

In vivo application of RNA leads to induction of specific cytotoxic T lymphocytes and antibodies

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To study the efficiency of RNA-based vaccines, RNA coding for the model antigen β -galactosidase (β -gal) was transcribed *in vitro* from a lacZ gene flanked by stabilizing *Xenopus laevis* β -globin 5' and 3' sequences and was protected from RNase degradation by condensation with the polycationic peptide protamine. The liposome-encapsulated condensed RNA-peptide complex, the condensed RNA-peptide complex without liposome or naked, unprotected RNA, was injected into BALB/c (H-2^d) mice. All preparations led to protein expression in the local tissue, activation of L^d-restricted specific cytotoxic T lymphocytes (CTL) and production of IgG antibodies reactive against β -gal. RNA-triggered CTL were as efficient in the lysis of lacZ-transfected target cells as CTL triggered by a lacZ-DNA eukaryotic expression vector. Immunization with RNA transcribed from a cDNA library from the β -gal-expressing cell line P13.1 again led to β -gal-specific CTL and IgG induction. Thus, both naked and protected RNA can be used to elicit a specific immune response *in vivo*, whereby the protected RNA is stable *in vitro* for a longer period of time. RNA vaccines can be produced in high amounts and have the same major advantages as DNA vaccines but lack the potentially harmful effect of DNA integration into the genome.

Key words: RNA vaccine / CTL induction / RNA protection / RNA library / β -Galactosidase

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1 Introduction

A large number of human tumor-specific antigens have been identified which can be utilized for tumor gene therapy [1]. In addition there are some approaches for inducing tumor immunity without the need to identify the tumor-specific antigens, such as immunization with heat-shock proteins [2] or multiepitopic RNA derived from tumor cells [3]. Nucleic acids are important tools for somatic gene therapy and prophylactic and therapeutic vaccination against infectious and malignant diseases. Typically, DNA vaccines are bacterial plasmids carrying genes for pathogen or tumor antigens that are transcribed in the injected host using a strong viral promoter

[4]. They offer concrete alternatives to conservative methods for generating CTL by priming *in vivo* with traditional live attenuated virus [5] with its associated risks or by immunization with peptides [6] which may be limited in their suitability for genetically variant populations.

Since RNA is very prone to hydrolysis by ubiquitous ribonucleases, gene therapy approaches involving nucleic acids have focused on DNA. A potentially unique advantage of using RNA rather than DNA is that no virus-derived promoter element has to be administered *in vivo* and no integration into the genome may occur randomly or as a result of homologous recombination leading to mutational inactivation of cellular genes, and, more importantly, the possibility of oncogenesis [7]. It has been shown that naked RNA injected into mouse skeletal muscle does indeed lead to gene expression *in vivo* [8] and that cytotoxic T cells can be primed *in vivo* with liposome-encapsulated mRNA, which so far could only be prepared by a complicated procedure [9]. It also has been shown that a carcinoembryonic antigen (CEA)-specific antibody response could be induced by CEA

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mRNA injection i.m. into mice. In this case, however, the procedure involved 10 injections within 5 weeks and challenge with syngeneic, CEA-expressing colon carcinoma cells [10]. These data suggested that RNA vaccines, despite their advantages, are insignificant in a practical approach when compared to DNA-based vaccines. To investigate the efficiency of RNA-based vaccines in inducing both humoral and cellular immune responses systematically we engineered a plasmid vector for *in vitro* transcription of lacZ RNA (β glacZ β g α_n RNA) containing a cap structure, a poly(A) tail which is flanked at the 5' end and the 3' end by untranslated regions (UTR) from β -globin of *Xenopus laevis* to enhance cytosolic protein expression [11, 12]. These constructs were injected into BALB/c mice under various conditions, and the CTL and antibody responses were measured.

2 Results

It is known that to become committed to proliferation, naive T cells require prolonged antigenic stimulation, a co-stimulus and a specific dose of antigen [13]. To test the capability of RNA-transfected HeLa-K^b cells to prime specific CTL [14], HeLa-K^b cells [15] were electroporated with β glacZ β g α_n RNA and injected i.p. into B6 (H2^b) mice. A specific CTL response against β -gal after stimulation of spleen cells *in vitro* with the synthetic peptide ICPMYARV corresponding to the K^b-presented epitope of *E. coli* β -galactosidase (amino acid sequence 497–504) [16] was observed that was comparable to CTL induction upon immunization with *in vitro* RNA-transfected syngeneic dendritic cells [3] (Fig. 1). To assess its immunogenic potential *in vivo*, we injected β glacZ β g α_n RNA directly into BALB/c mice. Protamine, a small naturally occurring, arginine-rich DNA-binding protein [17], was used to condense and protect RNA from RNase degradation. As shown in Fig. 2, the protamine RNA complex is less sensitive to RNase activity in 2.5 % fetal bovine serum (undegraded full-length RNA was visible after 60 min of incubation) as compared to naked RNA, which was degraded within minutes.

