Intranodal Vaccination with Naked Antigen-Encoding RNA Elicits Potent Prophylactic and Therapeutic Antitumoral Immunity

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Abstract

Although naked antigen-encoding RNA has entered clinical testing, basic knowledge on how to apply this promising novel vaccine format is still pending. By comparing different administration routes, we observed surprisingly potent antigen-specific T-cell immunity upon intranodal injection of naked antigen-encoding RNA. RNA was selectively uptaken by resident dendritic cells, propagated a T-cell attracting and stimulatory intralymphatic milieu, and led to efficient expansion of antigen-specific CD8+ as well as CD4+ T cells. By intranodal treatment of mice with repeated cycles of RNA, we achieved de novo priming of naïve T cells, which became potent cytolytic effectors capable of homing to primary and secondary lymphatic tissues as well as memory T cells. In tumor-bearing mice intralymphatic RNA vaccination elicited protective and therapeutic antitumor immune responses, resulting in a remarkable survival benefit as compared with other treatment regimens. This is the first report of strong systemic antigen-specific Th1-type immunity and cancer cure achieved with naked antigen-encoding RNA in preclinical animal models. Cancer Res; 70(22): 9031–40. ©2010 AACR.

Introduction

Cancer vaccines that induce potent protective and therapeutic T-cell immunity against defined antigens are under active investigation. One of the major research efforts is to determine the format in which the target antigen should be delivered.

Nucleic acids are particularly attractive as they can be engineered to deliver complete antigens with optimized properties for human leukocyte antigen (HLA)-independent antigen-specific immunization (1). As compared with gene transfer by viral or plasmid DNA, naked antigen-encoding RNA is considered a safer and superior pharmaceutical. Its major advantages are lack of integration into the genome, transient expression of the encoded proteins, and absence of interfering immunodominant viral antigens. Moreover, recombinant RNA generated by in vitro transcription is easy to produce in large amounts and with high purity (2).

Several in vitro and in vivo strategies utilize antigen-encoding RNA for vaccination. Two approaches are currently under clinical evaluation. The more widely used strategy is based on adoptive transfer of autologous dendritic cells transfected ex vivo with RNA (3, 4). Rapid introduction of RNA into cells via electroporation or lipofection rescues it from degradation by ubiquitous extracellular ribonucleases. The use of dendritic cells as host cells enables effective presentation of RNA-encoded antigens in the presence of key factors for induction of potent immune responses.

An alternative strategy pursues direct in vivo administration of antigen-encoding RNA, under the assumption that the cells at the administration site internalize, translate, and present the antigen. This circumvents the challenges of autologous cell transfer and allows extension of this approach to a wide number of patients.

The feasibility and safety of intradermal administration of RNA have been shown in preclinical and clinical investigations (5–7). However, induction of antigen-specific CD8+ T cells in patients was moderate (8), and CD4+ T-cell activity has not been reported. For optimization of the efficacy of naked RNA vaccines, a better understanding of in vivo pharmacology of directly administered RNA in animals and humans is required. Such knowledge is particularly important as the major limitation of RNA is its short extracellular half-life, which constrains pharmacologically effective dosing upon direct administration.

We had previously developed templates for in vitro transcription of RNA-encoded antigens with modified 3’ structures stabilizing the RNA and optimizing its translational performance as well as T-cell stimulating capacity (9). Moreover, we
achieved significant leverage of MHC class I and, importantly, class II presentation of the encoded antigen in dendritic cells by flanking it with a secretion signal (sec) and the transmembrane and cytosolic domains of MHC class I (MIFD) molecules (10). The current study, as a continuation of this work, explored the immunopharmacologic properties of such optimized RNA upon direct administration, as well as its mode of action and efficacy as part of a preclinical vaccine development program. Here we show that in comparison with other application routes, intranodal vaccination using naked antigen-encoding RNA generated by in vitro transcription induces stronger biologically relevant T-cell responses and superior antitumor immunity due to the bioavailability of RNA in the lymph node and RNA inherent adjuvant effects on the lymph node microenvironment. These unexpected findings are of high relevance for design of future RNA-based cancer vaccine trials.

