

Immunize and disappear—Safety-optimized mRNA vaccination with a panel of 29 allergens

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Background: The spread of type I allergic diseases has reached epidemic dimensions. The success of therapeutic intervention is limited, and hence prophylactic vaccination is now seriously considered. However, immunization of healthy individuals requires safety standards far beyond those applicable for therapeutic approaches. mRNAs encoding allergen molecules represent an attractive tool for preventive vaccination because of the inherent safety features of this vaccine type.

Objective: In the current study we investigated whether mRNA constructs would be capable of protecting against type I allergic reactions in a murine model using the grass pollen allergen Phl p 5 and 28 other major pollen, food, animal, mold, and latex allergens.

Methods: BALB/c mice were immunized intradermally either with conventional or replicase-based mRNA constructs. Subsequently, animals were sensitized by means of subcutaneous injection of allergen/alum, followed by airway provocation. IgG1/IgG2a/IgE titers were determined by using ELISAs. Allergen-specific functional IgE levels were assessed by using the basophil release assay. Measurement of cytokines in splenocyte cultures and bronchoalveolar lavage fluids were performed by using enzyme-linked immunosorbent spot assays/sandwich ELISAs. Eosinophil and CD8⁺ counts in bronchoalveolar lavage specimens were determined by means of flow cytometry. Airway hyperreactivity was assessed with whole-body plethysmography and invasive resistance/dynamic compliance measurement.

Results: mRNA vaccination proved its antiallergic efficacy in terms of IgG subclass distribution, functional IgE suppression, reduction of IL-4 and IL-5 levels, induction of IFN- γ -producing cells, and reduction of airway hyperreactivity and eosinophil counts in the lung.

Conclusion: Immunization with mRNA induces T_H1-biased immune responses similar to those elicited through DNA-based vaccination but additionally offers the advantage of a superior safety profile. (J Allergy Clin Immunol ■■■■;■■■:■■■-■■■.)

Key words: RNA vaccines, DNA vaccines, self-replicating vaccines, type I allergy, vaccination

During the last decades, type I allergic diseases have emerged as a major public health problem in Western industrialized countries, with about 25% of the population being affected.^{1,2} In addition to family predisposition, conditions of growing up and environmental factors have been demonstrated to be of great relevance for the development of atopic diseases.³⁻⁵

Specific immunotherapy currently represents the only available therapeutic intervention. However, because of the high doses administered, the risk of anaphylactic side effects is evident, and the use of crude, barely characterized allergen extracts implies the possibility for sensitization of the patient against previously unrecognized components.^{6,7}

Additionally, no preventive vaccination against type I allergy is available, although immunization of young children with increased hereditary risk of allergic diseases might be the most feasible approach because training of the naive immune system is easier to accomplish than diverting an already manifested allergic immune phenotype. Complete conversion of an established T_H2-type response, especially with respect to IgE production, seems to fail because of a persistent production of specific IgE secreted by plasma cells that survive in appropriate niches. These long-lived plasma cells represent an independent cellular component of immunologic memory.⁸

Recently, nucleic acid-based vaccines have become a promising approach to bias immune mechanisms underlying allergic diseases. It has been shown in numerous animal studies that DNA vaccines can prevent the induction of type I allergic responses and even downregulate an already established allergic T_H2 immune status.⁹ Nevertheless, concerns have been raised regarding the safety of DNA-based vaccines with respect to integration of the plasmid DNA into the host genome, adverse immunopathologic effects, induction of autoimmune diseases, and uncontrollable long-term expression of the encoded antigen.^{10,11}

Especially with respect to a vaccine against a not imminently life-threatening disease and considering prophylactic vaccination

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Abbreviations used

ELISPOT: Enzyme-linked immunosorbent spot
 Penh: Enhanced pause
 P5-RNA: mRNA encoding Phl p 5
 P5-repRNA: Self-replicating mRNA encoding Phl p 5
 TLR: Toll-like receptor

of healthy children, the safety of any antiallergic vaccine is of utmost importance. We therefore specify the requirements for an antiallergic vaccine as a minimal construct that is eliminated from the body after the induction of a desired immune response. We call this concept “immunize and disappear.”

A type of nucleic acid that could probably meet these enhanced safety requirements is mRNA. mRNA vaccines exhibit all the advantages attributed to DNA vaccines for the treatment of allergic diseases. They provide the allergen in its purest form—its genetic information—and at such low doses that neither induction of anaphylactic side effects caused by pre-existing IgE nor production of any novel allergen-specific IgE will occur.

In addition, mRNA vaccines offer several advantages over DNA vaccines. First, only the genetic information of the allergen itself has to be used, whereas no additional foreign sequences, such as plasmid backbone DNA or viral promoters, are included, thereby fulfilling the requirements of a minimal construct. Second, mRNA cannot integrate into the host genome, abolishing the risk of malignancies. Third, mRNA is translated in the cytoplasm of the cell, and hence the transcription machinery of the cell nucleus is not needed, rendering mRNA vaccines independent of transport into and out of the nucleus as well as of nuclear stages. Finally, because of the fast degradation of mRNA, expression of the foreign transgene is short-lived.¹²

However, because of the instability of mRNA, it was assumed that high amounts of the vaccine might be needed. Self-replicating mRNA vaccines have been developed to circumvent a possible dosage problem. Such vectors include replication elements derived from alphaviruses, whereas the structural virus proteins have been substituted for the gene of interest. Replicase-based mRNA vaccines induce antibody responses as well as cytotoxic responses at extremely low doses because of immune activation mediated by virus-derived danger signals.¹³

Although a number of articles deal with naked mRNA or mRNA replicon vaccines in the field of tumor immunology, no study investigating the potential of mRNA vaccines for prevention or treatment of type I allergy has been published thus far. In the current study, using the timothy grass pollen allergen Phl p 5, we exemplify for the first time that mRNA and replicase-based mRNA vaccines can effectively prevent allergic responses and represent promising tools for specific allergy immunization. In addition, we provide proof of principle for the wide applicability of this method by using a panel of 28 food, pollen, latex, dander, and mold allergen-encoding mRNAs.

METHODS**Plasmids used for mRNA transcription**

The Phl p 5 gene was excised from vector pCMV-Phlp5¹⁴ by *NheI/XbaI* and ligated into the *XbaI* restriction site of pTNT (Promega, Madison, Wis) and pSin-Rep5 (Invitrogen, Carlsbad, Calif), resulting in pTNT-P5 and pSin-Rep5-P5, respectively. Similarly, a panel of 28 food, pollen, latex, dander, and mold allergens has been cloned into pTNT (see Table E1 in this article's

Online Repository at www.jacionline.org). All inserts were sequence verified (Eurofins MWG Operon, Ebersberg, Germany). Sequences of all allergens used for RNA vaccination are shown in Table E2.

RNA transcription

Plasmids pTNT-P5 and pSin-Rep5-P5 were linearized, and templates were purified by means of phenol-chloroform-isoamyl alcohol extraction, followed by a single chloroform-isoamyl alcohol extraction. After addition of a 1:10 volume of 3 mol/L Na-acetate, pH 5.2, plasmids were precipitated with 2 volumes of 100% ethanol and washed 3 times with 70% ethanol.

All transcription reactions were performed with T7 or SP6 RiboMAX Large Scale RNA Production Systems (Promega). Residual template DNA was removed by means of digestion with RNase-free DNase (Promega).

RNAs were capped *in vitro* by using a ScriptCap m7 G Capping System (Epicentre Biotechnologies, Madison, Wis), according to the manufacturer's protocol, to maintain the stability and translational efficiency of the mRNA *in vivo*, except that we used 0.8 μ L of capping enzyme (instead of 4 μ L) and incubated at 37°C for 2 hours. mRNA was recovered by means of ammonium acetate precipitation, washed with 70% ethanol, and resuspended in nuclease-free H₂O.

Mice, immunization, and serology

Female BALB/c mice (Charles River, Wilmington, Mass) were immunized with different doses of mRNA or self-replicating mRNA in 200 μ L of PBS (Phl p 5 groups) or Ringer solution (102.7 mmol/L sodium chloride, 5.4 mmol/L potassium chloride, and 1.8 mmol/L calcium chloride; supplementary groups) 3 times in weekly intervals. The vaccine was applied by means of intradermal injection into the shaved back divided between 6 to 8 different spots. Two weeks later, mice were sensitized by means of subcutaneous injection of 1 μ g of recombinant allergen together with 100 μ L of Al(OH)₃ (Serva Electrophoresis, Heidelberg, Germany) in a total volume of 200 μ L of PBS 2 times in 2-week intervals. Sera were taken before and 2 weeks after sensitization. Two weeks later, mice underwent an airway challenge by means of intranasal instillation of 1 μ g of recombinant allergen in 40 μ L of PBS 3 times in daily intervals (see Fig 1 for the experimental schedule). A shortened schedule was used for groups receiving any of the other 28 allergens tested (see Table E1). All animal experiments were conducted according to local guidelines approved by the Austrian Ministry of Science.

