Preclinical Evaluation of TriMix and Antigen mRNA-Based Antitumor Therapy

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Abstract

The use of tumor-associated antigen (TAA) mRNA for therapeutic purposes is under active investigation. To be effective, mRNA vaccines need to deliver activation stimuli in addition to TAAs to dendritic cells (DC). In this study, we evaluated whether intranodal delivery of TAA mRNA together with TriMix, a mix of mRNA encoding CD40 ligand, constitutive active Toll-like receptor 4 and CD70, results in the in vivo modification and maturation of DCs, hence, priming of TAA-specific T cells. We showed selective uptake and translation of mRNA in vivo by lymph node resident CD11c+ cells. This process was hampered by codelivery of classical maturation stimuli but not by TriMix mRNA. Importantly, TriMix mRNA induced a T-cell-attracting and stimulatory environment, including recruitment of antigen-specific CD4+ and CD8+ T cells and CTLs against various TAAs. In several mouse tumor models, mRNA vaccination was as efficient in CTL induction and therapy response as vaccination with mRNA-electroporated DCs. Together, our findings suggest that intranodal administration of TAA mRNA together with mRNA encoding immunomodulating molecules is a promising vaccination strategy. Cancer Res; 72(7); 1–11. ©2012 AACR.

Introduction

The immune system can mount immune responses against tumor-associated antigens (TAAs). Such immune responses, mediated by CD4+ T-helper 1 (Th1) cells and CD8+ CTLs, can be enhanced or induced de novo by immunotherapeutic strategies using antigen-loaded dendritic cells (DCs refs. 1–3). Several strategies have been developed to deliver TAAs to DCs, including the use of mRNA (4–6). Autologous DCs loaded ex vivo with TAA mRNA have been extensively tested in preclinical studies, showing their ability to induce functional Th1 cells and CTLs (7–10). Moreover, clinical testing showed the induction of antigen-specific immune responses by DC vaccines (11). However, the logistics of developing a specific vaccine for each patient may be prohibitive. Therefore, direct administration of TAA mRNA has gained substantial interest (12–14). This method offers a number of advantages, mRNA is not patient-specific, available at all times, safe, and easy to produce at low cost (12–14).

The success of mRNA vaccination depends on the engulfment of mRNA by DCs and its potential to mature DCs. Consequently, the route of mRNA delivery and the modus of DC maturation are parameters that will critically impact on the efficiency of the mRNA vaccine. It was recently showed that intranodal delivery of mRNA results in the engulfment of mRNA by DCs, as well as the activation of Toll-like receptors (TLR refs. 15–17). Nevertheless, it is suggested that naked mRNA is insufficient to fully harness the stimulatory potential of DCs (9, 18). Therefore, codelivery of additional stimuli, such as lipopolysaccharide (LPS), CD40 ligand (CD40L), polynicotinic polycytydilic acid (polyI:C), and protamine-complexed mRNA, has been evaluated (18, 19). However, defining the optimal protocol for in vivo DC maturation, without abrogating the uptake/translation of mRNA has proven to be challenging. The use of mRNA encoding immunomodulating proteins might be an attractive alternative to potentiate DCs in situ.

We previously showed that electroporation of human DCs with CD40L mRNA and mRNA encoding a constitutive active form of TLR4 (caTLR4) induces DC maturation. We moreover introduced CD70 mRNA into these DCs to provide a costimulatory signal to CD27+ T cells. We showed that DCs modified with this so-called TriMix induce tumor-specific T-cell responses in vitro as well as in vaccinated patients with melanoma (20–23).

Here, we report on the delivery of TAA and TriMix mRNA in situ to generate T-cell-attracting and stimulating DCs, a strategy that was shown to be as efficient as vaccination with in vitro electroporated DCs in terms of CTL induction and antitumor therapy.

