis in a parallel ELISA.*

has been reported to be an effective method for the generation of protective antitumor T cell immunity.[35–37]

In our mouse model of type I allergy repeated immunization with βgal-protein adsorbed to alum lead to the induction of a strong Th2 immune response, characterized by high IgG1 and IgE Ab titers in the immune sera and high numbers of IL-4 producing T cells. Preventive vaccination of mice with AdCMV-βgal inhibited production of IgE Ab very efficiently (>99%) and long-lasting (more than 3 months). In parallel, IgG1 Ab titers were reduced as well, whereas IgG2a Ab production was strongly enhanced, so that the quotient of specific IgG1 *versus* IgG2a was decreased considerably as compared with control mice. These findings suggest that in prevaccinated animals allergen-specific immune deviation from a Th2- to a Th1-orientated immune response had occurred. This conclusion was supported by analysis of cytokine production following activation of $CD4^+$ T cells by *in vitro* culture with βgal-protein. The frequency of spleen cells that secreted IL-4 after stimulation with βgal was dramatically reduced by protective immunization with Ad vector, whereas IFN-γ producing T cells had expanded. Since IL-4 is known as the promoter of IgE isotype switch, whereas in contrast IFN-γ acts as an antagonist,[38,39] it is tempting to speculate that the shift in the balance of T cell subsets away from Th2-type (producing particularly IL-4) in favor of a Th1-type T lymphocyte response (with preferential production of IFN-γ) contributed to the inhibition of IgE Ab production observed in the vaccinated mice. Alternatively suppression of IgE Ab might be mediated by the observed induction of IFN-γ secreting $CD8^+$ effector T cells in AdCMV-βgal immunized mice, since it was previously demonstrated that $CD8^+$ CTL might possess inibitory capacity for IgE Ab production.[25] Our data do not resolve which of either explanation is more likely or whether both subpopulations of lymphocytes acted in a co-ordinated way. However, since the suppressive effect could be transferred with whole spleen cells from AdCMV-βgal immunized mice to naive recipients (data not shown), transfer experiments with isolated $CD4^+$ and $CD8^+$ T cells will probably solve this question.

Stämpfli *et al*[40] reported that intramuscular administration of replication-deficient Ad without transgene encoding sequence before intraperitoneal sensitization and subsequent aerosol challenge with ovalbumin (OVA) resulted in a reduction of local eosinophilic inflammation in the lung, which was accompanied by a shift from OVA-specific IL-4 production by spleen cells of sensit-

Table 3 Pattern of cytokine production and induction of IFN-γ producing $CD8^+$ effector T cells in mice of different experimental groups

First immunization	*Second immunization*	*Cytokine producing spleen cells (stimulation index)*[a]		*IFN-γ spots per 10^6 spleen cells*	
		IL-4	*IFN-γ*	*Medium*	*+ $\beta gal_{876–884}$*
βgal/alum	βgal/PBS	10	1	32 ± 8[b]	31 ± 8
βgal/alum	AdCMV-βgal	11	1	17 ± 8	860 ± 125
βgal/alum	AdCMV-EGFP	24	2	12 ± 5	22 ± 8

Results were obtained 67 days after beginning of secondary immunization.
[a]Stimulation index is defined as the quotient of the number of the respective cytokine producing cells in βgal-stimulated *versus* unstimulated spleen cell cultures.
[b]Data are presented as mean of quadruplicates ± s.e.m.

ized, PBS-treated mice to specific IFN-γ production by splenocytes of sensitized, Ad-infected mice. In our model of type I allergy, we did not observe similar changes in the lymphokine profile as the result of immunization with irrelevant Ad. Mice, prevaccinated with recombinant Ad expressing the antigen EGFP, exhibited the same IL-4 dominated cytokine pattern after immunization with βgal-protein as did untreated control mice. This discrepancy may be explained by the fact that in the allergic airway inflammation model the sensitization phase took place as early as 16 h after infection with Ad. At this early time, the acute anti-adenoviral immune response was in full progress, generating IFN-γ secreting effector cells which created a local cytokine microenvironment which favored the development of OVA-specific Th1 cells at the site of allergen exposure as a bystander effect. On the contrary, immunization with βgal-protein in our system started 42 days after Ad infection, when the inflammatory, anti-adenoviral reaction had declined, so that any unspecific effects on the βgal-induced immune response could be ruled out. Our finding of high IgE Ab levels in the sera of the sensitized mice, irrespective of previous immunization with irrelevant Ad, is in accordance with the study of Stämpfli *et al*.[40]

