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Effective induction of anti-melanoma immunity following genetic vaccination with synthetic mRNA coding for the fusion protein EGFP.TRP2

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Abstract RNA-based genetic immunization represents an alternative novel strategy for antigen-specific cancer vaccines. In the present paper we investigate the use of synthetic messenger RNA in an experimental melanoma model. We show that gene gun-based immunization using synthetic RNA mediates gene expression in the epidermis and effectively induces antigen-specific cellular and humoral immunity in mice *in vivo*. Importantly, bombardment of the skin with RNA coding for the melanocytic self-antigen TRP2 linked to the immunogenic protein EGFP was associated with protection against experimentally induced B16 melanoma lung metastases and vitiligo-like fur depigmentation. Our results provide a scientific basis for clinical trials using synthetic mRNA encoding melanocytic antigens linked to immunogenic helper proteins for vaccination of patients with melanoma.

Keywords RNA · Melanoma · Vaccine · TRP2

Abbreviations EGFP: Enhanced green fluorescent protein · mTRP2: Murine tyrosinase-related protein 2

Introduction

The molecular identification of tumor antigens recognized by specific cytotoxic T lymphocytes (CTL) has revolutionized the field of cancer immunotherapy, opening up new possibilities for the induction and measurement of antigen-specific immune responses. The concept of antigen-specific vaccination has generated great interest particularly for the immunotherapy of melanoma, a tumor entity, which has been the focus of interest of many tumor immunologists over years and for which a large series of different antigens have been defined. These include lineage specific differentiation antigens such as the tyrosinase family of enzymes involved in melanin synthesis, which are endogenously expressed in melanocytes and melanoma cells, cancer-germline antigens such as the MAGE family, overexpressed antigens such as telomerase and individually mutated proteins [1–4]. Currently, it is still unclear, which antigen and which vaccination strategy should be applied to optimally induce clinically effective immune responses against melanoma.

Our group has previously tested several vaccination strategies in an experimental mouse model targeting the melanocytic self-antigen TRP2, which has been identified as a tumor rejection antigen with melanoma-specific cytotoxic T cells [5]. We and others observed that peripheral immune tolerance limits the induction of potentially autoreactive CD8⁺ T cells [6–8]. Strong TRP2-specific cellular immunity could only be stimulated when immunogenic helper determinants were linked to the weakly immunogenic self-antigen TRP2 [9]. Here we investigated the use of synthetic messenger RNA as an alternative antigen format for a gene gun-based genetic immunization protocol. We show that gene gun immunization using synthetic mRNA coding for TRP2 linked to the immunogenic EGFP can also effectively induce antigen-specific cellular immunity in mice *in vivo* and mediate protection against B16 lung metastases and vitiligo-like fur depigmentation.

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Materials and methods

Animals, donors and cell lines

C57BL/6 mice (H-2^b), 6–12 weeks old, were bred at the Central Animal Facility of the University of Bonn. B16 (H-2^b) is a spontaneous murine melanoma and was maintained in DMEM supplemented with 10% heat-inactivated FCS, 2 mM L-glutamine, 50 μ M 2-mercaptoethanol, 100 IU/ml penicillin, and 100 μ g/ml streptomycin (all reagents were from Life Technologies GmbH, Eggenstein, Germany). All experiments with mice were approved by the local governmental authorities and carried out according to standard guidelines for the care of laboratory animals.

Plasmids and in vitro transcription and polyadenylation of RNA

The plasmids pCI- β -gal and pCI-EGFP.TRP2 expressing *Escherichia coli* β -gal and the fusion protein of EGFP and murine TRP2 (EGFP.TRP2) have been described previously [9]. Plasmids were grown in *E. coli* strain DH5 α and purified using Qiagen Plasmid Maxi Kits (Qiagen, Hilden, Germany). Plasmids were linearized with Bam H I (Roche, Mannheim, Germany). In vitro transcription was performed as described previously [10]. With the Ribomax large-scale RNA production system T7 (Promega, Mannheim, Germany) according to the manufacturer's instructions. Briefly, linearized DNA templates were incubated for 4 h at 37°C in a reaction containing T7 10 times reaction buffer, ATP, CTP, UTP (7.5 mM each), 1 mM GTP, 5 mM m⁷G(5')ppp(5')G cap-analogon (Invitrogen GmbH, Karlsruhe, Germany) and T7 RNA Polymerase. The RNA was resuspended in water and stored at –80°C. In vitro-transcribed RNA was polyadenylated using the Poly (A) Polymerase (USB Cleveland, OH, USA) according to the manufacturer's instructions. Briefly, in vitro-transcribed RNA was incubated for 1 h at 30°C in a reaction containing Poly (A) polymerase reaction buffer, ATP (Promega; 600 pmol per pmol RNA) and Poly (A) polymerase (USB; 12U per pmol RNA). mRNA was resuspended in water and stored at –80°C.