Data are shown for 1 experiment with 40, 8, and 1.6 μ g for conventional RNA and 8, 1.6, and 0.32 μ g for self-replicating RNA. Data of Figs 2 to 4 have been reproduced in 2 additional independent experiments by using either single doses (40 μ g for mRNA encoding Phl p 5 [P5-RNA] and 8 μ g for self-replicating mRNA encoding Phl p 5 [P5-repRNA]) or a dose range (100, 10, and 1 μ g) that produced comparable results for the respective doses.

Antigen-specific IgG1, IgG2a, and IgE antibody levels in sera were determined by using a luminescence-based ELISA, as previously described.¹⁵ Total IgE levels were measured with an IgE ELISA max kit (BioLegend, San Diego, Calif). Functional IgE levels were measured by using a basophil degranulation assay, as previously described.¹⁶

Lymphocyte cultures

Splenocytes from both immunized and control mice were cultured to harvest supernatants for IFN- γ and IL-5 sandwich ELISAs (BD PharMingen, San Diego, Calif) and for IL-13 sandwich ELISA (R&D Systems, Minneapolis, Minn) or for IL-4, IL-5, and IFN- γ enzyme-linked immunosorbent spot (ELISPOT) assay, as previously described.^{14,17} Splenocytes were cultured in the presence or absence of 10 μ g/mL recombinant allergen (Biomay AG, Wien, Austria). All allergen preparations contained less than 5 pg of LPS per microgram, as determined by using the Limulus amoebocyte assay.

Analysis of bronchoalveolar lavage specimens

Bronchoalveolar lavage was performed as previously described.¹⁴ Cells were stained with CD45-PECy7, Gr1-allophycocyanin, CD8-phycoerythrin, and CD4-PECy5 (BD PharMingen) and analyzed on a FACSCanto II flow cytometer. Eosinophils were distinguished from other leukocyte populations by their CD45^{med} Gr1^{low} side-scatter^{high} phenotype.

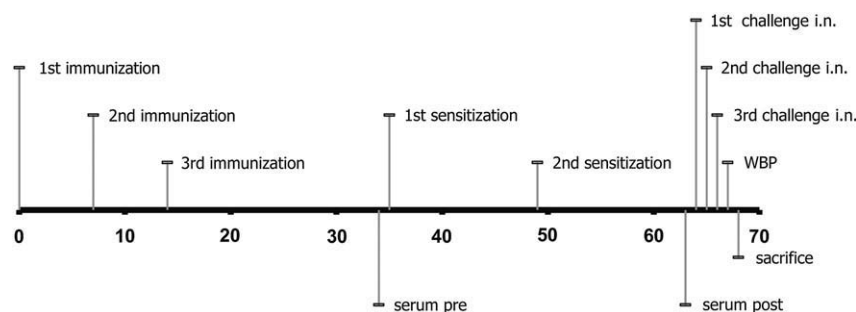


FIG 1. Experimental schedule. Numbers indicate days. Mice were immunized 3 times with mRNA vaccines and subsequently sensitized with recombinant allergen/alum. Sera were taken before (*pre*) and after (*post*) sensitization. Subsequently, mice were challenged by means of intranasal instillation of allergen, analyzed with whole-body plethysmography (WBP), and finally killed for cellular assays (cytokine profiles and bronchoalveolar lavage). i.n., Intranasal.

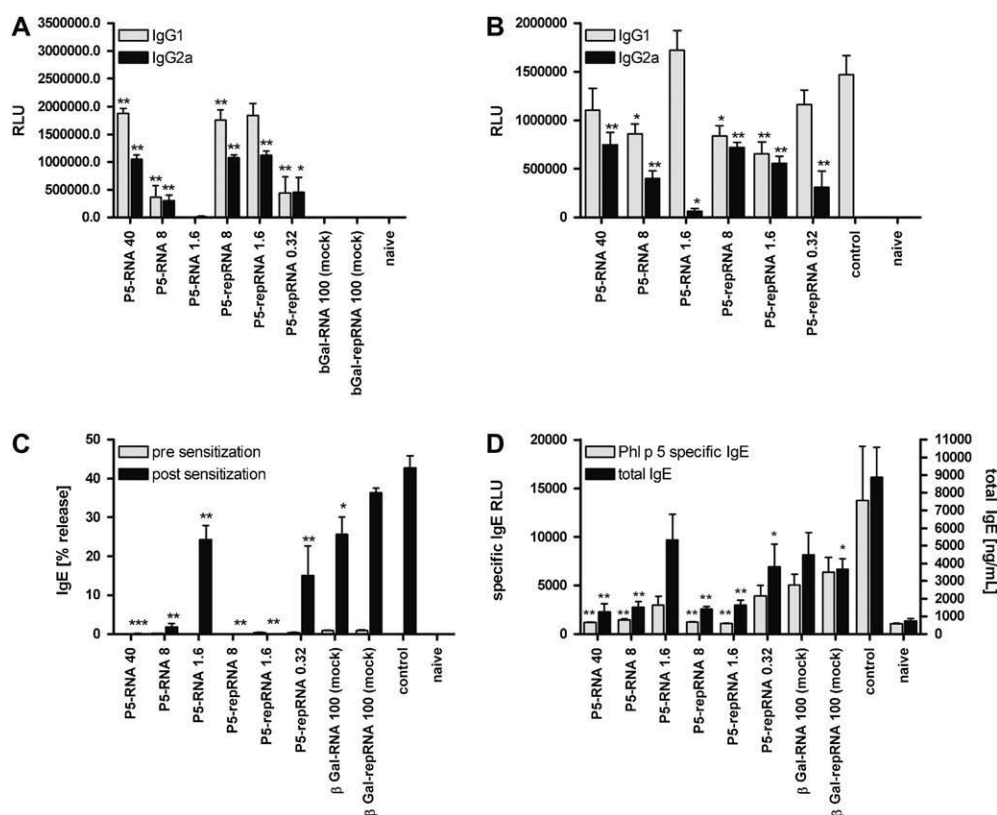


FIG 2. Phl p 5-specific IgG1, IgG2a, and IgE levels and total IgE levels after mRNA vaccination (**A** and **C**) and subsequent sensitization (**B**, **C**, and **D**). **Fig 2, A, B, and D**, show ELISA data; **Fig 2, C**, shows RBL assay data ($n = 5$). Sera were diluted 1:100 (**Fig 2, A**), 1:10,000 (**Fig 2, B**), and 1:100 (**Fig 2, C, pre**) or 1:200 (**Fig 2, C, post**, and **D**). Statistical significance was calculated compared with sera from naive mice (**Fig 2, A**) or sera from sensitization control animals (**Fig 2, B, C, and D; control**). * $P < .05$, ** $P < .01$, and *** $P < .001$.

Whole-body plethysmography

Mice were challenged with increasing doses of aerosolized methacholine, and enhanced pause (Penh) was measured by using unrestrained whole-body plethysmography (Buxco, Wilmington, NC).¹⁸

Statistical analysis

Statistical differences between means of immunized groups and nonimmunized control groups or sensitization-only groups were assessed by using the Mann-Whitney rank sum test. Groups that passed normality and equal variance tests were analyzed by using the Student *t* test. All data are shown as means $\pm$ SEMs. Methods concerning quality control, stability, and in

vivo persistence of mRNA, as well as resistance/compliance measurements, real-time-PCR, and MyD88 knockout mice can be found in this article's Online Repository at www.jacionline.org.