We investigated three vaccination protocols with (i) liposome-encapsulated, protamine-condensed RNA, (ii) protamine-condensed RNA and (iii) naked RNA.

(i) Protamine-condensed β glacZ β g α_n RNA (30 μ g) was encapsulated with the cationic liposome Unifectin by a very simple procedure and injected i.v. and s.c. into BALB/c mice. Here we obtained a specific CTL response against β -gal (Fig. 3 a, b) after stimulation *in vitro* with P13.1 cells (H-2^d, lacZ-transfected). Control mice were immunized intravenously with 30 μ g of Unifectin-encapsulated, protamine-condensed β ggfp β g α_n RNA,

10⁵ HeLa-K^b RNA transfected, i.p. injected

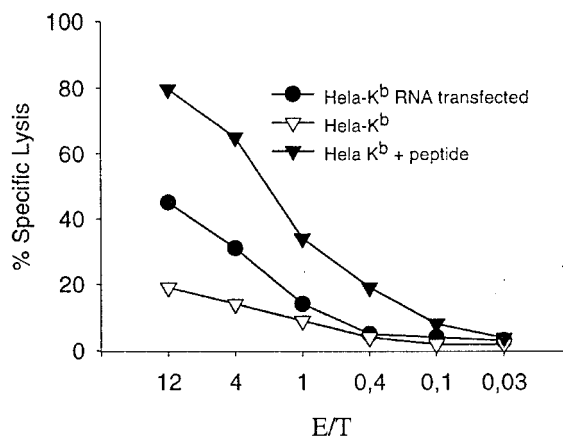


Fig. 1. Induction of β -gal-specific CTL activity. B6 mice were immunized i.p. with 10⁵ HeLa K^b cells electroporated with β glacZ β g α_n RNA. Spleen cells were stimulated *in vitro* with the synthetic peptide ICPMYARV corresponding to the amino acid sequence 497–504 of *E. coli* β -galactosidase. CTL activity was determined in a standard ⁵¹Cr-release assay 5 days after *in vitro* restimulation. The targets were HeLa (HeLa) K^b cells, HeLa K^b cells transfected with β glacZ β g α_n RNA and HeLa K^b cells incubated with synthetic peptide ICPMYARV.

encoding the gfp gene from *Aequoria victoria* (Fig. 3 c). Application of Unifectin-encapsulated, protamine-condensed β glacZ β g α_n RNA, in the form of i.m. and i.p. injection did not generate specific CTL (data not shown). (ii) To test whether injection of protamine-condensed RNA without any liposome is able to generate specific CTL, 30 μ g of protamine-condensed β glacZ β g α_n RNA was injected i.v., s.c. and i.m. With this approach, no

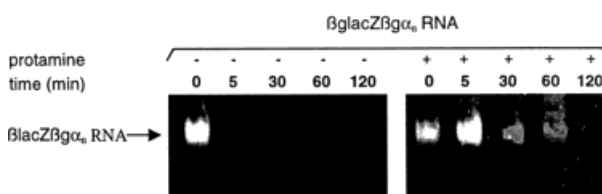


Fig. 2. RNA degradation protection assay. Arrowhead indicates 0.5 μ g of naked β glacZ β g α_n RNA or protamine-condensed β glacZ β g α_n RNA, respectively. RNA was incubated with 2.5 % fetal bovine serum in vaccination buffer at room temperature. At the indicated time, samples were withdrawn, treated with RNase inhibitor, frozen at -70°C and analyzed on a 1 % agarose gel.

