Delivery of Immunostimulatory RNA Oligonucleotides by Gelatin Nanoparticles Triggers an Efficient Antitumoral Response

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Summary: RNA oligonucleotides have emerged as a new class of biologicals that can silence gene expression but also stimulate immune responses through specific pattern-recognition receptors. The development of effective delivery systems remains a major challenge for the therapeutic application of the RNA oligonucleotides. In this study, we have established a novel biodegradable carrier system that is highly effective for the delivery of immunostimulatory RNA oligonucleotides. Formulation of RNA oligonucleotides with cationized gelatin nanoparticles potentiates immune activation through the Toll-like receptor 7 (TLR7) in both myeloid and plasmacytoid dendritic cells. Further, nanoparticle-delivered RNA oligonucleotides trigger production of the antitumoral cytokines IL-12 and IFN-α. Binding to gelatin nanoparticles protects RNA oligonucleotides from degradation by nucleases, facilitates their uptake by dendritic cells, and targets these nucleic acids to the endosomal compartment in which they are recognized by TLR7. In these effects, the nanoparticles are superior to the conventional transfection reagents lipofectamine, polyethylenimine, and DOTAP. In vivo, the delivery of TLR7-activating RNA oligonucleotides by gelatin nanoparticles triggers antigen-specific $CD8^+$ T-cell and antibody responses. Indeed, immunization with RNA-loaded nanoparticles leads to an efficient antitumoral immune response in two different mouse tumor models. Thus, gelatin-based nanoparticles represent a novel delivery system for immunostimulatory RNA oligonucleotides that is both effective and nontoxic.

Key Words: Toll-like receptor 7, RNA oligonucleotides, tumor immunotherapy, nanoparticles, delivery

(*J Immunother* 2010;33:935–944)

Upon viral infection, a strong immune response is elicited through recognition of the viral components by specific receptors of the innate immune system. Molecular patterns within viral nucleic acids are recognized by the pattern-recognition receptors TLR7 (single-stranded RNA sequences), TLR9 (CpG DNA sequences), RIG-I (retinoid acid inducible gene I, 5′-triphosphate RNA), and MDA-5 (double-stranded RNA).[1–5] Synthetic DNA and RNA oligonucleotides containing these molecular patterns are powerful tools to stimulate an immune response in a highly selective manner. It is for instance well established that binding of synthetic CpG DNA oligonucleotides to TLR9 both promotes innate immunity and triggers the generation of a protective Th1-type immune response.[6] In experimental models and in cancer patients, CpG oligonucleotides enhance cytotoxic T-cell responses to tumor antigens upon vaccination.[7–9] Furthermore, we have recently shown that RNA oligonucleotides can stimulate innate immunity through both the Toll-like receptor 7 and RIG-I receptors[3,10] and, thereby induce efficient antitumor responses.[11,12]

TLR7-activating RNA oligonucleotides act on several components of both the murine and the human immune systems: they potentiate T-cell and B-cell responses to antigen,[13] activate neutrophils[14] and antitumoral NK cells,[11,15] and block the suppressive function of regulatory T cells.[16] We have shown that these effects are orchestrated by TLR7-expressing dendritic cells that control immune activation through the production of a panel of cytokines.[11,13,16] The therapeutic potential of TLR7 agonists is supported by the clinical efficacy of the imidazoquinolines, a class of antitumor agents that acts in part through the activation of TLR7.[17] The lead compound, imiquimod, is approved for the treatment of skin tumors by topical use but is effective against solid tumors only when applied locally.[18] RNA oligonucleotides thus form a new class of TLR7 agonists with promising therapeutic potential. Furthermore, RNA oligonucleotides can be designed to include other antitumoral properties in the same molecule: introduction of an inhibitory siRNA sequence permits silencing of tumor-promoting genes that synergizes with the immunostimulatory activity of the RNA oligonucleotides to block tumor growth.[12]

A key challenge for the therapeutic application of the RNA oligonucleotides is the need for efficient in vivo delivery to protect RNA oligonucleotides from degradation, to promote cellular uptake, and to target the RNA to the desired intracellular compartment.[19] In this study, we have investigated the efficacy of cationized gelatin-based nanoparticles for the delivery of immunostimulatory RNA oligonucleotides. Gelatin presents the advantage of being biodegradable and nontoxic and has been used in patients as a plasma expander for decades.[20,21] Gelatin nanoparticles are stable during storage, show high stability after administration and can be easily scaled-up for manufacturing processes. Highly homogeneous gelatin nanoparticles of

Received for publication February 3, 2010; accepted August 1, 2010.
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The authors declared no conflict of interests.
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Supported by grants from the German Research Foundation (DFG En 169/7-2 and Graduiertenkolleg 1202 to C.B. and S.E., the excellence cluster CIPSM 114 to S.E.), from LMUexcellent (research professorship to S.E.), from the Else-Kröner Fresenius Foundation, and from BayImmuNet to C.B. and S.E. This work is part of the doctoral thesis of SF and SW at the Ludwig-Maximilian University of Munich.
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well-defined diameter can be generated by a two-step desolvation method.[22] We have earlier shown that the gelatin nanoparticle delivery enhances the $CD8^+$ T-cell response triggered by CpG DNA oligonucleotides.[23]

Here, we examined the immunostimulatory capacity of gelatin nanoparticle-delivered RNA oligonucleotides both in vitro and in vivo. We show that nanoparticle-delivered RNA oligonucleotides strongly activate immune responses in a TLR7-dependent manner. Gelatin nanoparticles enhance uptake of RNA oligonucleotides by immune cells and protect them from degradation. In addition, immunization with nanoparticle-bound RNA oligonucleotides and a model antigen protected from tumor growth. We thus characterize a new formulation for immunostimulatory RNA oligonucleotides that enhances their ability to elicit Th1 immunity and to trigger efficient antitumor responses.

MATERIALS AND METHODS

Mice

Female C57BL/6 mice were purchased from Harlan-Winkelmann (Borchen, Germany). Mice were 6 to 12 weeks of age at the onset of experiments. TCR transgenic OT-I mice were kindly provided by Dr T. Brocker (Munich, Germany). Animal studies were approved by the local regulatory agency (Regierung von Oberbayern, Munich, Germany).