RESULTS

mRNA quality control, stability, and *in vivo* persistence

Quality control of injected mRNA was done by means of agarose gel electrophoresis and *in vitro* transfection (see **Fig E1** in

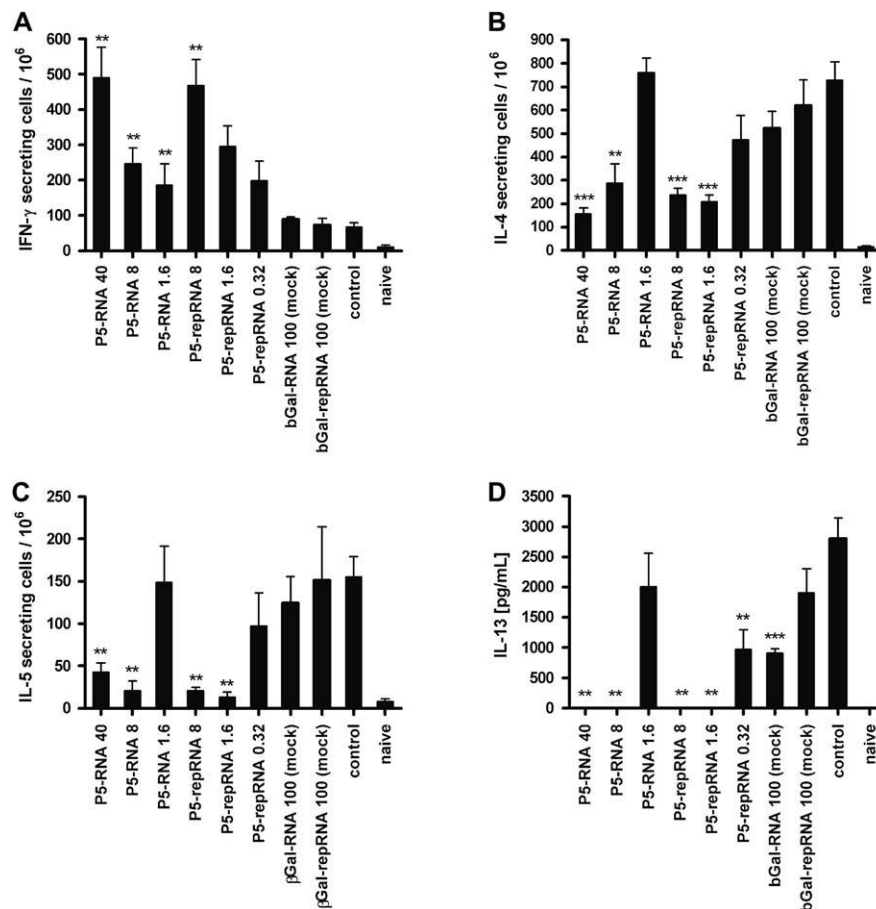


FIG 3. Number of IFN- γ -secreting (A), IL-4-secreting (B), and IL-5-secreting (C) splenocytes and levels of IL-13 (D) after *in vitro* restimulation with recombinant Phl p 5, as determined by means of ELISPOT assays and ELISAs. Data are shown as means $\pm$ SEMs ($n = 5$). Statistical significance was calculated compared with sensitization control animals (control). * $P < .05$, ** $P < .01$, and *** $P < .001$.

this article's Online Repository at www.jacionline.org). mRNA preparations showed practically no degradation over a storage period of 32 days, regardless of the temperature or storage condition (see Fig E2, A and B, in this article's Online Repository at www.jacionline.org). Yet translation efficacy of mRNA stored at room temperature started to decrease after 8 (liquid) or 16 (lyophilized) days, although not at the lower temperatures (see Fig E2, C).

In vivo expression of luciferase-encoding mRNA was short lived compared with that of DNA or self-replicating vaccines (see Fig E3 in this article's Online Repository at www.jacionline.org).

mRNA-based vaccines encoding the allergen Phl p 5 are immunogenic and prevent IgE induction

To estimate the potency of conventional (P5-RNA) and self-replicating (P5-repRNA) mRNA vaccines encoding Phl p 5, we performed dose-response studies using 1.6 to 40 μ g of mRNA and 0.32 to 8 μ g of self-replicating mRNA (see Fig 1 for the schedule).

Fig 2, A, shows that both types of mRNA vaccines induce Phl p 5-specific humoral immune responses in a dose-dependent manner. Both vaccine types displayed a clearly T_H1 -biased serologic profile characterized by low IgG1/IgG2a ratios and no induction of functional IgE, as measured with the RBL release assay (Fig 2,

C, gray bars). Of 29 conventional mRNA vaccines encoding major allergens tested, none induced detectable IgE responses (see Table E1).

After sensitization, the control group, which had not been preimmunized, showed a strictly T_H2 -biased serology with high IgG1 levels and complete absence of IgG2a, which is indicative of an allergic sensitization. In contrast, all vaccinated groups maintained a T_H1 -balanced immunophenotype, with suppression of IgG1 and induction of IgG2a (Fig 2, B). However, the low-dose groups (P5-RNA 1.6 μ g and P5-repRNA 0.32 μ g) showed only very weak IgG2a induction and no IgG1 suppression compared with the sensitization control groups. This induction of allergen-specific IgG2a by means of prevaccination with mRNA vaccines was observed for 16 of 28 additional allergens tested (see Table E1).

Prevaccination with both types of mRNA vaccines induced dose-dependent suppression of IgE induction (Fig 2, C, black bars, and Fig 2, D). IgE suppression was strictly antigen dependent because vaccination with 100 μ g of conventional or self-replicating mRNA encoding the irrelevant antigen β -galactosidase (mock) induced little or no suppression at all. IgE suppression was seen in 8 of 28 additional allergens tested (see Table E1 and Fig E4 in this article's Online Repository at www.jacionline.org). However, in 15 supplementary groups no IgE suppression could be demonstrated because no allergen-specific IgE was

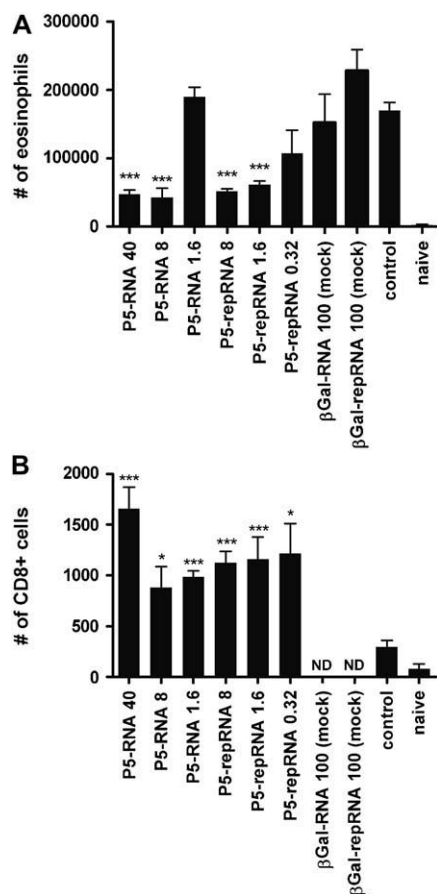


FIG 4. Number of eosinophils (**A**) and CD8⁺ cells (**B**) in bronchoalveolar lavage fluid of sensitized mice after intranasal application of allergen. Values are shown as means $\pm$ SEMs ($n = 5$). * $P < .05$, ** $P < .01$, and *** $P < .001$. ND, Not done.

induced in the respective control group. This might be due to a shorter sensitization protocol used for the supplementary groups. mRNA prevaccination never resulted in an increase in allergen-specific IgE levels compared with those seen in the sensitization control groups (data not shown).

mRNA-based vaccines induce T_H1-biased T-cell memory

Two weeks after the final sensitization, splenocytes were restimulated *in vitro* with recombinant Phl p 5 protein to assess their T_H1/T_H2 profile. For this purpose, the number of IFN- γ -, IL-4-, and IL-5-secreting cells was determined by using ELISPOT assays and ELISAs. IL-13 was determined by means of ELISA.

Groups prevaccinated with mRNA vaccines showed an induction of IFN- γ -secreting cells in a dose-dependent manner compared with the control group (Fig 3, A). Simultaneously, the amount of cells secreting the T_H2 type cytokines IL-4 (Fig 3, B) and IL-5 (Fig 3, C) and the levels of secreted IL-13 (Fig 3, D) were suppressed. ELISA data of secreted IFN- γ , IL-4, and IL-5 showed results comparable with those seen with ELISPOT assays (data not shown). Cytokine secretion was not detectable (<10 pg/mL) in supernatants of cells that had not been restimulated with recombinant protein. The induction of IFN- γ was found in 23 of 29 groups prevaccinated with different allergen-encoding mRNA vaccines (see Table E1 and Fig E4).

mRNA-based vaccines alleviate allergen-induced lung inflammation

Two weeks after the last sensitization, lung inflammation was induced by means of 3 daily intranasal applications of 1 μ g of recombinant Phl p 5. This protocol induced strong infiltration of eosinophils into the bronchoalveolar lavage fluid of sensitized mice (Fig 4, A, control). In contrast, prevaccinated mice had significantly reduced numbers of eosinophils (Fig 4, A, and see Fig E4). Prevaccination induced a small but significant lung infiltrate of CD8⁺ cells at all tested doses (Fig 4, B), whereas the numbers of CD4⁺ cells did not significantly differ from those seen in control animals (not shown). Prevaccination also prevented the induction of IL-5 in the bronchoalveolar lavage fluid with a concomitant increase in IFN- γ levels (see Fig E5 in this article's Online Repository at www.jacionline.org).