Materials and Methods

Animals

RAG-2−/− TCR-HA mice (H-2d; Thy1.2+) transgenic for the influenza virus hemagglutinin-A HA518-526 peptide-specific, I-Eα-restricted T cell receptor (TCR) (TCR-HA); BALB/c mice transgenic for HA518-526 peptide-specific, H-2Kb-restricted TCR (TCR-CL4); and BALB/c Thy1.1+ mice were from U. Hartwig (3rd Medical Department, University of Mainz, Germany; refs. 11, 12). RAG-2−/− C57BL/6, C57BL/6 Thy1.1+ mice, TCR transgenic OT-I mice recognizing the H-2Kd-restricted SIINFEKL epitope (OVA257-264) from chicken ovalbumin, and H-2Kd-restricted HA518-526, SIINFEKL (H2-Kb-restricted RNA vaccines

Plasmid templates for in vitro transcription of naked antigen-encoding RNAs (Supplementary Fig. S1A) were based on pSTI-A120 and pSTI-MIFD vectors (9). pSTI-MIFD features a signal sequence that directs the encoded antigen to endoplasmic reticulum and the MHC class I transmembrane and cytoplasmic domains improving the presentation of MHC Class I and II epitopes. The pSTI-SIINFEKLMIFD (SIINFEKL) and pSTI-NSO-I-MIFD (NY-ESO-I) vectors have been described (9, 10). pSTI-Influenza-HA-MIFD (HA) contains a codon optimized partial sequence of influenza HA (aa 60-285 fused to aa 517-527; influenza strain A/P/8/34), designed to combine all major immunodominant MHC epitopes. pSTI-Luciferase-A120 (Luc) contains the firefly luciferase gene (15).

In vitro transcription and pulsing

RNA was generated as described (16) and purified using the MEGAclear kit (Ambion). Cy3-UTP- or Cy5-UTP-labeling of RNA (Amersham) was conducted using the pSTI-Influenza-HA-MIFD (HA) vector template. Concentration and quality were assessed by spectrophotometry, gel electrophoresis, and BioAnalyzer (Agilent). For in vitro pulsing, 1 × 10^6 BMDCs generated with FLT3L were pelleted and incubated with RNA in a volume of 10 to 20 μL for 15 minutes at 37°C.

Immunization of mice

For intranodal or near-nodal (into soft tissue) immunizations, mice were anesthetized with xylazine/ketamine. The inguinal lymph node was surgically exposed, 5 to 10 μL RNA formulated in Rnase-free PBS (Ambion) were injected with a single-use 0.3-ML syringe with an ultrafine needle (31G, Becton Dickinson), and the wound was closed. For some experiments RNA was administered repetitively to the same lymph node, or 20 μg polyuridylic acid or polycytidylic acid [Poly(U) or Poly(C), Sigma] was injected. Subcutaneous, i.d., or near-nodal injections of 5 to 10 μL RNA (20 μg) were performed within the draining area of the inguinal lymph node. In some experiments, HA518-526 peptide (50 μg) in incomplete Freund’s adjuvant (IFA), SIINFEKL peptide (200 μg) with Cpg 1826 (25 μg; MWG Biotech), or 1 × 10^6 mature BMDCs transfected with 20 μg RNA were given s.c. into the upper thigh of the mice.

In vivo bioluminescence imaging

Bioluminescence imaging was performed using an IVIS Lumina imaging system (Caliper Life Sciences) as described previously (15).

In vivo cytotoxicity assay

Splenocytes (1 × 10^6) from TCR transgenic OT-I or CL4 mice were administered i.v. to C57BL/6 or BALB/c mice one day prior to immunization. Five days after immunization, splenocytes were labeled with either low (0.5 μmol/L) or high (5 μmol/L) concentrations of carboxyfluorescein diacetate, succinimidyl ester (CFSE) (Invitrogen). Of these target cells, 2 × 10^5 cells were adoptively transferred into mice in a 1:1 ratio (CFSElow; CFSEhigh) after pulsing the CFSEhigh cells with SIINFEKL or HA518-526 peptide (10 μmol/L). Controls were CFSElow cells pulsed with VSV-NP52-59 or gp70423-431. Splenocytes from host mice were analyzed 20 hours later by flow cytometry. Specific lysis was calculated as follows:

\[
\text{Specific lysis} = \frac{\text{CFSElow} - \text{CFSEhigh}}{\text{CFSElow}} \times 100\% 
\]
specific lysis = (1 – (percent of cells pulsed with peptide)/(percent of cells not peptide pulsed)) × 100.