Genetic immunization of mice

Genetic immunization with synthetic RNA or plasmid DNA using the gene gun has been performed as described previously [9]. Briefly, plasmid DNA was precipitated onto 1.6 μ m gold particles at a density of 2 μ g per mg of particles. Synthetic RNA was precipitated at a density of 30 μ g per mg of particles. Gold particles were resuspended in 100 μ l of 0.05 M spermidine (Sigma Chemical Co., St. Louis, MO, USA) and DNA or RNA

precipitated by the addition of 100 μ l of 1 M CaCl₂. Particles were washed with ethanol to remove H₂O, resuspended in ethanol containing 0.075 mg/ml of PVP (Sigma), and coated onto the inner surface of Tefzel tubing using a tube loader. The tubing was cut into 0.5 inch segments, each containing approximately 0.5 mg gold coated with 1 μ g plasmid DNA or 15 μ g of synthetic RNA. For immunizations, shaved skin of the abdomen was transfected in vivo by two shots with the Helios gene gun at a pressure of 400 psi, resulting in the delivery of a total of approximately 2 μ g of plasmid DNA or 30 μ g of synthetic RNA. Immunizations were repeated after 1 week. Gold particles, tubing, tube loader, and the Helios gene gun were purchased from BioRad (Munich, Germany).

Verification of antigen-expression in vivo

Skin sections were taken 24 h after gene gun bombardment with β -galactosidase-encoding or EGFP.mTRP2-encoding mRNA or plasmid DNA and cryo-conserved. 4–5 μ m section were prepared, fixed with acetone and analyzed. β -gal expression in keratinocytes of superficial epidermis and hair follicles was determined by standard β -gal staining using X-Gal substrate (1 mg/ml X-Gal in PBS/5 mM potassium ferri-cyanide/5 mM potassium ferrocyanide/2 mM magnesium chloride; Roth, Karlsruhe, Germany). EGFP expression was determined by fluorescence microscopy.

Sandwich ELISA assays

Antibodies against β -gal or EGFP present in serum samples obtained from mice after genetic immunization were measured by ELISA using recombinant EGFP or β -gal produced in *E. coli* and purified by affinity chromatography as a solid-phase antigen. Briefly, microtiter plates were coated with 5 μ g/ml protein in carbonate buffer (pH 9.6) for 16 h at 4°C and blocked with PBS containing 3% BSA and 0.05% Tween. Serum samples were serially diluted in PBS containing 1% BSA and 0.05% Tween (assay buffer) and added for 2 h. Bound antibodies were detected using peroxidase-conjugated donkey anti-mouse IgG (Jackson ImmunoResearch, Westgrove, PA, USA) at a 1:5000 dilution in assay buffer followed by color development with ABTS substrate system (Sigma). Plates were read in an ELISA reader at 405 nm.

Peptides and ELISPOT assays

The H-2K^b-binding peptide SVYDFVWL (TRP2_{aa180–188}) derived from the murine melanosomal protein TRP2 [5] and the H-2K^b-binding peptide IC-PMYARV (β -gal_{aa497–504}) derived from *E. coli* β -gal were purchased from Sigma Genosys. Peptides were dissolved at 10 mg/ml in PBS containing 10% DMSO