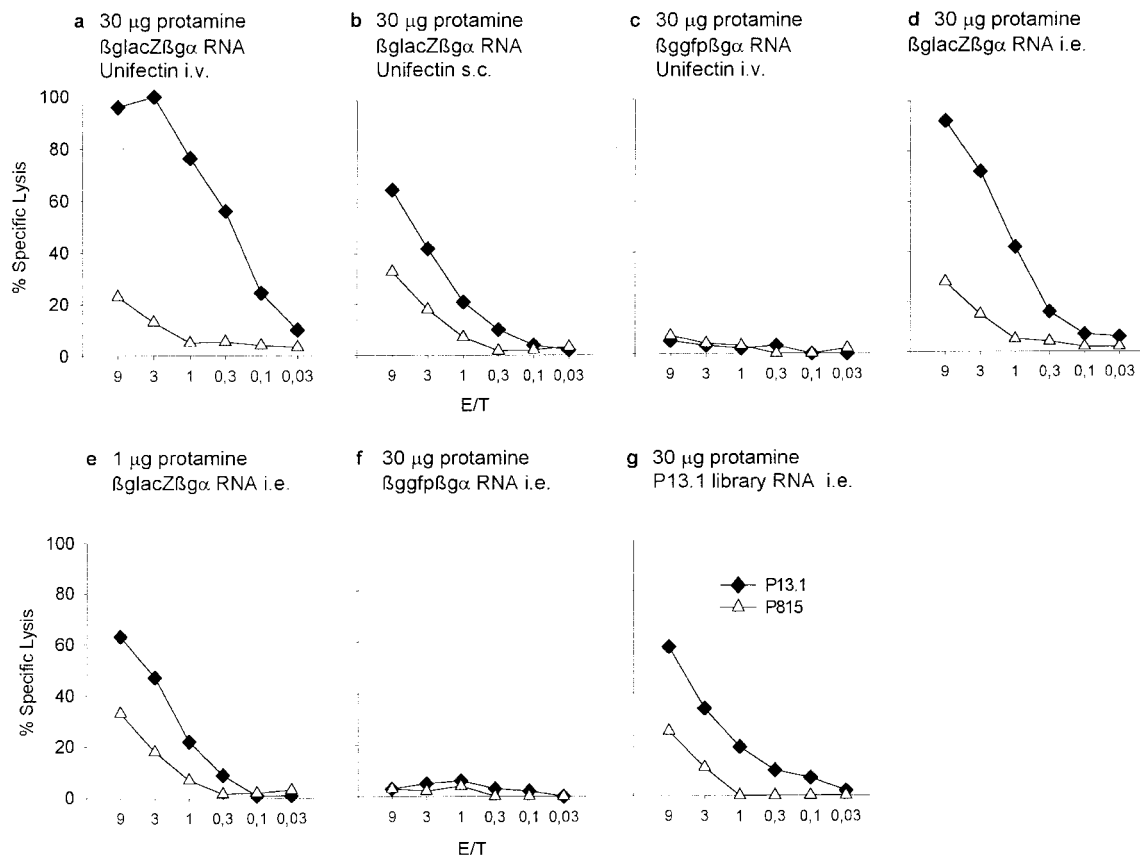


Fig. 3. Induction of β -gal-specific CTL activity. BALB/c mice were immunized as described with Unifectin-encapsulated protamine-condensed RNA or with protamine condensed RNA. Thirty micrograms of protamine- β glacZ β g α_n RNA + Unifectin was injected i.v. (a) or s.c. (b) and 30 μ g of protamine- β ggfp β g α_n RNA + Unifectin was injected i.v. as a control (c). Using the ear pinna as target site (i.e.), 30 μ g of protamine- β glacZ β g α_n RNA (d), 1 μ g of β glacZ β g α_n protamine-RNA (e), 30 μ g of protamine- β ggfp β g α_n RNA as a control (f) and 30 μ g of P13.1 protamine-library-RNA (g) were injected once, respectively. Spleen cells were stimulated twice *in vitro* with the synthetic peptide TPHPARIGL corresponding to the H-2^d epitope. *E. coli* β -gal (amino acid 876–884 sequence). CTL activity was determined in a standard ⁵¹Cr-release assay 5 days after *in vitro* restimulation. The targets were P13.1 (H-2^d lacZ transfected) and P815 cells (H-2^d).

CTL response could be obtained (data not shown). We investigated the efficiency of RNA vaccination using the ear pinna (i.e.) as injection site. Surprisingly a single injection of 30 μ g protamine-condensed β glacZ β g α_n RNA into the ear pinna triggered a specific CTL response that was recalled after stimulation *in vitro* using a synthetic peptide corresponding to the H-2^d β -gal epitope TPHPARIGL (amino acid sequence 876–884) [18] (Fig. 3 d). Only 1 μ g of protamine-condensed β glacZ β g α_n RNA (a low amount of nucleic acid compared to the normal application for DNA vaccination in mice which involves at least 50 μ g of DNA [19, 20] sufficed for *in vivo* CTL priming (Fig. 3 e). Control mice immunized i.e. with 30 μ g of protamine-condensed β ggfp β g α_n RNA did not develop anti- β -gal CTL (Fig. 3 f). These results empha-