Reagents and Cell Lines

The fully phosphorothioated 20-mer RNA oligonucleotides 9.2dr (5′-UGUCCUUCAAUGUCCUUCAA-3′)[10] and polyA were purchased from CureVac (Tübingen, Germany). For some experiments, fluorescein isothiocyanate -5′-tagged RNA oligonucleotides were used (Metabion, Martinsried, Germany). The unmodified 2.2 triphosphate RNA (PPP-5′-GCAUGCGACCUCUGUUUGA-3′) was produced by in vitro transcription in our laboratory.[3] The fully phosphorothioated CpG oligodeoxynucleotide 1826 (5′-TCCATGAC GTTCCTGACGTT-3′) was purchased from the Coley Pharmaceutical Group (Wellesley, MA). Gelatin type A from porcine skin (175 Bloom) and chicken egg ovalbumin (OVA) were purchased from Sigma-Aldrich (St Louis, MO). The OVA-transfected cell lines B16-F10 and Panc-02 were kindly provided by Dr T. Brocker and Dr M. Schnurr.

Preparation of Cationized Gelatin Nanoparticles

Gelatin nanoparticles were prepared in the Division of Pharmaceutical Technology and Biopharmaceutics at the University of Munich as described.[22] Subsequently, cationization of the nanoparticles was achieved through the introduction of a permanent quaternary amino group by covalent coupling of cholaminechloride hydrochloride onto the particle surface.[20] Cationized particles prepared by this protocol were shown by Limulus amoebocyte lysate assay to be endotoxin-free.[24] The size of the prepared nanoparticles was measured by dynamic light scattering using a Zetasizer Nano ZS (Malvern). Average diameter was 272 nm (SD 33 nm) with a polydispersity index below 0.1 and a ζ potential above 4 mV in PBS or above 19 mV in NaCl. Unloaded particles were stable in size and ζ potential when stored as stock dispersion at 4°C for several months. For formulation with RNA oligonucleotides, the particles were loaded with 5% (w/w) RNA oligonucleotides by shaking for 2 hours at 800 rpm at room temperature. To evaluate the physical stability of RNA oligonucleotide attachment to the cationic nanoparticle surface, dispersions were incubated in PBS at a final pH of 7.4 or 4.9 for up to 1 week. Dispersions were then centrifuged at 25,000 g for 30 minutes and the supernatant was analyzed spectrophotometrically at 260 nm for free RNA. Controls consisted of either free RNA or gelatin nanoparticles (NP) in the equivalent buffer. The fraction of nanoparticle-bound RNA was calculated as:

$$1 - \frac{(\text{OD of NP-RNA supernatant} - \text{OD of NP supernatant})}{\text{OD of RNA supernatant}}$$

and expressed in %.

Bone Marrow Cell Culture

Bone marrow cells were harvested from murine femur and tibia and erythrocytes were lysed with ammonium chloride buffer (BD Biosciences, Heidelberg, Germany). Cells were cultured in RPMI 1640 medium supplemented with 10% FCS, 2-mM L-glutamine, 100 μg/mL streptomycin, and 100 IU/mL penicillin and activated with 10 μg/mL RNA oligonucleotides complexed to 20-fold the concentration of gelatin nanoparticles if not stated otherwise. Alternatively, 10 μg/mL RNA oligonucleotides were complexed to 50 μg/mL DOTAP (Roche, Mannheim, Germany) or 2.5 μg/mL lipofectamine (Lipofectamine 2000; Invitrogen, Carlsbad) according to the manufacturers' instructions. PEI was kindly provided by Dr M. Ogris (Munich, Germany). A total of 10 μg/mL RNA oligonucleotides were complexed to 7.5 μg/mL PEI directly before stimulation. In some experiments, cells were activated with 3 μg/mL free CpG oligodeoxynucleotides or 1 μg/mL nanoparticle-bound 5′-triphosphate-RNA oligonucleotides.

Confocal Microscopy

Bone marrow cells were stimulated for 3 hours with fluorescein-5′-tagged RNA oligonucleotides and carriers as described above, washed 3 times, and adhered to poly-L-lysine-coated microscope slides. Cells were then fixed in 100% acetone for 10 minutes. Topro-3 (Invitrogen) was used for nuclear counterstaining. Stained cells were visualized using a confocal laser scanning microscope (LSM 510, Carl Zeiss) and Adobe Photoshop was used for the adjustment of contrast and size.

Quantification of Cytokines and Flow Cytometric Analyses

Concentration of IL-6 in culture supernatants was determined by ELISA according to the manufacturer's instructions (BD Biosciences). IFN-α was measured according to this protocol: rat monoclonal antibody to mouse IFN-α (clone RMMA-1) was used as the capture antibody, rabbit polyclonal antibody to mouse IFN-α for detection (both from PBL Biomedical Laboratories, Piscataway, NJ) together with HRP-conjugated donkey antibody to rabbit IgG as the secondary reagent (Jackson ImmunoLaboratories, Bar Harbor, ME). Recombinant mouse IFN-α (PBL Biomedical Laboratories) was used as standard (IFN-α concentration in IU/mL). For flow cytometric analyses, cells were stained with fluorochrome-conjugated monoclonal antibodies (B220, CD3, CD4, CD8, CD11b, CD11c, CD44, CD62L, CD69, and isotype controls) and propidium iodide from BD Biosciences. Data were acquired on a FACSCalibur or a FACSCanto II (BD Biosciences) and analyzed using FlowJo software (Tree Star, Ashland, OR).

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Induction of OVA-specific CD8+ T-cell Proliferation

For the in vivo induction of antigen-specific $CD8^+$ T-cell proliferation, splenocytes from OT-I mice were labeled with 15 nmol/mL carboxyfluorescein succinimidyl ester (CFSE; Molecular Probes, Eugene, OR) after erythrocyte lysis. 10 to 15×10^6 labeled OT-I cells were adoptively transferred into wild-type mice on day 0. On day 1, the mice were immunized s.c. with 30 µg OVA together with 100 µg free RNA oligonucleotides, 100 µg RNA oligonucleotides complexed to 2 mg gelatin nanoparticles, 50 µg 5′-triphosphate RNA complexed to 2 mg gelatin nanoparticles or 100 µg free CpG oligodeoxynucleotides. Eighty hours later, freshly isolated cells from draining and contralateral lymph nodes were analyzed by flow cytometry. Antigen-specific T-cell proliferation is expressed as percentage of highly dividing cells ($CFSE^{low}$) within all CFSE-positive $CD8^+$ cells. The phenotype of CFSE-positive $CD8^+$ T cells was defined as: naïve ($CD62L^{high}$, $CD44^{low}$) and effector/memory ($CD44^{high}$).