**In vivo functional assays**

TCR-HA and TCR-CL4 cells (1 × 10⁶) labeled with CFSE (5 μmol/L) were adoptively transferred into BALB/c Thy1.1 mice and quantified three days later by staining for Thy1.2+ CD4+ and Thy1.2+ CD8+ cells. SIINFEKL reactive cells were quantified by staining peripheral blood or spleen cells with H-2k/β2m/SIINFEKL tetramer (Beckman Coulter) and CD8 antibody (Caltag).

For experiments in which OT-I T cells of memory phenotype were required for adoptive transfer, Thy1.2+ OT-I spleen cells were boosted in vitro with SIINFEKL peptide (10 μmol/L) for two days and administered into RAG−/− C57BL/6 mice. After 32 days, CD8+ cells exhibiting memory phenotype (CD62L− CD127+ CD69+) were isolated from these mice, and 7 × 10⁵ OT-I CD8+ Thy1.2+ memory T cells were adoptively transferred to C57BL/6 Thy1.1 mice.

**Flow cytometric analysis**

Monoclonal antibodies for flow cytometry, including one against interleukin-12 (IL-12; p40/p70), were from BD Phar-mingen. Hypotonically lysed blood samples were incubated at 4°C with specific monoclonal antibodies. Lymph node cells were obtained by digestion with collagenase (1 mg/mL; Roche). Quantification of H-2 Kβ/SIINFEKL complexes (9) and intracellular cytokine staining (16) were previously described. Flow cytometric data were acquired on a FACSCalibur analytical flow cytometer and analyzed by using Cellquest-Pro (BD Biosciences) or FlowJo (Tree Star) software.

**Immunohistochemistry and immunofluorescence**

Immunohistochemical detection of Thy1.2+ cells was conducted on 6-μm-thin air-dried cryosections with a biotinylated secondary antirat antibody, streptavidin-alkaline phosphatase (Dako), fast Red substrate (Dako), and hematoxylin (Merck) counterstaining. Cells labeled with Cy3-RNA were visualized in 6-μm lymph node sections fixed with 4% paraformaldehyde (PFA), counterstained with Hoechst 33342 (Molecular Probes, Invitrogen), and mounted in fluorescent mounting medium (Dako), as was CD11b expression. Documentation was conducted with a Leica immunofluorescence microscope with a 40×/0.4 NA objective lens and the QFISH software.

**Enzyme-linked immunospot assay**

Enzyme-linked immunospot assay (16, 17) was adapted for the mouse system. Briefly, 1.5 to 4 × 10⁶ spleen cells pulsed with peptide (2 μg/mL) were added per well of microtiter plates coated with anti-IFN-γ antibody (10 μg/mL; clone AN18, Mabtech). After 18 hours at 37°C, cytokine secretion was detected with an anti-IFN-γ antibody (clone R4-6A2, Mabtech).

**Quantitative reverse transcriptase-PCR**

RNA extraction, first-strand cDNA synthesis, and real-time PCR using TaqMan Gene Expression Assays (Applied Biosystems) were described previously (18).

**Vaccination in mouse models**

Previous reports show intensified administrations in short intervals to be superior for nucleic acid vaccines (19–21). Therefore, to determine protective immunity, mice received three to four immunizations at 3-day intervals. Thereafter, 1 × 10⁶ A20-HA or 2 × 10⁵ B16-OVA tumor cells were inoculated s.c. into the flanks. For assessment of therapeutic immunity, first the tumor cells were inoculated. Treatment with four to five immunizations in 3-day intervals was initiated after tumors had reached a diameter of 2 to 3 mm. Tumor sizes were measured every three days.

**Statistics**

Statistical analysis was performed in GraphPad Prism software employing unpaired two-tailed Student’s t test or ANOVA with Tukey’s multiple comparison test.

**Results**

**Superiority of intranodal RNA vaccination for efficient expansion of T cells**

In current clinical trials naked RNA vaccines are administered i.d. (7, 8). However, administration routes have never been systematically compared in animal models or in patients. Therefore, we evaluated the relevance of the administration route for induction of immune responses by naked antigen-encoding RNA.

To this aim, we used immunopharmacologically optimized influenza hemagglutinin (HA) RNA as a model antigen (Supplementary Fig. S1A). Thy1.1+ BALB/c mice, which had received CFSE-labeled Thy1.2+ TCR-transgenic CD8+ HA-specific CL4 T cells, were immunized with HA-RNA either s.c. or i.d. within the draining region of the inguinal lymph node. To deliver naked RNA straight to the actual site of T-cell activation, we also assessed direct injection into the inguinal lymph node (intranodal) as well as into soft tissue close to the lymph node (near nodal).