Measurement of Penh with whole-body plethysmography showed a dose-dependent reduction in groups that had received the conventional mRNA vaccine (Fig 5, A), whereas the self-replicating vaccine displayed similar suppression at all tested doses (Fig 5, B). In a separate experiment groups receiving the highest doses were reproduced, and invasive lung resistance and dynamic compliance were measured (see Fig E6 in this article's Online Repository at www.jacionline.org).

mRNA-based vaccines have similar T_H1-inducing potency compared with DNA vaccines

Based on these results, we compared the efficacy of mRNA vaccines (P5-RNA and P5-repRNA) with plasmid DNA vaccination (DNA plasmid encoding Phl p 5 and self-replicating DNA plasmid encoding Phl p 5) at a dose that provided effective protection with mRNA vaccines (ie, 40 μ g of the conventional and 8 μ g of the self-replicating vaccines). The immunization/sensitization schedule was the same as shown in Fig 1.

DNA plasmid encoding Phl p 5 induced significantly higher levels of IgG2a after sensitization compared with P5-RNA, whereas there was no difference in the groups immunized with self-replicating vaccines (Fig 6, A). In general, at the tested dosage, there was no significant difference in IgE suppression (Fig 6, B), T_H1 memory induction (Fig 6, C), or suppression of lung inflammation (Fig 6, D) in all DNA- or mRNA-vaccinated groups.

DISCUSSION

Over the past decade, the worldwide rapidly growing incidence and epidemic dimension of allergic diseases triggered a serious discussion about prophylactic allergen-specific vaccination approaches. The hygiene hypothesis has shown that an early T_H1 priming of the immune system is the key to prevention of atopic diseases.^{19,20} Vaccinating against a panel of major allergens (adapted to regional needs) that display high cross-reactivity would enable us to induce a broad range of allergen-specific T_H1 priming in individuals with a genetic predisposition to allergy. We have observed in numerous experiments that even very weak (allergen-specific) T_H1 priming is efficient in preventing the sensitization against allergens because, once induced, T_H1 memory cells are reactivated and expanded by later allergen contact, which recalls and strengthens the T_H1 bias.^{14,17,21,22} Translated into a clinical setting, this means that even a subtle T_H1 memory would be prolonged and further strengthened by later natural exposure to the respective allergen.

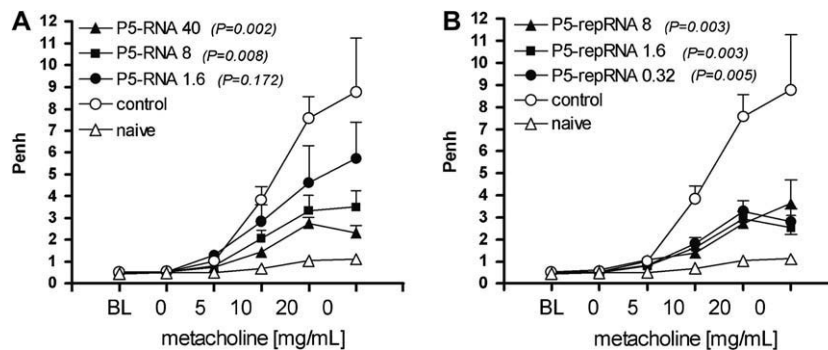


FIG 5. Penh values of mice prevaccinated with P5-RNA (A) and P5-repRNA (B) after sensitization and intranasal allergen challenge. Mice were exposed to escalating doses of methacholine with a final recovery interval. BL, Baseline measurement. Values are shown as means $\pm$ SEMs ($n = 5$). P values are shown for the 20 mg/mL dose.

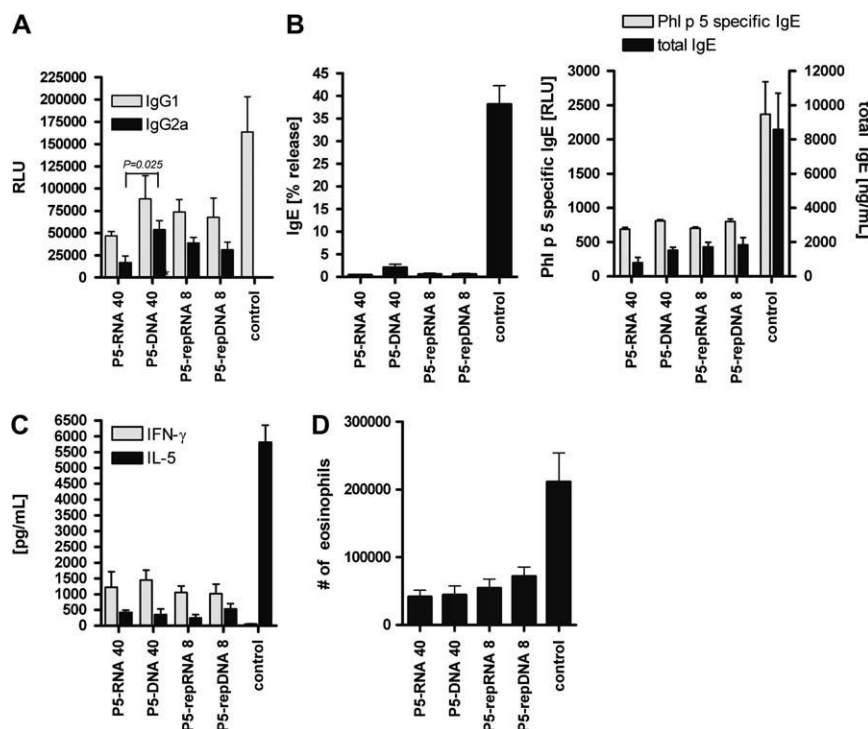


FIG 6. Comparison of mRNA and DNA vaccines at equal doses. IgG2a induction and IgG1 suppression (A), as well as IgE suppression after sensitization (B), are shown. C, IFN- γ and IL-5 levels in supernatants of restimulated splenocytes after sensitization. D, Number of eosinophils in bronchoalveolar lavage fluid of challenged mice. All data are shown as means $\pm$ SEMs ($n = 4$).

Current formulations used for specific immunotherapy are most likely not applicable for such prophylactic approaches because they lack a sufficient T_H1 -inducing component and in the worst case could sensitize a healthy individual against an allergen.

In contrast, DNA vaccines hold great promise for the prevention and treatment of allergic diseases and are safe with respect to unwanted T_H2 induction.⁹ However, other hypothetical risks associated with DNA vaccines (eg, genomic integration and long-term persistence) question the use of this novel type of vaccine for clinical use in healthy adults and especially in children.¹⁰ Ideally, a prophylactic vaccine should be self-removing once it has induced immunity against a certain allergen ("immunize and disappear"). Although self-replicating (DNA) vaccines fulfill this

premise by driving the transfected cells into apoptosis,²³ this type of vector has other limitations (ie, the potentially unwanted expression of a virus-derived protein [replicase]). The obvious alternative to DNA vaccines, namely mRNA vaccines, has been largely ignored because of higher expenses in the preparation and storage of mRNA vaccines. The few studies reporting on mRNA vaccination mostly describe tumor vaccination with a special focus on therapies using *ex vivo* transfected dendritic cells.²⁴ In principle, mRNA vaccines meet the same requirements for antiallergic vaccination as DNA vaccines but without any safety disadvantages. However, mRNA vaccines address different danger signals compared with their DNA counterparts (Toll-like receptor [TLR] 7/8 in contrast to TLR9 activation).

Indeed, Carralot et al²⁵ have suggested that mRNA vaccines rather induce T_H2-type immune responses that can be converted to T_H1 only through addition of GM-CSF. Such an inherent T_H2 bias, similar to what we observed for gene gun immunization,²² would contraindicate the use of mRNA vaccines for prophylaxis of allergy.

In contrast to the assumption of Carralot et al,²⁵ our data clearly prove that naked mRNA vaccination with a clinically relevant allergen can prevent induction of allergy to the same extent as a comparable DNA vaccine applied at the same dose. The results of experiments with 29 different allergens strongly point to a general validity of this approach.