Materials and Methods

Mice

Female, 6- to 12-week-old C57BL/6, DBA/2, and BALB/c mice were purchased from Harlan. Transgenic mice were...
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A

CD70  CD40  CD86

B

IL-6  TNF-α

IL-12p70

P = 0.0003

P = 0.0415

P < 0.0001

P < 0.0001

P < 0.0001

P = 0.0004

P = 0.0006

C

D

CD8

Pentamer

OVA  LPS  TriMix

E

CFSE

CD8

F

IRFγ

G

CFSE

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Figure 1. DCs matured through electroporation of TriMix efficiently stimulate antigen-specific T cells. The histogram overlays in (A) show the phenotype of DCs electroporated with mNGFR mRNA and left immature or matured by coelectroporation of TriMix or addition of LPS (n = 10). The graphs in (B) show the cytokines secreted by these DCs (n = 6). The graph in (C) depicts the incorporation of 3H thymidine by allogeneic spleen cells cultured with these DCs (n = 3). D–F, mice were immunized intravenously with 5 × 10⁶ DCs electroporated with OVA mRNA and matured by coelectroporation of TriMix mRNA or addition of LPS. Five days later, the expansion of functional OVA-specific CD8⁺ T cells was assessed. The results of (D) the pentamer staining, (E) the in vivo cytotoxicity assay, and (F) the intracytoplasmatic staining of IFN-γ on spleen cells restimulated with SINFEKL-presenting DCs are shown (n = 2). G, mice, immunized with Trp2-presenting DCs, were subjected to an in vivo cytotoxicity assay to evaluate the stimulation of Trp2-specific CD8⁺ T cells (n = 2).

Figure 2. Formulation and pharmacokinetics of mRNA. A, mouse DCs were pulsed with FLuc mRNA in the indicated buffer. Luminescence was measured 4 hours later. The graph depicts the photon emission (n = 4). B and C, mice were injected intranodally with FLuc mRNA. In vivo bioluminescence imaging was conducted at the indicated time points (n = 4). C, to evaluate the stability of FLuc mRNA in vivo, lymph nodes were isolated 6, 12, and 24 hours after injection and PCR carried out on cDNA synthesized from extracted mRNA (n = 4). D, mice received an intranodal injection of eGFP mRNA formulated in 0.8 RL. Four hours later, the lymph node was resected, a single-cell suspension prepared and stained for CD11c. The graph obtained by fluorescence microscopy shows eGFP (green) expression by CD11c⁺ cells (red, n = 4). E, transgenic CD11c-DTR mice, which were pretreated with PBS or DT, received an intranodal injection with FLuc mRNA. In vivo bioluminescence imaging was conducted 4 hours later. Single-cell suspensions were prepared from the lymph nodes and analyzed by flow cytometry for the presence of CD11c⁺ cells (n = 3). F, mice, of which the skin was pretreated with PBS or GM-CSF, were injected intradermally with FLuc mRNA. In vivo bioluminescence imaging was conducted 6 hours later (n = 3).

Mouse cell lines and DCs

The melanoma M04, the T-cell lymphoma EG7-OVA, the mastocytoma P815, and the myeloid leukemia C1498-WT1 were obtained from the American Type Culture Collection, C. Uyttenhove (Université Catholique de Louvain, Brussels, Belgium), and H.E. Kohrt (Stanford University Medical Centre, Stanford, CA), respectively. No full authentication was carried out. Cell lines were evaluated for the expression of provided by B. Lambrecht (University of Ghent, Ghent, Belgium) and include OT-I mice that carry a transgenic CD8 T-cell receptor (TCR) specific for the MHC I-restricted ovalbumin (OVA) peptide SIINFEKL, OT-II mice that carry a transgenic CD4 TCR specific for the MHC II-restricted OVA peptide ISQAVHAAHAEINEAGR, and CD11c-diphtheria toxin receptor (DTR) mice in which CD11c⁺ cells are depleted upon treatment with 4 ng diphtheria toxin (DT)/g mouse (Sigma). Where indicated mice received an intravenous hydrodynamic injection with 10 µg of a plasmid encoding Flt3 ligand (a gift from O. Leo, Universiteit Libre de Bruxelles, Brussels, Belgium) in 0.9 NaCl in a final volume equal to 10% of the mouse body weight. Animals were treated according to the European guidelines for animal experimentation. Experiments were reviewed by the Ethical committee for use of laboratory animals of the Vrije Universiteit Brussel (Jette, Belgium).
p-eGFP-N1 as a Hin sequence encoding enhanced GFP (eGFP) was isolated from linearized with Spe shrimp alkaline phosphatase cloned as a Bam from Fermentas.