and stored at -20°C . The induction of peptide-specific T-cells was measured using the ELISPOT technique. Briefly, splenocytes were harvested 1 and 2 weeks after immunization and red blood cells depleted. 10^6 splenocytes/well were restimulated in 200 μl of CM containing 1 $\mu\text{g}/\text{ml}$ synthetic peptide and 25 IU/ml rhIL-2 in Millipore HA plates, which were coated overnight with 10 $\mu\text{g}/\text{ml}$ (50 $\mu\text{l}/\text{well}$) of anti-mIFN γ mAb (R4-6A2, Pharmingen, Heidelberg, Germany) in PBS. After 22 h cells were washed out of the plates and bound cytokines visualized by incubation with 2.5 $\mu\text{g}/\text{ml}$ (50 $\mu\text{l}/\text{well}$) of biotinylated anti-mIFN γ mAb (XMG1.2, Pharmingen) for 1.5 h at 37°C , followed by 100 $\mu\text{l}/\text{well}$ streptavidin-peroxidase (Roche, 1:1500 dilution in PBS containing 1% BSA, and 0.05% Tween 20) for 1/2 h at RT, and premixed peroxidase substrate kit DAB (Vector Laboratories, Heidelberg, Germany). The number of spots was counted under a dissecting microscope and expressed as mean number of spots of repeated duplicate or triplicate determinations. All experiments were performed three to five times.

Tumor protection assays and detection of vitiligo

Induction of antitumor immunity was assessed 2 weeks after immunization. Mice were challenged by intravenous injection of 4×10^5 B16 melanoma cells, which experimentally induces heavily pigmented lung metastases. The number of macroscopically visible metastases on the surface of the lungs was counted with the help of a dissecting microscope 14 days after challenge. All experiments included six mice per group and were performed three times with similar results. Alternatively, mice were followed for the appearance of coat color changes in the bombarded areas of the shaved abdomen.

Results

Expression of antigen in the skin following gene gun bombardment with synthetic RNA

Initially, we wished to optimize expression of antigen in the epidermis following bombardment of the skin with mRNA-coated gold particles. Based on our previous experience with plasmid DNA we used the bacterial protein *E. coli* β -galactosidase (β -gal) and the melanosomal enzyme TRP2 fused to enhanced green fluorescent protein (EGFP) as model antigens in C57BL/6 mice. β -galactosidase-encoding and EGFP.mTRP2-encoding mRNA were transcribed in vitro from expression plasmids as described in materials and methods and subsequently polyadenylated. Synthetic mRNA was precipitated onto gold beads, loaded into plastic cartridges and used for bombardment of mice into the shaved abdominal skin using the gene gun. Each shot resulted in the delivery of 15 μg mRNA, which had

previously been determined to work efficiently. Control mice received 1 μg plasmid DNA encoding β -gal or EGFP.mTRP2 as described [9]. Skin was harvested 24 h after gene gun bombardment and snap frozen. Cryosections were stained with X-gal to visualize expression of β -gal or investigated with a fluorescence microscope to visualize expression of EGFP. Antigen expression by in vivo transfected keratinocytes following bombardment of the skin with synthetic mRNA could clearly be demonstrated (Fig. 1).

Stimulation of antigen-specific humoral and cellular immunity

In subsequent experiments, we sought to determine whether gene gun bombardment of the skin with synthetic mRNA was able to induce humoral and cellular immunity to the encoded antigens. Groups of four mice were each bombarded with two shots delivering a total of 30 μg RNA coding for either β -gal or the fusion protein EGFP.mTRP2 and boosted 1 week later. Control groups of mice received 1 μg plasmid DNA encoding β -gal or EGFP.mTRP2. Two weeks later, serum was harvested and tested for the presence of antibodies specifically recognizing recombinant β -gal or EGFP protein using a sandwich ELISA. Alternatively, splenocytes were harvested and tested for the induction of antigen-specific T cells recognizing the H-2K^b-binding peptides ICPMYARV derived from amino acid 497–504 of β -gal or SVYDFFVWL derived from amino acid 180–188 of murine TRP2 using the ELISPOT technique. Importantly, we could verify the in vivo induction of antigen-specific humoral and cellular immunity in mRNA-immunized mice as illustrated in Fig. 2. In direct comparison, we could not detect significant differences between mice vaccinated with synthetic RNA or plasmid DNA.