Among the 2 mRNA vaccine types, self-replicating mRNA vaccines were superior. Because of their self-amplification and immunostimulatory capacities (TLR3, melanoma differentiation-associated antigen 5 [MDA-5], and retinoic acid inducible gene I [RIG-I]), they are efficient at extremely low doses.^{13,26,27} However, they use a virus-derived replicase molecule, which can be considered problematic for a prophylactic vaccine in which safety is the highest concern. Therefore we addressed the question of whether a conventional mRNA vaccine that contains no foreign genetic material beside the allergen of interest would be equally efficient when applied at an appropriate dose. Surprisingly, we could show that an mRNA vaccine encoding Phl p 5 can induce similar T_H1-biased immune responses (Figs 2 and 3) and is equally efficient in preventing IgE induction (Fig 2, C) and lung inflammation (Figs 4 and 5) compared with a self-replicating mRNA vaccine when applied at an approximately 5-fold higher dose. Reduction of lung eosinophilia (Fig 4, A) correlated with a reduction in IL-5 levels and induction of moderate levels of IFN- γ in the lung (see Figs E4 and E5), indicating that the vaccine-induced generation of T_H1 cells also affects the *in vivo* T_H1/T_H2 cytokine balance in the lung. Although in viral models IFN- γ in the lung can have detrimental effects on asthma and lung pathology, this seems to be an indirect effect because IFN- γ can activate lung epithelial cells to recruit more T_H2 cells into the tissue.²⁸ Indeed, in allergy models it could be shown that redirecting T_H2 immunity toward a more balanced T_H1 milieu has a beneficial effect on lung inflammation and airway hyper-reactivity, mainly by counterregulating IL-5 and IL-13.²⁹ In fact, we could also measure a significant reduction in Penh when using whole-body plethysmography (Fig 5). Although Penh is no direct measurement of airway hyperreactivity and is controversial,³⁰ we could also show a reduction in lung resistance and increased dynamic compliance when using invasive measurements (see Fig E6). Interestingly, we also observed an influx of CD8⁺ lymphocytes into the lung (Fig 4, B). There are some publications that showed that CD8⁺ cells can prevent allergic diseases independently of CD4⁺ cells.^{31–33} However, in our model similar numbers of CD8⁺ cells in the lung could be observed in low-dose groups that were no longer protected from IgE induction or lung inflammation and therefore question a significant effect of CD8⁺ cells on protection. Injection of a conventional mRNA vaccine rapidly triggered TLR7- and IFN- γ -dependent pathways, which might account for the observed T_H1 induction (see Fig E7 in this article's Online Repository at www.jacionline.org). Nonetheless, MyD88^{−/−} mice mounted significantly reduced but still substantial T_H1-biased immune responses after vaccination with conventional, as well as self-replicating, mRNA before and after sensitization (see Fig E8 in this article's Online Repository at www.jacionline.org) and also mounted no IgE responses

after sensitization (data not shown). These results indicate that alternative pathways for mRNA-induced T_H1 activation beside TLR3, TLR7, TLR8, and TLR9 exist, which was also recently confirmed by Diebold et al.³⁴

A comparison of mRNA vaccines with their DNA counterparts revealed that DNA vaccines are efficient at an approximately 10-fold lower dose compared with mRNA vaccines,¹⁴ induce higher levels of antigen secretion over a longer period of time (see Fig E3),¹¹ and induce stronger humoral immune reactions. Nevertheless, at a dose of 40 μ g (mRNA) or 8 μ g (self-replicating mRNA), both types of mRNA vaccines showed an equal or even higher protective capacity compared with the same dose of the respective DNA vaccines (Fig 6), although the latter induces higher levels of intact antigen. This indicates that a vaccine-induced long-lasting secretion of the allergen is not necessarily an advantage compared with short-term vaccine expression, as seen with mRNA and self-replicating vaccines. Despite the short *in vivo* persistence, mRNA vaccines were surprisingly stable over a prolonged period, even at room temperature (see Fig E2). This clearly shows that the stability of mRNA is no issue when it comes to shelf life as long as the preparations are pure enough (ie, RNase free).

Taken together, we could demonstrate for the first time that mRNA-based vaccines can induce effective protection from allergic sensitization. Not a single allergen of 29 tested induced IgE or enhanced IgE induction on sensitization, underscoring the excellent safety profile of mRNA vaccines. Indeed, all tested allergens except Art v 1 and Ole e 1 induced more or less potent T_H1 reactions, IgE suppression, or both. The data open the door to clinical investigation of mRNA for vaccination against allergic disease.

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Clinical implications: The present data demonstrate that mRNA-based immunization fulfills all the necessary criteria for an effective and safety-optimized method to prevent type I allergic reactions.

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METHODS

Quality control of Phl p 5 mRNA

The overall quality of *in vitro* synthesized, capped, and polyadenylated P5 mRNA preparations was assessed by means of electrophoresis on a denaturing agarose gel (1% agarose, 7.5% formaldehyde in 25 mmol/L 3-(N-morpholino)-propanesulfonic acid [MOPS], 15 mmol/L sodium acetate, and 2 mmol/L EDTA buffer). Integrity and accurate size of transcripts were examined.

BHK-21 cells were transfected with P5-RNA and P5-repRNA by using TurboFect, according to the manufacturer's recommendations (Fermentas International, Burlington, Ontario, Canada), to exemplify the translation of the mRNA-encoded allergen Phl p 5. Transfected BHK-21 cells were harvested and prepared for Western blot analysis by performing an SDS-PAGE run and subsequent transfer onto a nitrocellulose membrane according to standard molecular biology protocols. Immunostaining was done with pooled sera of mice immunized with recombinant Phl p 5 and a goat anti-mouse IgG secondary antibody labeled with horseradish peroxidase (Bio-Rad).

Stability of mRNA preparations

mRNA encoding luciferase was synthesized and capped as described and diluted to 1 mg/mL in nuclease-free water. Five-microliter aliquots of mRNA were stored as liquid or were lyophilized at room temperature with a SC100 SpeedVac (Savant, Farmingdale, NY). Three aliquots per time point were stored at the indicated temperature and subsequently moved to -196°C until analysis. At the end of the experiment, all samples were thawed on ice, lyophilized samples were resuspended in 5 μL of nuclease-free water, and triplicates were pooled. Pooled samples were analyzed by denaturing agarose gel electrophoresis (1 μL per lane) and transfection of BHK-21 cells with TurboFect. Cell lysates were analyzed in triplicate for luciferase expression by using a Sunrise luminometer (Tecan, Männedorf, Switzerland).

In vivo luminescence

In vivo expression of DNA and mRNA vaccines was measured by using *in vivo* luminescence.

BALB/c mice ($n = 3$) were immunized with 20 μg of conventional mRNA or DNA vaccines or 4 μg of self-replicating mRNA/DNA vaccines encoding luciferase divided between 2 spots (50 μL per spot) at the shaved back. *In vivo* luminescence was assessed 3 minutes after intraperitoneal injection of 200 μL of luciferin/PBS (15 mg/mL; Biosynth International, Inc, Itasca, Ill) with an Ivis Lumina imaging system (Caliper LifeSciences, Hopkinton, Mass). Expression is shown as a relative percentage of luminescence compared with the luminescence of naive control mice.

Resistance/compliance measurement

Resistance and dynamic compliance were measured with a Finepointe Series RC site (Buxco), according to the manufacturer's instructions. Briefly, mice were anesthetized by means of intraperitoneal ketamine/xylazine injection, and the trachea was exposed and cannulated and connected to the ventilator. Transpulmonary pressure was measured by inserting an esophageal canula. Baseline signals for resistance and dynamic compliance were recorded, and mice were exposed to aerosolized PBS containing increasing amounts of methacholine (0, 5, and 10 mg/mL). Values for each dose are expressed as a percentage of baseline values.

Real-time PCR

Total RNA was extracted from draining lymph nodes 0, 5, and 24 hours after vaccination with 40 μg of P5-RNA by using a SurePrep true total RNA purification kit (Fisher Scientific, Hampton, NH), and cDNA was reverse transcribed with RevertAid M-MuLV reverse transcriptase (Fermentas International) and oligo-dT primers, according to the manufacturers' instructions. Real-time PCR analysis was done on a Rotorgene 2000 (Corbett Research, Sydney, Australia) by using iQ SYBR Green Supermix (Bio-Rad). Primers used for real-time analysis of interferon regulatory factor 7 expression were 5'-AGTGCAAGGTGTACTGGGAGGTAGGCAGCC-3' (forward primer) and 5'-ATGGTGTAGTGTGGTGACCCCTTGCCGC-3' (reverse primer). For CXCL10, 5'-CCTGCCCCACGTGTTGAGATCATTGCC-3' (forward primer) and 5'-TGGGTAAAGGGGAGTGATGGAGAGAGGC-3' (reverse primer) were used, and 5'-TGATGACATCAAGAAGGTGGTGAAGCAGGC-3' (forward primer) and 5'-ATGAGGTCCACCACCCTGTTGCTGTAGCC-3' (reverse primer) were used for glyceraldehyde-3-phosphate dehydrogenase, which was used as a reference gene. The specificity of the PCR was checked by recording a melting curve and carrying out agarose gel electrophoresis. Induction ratios (x) were calculated by using the formula

$$x = 2^{-\Delta Ct},$$

where Ct represents the threshold cycle of a given gene and ΔCt represents the difference between the Ct values of the gene in question and the Ct value of the reference gene.

MyD88 knockout mice

MyD88 knockout mice were a kind gift from Dr André Gessner (University of Erlangen, Erlangen, Germany). Knockout and matched wild-type mice were backcrossed from a C57/BL6 background to a C3H/HeJ background over 7 generations. MyD88^{+/+} and MyD88^{-/-} mice were immunized with 40 μg of P5-RNA or 8 μg of P5-repRNA and sensitized as described for the other experiments.