Immunization With OVA

For immunization of mice, 75 µg OVA were injected s.c. together with 100 µg free RNA oligonucleotides or 100 µg RNA oligonucleotides complexed to 2 mg nanoparticles 2 or 3 times at a 14-day interval. For the treatment of established tumors, 75 µg OVA were injected s.c. together with 200 µg RNA oligonucleotides complexed to 4 mg nanoparticles 3 times at a 7-day interval. For the detection of OVA-specific antibodies, serum was collected 1 week after the second immunization and serum IgG concentrations were determined by ELISA: plates were coated overnight with 10 µg/mL OVA in PBS and blocked 1 hour with 1% BSA in PBS. After incubation of the serum samples for 1 hour at a dilution of 1:200, plates were washed with PBS/0.05% Tween 20. Goat-anti-mouse IgG conjugated to HRP (SouthernBiotech, Birmingham, AL) was added at 1 µg/mL for 1 hour. Plates were again washed and ELISA was developed by o-phenylenediamine (Sigma-Aldrich). Reaction was stopped by 1 M H_2SO_4 and OD was read by photometer at 490 nm.

Tumor Challenge

In the prophylactic setting, 1 week after the third immunization 10^6 B16-OVA cells were injected s.c. in the right flank. Tumor growth was monitored 3 times a week and is expressed as the product of the perpendicular diameters of individual tumors. The experiment was terminated at day 36 after tumor induction. For the treatment of established tumors, 5×10^5 Panc-OVA cells were injected s.c. in the right flank. Immunization was initiated 8 days post-tumor challenge when all the tumors were palpable. Tumor growth was monitored 3 times a week and is expressed as the product of the perpendicular diameters of individual tumors. According to requirements of the local regulatory agency, mice were killed when the tumor size exceeded 225 mm^2. The experiment was terminated at day 46 after tumor induction.

Statistics

Statistical analyses were done by unpaired, one-way analysis of variance (ANOVA) with the Newman-Keuls multiple comparison test. Significance was set at $P < 0.05$. Comparisons between groups regarding day of onset of tumors were made using the log-rank test. Mean tumor size curves were plotted until 4 mice per group died. The last measured value for each mouse was included in the calculation of the mean for remaining time points. Statistical analyses were done using SPSS software (SPSS, Chicago, IL).

RESULTS

Nanoparticle-delivered RNA Oligonucleotides Efficiently Trigger an Innate Immune Response

We have earlier described immunostimulatory sequences within RNA oligonucleotides that activate both innate and adaptive immune responses through TLR7.[10,11,13] To assess the delivery potential of cationized gelatin nanoparticles for these immune-activating RNA oligonucleotides, murine bone marrow cells were stimulated with nanoparticle-bound RNA 9.2dr oligonucleotides containing a phosphorothioate-modified backbone (PTO).[13] Nanoparticle-bound RNA induced production of the proinflammatory cytokines IL-6 and IFN-α in a dose-dependent manner in the culture (Fig. 1A). In contrast, no cytokine production was detected upon stimulation with free RNA oligonucleotides. Nanoparticles alone induced no IL-6 and only low levels of IFN-α. Cytokine induction by nanoparticle-bound RNA oligonucleotides was abolished in the bone marrow cells from TLR7-deficient mice, confirming that the immunostimulatory activity was mediated by TLR7 (data not shown). Dendritic cells within the culture were activated by nanoparticle-bound 9.2dr PTO RNA with over 40% of both myeloid and plasmacytoid dendritic cells expressing the early activation marker CD69 (Fig. 1B). Indeed, nanoparticle-delivered RNA induced a more potent activation of dendritic cells than RNA bound to the transfection reagents lipofectamine or polyethylenimine (PEI) (Fig. 1B). CD69 upregulation was absent in TLR7-deficient bone marrow cells, showing that immune stimulation by 9.2dr RNA oligonucleotides is TLR7-dependent. Furthermore, cell activation was dependent on the RNA sequence as no activation was detected after stimulation with a PolyA oligonucleotide of the same length (Fig. 1B). In contrast to the sequence-dependent activation of TLR7 by RNA oligonucleotides, we have earlier shown that RNA oligonucleotides bearing a triphosphate at the 5′ end target the cytosolic receptor RIG-I.[3] It is interesting to note that, nanoparticle-bound 5′-triphosphate RNA 2.2 oligonucleotides with a phosphodiester backbone (3pRNA) also induced an activated phenotype in dendritic cells, but did not induce cytokine secretion (Fig. 1C). Stimulation by 5′-triphosphate oligonucleotides was independent of TLR7 (Fig. 1C), in keeping with the role of 5′-triphosphate RNA oligonucleotides as ligand for the RIG-I receptor.

Nanoparticle Formulation of Immunostimulatory RNA Oligonucleotides Promotes Their Uptake Into Intracellular Compartments

We have earlier shown that efficient endosomal delivery of RNA oligonucleotides is required to induce TLR7-mediated immune activation.[25] Here, we examined the efficacy of cationized gelatin nanoparticles for the intracellular delivery of fluorescently labeled RNA oligonucleotides in direct comparison with the transfection reagents DOTAP, PEI, and lipofectamine. RNA oligonucleotides bound to gelatin nanoparticles were rapidly taken up by bone marrow cells with over 80% of cells