Suppression of an ongoing IgE Ab response represents a major challenge. Successful inhibition seems to depend on the immunization protocol used for priming mice. Suzuki *et al*[41] demonstrated that intranasal instillation of Ad after two sensitizing intraperitoneal injections with OVA lead to decreased IgE Ab production. In contrast, after subsequent aerosol challenge the IgE Ab titers were not significantly different from those of untreated control mice, although profound effects on eosinophil infiltration in the airways and local cytokine production were observed. By preventive DNA immunization with naked plasmid DNA IgE Ab production was inhibited in a number of studies,[7,8,12–16] whereas effective therapeutic treatment of pre-existing IgE Ab responses with plasmid DNA was achieved by three groups only.[7,14,15] The reason for successful inhibition of pre-existing IgE responses may have been that Raz *et al*[7] used a relatively weak immunization protocol for priming, while Maecker *et al*[14] vaccinated with an allergen-IL-18 fusion DNA construct to enhance the efficiency of vaccination. Jilek *et al*,[15] on the other hand, showed significant reduction of specific IgE production in sensitized mice with plasmid vectors lacking any transgene encoding sequence. Likewise, in a related approach to modulate IgE-mediated allergic reactions, namely the vaccination with mixtures or conjugates of immunostimulatory CpG oligodeoxynucleotides and allergen, attempts to down-regulate IgE Ab production lead to contrasting results.[17,42–45] For our model of type I allergy we chose an immunization protocol with three repeated injections of βgal-protein in alum which induced strong and sustained IgE Ab secretion and thus resembled closely the clinical situation in allergic patients. We were not successful in manipulating the Th2 immune response by a single therapeutic administration of recombinant Ad, neither in terms of inhibition of IgE Ab production nor in skewing the cytokine release profile to a Th1 phenotype. The only significant difference between the experimental group of animals treated with AdCMV-βgal and the two control groups was the induction of considerable numbers of βgal-specific IFN-γ producing $CD8^+$ effector T cells, but interestingly these cells had no inhibitory capacity for production of IgE Ab.

The insufficient efficacy of AdCMV-βgal to inhibit ongoing IgE Ab production might be explained by only transient expression of the encoded antigen which is due to the fact that Ad vectors are highly immunogenic. The elicitation of MHC class I-restricted $CD8^+$ CTL responses causes the destruction of the virus-infected cells and therefore leads to the rapid loss of transgene expression.[46,47] An additional immunologic limitation is the development of Ad-specific neutralizing antibodies following primary infection, which hinder repeated administration of the vector.[48,49] Consequently the transgene-specific immune reaction after a single therapeutic vaccination with AdCMV-βgal is probably not potent enough to convert the manifest Th2 immune response, induced by three consecutive immunizations with βgal-protein, and to suppress IgE Ab production. The use of Ad vectors with different serotypes[50] or modified Ad with reduced expression of viral genes[51] might help to circumvent the problems arising from the host–immune response against Ad and Ad-infected cells, respectively, and therefore might increase the efficiency of successful gene transfer. An alternative approach, although labor-intensive and expensive, to Ad-mediated transfection *in vivo* is transduction of DC *in vitro* with βgal-encoding recombinant Ad and the subsequent administration of modified DC into the host,[52] as has been successfully employed in a melanoma animal model with DC transduced by recombinant Ad expressing a clinically relevant tumor antigen.[37,53]

Materials and methods

Mice and immunization protocol for induction of IgE Ab production

Six- to 8-week-old female BALB/c mice, bred in the animal facilities in Mainz, were immunized at intervals of 2 weeks by intraperitoneal injection of 1 μg recombinant βgal (Sigma-Aldrich, Deisenhofen, Germany), dissolved in 100 μl PBS and adsorbed to an equal volume of Imject Alum (Pierce, Rockford, IL, USA) as adjuvant, according to the manufacturer's instructions. At various times of the immunization period, mice were bled from the retroorbital plexus and sera were analyzed for βgal-specific antibodies. At the end of the experiment, mice were killed by cervical dislocation, spleens were removed and single cell suspensions were prepared by teasing the tissue apart. Erythrocytes were lysed by incubating the cells for 1 min in lysis buffer (155 mM NH_4Cl, 10 mM $KHCO_3$, 100 μM EDTA-disodium, pH 7.4). After extensive washing splenocytes were passed through a 100-μm cell strainer (Becton Dickinson Labware, Franklin Lakes, NJ, USA) to remove cell debris and were subsequently analyzed for cytokine production.