size the importance of choosing the most suitable injection site for successful nucleic acid vaccination. After demonstrating that it is possible to raise CTL *in vivo* directed against a single protein encoded by a homogeneous RNA population, we explored the assumption that a complex mRNA mixture could trigger antigen-specific CTL as well. It has previously been shown that RNA transcribed *in vitro* from a cDNA library can be taken up by dendritic cells (DC) *in vitro* and that RNA-loaded DC induce CTL responses *in vivo* [3]. The possibility of triggering a CTL response directed against β -gal by employing a complex mixture of RNA was evaluated using RNA transcribed *in vitro* from a cDNA library of P13.1 cells (H-2^d, lacZ transfected). Immunization (i.e.) with protamine-condensed P13.1 library-RNA, indeed led to

a specific CTL response against β -gal (Fig. 3 g), even though only a low amount of lacZ-encoding RNA can be expected in the 30 μ g of total unfractionated P13.1 RNA.

(iii) To test whether protection of RNA by protamine is essential for *in vivo* CTL priming, we immunized BALB/c mice i.e. with 30 μ g of unprotected β glacZ β g α_n RNA immediately after preparation (Fig. 4 a). Surprisingly, naked RNA immunization also generated a β -gal-specific CTL response that was comparable to the injection of 30 μ g of naked pCMV β plasmid DNA i.e., for example (Fig. 4 b). On the contrary, injection of unprotected β ggfp β g α_n RNA, as well as RNase-treated β glacZ β g α_n RNA did not induce β -gal specific CTL (Fig. 4 c). Furthermore, the injection of unprotected β glacZ β g α_n RNA stored for 1 h at room temperature in

buffer that was not specifically RNase free did not induce CTL (data not shown).

The development of humoral immunity upon naked RNA immunization was tested by β -gal ELISA plate assay using sera 2 weeks after immunization. IgG antibodies specific for β -gal are detectable in sera of mice immunized i.e. either with 30 μ g of pCMV β plasmid DNA, 30 μ g of unprotected β glacZ β g α_n RNA, or 30 μ g of protamine-condensed β glacZ β g α_n RNA. Sera of mice injected i.e. with β ggfp β g α_n RNA was used as control (Fig. 4 e).