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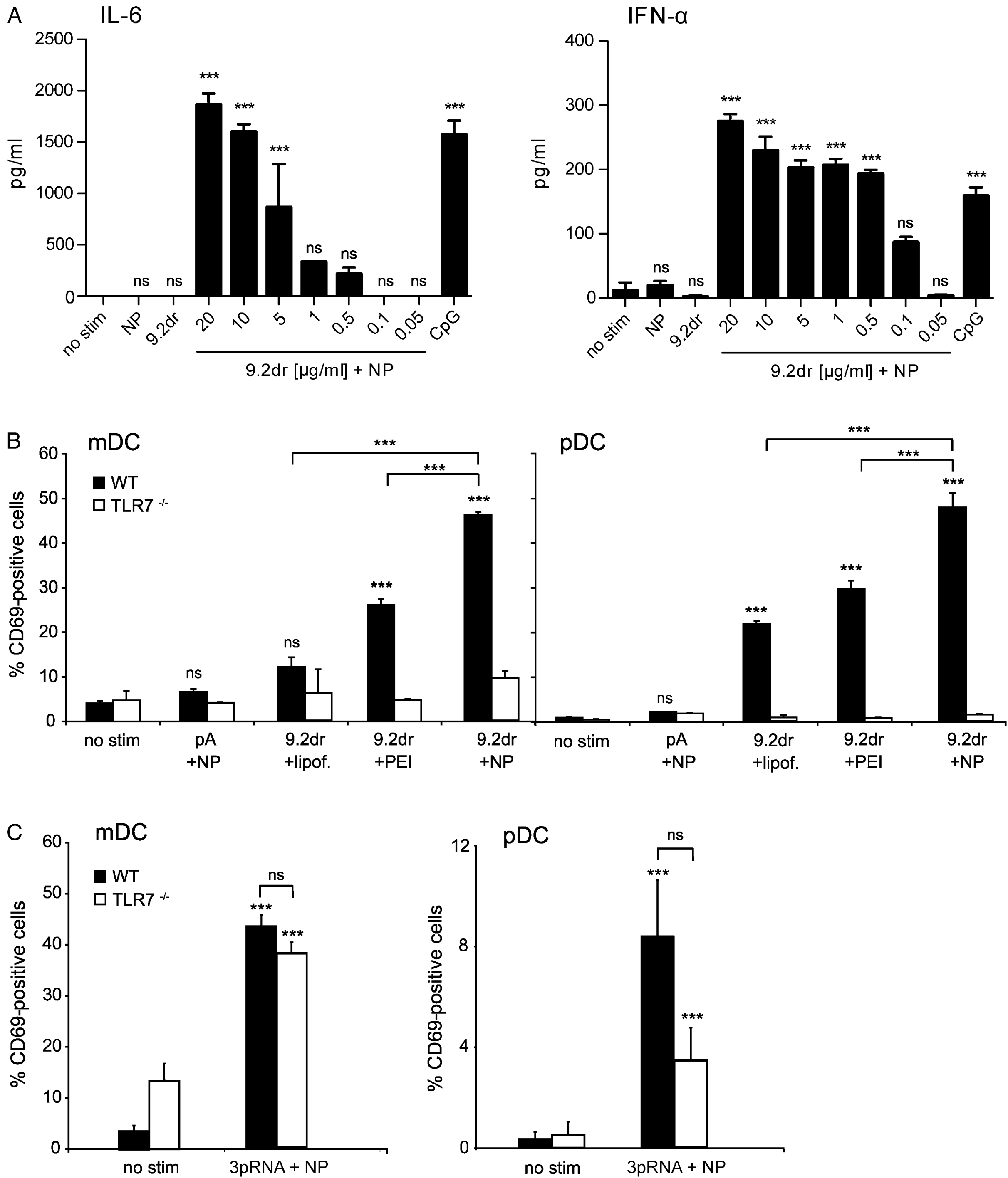

FIGURE 1. Nanoparticle-bound RNA oligonucleotides activate innate immune responses. Murine bone marrow cells from wild-type or TLR7-deficient mice were activated for 18 hours with RNA oligonucleotides complexed to different carriers or with the CpG oligodeoxynucleotide 1826 as indicated. A, Culture supernatants were analyzed by ELISA for IL-6 and IFN-α production. B and C, Surface expression of the activation marker CD69 on CD11c+ B220− myeloid DC (dendritic cell, mDC) and CD11c+ B220+ plasmacytoid DC (pDC) within the culture was analyzed by flow cytometry. Data are expressed as % CD69-positive cells within the indicated populations. All results show the mean of triplicate samples ± SEM. Data are representative of 4 independent experiments. Asterisks without brackets indicate comparison with unstimulated cells. *$P < 0.05$, ***$P < 0.001$, ns indicates not significant.

staining positive for the labeled RNA after 6 hours (Figs. 2A, B). Free RNA oligonucleotides were not taken up by the bone marrow cells (less than 1% 9.2dr RNA-positive cells). Uptake of the RNA oligonucleotides complexed to the transfection reagents DOTAP, PEI, or lipofectamine was less efficient with a maximum of 38% of bone marrow

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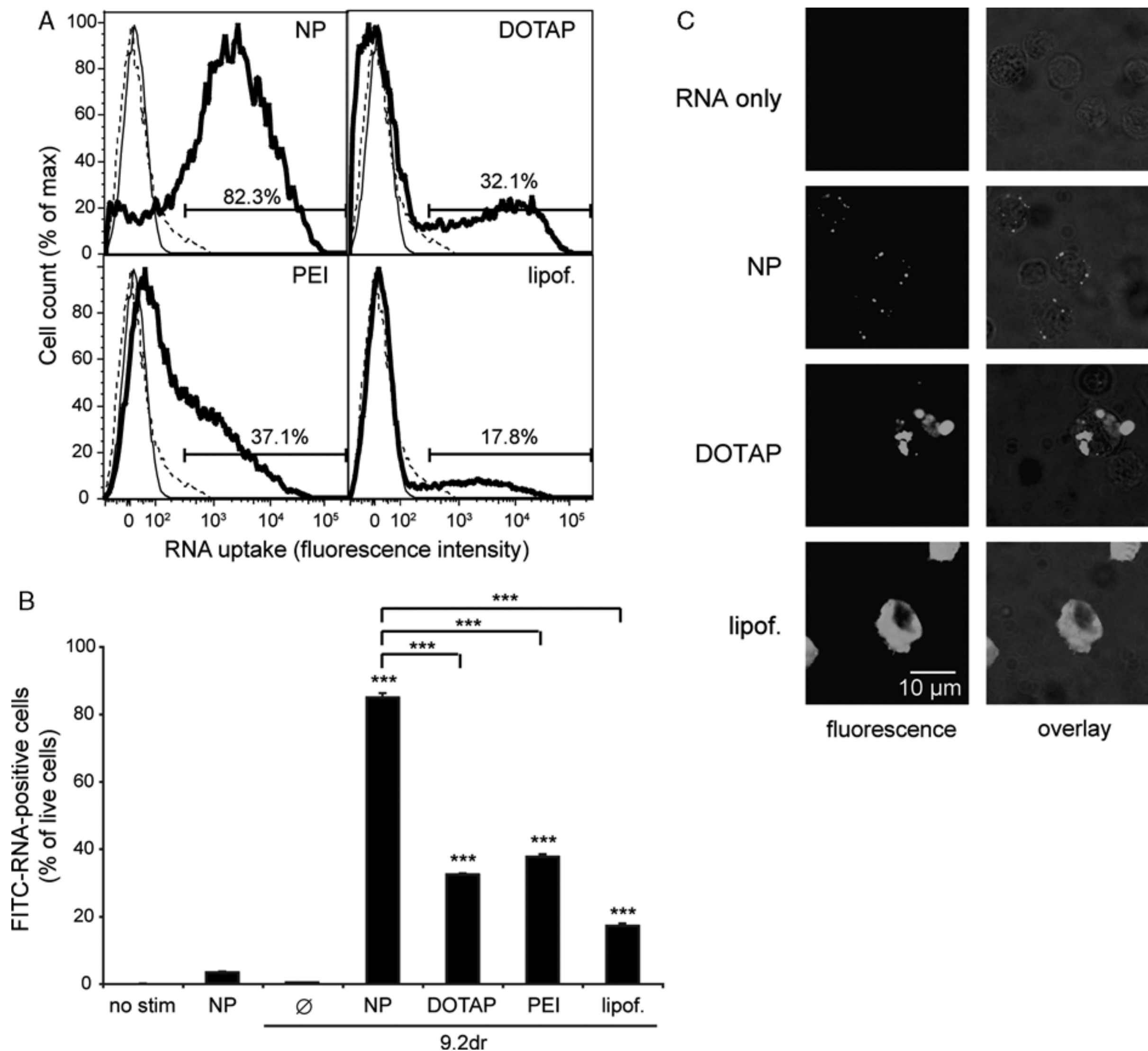