Recombinant adenoviruses and vaccination protocols

Recombinant adenovirus encoding *E. coli* βgal (AdCMV-βgal) was kindly provided by Dr A Gambotto (University of Pittsburgh, PA, USA) and propagated on 293 cells. Recombinant adenovirus expressing EGFP (AdCMV-EGFP) was constructed through Cre-lox recombination[54] and propagated on 293 cells. Adenoviruses were purified by cesium chloride gradient centrifugation and subsequent dialysis according to standard protocols.[55]

AdCMV-βgal or control vector AdCMV-EGFP (5×10^8 p.f.u. in a volume of 200 μl PBS) were injected intraperitoneally once, 42 days before immunization with βgal-protein in case of preventive vaccination (Figure 2a) or 14 days after the third immunization with βgal-protein for therapeutic vaccination (Figure 2b). Representative data of a set of two to four independent experiments are shown.

ELISA for determination of βgal-specific antibodies in serum

βGal-specific antibodies of different isotypes (IgG1, IgG2a, IgE) were measured using an antigen capture ELISA as reported[56] with modifications. All reagents except the blocking buffer were used in volumes of 100 μl/well. The incubation time for all reagents was 1 h at room temperature unless indicated otherwise. After each incubation step, wells were washed three times with PBS (pH 7.2) containing 0.1% Tween 20 (Sigma-Aldrich). MaxiSorp ELISA plates (Nunc, Wiesbaden, Germany) were incubated overnight at 4°C with recombinant βgal (5 μg/ml) in coating buffer (0.1 M $NaHCO_3$, pH 8.2). After coating, 200 μl PBS containing 1% BSA (PBS-B) (Sigma-Aldrich) were added to each well to block residual protein binding sites. In the next step, serial dilutions of serum samples in PBS-B were added. Subsequently, βgal-specific IgG1 and IgG2a were detected by incubating the plates with biotinylated goat anti-mouse IgG1 and goat anti-mouse IgG2a, respectively (0.1 μg/ml in PBS-B; Southern Biotechnology, Birmingham, AL, USA). βGal-specific IgE Ab were detected using purified rat anti-mouse IgE mAb EM95.3[57] (1:1000 in PBS-B), followed by biotinylated polyclonal mouse anti-rat IgG (1 μg/ml in PBS-B; Dianova, Hamburg, Germany). Biotinylated antibodies were detected using ExtrAvidin-peroxidase conjugate (1:1000 in PBS-B; Sigma-Aldrich) followed by addition of *o*-phenylenediamine (1 mg/ml; Sigma-Aldrich) and 30% H_2O_2 (1 μl/ml; Merck, Darmstadt, Germany) in substrate buffer (0.2 M NaH_2PO_4, 0.1 M sodium citrate, pH 5.0). The reaction was stopped by addition of 1 M H_2SO_4. The absorption was measured in a microplate reader E_{max} (MWG-Biotech, Ebersberg, Germany) at 490 nm.

Two controls were included for each serum sample: the blank control, to which no serum was added, was substracted from every absorbance value, and the serum control, in which serum was incubated in wells that had not been coated with the antigen, to detect and evaluate unspecific binding activity. The antibody titer was defined as the reciprocal serum dilution, yielding an absorbance value of OD = 0.2 after linear regression analysis. Serum samples were normalized using a standard reference serum, obtained and pooled from mice immunized five times with βgal in alum. The antibody titer of the standard serum was defined as 1 U/ml. The ratio of specific IgG1 *versus* IgG2a was expressed as the quotient of IgG1 and IgG2a antibody titers determined in a parallel ELISA.