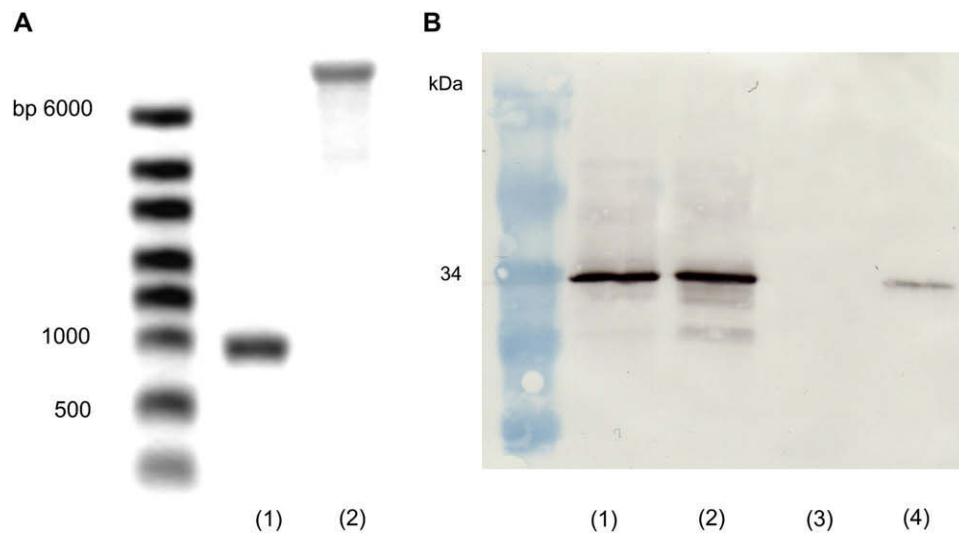


FIG E1. RNA quality control. **A**, Agarose gel electrophoresis of *in vitro* transcribed P5-RNA (approximately 990 bp, *lane 1*) and P5-repRNA (approximately 8900 bp, *lane 2*). **B**, Western blot of cell lysates of BHK-21 cells transfected with P5-RNA (*lane 1*), P5-repRNA (*lane 2*), or no RNA (*lane 3*). Four nanograms of recombinant Phl p 5 were applied to *lane 4*.

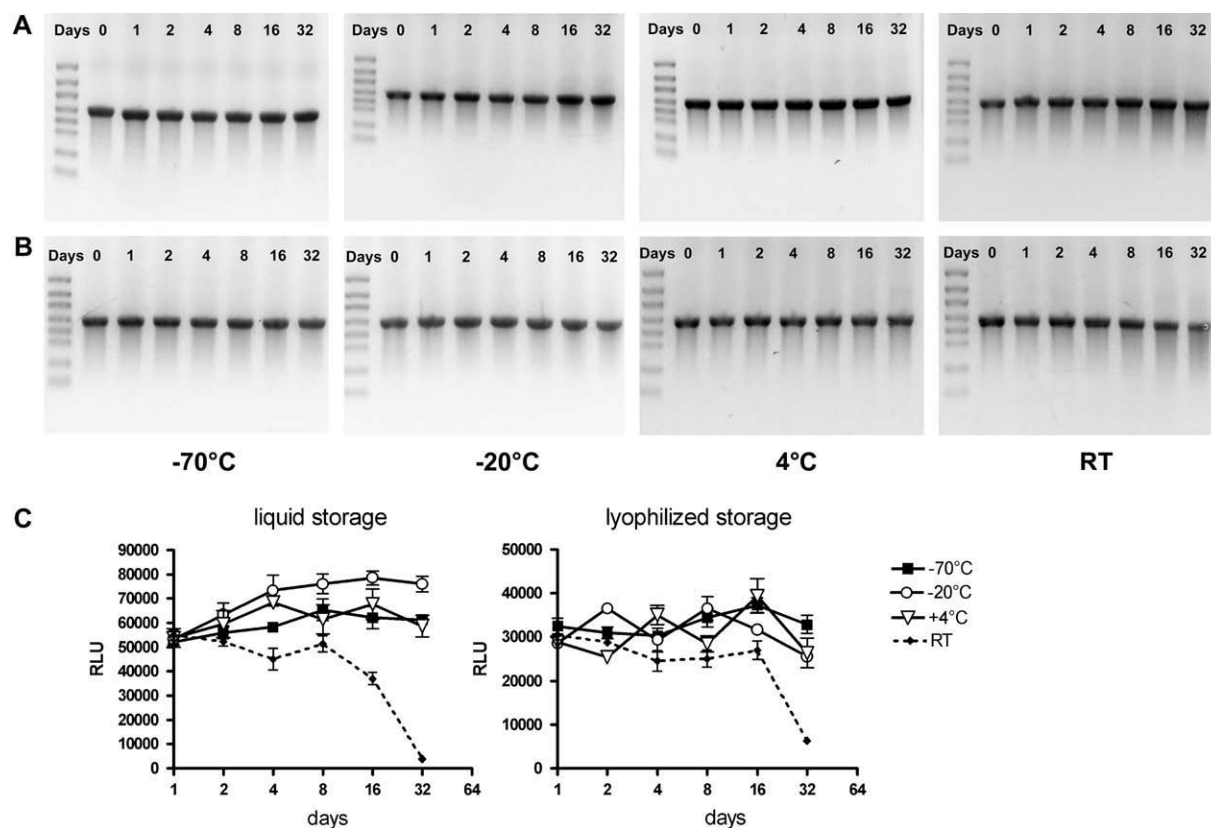


FIG E2. Stability of RNA preparations. mRNA encoding luciferase was stored for 32 days at the indicated temperatures either in water (**A**) or lyophilized (**B**). Integrity of RNA was assessed by using denaturing agarose gel electrophoresis (Fig E2, **A** and **B**) and luciferase expression in transfected BHK-21 cells (**C**). *RT*, Room temperature.

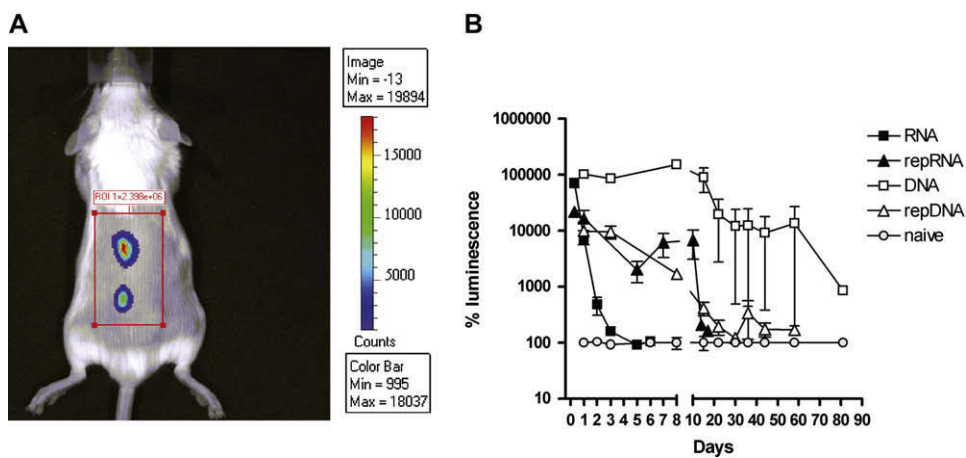


FIG E3. *In vivo* RNA expression is short lived. *In vivo* expression of luciferase in mice ($n = 3$) was quantitated on an Ivis Lumina imaging system (**A**) and expressed as a percentage of luminescence compared with that of naive mice injected with luciferin (**B**).