Surprisingly, quantification of cells in lymph nodes, blood, and spleen five days later showed strongly elevated frequencies of Thy1.2+ CL4 T cells exclusively in animals immunized intranodally (Fig. 1A). The vast majority (in lymph node and blood almost 100%) of these antigen-specific T cells underwent several cycles of cell division (Supplementary Fig. S1B), indicating an enduring T-cell stimulatory capacity of naked RNA when introduced into the lymphatic compartment. In contrast, administration of HA-RNA via all other routes had barely an effect on antigen-specific T cells.

**Bioavailability of the encoded antigen by selective uptake of naked RNA by lymph node resident dendritic cells**

Antigen dose is a key factor for the efficient induction of antigen-specific T-cell responses and eventually also for clinical success of an antitumor vaccine. We determined the bioavailability of antigens encoded by directly administered naked RNA by in vivo bioluminescence imaging. Luciferase encoding RNA (Luc-RNA) was injected into Thy1.1+ BALB/c mice s.c., i.d., and intranodally, and the bioluminescence signal produced upon injection of luciferin, the substrate
for the RNA-encoded luciferase enzyme, was quantified (Fig. 1B). In mice treated intranodally, we detected high levels of luciferase confined to the injection side 16 or 24 hours after injection, indicating efficient translation and robust delivery of the encoded protein. In contrast, photon emission was significantly weaker in mice treated i.d. and barely detectable in those injected s.c. This implies that the superiority of the intranodal route for T-cell expansion may be ascribed to higher bioavailability of the antigen in the lymph node through translation of the internalized RNA.

Figure 1. Potent expansion of antigen-specific T cells induced by intranodal administration of naked RNA. A, Thy1.1+ BALB/c mice (n = 6) were immunized with HA-RNA or NY-ESO-1 control RNA one day after adoptive transfer of CFSE-labeled Thy1.2+ CD8+ T cells. Five days later Thy1.2+ CD8+ cells harvested from compartments were quantified by flow cytometry. i.n., intranodal; n.n., near nodal. B, bioluminescence imaging was performed 16, 24, and 48 hours after injection of Luc-RNA into BALB/c Thy1.1+ mice (n = 6). Photon counts are represented by columns (left) or color scales (right). C, flow cytometry of Cy5-RNA (20 μg) in dendritic cell subpopulations 1 hour after administration to C57BL/6 mice (n = 2). Mean Cy3 signal after injection of pure Cy5-labeled nucleotide triphosphates was subtracted from that of Cy5-RNA to obtain the mean fluorescence intensity (MFI). D, fluorescence microscopy of inguinal lymph nodes from BALB/c mice (n = 5) resected 30 minutes after injection with Cy3-RNA (20 μg; red). CD11b (green) and Hoechst 33342 (blue) counterstaining. Magnification, top, ×40; bottom, ×400. Paracortical zone is demarcated with dashed line. Data in A, B, and C (mean ± SD) are from at least two independent experiments. **, P < 0.01 (ANOVA with Tukey’s multiple comparison test in B).
We then investigated the fate of the naked RNA by fluorophore-labeling of the RNA. Flow cytometric phenotyping of cell suspensions derived from the draining lymph node harvested 1 hour after injection of Cy5-labeled RNA (Cy5-RNA) via different routes showed exclusive enrichment of signals in lymph node resident dendritic cells, in particular CD11c+CD11b+ and CD11c+CD8+ dendritic cell subpopulations (Fig. 1C), which are known to be efficient stimulators of lymph node T cells (22). Cy5-RNA was hardly detected in these cells after administration via other routes. This was further confirmed by immunohistochemical analysis of lymph node sections after intranodal injection of Cy3-labeled RNA (Cy3-RNA). The intracellular Cy3-RNA gave a strong and distinct fluorescence signal, was localized predominantly in the paracortical zone, and exhibited co-staining with the myeloid dendritic cell marker CD11b (Fig. 1D).