FIGURE 2. Nanoparticle formulation of immunostimulatory RNA oligonucleotides promotes their uptake into intracellular compartments. Murine bone marrow cells were stimulated for 6 hours with 10 μg/mL fluorescein-labeled 9.2dr RNA oligonucleotides complexed to different carriers. A, Histograms illustrate uptake of fluorescently labeled RNA by bone marrow cells analyzed by flow cytometry (bold line: RNA with indicated carrier; fine line: free RNA; dashed line: nanoparticles without RNA). B, Graph shows percentage of FITC-RNA-positive cells as mean ± SEM of triplicate samples. Asterisks without brackets indicate comparison with unstimulated cells. ***$P < 0.001$. C, Confocal microscopy of RNA oligonucleotide-stimulated cells shows intracellular localization of the fluorescently labeled RNA after 3 hours. Left panel shows fluorescence images, right panel shows differential interference contrast (DIC) pictures merged with fluorescence. All results are representative of 2 independent experiments.

cells staining positive for the fluorescently labeled RNA complexed to PEI (Figs. 2A, B). Confocal microscopy showed that nanoparticle-delivered RNA oligonucleotides can be observed as distinct dots after internalization, suggesting an endosomal uptake (Fig. 2C). Indeed, we have earlier shown the localization of gelatin nanoparticles to the endosome after uptake by dendritic cells.[20,24] Complexation of RNA oligonucleotides to DOTAP resulted in a similar localization pattern, whereas RNA oligonucleotides bound to PEI or lipofectamine were evenly distributed in the cytoplasm of the transfected cells. Thus, nanoparticle-bound RNA oligonucleotides are efficiently taken up by immune cells and accumulate in the intracellular compartments.

Nanoparticle Formulation Protects Immunostimulatory RNA Oligonucleotides From Degradation by Ribonucleases

In addition to facilitating the uptake of RNA oligonucleotides into the appropriate intracellular compartment, an effective delivery system must protect nucleic acids from degradation. This is an important requirement for the application of immunostimulatory RNA oligonucleotides in a therapeutic setting, as the widespread distribution of

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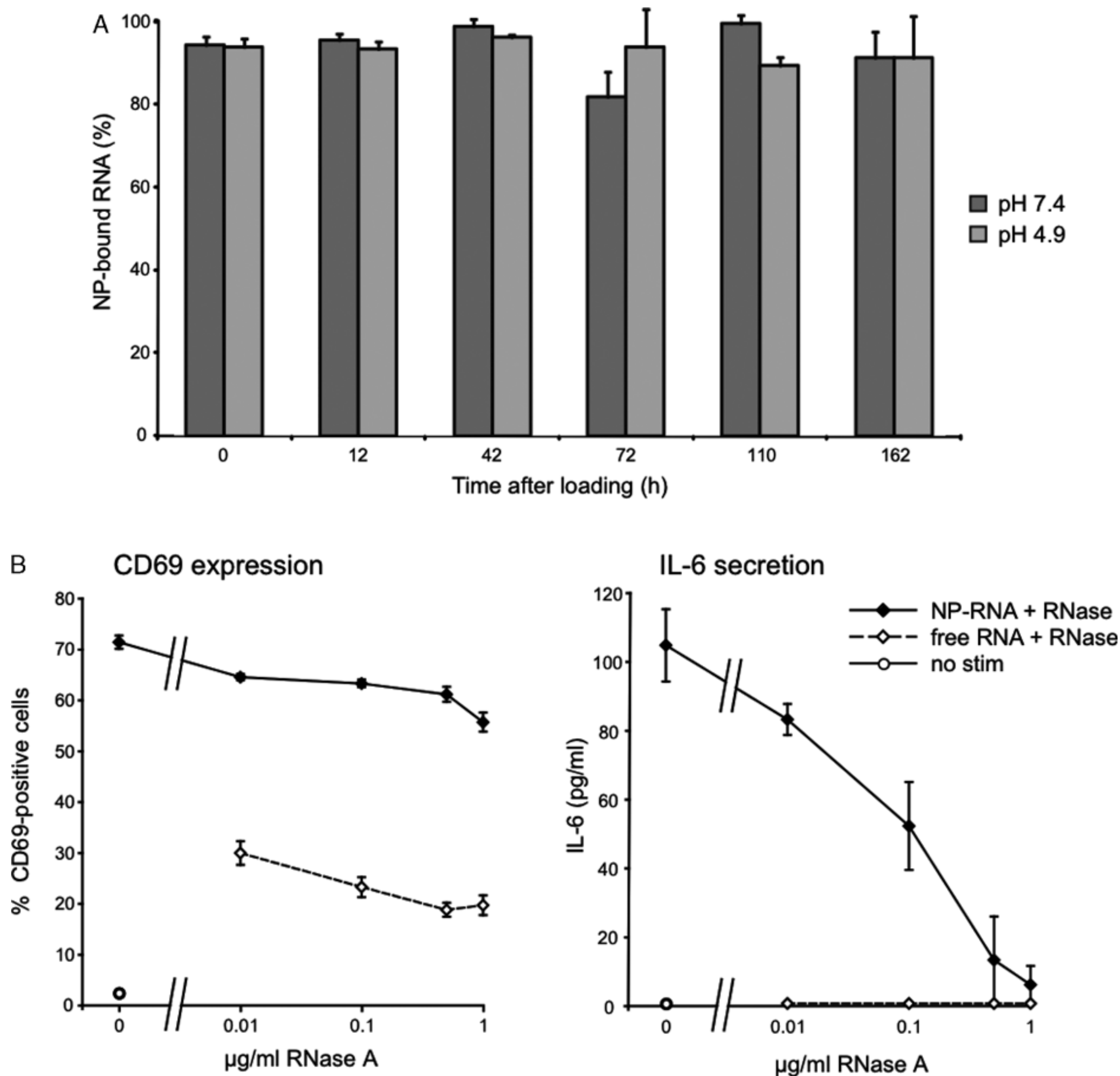