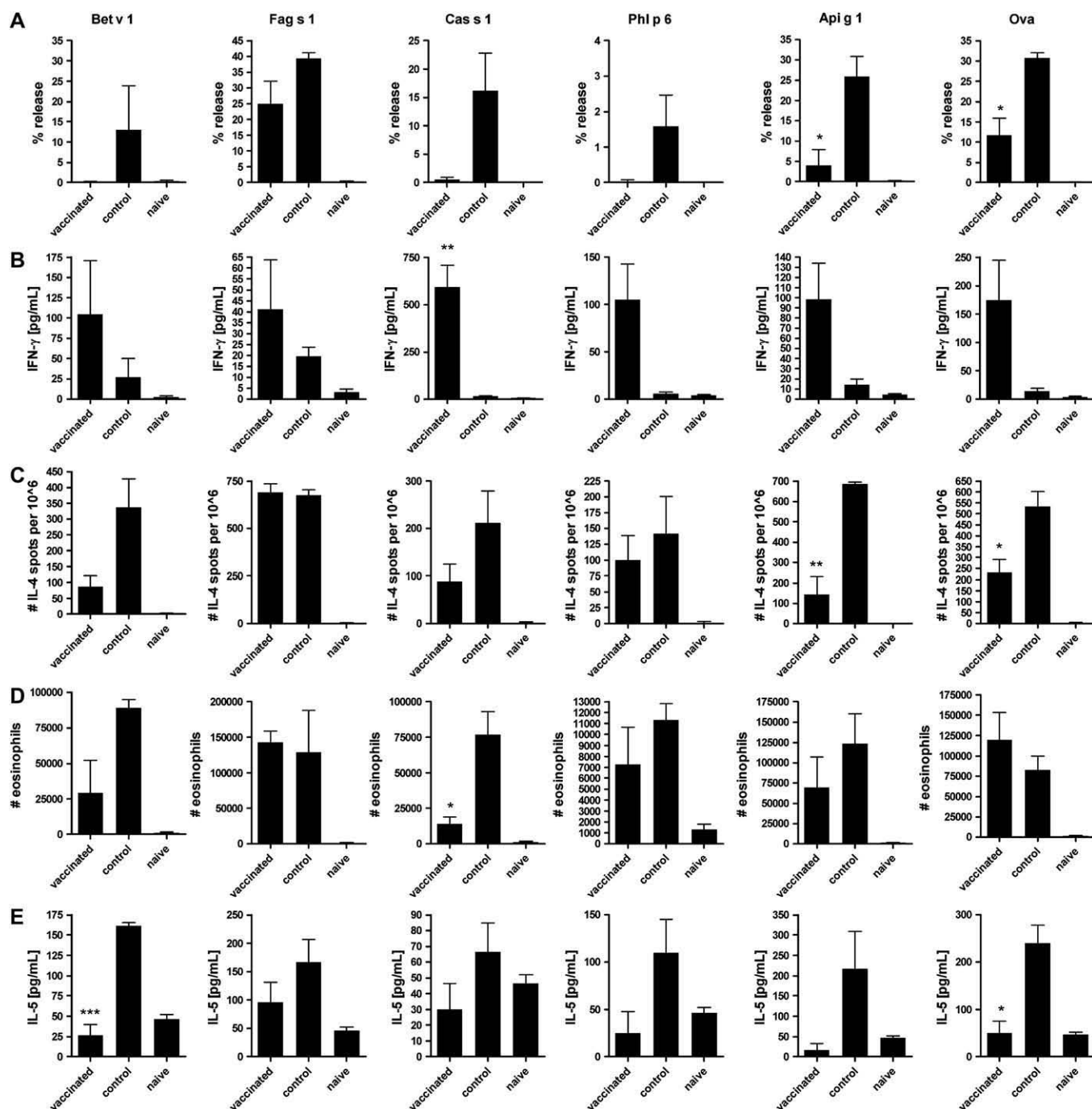


FIG E4. Selected groups of mice ($n = 3$) from Table E1 representing Fagales (Bet v 1, Cas s 1, and Fag s 1), timothy grass (Phi p 6), and food (Api g 1 and ovalbumin [Ova]) allergens were reproduced as described. Suppression of allergen-specific IgE (A, RBL assay), induction of IFN- γ (B, ELISA), suppression of IL-4 (C, ELISPOT assay) in splenocytes restimulated with the respective allergen, invasion of eosinophils into bronchoalveolar lavage fluid (D), and IL-5 levels in bronchoalveolar lavage fluid (E) were measured after sensitization and intranasal challenge. * $P < .05$ and ** $P < 0.01$ compared with the sensitization control groups.

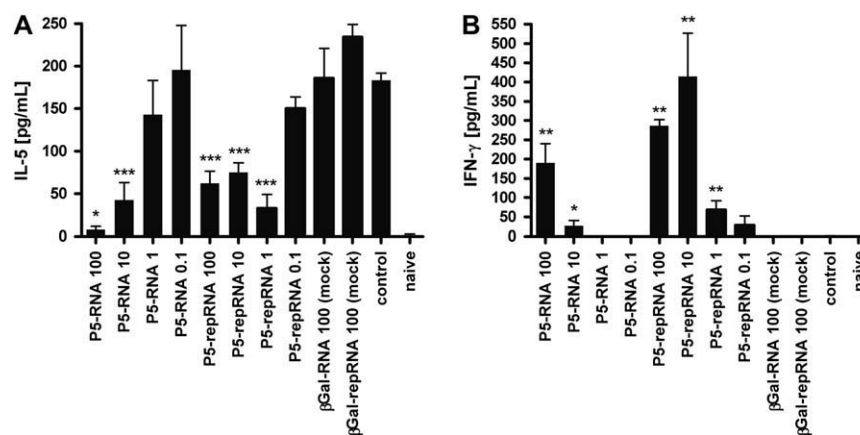


FIG E5. IL-5 (**A**) and IFN- γ (**B**) levels in bronchoalveolar lavage fluid of mice ($n = 5$) prevaccinated with escalating doses (0.1-100 μ g) of mRNA vaccines encoding Phl p 5 or β -galactosidase (mock control) after sensitization and intranasal challenge. * $P < .05$, ** $P < .01$, and *** $P < .001$ compared with the sensitization control groups.

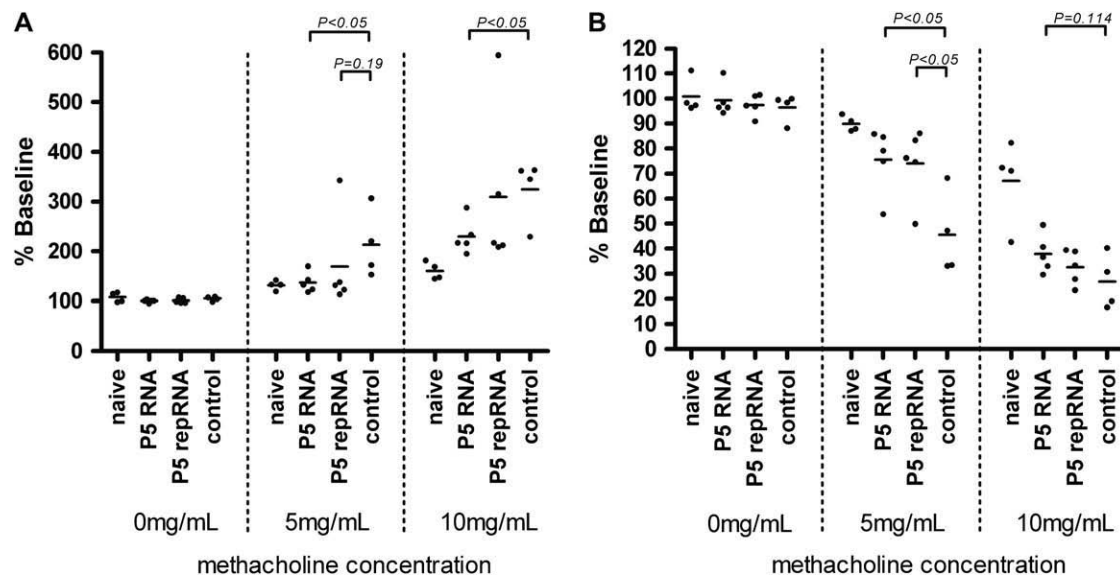


FIG E6. Lung resistance (A) and dynamic compliance (B) after sensitization and intranasal provocation of mice prevaccinated with 40 μ g of P5-RNA or 8 μ g P5-repRNA or sensitization control animals.

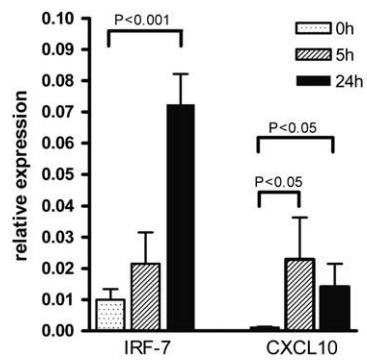


FIG E7. *In vivo* expression of interferon regulatory factor 7 (IRF-7) and CXCL10 in draining lymph nodes of mice (n = 4) 0, 5, and 24 hours after vaccination with 40 µL of P5-RNA.