Induction of protective immunity to melanoma and autoimmune vitiligo-like depigmentation of the skin

Since gene gun immunization with synthetic RNA was able to induce cellular and humoral immunity, we subsequently investigated the ability to promote protective anti-melanoma immunity against experimentally induced B16 melanoma lung metastases. Groups of four C57BL/6 mice were shaved on the abdomen and bombarded on days 1 and 8 with 30 μg mRNA encoding either β -gal or EGFP.mTRP2. On day 14, mice were challenged intravenously with 4×10^5 cultured B16 melanoma cells and metastases on the lung surfaces determined 2 weeks later. As illustrated in a representative experiment in Fig. 3a, mice vaccinated with mRNA encoding the fusion protein EGFP.mTRP2 were significantly protected against growth of B16 melanoma lung metastases. Alternatively, mice immunized with mRNA coding for β -gal or EGFP.mTRP2 were monitored for

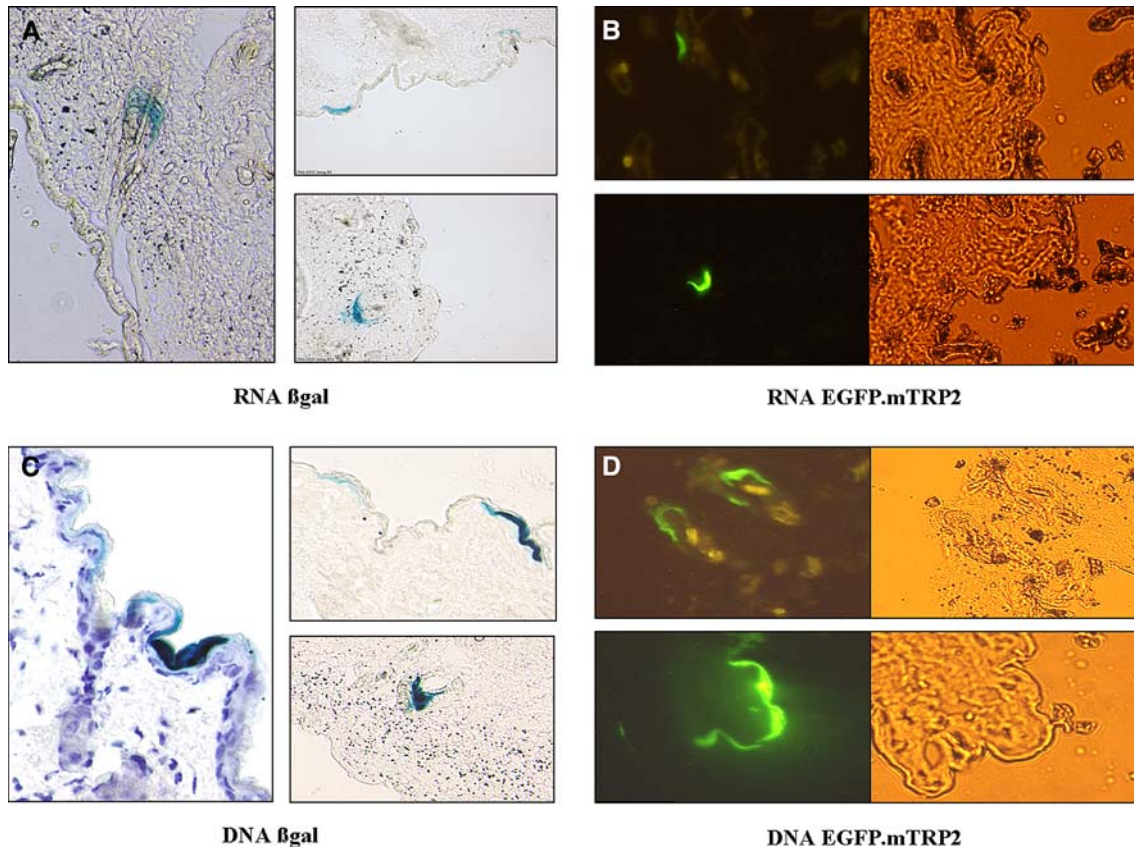


Fig. 1 Antigen expression in keratinocytes of the superficial epidermis and the hair follicles following gene gun bombardment with plasmid DNA or synthetic mRNA. **a, c** β -gal galactosidase expression was verified by X-gal staining of mouse skin 24 h after gene gun immunization as indicated. **b, d** EGFP expression was verified by fluorescence microscopy of mouse skin 24 h after gene gun immunization as indicated