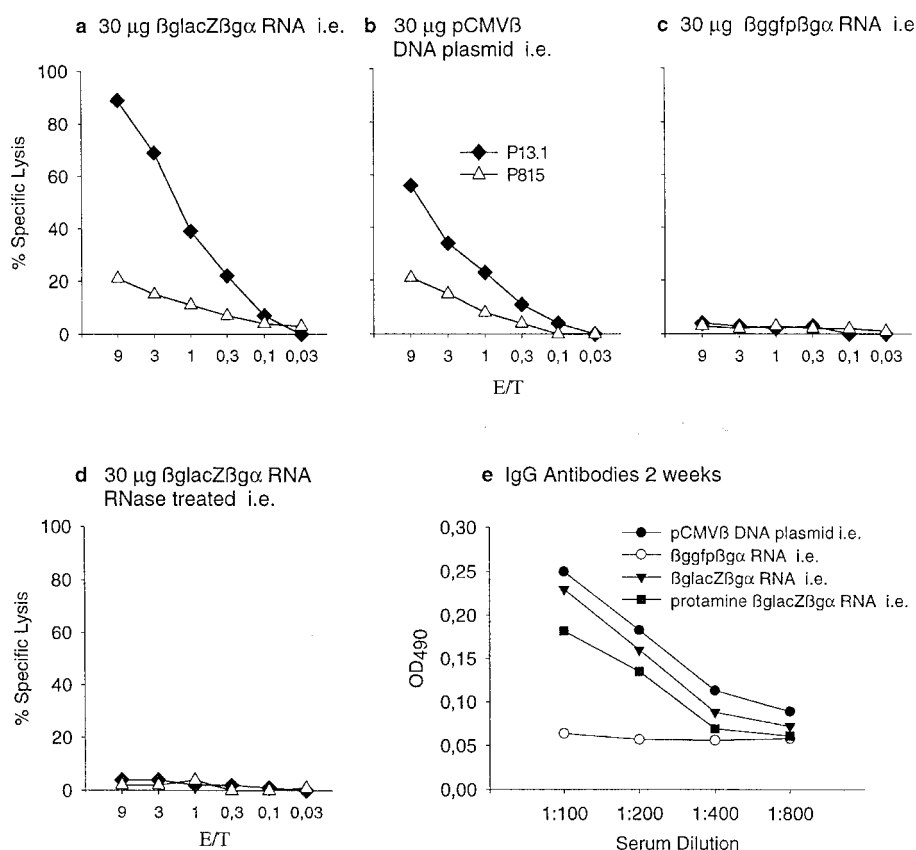


Fig. 4. Induction of β -gal specific CTL activity. BALB/c mice were immunized i.e. with 30 μ g of unprotected β glacZ β g α_n RNA (a) or with 30 μ g of pCMV β DNA plasmid (b) as described [12]. Control mice were immunized i.e. with 30 μ g of unprotected β ggfp β g α_n RNA (c) or with 30 μ g of RNase-treated β glacZ β g α_n RNA (d). Spleen cells were stimulated twice *in vitro* with the synthetic peptide TPHPARIGL. CTL activity was determined in a standard ^{51} Cr-release assay 5 days after *in vitro* restimulation. The targets were P13.1 cells (H-2^d lacZ-transfected) and P815 cells (H-2^b). The humoral IgG anti- β -gal immune response was detected by ELISA from sera 2 weeks after immunization (e). Average values were estimated by measuring the A₄₉₀ of 1:100 to 1:800 diluted sera. Mice were immunized once i.e. with 30 μ g of pCMV β DNA plasmid, 30 μ g of β ggfp β g α_n RNA, 30 μ g of unprotected β glacZ β g α_n RNA or 30 μ g of protamine- β glacZ β g α_n RNA.

3 Discussion

Our observations lead us to draw conclusions for vaccine development, especially with respect to therapeutic vaccination against cancer. Although the efficiency of freshly prepared naked RNA for vaccination is unlikely to promote its use in a clinical setting, protamine-protected RNA is more stable, easy to handle and appears to have all the advantages of DNA vaccination, but without the intrinsic risk of DNA integration into the genome and the induction of pathogenic anti-DNA antibodies [21].

Before protected RNA can be used as a vaccine, however, a number of issues have to be clarified. A suitable route for immunization in humans has to be found, since s.c. and i.m. immunization of mice with RNA was not effective, whereas immunization into the ear pinna was highly efficient, and can potentially be compared to i.d. injection in humans. However, this has to be clarified in future trials. Ear pinna tissue was tested 24 h after injection with protamine-condensed β glacZ β g α_n RNA for expression of the lacZ gene by X-gal staining. The intensity of β -gal expression was strong and not distinguishable from that of a control pCMV- β DNA plasmid (data not shown). This is reminiscent of similar observations with DNA immunizations [22].

In addition, the reason for the expression of RNA which occurs so readily in an immunogenic way, needs to be elucidated (RT-PCR confirmed that β glacZ β g α_n RNA was present in the ear pinna for more than 48 h after injection, data not shown). One possibility is that RNA transduces DC directly *in vivo*. It is certainly an advantage that the *in vivo* uptake of RNA is mediated without the need for any adjuvants or transfection vehicles, such as cationic liposomes, which often cause latent toxicity in higher doses. Cationic liposomes are highly toxic toward macrophages and induce a drop in the production of at least two immunomodulators by activated macrophages [23]. The RNA-protective protamine does not appear to be immunogenic since no protamine-specific IgG antibodies were detected in sera of mice (data not shown).

As a strategy for prophylactic or therapeutic vaccination, RNA can be used as multiepitopic RNA coding for known antigens, without regard for HLA expression in the vaccinee (as is required for peptide vaccines [6]). RNA prepared from a cDNA library established from a small tumor sample [3] seems to present a way of inducing immunity against certain types of cancer in patients in which tumor rejection antigens have not been identified, although it bears the potential risk of inducing autoimmunity, as does any other complex, tumor-derived vaccine, such as gp96 [24]).

In conclusion, we have demonstrated that RNA preparations are suitable tools for vaccination. RNA can be administered naked when freshly synthesized or protamine-protected when stored before injection. For practical purpose, RNA can be stored in precipitated form and solubilized in transfection buffer containing protamine directly before vaccination.