FIGURE 3. Nanoparticle-bound immunostimulatory RNA oligonucleotides are protected from RNase degradation. A, Stability of RNA oligonucleotide binding to gelatin nanoparticles at the indicated pH was examined at different time points after initial loading. After centrifugation, residual free RNA was determined photometrically in the supernatant. The fraction of nanoparticle-bound RNA is expressed in %. B, Bone marrow cells were activated with nanoparticle-bound 9.2dr RNA. Oligonucleotides were digested with RNase A in the indicated concentrations either before or after the complexation of RNA to nanoparticles. Surface expression of the activation marker CD69 was analyzed by flow cytometry (left) and IL-6 concentration in the supernatants was analyzed by ELISA (right). Data show mean and SEM of triplicate wells and are representative of 5 independent experiments.

ribonucleases results in a very short lifespan for unprotected RNA.[26] We assessed whether the complexation of RNA oligonucleotides to the nanoparticles provided protection from degradation by RNases. We observed that RNA loaded onto the cationic nanoparticle surface was stable for at least 1 week at both cytosolic (7.4) and endosomal pH (4.9) (Fig. 3A). Furthermore, binding to gelatin nanoparticles protected immunostimulatory RNA oligonucleotides from digestion by RNase A: RNA oligonucleotides complexed with cationized gelatin nanoparticles still induced an activated $CD69^+$ phenotype and cytokine production in a bone marrow cell culture after a 1-hour RNase treatment (Fig. 3B). In contrast, free RNA oligonucleotides treated with RNase A before complexing with gelatin nanoparticles lost their immunostimulatory potential.

Nanoparticle-bound RNA Oligonucleotides Trigger Antigen-specific $CD8^+$ T-cell and Antibody Responses

To investigate the effect of nanoparticle-bound RNA oligonucleotides on the development of an immune response to antigen, CFSE-labeled OVA-specific T cells from OT-I mice were adoptively transferred into wild-type mice.

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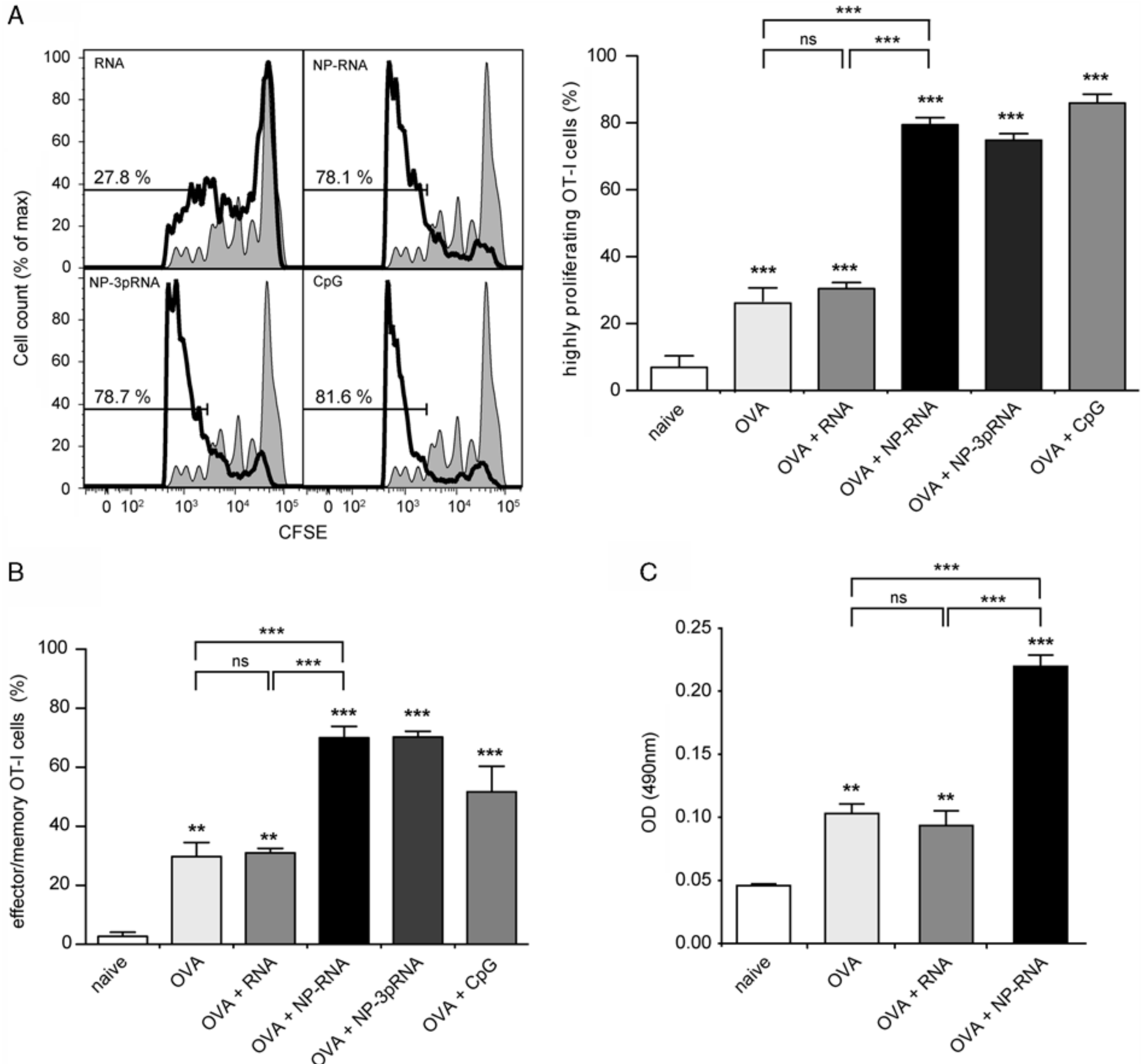

FIGURE 4. Nanoparticle-bound RNA oligonucleotides trigger an antigen-specific CD8+ T-cell and IgG response. A and B, CFSE-labeled OT-I splenocytes were adoptively transferred into wild-type mice. One day later, mice were injected with OVA alone, together with free RNA oligonucleotides, RNA oligonucleotides complexed to nanoparticles, or free CpG oligodeoxynucleotides (n = 3 to 5). CD8+ T-cell proliferation was analyzed by flow cytometry 80 hours after immunization. A, Graph shows percentage of highly dividing (CFSE^{low}) cells from one representative mouse per group (top left; bold line: mice immunized with OVA and the indicated free or nanoparticle-bound oligonucleotides; fine line: naive mice) and the mean of individual mice + SEM (top right). B, Frequency of effector/memory CD44^{hi} cells within CFSE-labeled CD8+ T cells. Results are representative of 2 independent experiments. C, Mice were immunized twice with OVA together with free RNA oligonucleotides or RNA oligonucleotides bound to nanoparticles (n = 5). One week after the second immunization, OVA-specific IgG in the serum was measured by ELISA. Data show the mean of individual mice + SEM. Asterisks without brackets indicate comparisons with naive mice. Results are representative of 4 independent experiments. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ns indicates not significant.