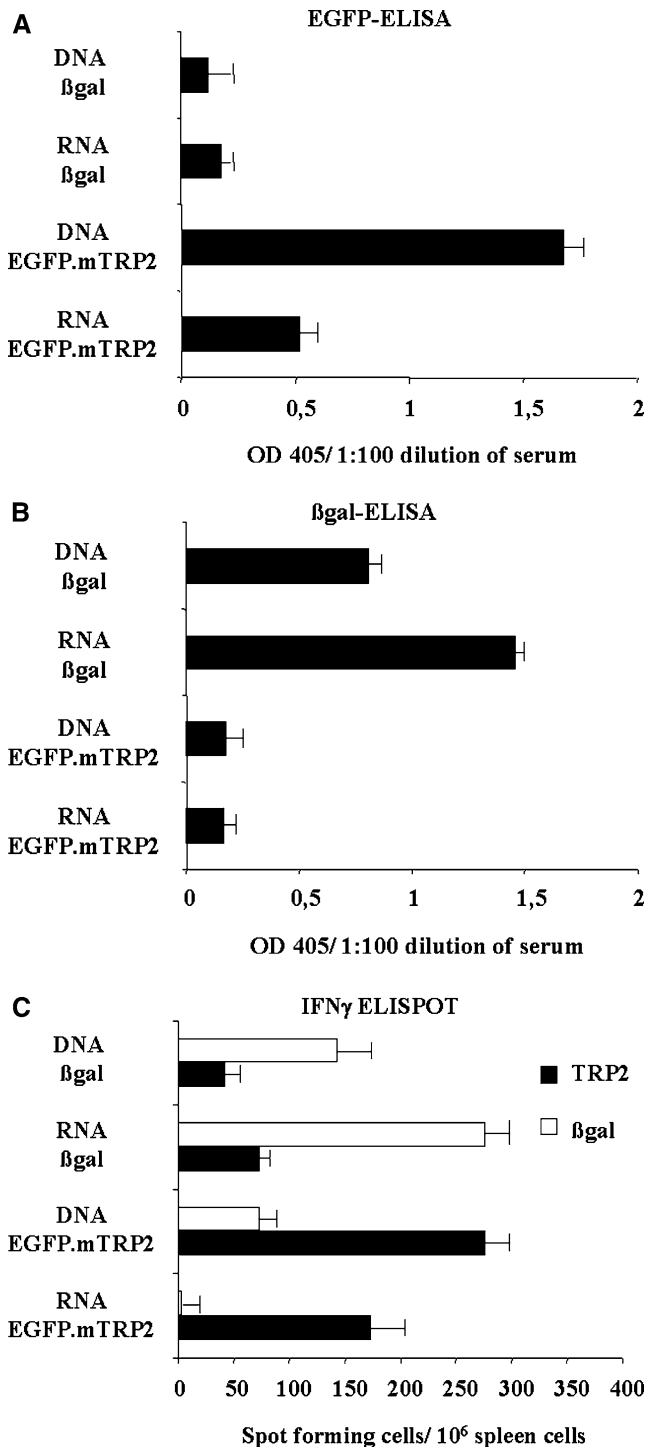
the appearance of coat color changes in the gene gun-transfected skin of the abdomen. Importantly, bombardment with mRNA encoding EGFP.mTRP2 was associated with vitiligo-like fur depigmentation indicating autoimmune destruction of melanocytes in the hair follicles (Fig. 3b). Consistent with our own previous work and that of other research groups using plasmid DNA and recombinant viruses [6, 7, 9, 11, 13, 50] we never observed vitiligo-like coat color changes in mice immunized with synthetic RNA encoding β -galactosidase.

Discussion

In the present study we provide experimental evidence that synthetic messenger RNA can be used as an alternative antigen format for gene gun-based genetic melanoma vaccination. We show that bombardment of the skin with mRNA encoding a fusion protein between the melanocytic differentiation antigen TRP2 and the immunogenic helper determinant EGFP leads to

cutaneous antigen-expression in vivo that induces potent cellular and humoral immune responses. Various melanoma vaccine strategies have recently been experimentally evaluated using TRP2 as a clinically relevant melanocytic self-antigen. Xenogeneic human TRP2 could be used effectively for genetic immunization, which presumably contains strong CD4+ T helper cell epitopes [7, 8]. Alternatively, cultured dendritic cells were used as a biological adjuvant to circumvent peripheral immune tolerance [6, 10, 14–17]. It has been shown that particle-bombardment of the skin with the gene gun is not only able to directly transfect keratinocytes but also dendritic cells in the epidermis [18, 19]. Direct transfection and activation of dendritic cells is thought to be critical for the induction of immune responses in the skin. Therefore, gene gun immunization targeting the skin may potentially be able to circumvent the laborious work to culture dendritic cells in vitro and use them as a biological vaccine adjuvant.