4 Materials and methods

4.1 Plasmids and RNA synthesis and electroporation

The lacZ ORF was excised from pCH110 (Pharmacia) and subcloned into pSpjC-1 (J. Lord, Warwick, GB) to generate pSpjC- β glacZ β g α_n , possessing *Xenopus laevis* β -globin untranslated 5' and 3'-sequences flanking the insert. The gfp cDNA from pGFP (Clontech Inc.) was inserted into pT7TS (P. A. Krieg, Austin, TX) to create pT7TS β -ggfp β g α_n for the synthesis of GFP transcripts flanked by untranslated 5' and 3'-sequences from β -globin.

The cDNA library from P13.1 cells [25] was constructed with the ZAP-cDNA Synthesis Kit (Stratagene Inc.), capped mRNA were prepared by *in vitro* transcription with the Sp6 Cap-Scribe (Boehringer-Mannheim), treated with DNase (2 U/ μ g template DNA) phenol extracted, precipitated, solubilized in DEPC-treated water and stored at -80°C .

Hela-K^b cells were electroporated in PBS at a density of 5×10^6 cells/500 μ l/cuvette with a Gene Pulser (Bio-Rad) set at 850 V, 50 μ F, $\infty \Omega$.

4.2 Immunization with nucleic acids

Protamine and the cationic liposome Unifectin were obtained from A. Surovoy, Tübingen. A protamine:RNA-mixture (1:2) was incubated in 100 μ l vaccination buffer (150 mM NaCl, 10 mM Hepes, pH 7.4) at room temperature for 5 min before injection. Unifectin-protamine-encapsulated RNA was prepared in a protamine:RNA:liposome ratio (μ g) of 1:1:2. Unifectin was added 10 min after incubation of protamine-RNA in 60 μ l vaccination buffer at room temperature. The vaccination solution (100 μ l) was injected i.p., i.v., s.c., and i.m. Naked RNA and DNA plasmid controls were prepared in 100 μ l DEPC-treated vaccination buffer and applied immediately. Alternatively, 50 μ l vaccination solution was injected into each ear pinna of anesthetized mice (Metofane, Janssen).

4.3 Cell culture, ^{51}Cr -release assay and ELISA

P815 and P13.1 cells [25] were cultured in α -MEM containing 10 % FCS, 2-ME, L-glutamine, and antibiotics. Seven to nine days after immunization of mice, recipient spleens were

removed and the splenocytes were restimulated with synthetic β -gal peptide TPHPARIGL (ICPMYARV for the Hela-K^b approach) at 50–100 mM. CTL lines were generated by weekly restimulation with syngeneic, irradiated spleen cells and synthetic peptides as described [26].

Lysis of target cells was tested in a standard 5-h ⁵¹Cr-release assay described previously [27]. Where applicable, target cells were pulsed with peptide during the ⁵¹Cr labeling period. After incubation of effector and target cells in round-bottom 96-well plates for 5 h, 50 μ l of 200 μ l culture supernatant were removed and radioactivity was measured in a microplate format scintillation counter (1450 Microbeta Plus), using solid-phase scintillation (Luma Plate-96, Packard). Percent specific release was determined from the amount of ⁵¹Cr released into the medium (A), corrected for spontaneous release (B), and compared to the total ⁵¹Cr content of the 1 % Triton-X-100 lysed target cells (C): % Specific lysis = 100(A–B)/(C–B).

For ELISA, blood samples were obtained from the tail vein of mice. Sera were prepared and ELISA plates (Greiner) were coated overnight with β -galactosidase (Sigma) in PBS buffer at 4 °C. After washing with 0.05 % Tween-20/PBS the plates were blocked with 1 % bovine serum albumin/PBS for 1 h at 37 °C. Sera were diluted in 0.05 % Tween-20/PBS and administered to the plates for 1 h at 37 °C. After further washing steps, bound antibodies were spotted with a peroxidase-conjugated goat anti-mouse IgG immunoglobulin (Sigma). The plates were washed and developed with 5-aminosalicylic acid (Sigma) in phosphate buffer and read at 490 nm.

4.4 RNA degradation protection assay

β glacZ β gal RNA (0.5 μ g) was incubated for 5 min in 5 μ l vaccination buffer containing 0.25 μ g of protamine. Five microliters of vaccination buffer containing 5 % fetal bovine serum was added and incubated at room temperature. At an indicated period of time, samples were withdrawn and mixed with 2 μ l vaccination buffer containing 10 U of RNase inhibitor (Sigma) and frozen at –70 °C. DEPC-treated vaccination buffer (4 μ l) containing 10 U of proteinase K and 4 μ l DEPC-treated vaccination buffer containing 20 % SDS and loading dye was added. Samples were analyzed on a 1 % agarose gel in DEPC-treated Tris-acetate-EDTA buffer.

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