**Activation of resident cell populations by administration of naked RNA into the lymph node**

Our data reveal that directly administered RNA has the intrinsic capability of targeted antigen delivery into professional antigen-presenting cells. Presentation of the antigen, however, has to take place in an adequate milieu to ensure efficient induction of potent T-cell responses. Therefore, we assessed costimulatory signals of lymph node cells following intranodal RNA administration. Flow cytometric phenotyping of CD11c+ dendritic cells harvested from RNA-injected lymph nodes revealed upregulation of maturation markers such as CD86, MHC II, and CD40 within 24 hours (Fig. 2A; Supplementary Fig. S3A). We also observed a dose-dependent increase in the percentage of dendritic cells with high expression of CD86 (Supplementary Fig. S3B), as well as a generally higher cellularity of lymph nodes treated with RNA (Supplementary Fig. S3C). RNA-induced activation seemed to be TLR3 and
TLR7 dependent as BMDCs from mice lacking signaling pathways for either TLR3 or TLR7 (TRIF$^{-/-}$ and MyD88$^{-/-}$ knockout mice, respectively) or both of these receptors (TRIF$^{-/-}$ MyD88$^{-/-}$ double knockout mice) were severely impaired in their capability to upregulate CD86 when pulsed with RNA (Fig. 2B). Moreover, RNA injected into the lymph node augmented the number of IL-12–secreting dendritic cells, confirming dendritic cell activation on the functional level (Fig. 2C). In accordance with high IL-12 levels, we also detected activation of NK1.1$^+$ natural killer (NK) cells (Supplementary Fig. S3D).

These findings prompted us to analyze the impact of RNA on intercellular cross-talk within the lymph node by profiling expression levels of cytokines, chemokine ligands, and costimulatory molecules by quantitative real-time reverse transcriptase-PCR (RT-PCR). We observed profound upregulation of proinflammatory cytokines (IL-6, IL-1b, and IL-1a), chemokine ligands (IP-10, CXCL9, CCL4, and CCL3), and the costimulatory molecule CD40 already 8 hours after intranodal HA-RNA administration (Fig. 2D). Expression levels of inhibitory molecules IL-10, IL-17, and transforming growth factor β (TGFβ), in contrast, remained unchanged. Noteworthy, intranodal injection of polyuridylic acid (Poly U), a known TLR7 ligand, induced an expression pattern, which was comparable with that of antigen-encoding RNA, whereas polycytidylic acid (Poly C), which does not induce TLR7 signaling, had modest effects. In summary, these findings reveal the induction of a Th1-type environment by the presence of naked antigen-encoding RNA.

**Rapid systemic expansion of antigen-specific CD4$^+$ as well as CD8$^+$ T cells with potent effector functions by RNA immunization**

As the efficient uptake of RNA by resident dendritic cells and its proinflammatory effects in the lymph node are prerequisites for induction of T-cell immunity, we characterized RNA vaccination-induced T-cell responses in more detail. To this aim, Thy1.1$^+$ BALB/c mice were immunized intranodally with HA-RNA after adoptive transfer of Thy1.2$^+$ CD8$^+$ TCR-CL4 or CD4$^+$ HA-specific T cells. Thy1.2-staining of lymph node cryosections five days poststimulation revealed a brisk infiltration of HA-specific CD8$^+$ as well as CD4$^+$ T cells in HA-RNA but not control RNA-injected lymph nodes, implying local expansion of naive T cells (Fig. 3A). Flow cytometric analysis of CFSE-labeled TCR transgenic CD8$^+$ TCR-CL4 cells harvested from lymph nodes showed considerable and dose-dependent proliferation of antigen-specific T cells in *situ* consecutive to intranodal immunization with HA-RNA (Fig. 3B).

A time course analysis of these cells in different compartments showed actively proliferating CD8$^+$ T cells in the injected lymph node already three days after immunization (Supplementary Fig. S4). A fraction of these left the lymph node between days 3 and 5 and migrated via the blood.
stream to the spleen and nondraining lymph nodes. (Supplementary Fig. S4). These T cells secreted IFNγ upon re-encountering the antigen (Fig. 3C) and exhibited a strong cytolytic activity in in vivo cytotoxicity assays (Fig. 3D).

In summary, these findings document that local intranodal RNA immunization induces a rapid and profound expansion of fully differentiated T-cell effectors. Most importantly, both antigen-specific CD4+ and CD8+ were efficiently expanded. RNA-induced T cells were capable of homing to primary and secondary lymphatic tissues and thereby establishing a systemic antigen-specific Th1-type immune response.