Immunization with synthetic mRNA has one very important advantage over naked DNA and recombinant viruses: it avoids the risk of random integration in the genome leading to mutational inactivation of cellular genes or, more importantly, to oncogenic transformation. When choosing mRNA as alternative antigen-format one biological property has always to be taken into account. mRNA may be rapidly hydrolysed by ubiquitous ribonucleases and therefore may not be an optimal alternative antigen-format for vaccine preparations. Despite these great concerns the injection of mRNA into



muscle has been shown to efficiently mediate gene expression in vivo and effectively induce antigen-specific immune responses [20]. The use of synthetic messenger RNA for gene gun-based genetic immunization targeting the skin has first been reported by Qiu et al. [21] using β -gal as a model antigen. Methods that stabilize mRNA in vivo like the introduction of *Xenopus laevis* beta-globulin 5' and 3' flanking sequences or that confer protection from RNase degradation are now being

introduced and may further help to increase the half-life of transferred mRNA and the efficacy of mRNA-based cancer vaccines [22]. RNA as an antigen format has been extensively tested preclinically in mouse models to support clinical RNA-vaccination trials in cancer patients. Gilboa and colleagues showed that dendritic cells pulsed with tumor RNA efficiently induce antitumor immunity in mice against OVA-expressing tumor cells [23] or murine B16 melanoma [24, 25]. Immunization of mice with dendritic cells pulsed with RNA encoding telomerase resulted in the induction of cytotoxic T lymphocytes, which lysed melanoma cells and inhibited the growth of unrelated tumors in mice of distinct genetic backgrounds [26]. Granstein et al. [27] showed that immunization of mice with freshly isolated epidermal cells enriched for Langerhans cells and pulsed with crude tumor cell-derived RNA are able to induce tumor-specific immunity. The experience gathered in animal models have been complemented by comprehensive in vitro data from the human system. It has been shown by multiple investigators that both in vivo primed as well as naïve CD8⁺ and CD4⁺ T cell responses can be efficiently stimulated in vitro from peripheral blood mononuclear cells of patients and healthy human donors after stimulation with RNA-transfected DC. This holds true for the stimulation of T lymphocytes using DC loaded with mRNA coding for defined viral antigens, such as the influenza matrix protein [28], EBV E6/E7 [29], EBV LMP2 [30] or HIV gag [31]. Efficient induction of antigen-specific CD8⁺ and CD4⁺ T lymphocytes was also achieved for mRNA of various autoantigens known to be expressed on human tumors, such as CEA [32], PSA [33], telomerase [26, 34], survivin [35], oncofetal antigen-immature laminin receptor [36] or Melan A Mart [37].

Immunization protocols can be based on vaccination with a single defined antigen or a mixture of more than one defined antigen. As mRNA can be extracted and further amplified from tumor tissue it is possible to obtain a vaccine covering the whole range of individual tumor-expressed antigens from a small tissue sample [27, 38]. In preparation of vaccination trials including this broader approach, preclinical experiments were con-

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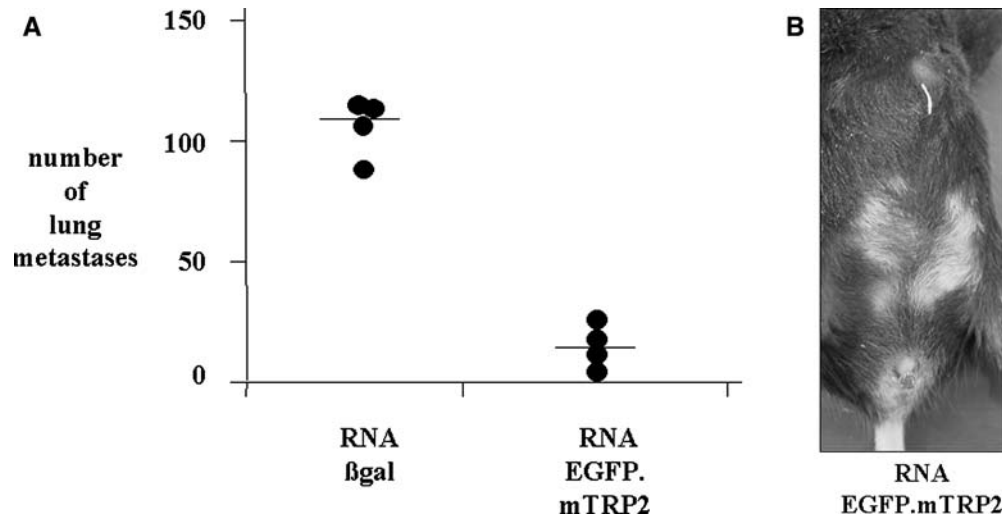