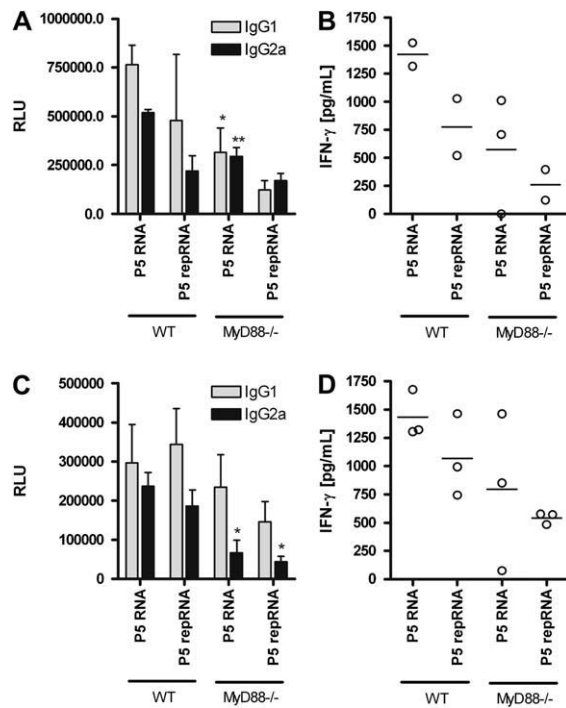


FIG E8. T_H1 immunogenicity of mRNA vaccines is reduced but still intact in $MyD88^{-/-}$ mice. Antibody subclasses (**A** and **C**) and $IFN-\gamma$ secretion (**B** and **D**) from restimulated splenocytes 2 weeks after vaccination (Fig E8, **A** and **B**) and after the last sensitization (Fig E8, **C** and **D**) are shown. Fig E8, **A**: $n = 4$ to 6, serum dilution 1:500. Fig E8, **C**: $n = 3$, serum dilution 1:10,000. Statistical differences between wild-type (*WT*) and corresponding knockout groups: * $P < .05$ and ** $P < .01$.

TABLE E1. Overview of allergens used for preventive mRNA vaccination*

Encoded allergen†	Type of allergen	IgG2a‡	IFN-γ§	IgE Induction	IgE¶ prevention
	Pollen allergen				
Aln g 1	Alder	+ NS	++	—	—
Amb a 1	Short ragweed	—	+	—	ND
Art v 1	Mugwort	—	—	—	ND
Bet v 1	Birch	++	++	—	++
Car b 1	European hornbeam	++	+++	—	+++
Cas s 1	Chestnut	++	+++	—	+++
Cor a 1	European hazel	+/-	++ NS	—	—
Cry j 1	Japanese cedar	+/-	+++	—	ND
Fag s 1	Buckwheat	+	+++	—	+/-
Jun a 1	Ozark white cedar	+/- NS	+++	—	ND
Ole e 1	Olive	—	—	—	ND
Que a 1	White oak	+ NS	++	—	ND
Phl p 1	Timothy grass	+	+	—	ND
Phl p 2	Timothy grass	—	+/-	—	ND
Phl p 6	Timothy grass	+++	+++	—	+++
Phl p 7	Timothy grass	+/- NS	+/-	—	ND
	Food allergens				
Api g 1	Celery	+	+++	—	++
Ara h 2.02	Peanut	+ NS	+++ NS	—	ND
β-casein	Milk	—	+/-	—	ND
Cyp c 1.01	Carp parvalbumin	+/-	—	—	ND
Dau c 1	Carrot	+++	+++	—	—
Mal d 1	Apple	+/-	+++	—	+
OVA	Ovalbumin (egg white)	+/-	+++	—	+
	Animals/molds/latex				
Alt a 1	Mold	+/-	+/-	—	ND
Cla h 8	Mold	—	+++	—	ND
Der p 2	House dust mite	+/- NS	+++ NS	—	ND
Fel d 1	Cat	+	+	—	—
Hev b 6.02	Hevein (rubber tree)	+/-	+/-	—	—

NS, Not specific for vaccinated group; in these groups, similar increased IgG2a responses, IFN-γ responses, or both were seen in the vaccinated and sensitization control groups.

*BALB/c mice (n = 3) were vaccinated with 200 μg of various allergen-encoding conventional mRNAs and sensitized as described for Phl p 5, except sensitization was started 1 week after the final vaccination and was done in a 1-week interval. IgG2a, IgE, and IFN-γ secretion was evaluated 1 week after the last sensitization.

†The respective allergen sequences were cloned into vector pTNT by means of PCR and sequence verified. For Bet v 1 and Ara h 2.02, codon-optimized sequences were used. Sequence variations compared with GenBank sequences and sequences of recoded allergens are shown in Table E2.

‡Allergen-specific IgG2a levels were measured 1 week after the final sensitization. Specific induction of IgG2a in the vaccinated group compared with the sensitization control animals is indicated.

§Specific IFN-γ induction in vaccinated groups compared with that seen in sensitization control animals was measured by means of ELISA.

||Specific IgE levels in vaccinated groups 1 week after the final vaccination.

¶Suppression of IgE induction in vaccinated groups compared with the respective control groups 1 week after the final sensitization. In many groups no allergen-specific IgE could be detected in vaccinated groups, as well as sensitization control groups (ND).

TABLE E2. Sequences used for mRNA vaccination

Allergen/GenBank no.	Sequence used*	Sequence variation compared with GenBank sequence†
Aln g 1 (S50892)	1-483	
Amb a 1 (M80560.1)	89-1207	G103 → A103, CT155-156 → AT155-156, G598 → T598, A793 → C793, T1178 → C1178
Art v 1 (AF493943)	39-437	G206 → A206
Bet v 1 (recoded)		ATGGGGCGTGTTCAACTACGAGACGGAGACCACGAGCGTCATCCCTGCCGCCAGACTCTT CAAGGCCCTTCATCCTCGACGGCGACAACCTCTTTCCCAAGGTCGCCCCCAGGCGATC TCCAGTGTGGAGAACATCGAAGGGAACGGCGGCCCGGTACTATCAAGAAGATCTCTT TCCCGAGGGCTTCCCATTCAGTACGTGAAGGACCGGGTGGACGAGGTTCGATCACAC TAATTTCAAGTACAATTACAGCGTGATTGAAGGCGGGCCAATTGGCGACACCCTGGAGA AGATCAGCAACGAGATCAAGATCGTGGCCACCCCTGACGGCGGGTCCATTCTGAAGAT TTCCAACAAGTACCACACCAAAGGAGACCATGAGGTGAAAGCCGAACAAGTGAAAGC TTCCAAGAGATGGGAGAAACCTGCTGCGGGCGGTGCGAAAGCTATCTGCTGGCACA CAGCGACGCTTATAACTAA
Car b 1 (EU283857)	1-483	
Cas s 1 (FJ390843)	1-483	
Cor a 1 (X70999)	1-483	
Cry j 1 (D34639)	179-1240	A190 → C190, G304 → A304, G310 → A310, C325 → T325
Fag s 1 (FJ390846)	1-483	
Jun a 1 (AF10662)	64-1104	T501 → C501, C606 → T606, C783 → T783
Ole e 1 (Y12428)	1-441	
Que a 1 (EU283863)	1-483	
Phl p 1 (X78813)	694-792	
Phl p 2 (X75925)	103-369	
Phl p 6 (Y16956)	102-434	
Phl p 7 (Y17835)	49-285	
Api g 1 (Z48967)	1-465	
Ara h 2.02 (recoded)		ATGGCCAAACTGACGATCCTGGTTGCTCTGGCTCTGTTTCTGCTGGCTGCTCATGCTTCT GCCCCCAACAATGGGAACTGCAAGGCGACCGTCGTTGCCAATCTCAACTGGAACGT GCCAATCTGCGCCCGTGTGAACAACATCTGATGCAAAAAATCCAACGTGACGAAGAT TCTTATGGTCGTGATCCGTATTCTCCGAGTCAAGATCCGTATAGCCCGTCCCAAGATCC GGACCGTCGTGACCCGTATAGTCCGAGTCCGTATGATCGCCGTGGTGTGTTCTTCT CAACACCAAGAACGTTGCTGTAACTGAACGAATTTGAAAACAATCAACGCTGT ATGTGTGAAGCGCTGCAACAAATCATGGAAAACCAATCCGACCGCTGCAAGGCCGT CAACAGGAACAACAATTCAAACGTGAACGCAACCTGCCGCAACAATGTGGCCTG CGTGCACCGCAACGCTGTGATCTGGAAGTGAATCCGGTGGTCGTGACCGCTATTAA
β-Casein (NM18108)	105-734	A422 → C422
Cyp c 1.01 (AJ292211)	55-384	
Dau c 1 (DCZ84376)	1-465	
Mal d 1 (Z48969)	89-568	
OVA (AY223553)	27-1187	C875 → A875
Alt a 1 (AAU82633)	75-474	C96 → G96
Cla h 8 (AY1918169)	52-855	
Der p 2 (DQ185510)	1-387	C324 → T324, T331 → A331, C354 → T354, additional bases 388-402: catcaccatcaata
Fel d 1 (published)		Grönlund H, et al. J Biol Chem. 2003;278:40144-51
Hev b 6.02 (EU185059)	61-189	

*Sequence cloned into pTNT. Numbers refer to the respective GenBank file.

†Sequence variation of the used constructs compared with the published GenBank data is shown. Numbers refer to the base positions of the respective GenBank files. Sequences of Bet v 1 and Ara h 2.02 were synthesized with optimized codon use (Mr Gene, Regensburg, Germany).