ELISPOT assay for enumeration of Ig producing B cells

The frequency of specific Ig producing B cells among spleen cells was determined *ex vivo* by ELISPOT assay with slight modifications as described.[27] All reagents except the blocking buffer and the substrate solution were used in volumes of 100 μl/well. After each incubation step, wells were washed three times with PBS (pH 7.2) containing 0.1% Tween 20 (Sigma-Aldrich). Nylon filter (Biodyne B membrane)-bottomed 96-well Silent Screen plates (Nunc) were incubated overnight at 4°C with βgal (20 μg/ml in coating buffer). Then the filters were blocked with 200 μl of 10% (w/v) non-fat dry milk in Tris-buffered saline (20 mM Tris, 137 mM NaCl, 1 M HCl, pH 7.6). Appropriate numbers of freshly isolated spleen cells, suspended in culture medium (IMDM supplemented with 10% fetal calf serum (PAN Biotech, Aidenbach, Germany), 50 μM 2-mercaptoethanol (Carl Roth, Karlsruhe, Germany), 2 mM L-glutamine (Carl Roth), 100 μg/ml penicillin and 100 IU/ml streptomycin (Life Technologies, Karlsruhe, Germany)), were added, serially diluted and incubated for 4 h at 37°C in a humidified 10% CO_2 incubator. As in the ELISA protocol, wells were overlaid with rat anti-mouse IgE mAb EM95.3 and biotinylated mouse anti-rat IgG for the determination of IgE-producing cells, or with biotinylated goat anti-mouse IgG1 and IgG2a, respectively, for the detection of antibody producing cells of the respective IgG subclass, followed by an incubation step with ExtrAvidin-peroxidase conjugate. The incubation time for all reagents was 1 h at 37°C. The substrate 3-amino-9-ethylcarbazole (AEC; Sigma-Aldrich) was prepared by dissolving one tablet in 2.5 ml dimethylformamide and subsequently adding 50 ml 0.1 M sodium acetate buffer (pH 5.0). To remove aggregates, the solution was filtered through a 0.2-μm membrane (Schleicher and Schuell, Dassel, Germany). Before use, 30% H_2O_2 (0.5 μl/ml) was added, and 50 μl of substrate solution was placed into the wells. After 1 to 5 min at room temperature, macroscopic red spots became visible. The substrate solution was removed by flicking the plates, and membranes were rinsed with water. After drying, the spots were counted under a stereomicroscope (Olympus, Hamburg, Germany) using 10- to 20-fold magnification.

ELISPOT assay for enumeration of IFN-γ producing $CD8^+$ effector T cells

The frequency of $CD8^+$ effector T cells among spleen cells recognizing the H-2L^d-binding βgal-derived nonamer peptide TPHPARIGL (βgal$_{876-884}$)[58] was determined by a modified ELISPOT assay measuring secretion of IFN-γ.[59] Briefly, 96-well MultiScreen microtiter plates (Millipore, Bedford, MA, USA) were coated overnight at 4°C with anti-IFN-γ mAb (clone R4-6A2; 10 μg/ml in PBS; PharMingen, San Diego, CA, USA) and then blocked for 1 h at 37°C with culture medium. Subsequently, 10^6 freshly isolated spleen cells or co-cultures of 10^5 $CD8^+$ T cells, purified by magnetic separation with the MiniMACS system[60] (Miltenyi Biotec, Bergisch-Gladbach, Germany), and 10^6 irradiated spleen cells from naive BALB/c mice as APC, were incubated in culture medium, containing 1 μg/ml βgal$_{876-884}$ peptide and 250 U/ml recombinant IL-2 (Cetus, Emeryville, CA, USA), for 22 h at 37°C in a humidified 10% CO_2 incubator. Then wells were overlaid with biotinylated anti-IFN-γ mAb (clone XMG1.2; 10 μg/ml in PBS-B; PharMingen) and thereafter ExtrAvidin-peroxidase conjugate (1:1000 in PBS-B) was added. The incubation time for all reagents was 1 h at 37°C. The ELISPOT assay was developed by addition of the substrate AEC as described above. The number of specific $CD8^+$ effector T cells was calculated as the difference of the

number of spots in cultures incubated with and without the βgal-derived peptide, respectively.