Fig. 3 Induction of protective immunity to melanoma and vitiligo-like fur depigmentation. Groups of C57BL/6 mice were immunized by particle-mediated gene transfer using the indicated synthetic mRNA. **a** The induction of protective tumor immunity against experimentally induced B16 melanoma lung metastases was assessed 2 weeks later. Shown are the number of lung metastases in individual mice (*dots*) and the mean number of metastases in each group (*horizontal bar*). This experiment was repeated three times with similar results. **b** Alternatively, mice were followed for the appearance of vitiligo-like fur depigmentation. Shown is a representative picture of a mouse immunized with mRNA coding for EGFP.mTRP2

ducted that applied autologous DCs loaded with RNA preparations amplified from tumor cells for the stimulation of human T cells. Resulting T cell populations showed antitumor reactivity and were able to specifically lyse various tumor cells used for stimulation, such as prostate cancer [39], multiple myeloma [40], colorectal Cancer [41] or CLL [42].

Based on the results of preclinical models, first immunotherapy trials with RNA as a vaccine for cancer patients have recently been initiated. Phase I clinical trials using RNA-transfected autologous DC showed safety and feasibility, as well as immunological and some clinical responses for the new antigen format. Heiser and colleagues conducted two initial trials in patients with metastatic prostate cancer and could achieve in vivo induction of T cell responses against prostate-specific antigen by vaccination DCs pulsed with mRNA encoding prostate-specific antigen [43] or transfected with RNA amplified from microdissected tumor cells [39]. These trials were followed by immunization trials initiated for patients with metastatic renal cell cancer. Administration of autologous DC transfected with total renal tumor RNA to patients had the potency to induce T cell reactivities directed against a broad set of renal tumor-associated antigens but not against self-antigens expressed by normal renal tissues [44]. Tumor-specific immune responses that are capable of recognizing and lysing autologous tumor cells have also been successfully induced in a patient with adenocarcinoma of the lung after immunization with autologous DC transfected

with total tumor RNA [45]. Kobayashi and colleagues recently demonstrated that cytotoxic T cells, which were able to kill autologous glioma cells, could be elicited in a small group of patients by administration of tumor mRNA-loaded DC [46]. Although the trials published until now provide evidence on vaccine safety and in vivo bioactivity of RNA-loaded DC in a small number of patients with various tumor types, the proof for a broad clinical benefit remains to be delivered in future immunization trials. Further RNA-based immunization protocols are now being developed, that will introduce modifications to the RNA sequence to guarantee for higher in vivo stability, improved antigen-presentation through endosomal targeting or a more efficient T cell induction by the fusion of helper-sequences. It will also have to be shown if new administration routes, such as the direct injection of RNA into skin or draining lymph nodes, will avoid the need for the laborious and expensive generation of cellular carriers.

Independently of the approach used, it has to be kept in mind that a majority of tumor-antigens represent autoantigens and that immunity against these antigens is effectively blocked by peripheral tolerance mechanisms. Recent insights in the immunostimulatory effects of therapeutic nucleic acids suggest that the use of mRNA may activate innate antiviral pathways, which may be different from those stimulated by plasmid DNA. Specifically, bacterial DNA containing CpG motifs is recognized by TLR9, while single stranded RNA is recognized by TLR7 and 8 [47–49]. Future experiments will have to address the question whether the use of synthetic mRNA might be able to directly enhance the immunogenicity of TRP2 and substitute for linkage of immunogenic helper proteins.

Taken together, we demonstrate here that genetic immunization with synthetic mRNA encoding melanocytic antigens linked to immunogenic helper proteins can be successfully employed for the induction of anti-melanoma immunity in mice in vivo. Our results provide proof of principle for melanoma vaccines employing synthetic RNA in an experimental mouse model and

provide a scientific basis for the translation of this strategy into future clinical trials.

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