Twenty-four hours later, the mice were immunized s.c. with OVA protein together with free RNA oligonucleotides or nanoparticle-bound RNA oligonucleotides. Eighty hours after immunization, proliferation of CFSE-labeled cytotoxic T cells in lymph nodes was analyzed by flow cytometry. In mice immunized with OVA plus nanoparticle-bound RNA oligonucleotides, over 80% of transferred CD8$^+$ T cells had highly proliferated (more than 3 divisions), similar to the proliferation induced by immunization with the highly potent TLR9 ligand CpG (Fig. 4A). In contrast, free RNA oligonucleotides did not promote OVA-specific proliferation. It is interesting to note that, proliferation of OT-I T cells in mice immunized with OVA plus nanoparticle-bound 5′-triphosphate RNA oligonucleotides was also strongly enhanced, indicating that gelatin nanoparticles are an efficient in vivo delivery system for these RIG-I ligands. Determination of the naive (CD62L^{high}, CD44^{low}) and effector/memory (CD44^{high}) phenotype of the antigen-specific cells showed that the effector/memory cells were predominant in the treatment groups with highly proliferating cells (Fig. 4B). To examine the effect of immunization with nanoparticle-bound RNA oligonucleotides on the development of an antibody

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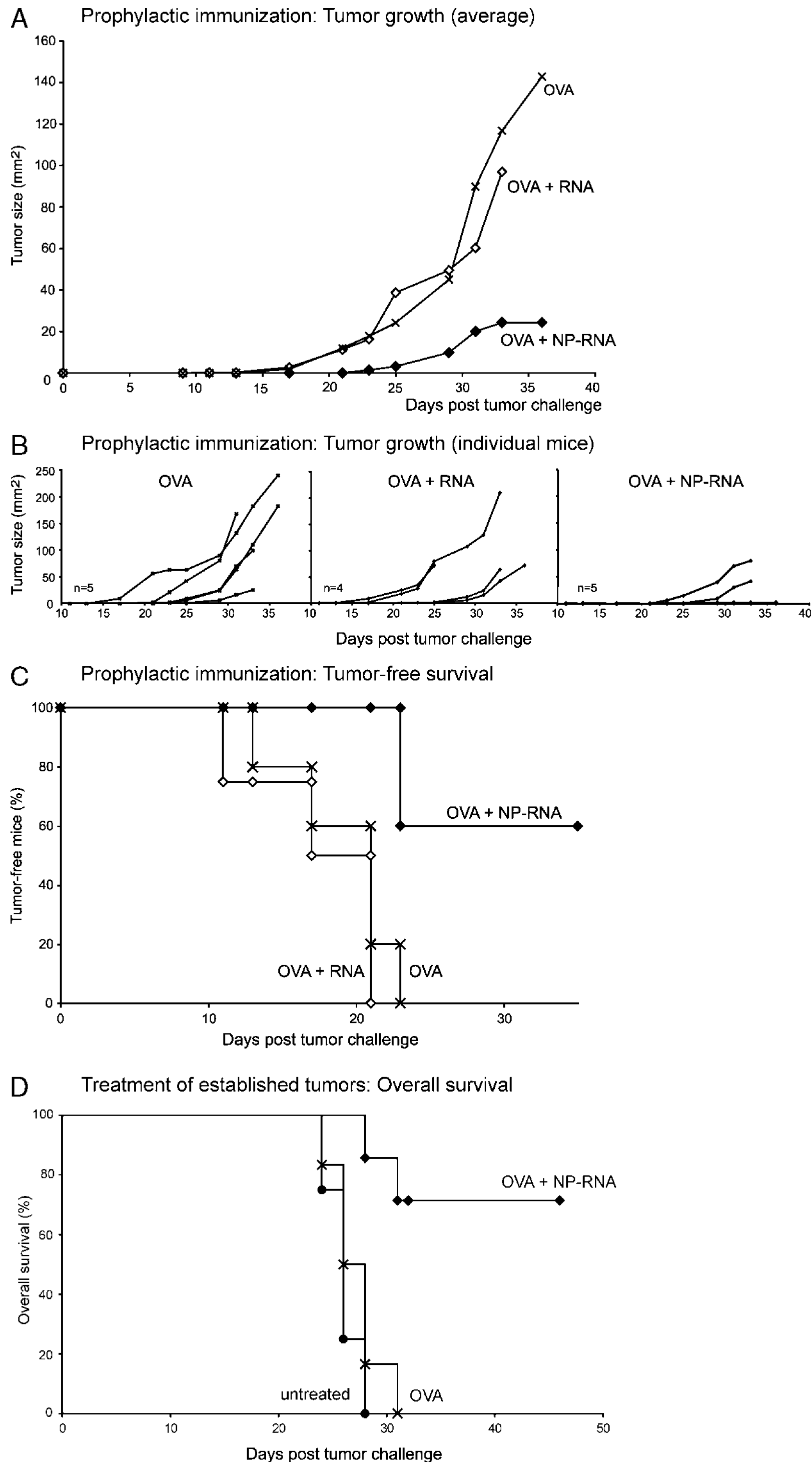

FIGURE 5. Nanoparticle-bound RNA oligonucleotides induce an antigen-specific antitumor immune response. A to C, Mice were immunized 3 times with OVA together with free RNA oligonucleotides or RNA oligonucleotides bound to nanoparticles. One week after the last immunization, mice were injected with OVA-expressing B16 melanoma cells and tumor growth was monitored. Results are shown as (A) mean tumor sizes per group and (B) tumor growth of individual mice. C, Immunization with OVA together with nanoparticle-bound RNA oligonucleotides increased tumor-free survival compared with OVA with free RNA oligonucleotides ($P=0.004$) or with OVA alone ($P=0.009$). Similar results were obtained in 2 independent experiments. D, Mice were injected subcutaneously with OVA-expressing Panc-02 carcinoma cells. Starting on day 8 after tumor induction, the mice were treated with 3 weekly injections of OVA and nanoparticle-bound RNA oligonucleotides. Immunization with OVA together with NP-RNA inhibited tumor growth and improved overall survival compared with nontreatment ($P=0.002$) and with OVA treatment ($P=0.003$).