De novo priming of T cells and generation of T-cell memory by repetitive intranodal RNA immunization

Next, we determined the capability of intranodal RNA immunization to prime naïve T cells. To this end C57BL/6 mice were immunized repetitively with RNA encoding the SINFEKL epitope of chicken ovalbumin gene (SIINFEKL-RNA). Antigen-specific T-cell expansion as determined by MHC-peptide tetramer staining of ovalbumin-specific CD8+ T cells and cytolytic effector function measured in vivo positively correlated with the number of sequential immunizations (Fig. 4A and B). Notably, three immunization cycles were sufficient to amplify the amount of ovalbumin-specific CD8+ T cells beyond 10% of all peripheral CD8+ T cells. No apparent morphologic changes were found in lymph node anatomy despite repetitive injections (data not shown).

Protective immunity relies on the efficient generation of memory T cells. SIINFEKL peptide rechallenge of mice 60 days after the last intranodal RNA immunization induced rapid expansion of MHCI-peptide tetramer–positive cells only in mice preimmunized with SIINFEKL-RNA but not control RNA, indicating generation of CD8+ memory T cells from the endogenous T-cell repertoire (Fig. 4C). Likewise, we observed a strong increase in the number of preformed Thy1.2+ OT-1 memory T cells adoptively transferred to Thy1.1+ hosts upon intranodal immunization with SIINFEKL-RNA (Fig. 4D). Taken together, these establish the capability of intranodal RNA immunization to induce T-cell de novo priming and to restimulate pre-existing memory T cells, which are features crucial for enduring potency of an antitumoral vaccine.

Prophylactic and therapeutic antitumor immunity by repetitive intranodal RNA immunization

To assess the tumor-protective capability of intranodally administered RNA we challenged BALB/c mice with A20-HA lymphoma cells. Four cycles of intranodal immunization with HA-RNA prior to tumor challenge protected 90% of immunized mice without any sign of tumor growth for up to 300 days, whereas untreated mice died within 40 days (Fig. 5A). As expected, protection by s.c., i.d., or near nodal administration of HA-RNA was insufficient.

To explore the therapeutic potential of intranodal RNA, mice were inoculated s.c. with A20-HA tumor cells. When tumors reached macroscopic size (day 16), five cycles of immunization were carried out either with naked HA-RNA intranodally, HA-RNA–transfected BMDCs s.c., or HA\textsubscript{A218-226} peptide in IFA. Of the mice treated by intranodal HA-RNA
immunization, 60% showed complete regression of the tumors and survived. Survival benefit provided by all other vaccination strategies was <40% and all untreated mice died 45 days after tumor inoculation (Fig. 5B).

Similar observations were made in a mouse melanoma model. Repeated cycles of intranodal immunization with SIINFEKL-RNA protected C57BL/6 mice completely against challenge with the aggressive B16-OVA melanoma (Fig. 5C). Moreover, therapeutic vaccination with repetitive intranodal immunizations using SIINFEKL RNA was effective in treating 11-day implanted B16-OVA melanomas, inducing rejection of established tumors in 40% of mice (Fig. 5D). Cumulatively, these data show that intranodal immunization with naked RNA provides tumor protection and significantly prolongs survival of mice in advanced tumor models.

Discussion

The success and the ultimate clinical usefulness of naked antigen-encoding RNA depend on the induction of potent and enduring T-cell immunity. Our previous work was aimed at optimizing the immunobioavailability of RNA-encoded antigens. This was achieved by engineering antigen-encoding RNA of considerably higher stability and translational efficiency (9) and by routing antigens into MHC class I and II processing compartments (10). We have reported that the use of dendritic cells genetically modified with such RNA allows for efficient and polypepitopic expansion of antigen-specific CD8+ as well as CD4+ T cells with remarkably improved effector functions (9, 10). The objective of the current study was the evaluation of immune-pharmacologically optimized RNA in in vivo preclinical models, which resulted in several unexpected findings.

First, we identified the administration route as a critical success-limiting factor for naked RNA vaccines. Intranodal delivery of RNA was far more efficient in ensuring bioavailability of the encoded antigen than s.c. and i.d. application. Surprisingly, this was attributable to uptake of administered RNA exclusively by lymph node resident dendritic cells. It has been known for more than a decade that dendritic cells are able to present RNA encoded epitopes when coincubated with antigen-encoding RNA in vitro (23). This study shows for the first time the efficiency and selectivity of this process in vivo. Meanwhile, we have evidence that this process is based on macropinocytosis.4

Second, we showed that administration of naked RNA into lymph nodes creates a microenvironment that favors the induction of potent and sustained immune responses. Lymph

Figure 5. Superiority of intranodal immunization with RNA for induction of potent antitumor immunity.