ELISPOT assay for enumeration of cytokine producing cells

Freshly isolated spleen cells (5×10^6/well) were cultured on 24-well tissue culture plates (Corning Costar, Bodenheim, Germany) in a volume of 1 ml culture medium with or without recombinant βgal (25 μg/ml) for 72 h at 37°C in a humidified 10% CO_2 incubator. Then cells were resuspended and appropriate aliquots of suspensions were transferred on to 96-well MultiScreen microtiter plates (Millipore), which had been coated overnight at 4°C with anti-IFN-γ mAb (see above) or anti-IL-4 mAb (clone 11B11; 10 μg/ml in PBS; PharMingen) and subsequently had been blocked for 1 h with culture medium. Splenocytes were cultured for another 24 h at 37°C. Afterwards wells were overlaid with biotinylated anti-IFN-γ mAb (see above) and biotinylated anti-IL-4 mAb (clone BVD6-24G2; 10 μg/ml in PBS-B; PharMingen), respectively. Biotinylated antibodies were detected by incubating the plates with ExtrAvidin-peroxidase conjugate (1:1000 in PBS-B). The incubation time for all reagents was 1 h at 37°C. ELISPOT assay was developed by addition of the substrate AEC as described above. The ELISPOT data were used to calculate the stimulation index, which was defined as the quotient of the number of cytokine producing cells in spleen cell cultures restimulated with βgal *versus* unstimulated cultures.

Statistical analysis of data

Statistical evaluation of the experimental data was performed by Student's *t* test using SigmaPlot software. A value of $P < 0.05$ was considered statistically significant.

Acknowledgements

We thank Dr Z Eshhar (Weizmann Institute of Science, Rehovot, Israel) for generously providing the EM95.3 hybridoma cell line and Dr A Gambotto (University of Pittsburgh, PA, USA) for the kind gift of AdCMV-βgal. This work was supported by the Deutsche Forschungsgemeinschaft, SFB548 and SFB432.

References

1 Cookson WO, Moffatt MF. Asthma: an epidemic in the absence of infection? *Science* 1997; **275**: 41–42.
2 Lerch E, Müller UR. Long-term protection after stopping venom immunotherapy: results of re-stings in 200 patients. *J Allergy Clin Immunol* 1998; **101**: 606–612.
3 Durham SR *et al*. Long-term clinical efficacy of grass-pollen immunotherapy. *N Engl J Med* 1999; **341**: 468–475.
4 Abramson M, Puy R, Weiner J. Immunotherapy in asthma: an updated systematic review. *Allergy* 1999; **54**: 1022–1041.
5 Greineder DK. Risk management in allergen immunotherapy. *J Allergy Clin Immunol* 1996; **98**: S330–S334.
6 Weber RW. Immunotherapy with allergens. *JAMA* 1997; **278**: 1881–1887.
7 Raz E *et al*. Preferential induction of a Th1 immune response and inhibition of specific IgE antibody formation by plasmid DNA immunization. *Proc Natl Acad Sci USA* 1996; **93**: 5141–5145.
8 Hsu CH *et al*. Immunoprophylaxis of allergen-induced immunoglobulin E synthesis and airway hyperresponsiveness *in vivo* by genetic immunization. *Nat Med* 1996; **2**: 540–544.
9 Slater JE, Paupore E, Zhang YT, Colberg-Poley AM. The latex allergen Hev b 5 transcript is widely distributed after subcutaneous injection in BALB/c mice of its DNA vaccine. *J Allergy Clin Immunol* 1998; **102**: 469–475.
10 Hartl A *et al*. Immune responses after immunization with plasmid DNA encoding Bet v 1, the major allergen of birch pollen. *J Allergy Clin Immunol* 1999; **103**: 107–113.