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response, the mice were immunized with the model antigen ovalbumin together with the nanoparticle-bound RNA oligonucleotides or free RNA oligonucleotides twice at a 14-day interval. One week after the second immunization, the serum levels of OVA-specific antibodies were measured. In mice immunized with OVA and nanoparticle-bound RNA oligonucleotides, levels of OVA-specific IgG were significantly increased compared with the mice immunized with either OVA alone or OVA with free RNA oligonucleotides (Fig. 4C). Nanoparticle-bound RNA oligonucleotides did not induce a generalized immune activation, as we detected neither unspecific activation of immune cells in the spleen nor elevated serum cytokine levels after s.c. injection of nanoparticle-bound RNA. Thus, nanoparticle-bound RNA oligonucleotides act as potent adjuvants and elicit efficient antigen-specific immune responses when administered with antigen while at the same time preventing an indiscriminate systemic activation of the immune system.

Nanoparticle-bound RNA Oligonucleotides Induce an Efficient Antitumor Response

The ability to stimulate cytotoxic T-cell responses is a prerequisite for obtaining an efficient antitumoral immune response. To assess whether the antigen-specific responses induced by nanoparticle-bound RNA oligonucleotides protected from the development of a tumor, wild-type mice were immunized with OVA together with nanoparticle-bound RNA oligonucleotides or free RNA oligonucleotides or with OVA alone before s.c. challenge with OVA-expressing B16 melanoma cells. Although all mice immunized with OVA or OVA plus free RNA oligonucleotides developed a measurable tumor from day 11 onward, only 2 of 5 mice immunized with OVA and nanoparticle-bound RNA oligonucleotides developed a tumor, leading to improved tumor-free survival in this group ($P = 0.004$; Figs. 5A–C). To examine the potential of this immunization in a therapeutic setting, mice bearing established Panc-OVA tumors were treated with OVA together with nanoparticle-bound RNA oligonucleotides or with OVA alone 3 times at weekly intervals. Whereas untreated mice and mice treated with OVA alone survived for a maximum of 31 days, mice treated with OVA together with nanoparticle-bound RNA showed reduced tumor growth and increased survival (Fig. 5D). Thus, immunization with nanoparticle-bound RNA oligonucleotides induces an effective antitumor response.

DISCUSSION

RNA oligonucleotides containing specific molecular patterns trigger immune activation through a broad range of mechanisms and can potentiate antigen-specific immune responses, block immunosuppressive mechanisms, and inhibit tumor growth.[11–14,16,27] Furthermore, RNA oligonucleotides have emerged as a powerful tool to silence the expression of specific genes, raising high expectations for the development of novel therapeutics.[28] The combination of both gene-silencing and immunostimulation in one RNA molecule may further enhance the therapeutic potential of the RNA oligonucleotides.[12] A major issue for the application of RNA oligonucleotides in the clinic is however the development of suitable drug delivery systems that are both safe and effective.[19]

An efficient delivery system must protect RNA oligonucleotides from degradation, facilitate uptake into target cells, and deliver the RNA to the appropriate intracellular localization.[19] Dendritic cells play a critical role in orchestrating the immune response to RNA oligonucleotides and therefore represent an important cellular target.[11,16] We show here that the gelatin nanoparticle-delivered RNA oligonucleotides activate dendritic cells more efficiently than RNA delivered by frequently used transfection reagents such as lipid-based formulations or PEI. Indeed, gelatin-based nanoparticles greatly enhance uptake of immunostimulatory RNA oligonucleotides into dendritic cells with over 80% of cells positive for fluorescently labeled RNA. This highly efficient uptake was not achieved by the other transfection reagents examined. We further show that complexes of gelatin nanoparticles and RNA oligonucleotides are highly stable and that gelatin nanoparticles protect RNA from degradation by nucleases.

The mode of delivery of RNA oligonucleotides plays an important role in the type of immune response induced and directs the subsequent cytokine response.[25] In particular, targeting of the appropriate intracellular compartment is essential for optimal efficacy. Nanoparticle-delivered RNA accumulates in endosomal compartments in which the TLR7 is located,[29] whereas RNA oligonucleotides delivered by lipofectamine or PEI are found diffusely in the cytosol. DOTAP, a lipid-based transfection reagent, also enhances delivery to the endosome.[30] We have earlier shown that the RNA oligonucleotides delivered by DOTAP induce TLR7-dependent immune responses in vitro and in vivo.[11,13] However, DOTAP is highly toxic and forms unstable complexes that preclude an application in a clinical setting.[31] It is interesting to note that, we observed that 5′-triphosphate RNA bound to nanoparticles also activates dendritic cells. As this activation is independent of TLR7 and 5′-triphosphate RNA is a known ligand for the cytosolic RIG-I receptor,[3] it is probable that the observed stimulation is mediated by RIG-I. Thus, gelatin nanoparticles may also deliver RNA oligonucleotides to the cytosol in amounts sufficient for immune activation of RIG-I.

Systemic administration of particulate vehicles often results in accumulation in the liver, kidneys, and lungs.[19] Importantly, we show here that antitumoral protection can be achieved by subcutaneous injection of nanoparticle-bound RNA together with antigen, bypassing the need for systemic delivery. We have earlier shown that the gelatin-based nanoparticles target oligonucleotides almost exclusively to the draining lymph nodes after subcutaneous injection, thus directing the oligonucleotides to the initiation site of the immune response.[23] This enables the selective triggering of antigen-specific T-cell and B-cell responses without resulting in systemic immune activation.

In addition to their essential role for the in vivo delivery of nucleic acids, gelatin nanoparticles may have an adjuvant effect of their own. Indeed, particulate adjuvants such as alum, poly(lactide-co-glycolyde), and polystyrene microparticles enhance TLR-induced secretion of proinflammatory cytokines by dendritic cells through stimulation of the NALP3 (NACHT-, LRP-containing and PYD-containing protein 3) inflammasome receptor complex.[32,33] It is thus possible that the gelatin nanoparticles may themselves activate the NALP3 inflammasome. The induced immune response may synergize with TLR7 activation by RNA oligonucleotides and contribute to the adjuvanticity of gelatin nanoparticle-delivered RNA oligonucleotides.

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In conclusion, we show that gelatin nanoparticles are an efficient delivery system for TLR7-activating RNA oligonucleotides both in vitro and in vivo. As gelatin nanoparticles are also a suitable carrier for antigen,[23] it may be possible in the future to load immunostimulatory oligonucleotides together with antigen onto the same particles. This may further improve immunization outcome, as a conjugation of TLR ligands and antigen results in more effective activation of T-cell responses.[34] Thus, gelatin nanoparticles may be used to simultaneously deliver antigen and immunostimulatory RNA oligonucleotides, representing a safe and effective all-in-one vaccine formulation.

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