A, BALB/c mice (n = 7) received four (days 0, 3, 6, 9) immunizations with HA-RNA (20 μg) or HA-peptide518-526 and S1-peptide110-119 (each 50 μg) plus CpG (25 μg) s.c. On day 12, A20-HA tumor cells were injected s.c. into the flanks.

B, A20-HA tumor cells were inoculated s.c. into the flanks of BALB/c mice (n = 10). On day 17 (tumor diameter 2–3 mm), mice received five immunizations in 3-day intervals with HA-RNA (20 μg) intranodally. BMDCs electroporated with HA-RNA (20 μg) s.c., or HA-peptide518-526 (50 μg) plus IFA s.c. C, C57Bl6 mice (n = 12) received three immunizations with SIINFEKL-RNA (20 μg) intranodally. On day 10, B16-OVA tumor cells were injected s.c. into the flanks. D, B16-OVA tumor cells were inoculated s.c. into the flanks of C57Bl6 mice (n = 10, d0). At day 11 (tumor diameter 2–3 mm) mice received four immunizations of SIINFEKL-RNA (20 μg) in 3-day intervals.

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4 Submitted for publication.
node resident dendritic cells upregulate CD86, a costimulatory molecule required for efficient activation of naïve T cells and for immunologic memory (24). Moreover, proinflammatory cytokines such as IL-12p40, IL-1α, and IL-1β, which foster T-cell responses (25), and IL-6, which supports expansion and survival of primed T cells (26, 27), are strongly induced, whereas regulatory T-cell promoters, such as IL-10 and TGFβ (28) remained on baseline level. Chemokine ligands for attraction of memory T cells (CCL3 and CCL4; refs. 29, 30) and NK cells (CXCL9, IP-10) to the lymph node (31) were strongly upregulated. This may explain the influx of pre-existing memory T cells and the increase of activated NK cells observed after intranodal RNA administration. In principle, RNA may trigger signaling through TLR3 via spontaneously formed double-stranded secondary structure as well as TLR7 by peculiar single-stranded nucleotide sequence motifs. By exploiting dendritic cells from knockout mice, we showed that the observed effects are mediated by both TLRs. The strong inflammatory effect of naked RNA when administered intranodally is surprising, as in previous in vitro studies TLR3 and TLR7 signaling by single-stranded RNA required protection of the RNA from degradation by ubiquitous RNAses either by complexing with polymers such as polyethyleneimine (32) or by utilization of nuclease-resistant ribonucleotide analogs (33). Instant rescue of the RNA in the lymph node by uptake seems to ensure direct access to endosomal compartments rich in TLR7 and TLR3.

Third and most importantly, we showed that naked antigen-encoding RNA has the capability to induce potent systemic T-cell immunity in vivo. This is most likely attributable to the fact that injecting optimized antigen-encoding RNA into the lymph node ensures spatiotemporal synchronization of antigen delivery and efficient presentation by resident dendritic cells providing essential immunologic signals mediating attraction and costimulation of T lymphocytes. Due to the use of MHC class II leveraging routing signals, not only CD8+ but also CD4+ T cells were substantially expanded. Moreover, RNA allows rapid and potent priming of T cells as well as repeated boosting of systemic antigen-specific T-cell responses. The capability of intranodal RNA immunization to generate fully differentiated highly cytotoxic effector as well as memory T cells may be associated with enduring potency of an antitumoral vaccine. By further revealing that RNA-induced immune responses protect mice from tumor challenge and have substantial therapeutic impact on advanced tumors, we provide the preclinical proof of concept that intranodal immunization with naked antigen-encoding RNA is a feasible, safe, and a powerful approach for antitumoral vaccination.

This study is, to our knowledge, the first report of potent systemic T-cell immunity in animal models induced by direct application of naked antigen-encoding RNA. Our findings will support rational clinical development of RNA as an active pharmaceutical ingredient.

Disclosure of Potential Conflicts of Interest

U. Sahin (founder, chief executive officer), C. Huber (founder), and C. Britten (employee) are associated with Ribological, Biontech AG (Mainz, Germany), a company that develops RNA-based cancer vaccines in the indicated functions. U. Sahin, S. Kreiter, Ö. Türeci, and A. Selmi are inventors on a patent application, in which parts of this article are covered.

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