11 Li X *et al*. Strain-dependent induction of allergic sensitization caused by peanut allergen DNA immunization in mice. *J Immunol* 1999; **162**: 3045–3052.
12 Toda M *et al*. 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* 2000; **99**: 179–186.
13 Horner AA *et al*. DNA-based vaccination reduces the risk of lethal anaphylactic hypersensitivity in mice. *J Allergy Clin Immunol* 2000; **106**: 349–356.
14 Maecker HT *et al*. Vaccination with allergen-IL-18 fusion DNA protects against, and reverses established, airway hyperreactivity in a murine asthma model. *J Immunol* 2001; **166**: 959–965.
15 Jilek S, Barbey C, Spertini F, Corthésy B. Antigen-independent suppression of the allergic immune response to bee venom phospholipase A_2 by DNA vaccination in CBA/J mice. *J Immunol* 2001; **166**: 3612–3621.
16 Hochreiter R *et al*. 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 β-galactosidase. *Int Arch Allergy Immunol* 2001; **124**: 406–410.
17 Roy K, Mao HQ, Huang SK, Leong KW. Oral gene delivery with chitosan-DNA nanoparticles generates immunologic protection in a murine model of peanut allergy. *Nat Med* 1999; **5**: 387–391.
18 Janssen R *et al*. Analysis of recombinant mycobacteria as T helper type 1 vaccines in an allergy challenge model. *Immunology* 2001; **102**: 441–449.
19 Hsu CH *et al*. Inhibition of specific IgE response *in vivo* by allergen-gene transfer. *Int Immunol* 1996; **8**: 1405–1411.
20 Lee DJ *et al*. Inhibition of IgE antibody formation by plasmid DNA immunization is mediated by both CD4+ and CD8+ T cells. *Int Arch Allergy Immunol* 1997; **113**: 227–230.
21 Imler JL. Adenovirus vectors as recombinant viral vaccines. *Vaccine* 1995; **13**: 1143–1151.
22 Sandmair AM, Vapalahti M, Ylä-Herttuala S. Adenoviruses as gene delivery vectors. *Adv Exp Med Biol* 2000; **465**: 423–429.
23 Juillard V *et al*. Long-term humoral and cellular immunity induced by a single immunization with replication-defective adenovirus recombinant vector. *Eur J Immunol* 1995; **25**: 3467–3473.
24 Tüting T *et al*. Co-delivery of T helper 1-biasing cytokine genes enhances the efficacy of gene gun immunization of mice: studies with the model tumor antigen β-galactosidase and the BALB/c Meth A p53 tumor-specific antigen. *Gene Therapy* 1999; **6**: 629–636.
25 Kemeny DM. $CD8^+$ T cells in atopic disease. *Curr Opin Immunol* 1998; **10**: 628–633.
26 Rolland J, O'Hehir R. Immunotherapy of allergy: anergy, deletion, and immune deviation. *Curr Opin Immunol* 1998; **10**: 640–645.
27 Sudowe S, Rademaekers A, Kölsch E. Antigen dose-dependent predominance of either direct or sequential switch in IgE antibody responses. *Immunology* 1997; **91**: 464–472.
28 Chen PW *et al*. Therapeutic antitumor response after immunization with a recombinant adenovirus encoding a model tumor-associated antigen. *J Immunol* 1996; **156**: 224–231.
29 Steitz J *et al*. Genetic immunization of mice with human tyrosinase-related protein 2: implications for the immunotherapy of melanoma. *Int J Cancer* 2000; **86**: 89–94.
30 Van Ginkel FW *et al*. Intratracheal gene delivery with adenoviral vector induces elevated systemic IgG and mucosal IgA antibodies to adenovirus and β-galactosidase. *Hum Gene Ther* 1995; **6**: 895–903.
31 Brubaker JO *et al*. Th1-associated immune responses to β-

galactosidase expressed by a replication-defective herpes simplex virus. *J Immunol* 1996; **157**: 1598–1604.
32 Prasad SA *et al*. Recombinant adenoviruses induce CD8 T cell responses to an inserted protein whose expression is limited to nonimmune cells. *J Immunol* 2001; **166**: 4809–4812.
33 Carbone FR *et al*. Cross-presentation: a general mechanism for CTL immunity and tolerance. *Immunol Today* 1998; **19**: 368–373.
34 Sigal LJ, Crotty S, Andino R, Rock KL. Cytotoxic T-cell immunity to virus-infected non-haematopoietic cells requires presentation of exogenous antigen. *Nature* 1999; **398**: 77–80.
35 Song W *et al*. Dendritic cells genetically modified with an adenovirus vector encoding the cDNA for a model antigen induce protective and therapeutic antitumor immunity. *J Exp Med* 1997; **186**: 1247–1256.
36 Gong J *et al*. Induction of antigen-specific antitumor immunity with adenovirus-transduced dendritic cells. *Gene Therapy* 1997; **4**: 1023–1028.
37 Tüting T *et al*. Dendritic cell-based genetic immunization in mice with a recombinant adenovirus encoding murine TRP2 induces effective anti-melanoma immunity. *J Gene Med* 1999; **1**: 400–406.
38 Snapper CM, Paul WE. Interferon-γ and B cell stimulatory factor-1 reciprocally regulate Ig isotype production. *Science* 1987; **236**: 944–947.
39 Pène J *et al*. IgE production by normal human lymphocytes is induced by interleukin 4 and suppressed by interferons γ and α and prostaglandin E_2. *Proc Natl Acad Sci USA* 1988; **85**: 6880–6884.
40 Stämpfli MR *et al*. Adenoviral infection inhibits allergic airways inflammation in mice. *Clin Exp Allergy* 1998; **28**: 1581–1590.
41 Suzuki M *et al*. Immune responses against replication-deficient adenovirus inhibit ovalbumin-specific allergic reactions in mice. *Hum Gene Ther* 2000; **11**: 827–838.
42 Sur S *et al*. Long-term prevention of allergic lung inflammation in a mouse model of asthma by CpG oligodeoxynucleotides. *J Immunol* 1999; **162**: 6284–6293.
43 Kohama Y *et al*. Immunostimulatory oligodeoxynucleotide induces TH1 immune response and inhibition of IgE antibody production to cedar pollen allergens in mice. *J Allergy Clin Immunol* 1999; **104**: 1231–1238.
44 Tighe H *et al*. Conjugation of immunostimulatory DNA to the short ragweed allergen Amb a 1 enhances its immunogenicity and reduces its allergenicity. *J Allergy Clin Immunol* 2000; **106**: 124–134.
45 Peng Z *et al*. CpG oligodeoxynucleotide vaccination suppresses IgE induction but may fail to down-regulate ongoing IgE responses in mice. *Int Immunol* 2001; **13**: 3–11.
46 Yang Y *et al*. Immune responses to viral antigens *versus* transgene product in the elimination of recombinant adenovirus-infected hepatocytes *in vivo*. *Gene Therapy* 1996; **3**: 137–144.
47 Michou AI *et al*. Adenovirus-mediated gene transfer: influence of transgene, mouse strain and type of immune response on persistence of transgene expression. *Gene Therapy* 1997; **4**: 473–482.
48 Kass-Eisler A *et al*. The impact of developmental stage, route of administration and the immune system on adenovirus-mediated gene transfer. *Gene Therapy* 1994; **1**: 395–402.
49 Yang Y, Li Q, Ertl HC, Wilson JM. Cellular and humoral immune responses to viral antigens create barriers to lung-directed gene therapy with recombinant adenoviruses. *J Virol* 1995; **69**: 2004–2015.
50 Mack CA *et al*. Circumvention of anti-adenoviral neutralizing immunity by administration of an adenoviral vector of an alternate serotype. *Hum Gene Ther* 1997; **8**: 99–109.
51 Yeh P, Perricaudet M. Advances in adenoviral vectors: from genetic engineering to their biology. *FASEB J* 1997; **11**: 615–623.
52 Inoue N *et al*. Production of specific antibody and T helper 1-dominant cytokine elicited by dendritic cells genetically modified with an adenovirus vector. *Immunol Lett* 1999; **70**: 77–81.
53 Steitz J, Brück J, Knop J, Tüting T. Adenovirus-transduced DC stimulate cellular immunity to melanoma via a $CD4^+$ T cell-dependent mechanism. *Gene Therapy* 2001; **8**: 1255–1263.
54 Hardy S *et al*. Construction of adenovirus vectors through Cre-*lox* recombination. *J Virol* 1997; **71**: 1842–1849.
55 Graham FL, Prevec L. Adenovirus-based expression vectors and recombinant vaccines. *Biotechnology* 1992; **20**: 363–390.
56 Jarman ER *et al*. Inhibition of murine IgE and immediate cutaneous hypersensitivity responses to ovalbumin by the immunomodulatory agent leflunomide. *Clin Exp Immunol* 1999; **114**: 221–228.
57 Baniyash M, Eshhar Z. Inhibition of IgE binding to mast cells and basophils by monoclonal antibodies to murine IgE. *Eur J Immunol* 1984; **14**: 799–807.
58 Gavin MA *et al*. Alkali hydrolysis of recombinant proteins allows for the rapid identification of class I MHC-restricted CTL epitopes. *J Immunol* 1993; **151**: 3971–3980.
59 Miyahira Y *et al*. Quantification of antigen specific $CD8^+$ T cells using an ELISPOT assay. *J Immunol Meth* 1995; **181**: 45–54.
60 Sudowe S, Arps V, Vogel T, Kölsch E. The role of interleukin-4 in the regulation of sequential isotype switch from immunoglobulin G1 to immunoglobulin E antibody production. *Scand J Immunol* 2000; **51**: 461–471.