Hin injected with FLuc mRNA alone or the latter together with TriMix mRNA or LPS (\(n = 3\) 0 -0 following primers: 5'-GGGGATCCGTGCACACGTCACACTCGTTC-5' fragment in the pST1 vector. A fragment of the mouse Trp2 or CD70 were obtained from Geneart and cloned as a Ii80tOVA. The codon-optimized cDNA encoding mouse CD40L pGEM-Ii80P1A was cloned analogous to the cloning of pGEM-

dIII-NotI digested pST1 vector. All enzymes were purchased from Fermentas.

MHC molecules and antigens (OVA, MO4 and EG7-OVA; P1A, P815; and WT1, C1498-WT1) by reverse transcriptase PCR (RT-PCR) or flow cytometry. Bone marrow–derived DCs were generated as described (9).

**Messenger RNA**

The vector, pST1 was provided by U. Sahin (Johannes-Gutenberg University, Mainz, Germany). The vectors pGEM-li80OVA, pST1-tyrosinase-DC-LAMP, pST1-sig WT1-\(\Delta\)NLSDC-LAMP, pST1-caTLR4, and pGEM-tNGFR have been described (Benteyn and colleagues; manuscript in preparation; refs. 9, 21, 24). The sequence encoding firefly luciferase (FLuc) was cloned into pST1 with minor modifications. The vector pGEM-li80P1A was cloned analogous to the cloning of pGEM-li80OVA. The codon-optimized cDNA encoding mouse CD40L or CD70 were obtained from Geneart and cloned as a SpeI-Xhol fragment in the pST1 vector. A fragment of the mouse Trp2 gene that encodes SYDFFVWL was amplified with the following primers: 5'-GGGGATCCGCGCATCTAAGACGG-3' and 3'-GGGGATCCGCGCAACGTCACACTCGTTC-5' and cloned as a BamHI fragment in the BamHI linearized and shrimp alkaline phosphatase–treated pST1-sig-DC-LAMP. The sequence encoding enhanced GFP (eGFP) was isolated from p-eGFP-N1 as a HinIII-NotI fragment and cloned into the HindIII-NotI digested pST1 vector. All enzymes were purchased from Fermentas.

Before in vitro transcription, pGEM and pST1 vectors were linearized with SpeI and SapI, respectively. In vitro transcription was carried out as described (9). The mRNA was dissolved in PBS, Ca\(^{2+}\)-containing Hank’s balanced salt solution (HBSS, Lonza), or 0.8 Ringer lactate (0.8 RL; Baxter).

**Passive pulsing and electroporation of mRNA**

To pulse DCs with mRNA, 5 x 10^6 DCs were pelleted and incubated for 15 minutes with 10 µg tNGFR or FLuc mRNA in 15 µL. Where indicated pulsing was carried out in the presence of 1 ng/mL LPS from *Escherichia coli* serotype 055: B5 (Sigma-Aldrich), 10 µg/mL polyIC (Sigma), or 100 ng/mL monophosphoryl lipid A (MPL; GlaxoSmithKline). DCs were cultured in RPMI-1640 medium supplemented with 5% FCI (Harlan), 50 µM 2-mercaptoethanol, and 20 ng/mL mouse granulocyte macrophage colony-stimulating factor (GM-CSF; prepared in-house) at a cell density of 10^6 DCs per mL. Four hours later, DCs were lysed using the reporter lysis buffer from Promega. β-Luciferin (Xenogen) was added, luminescence measured with the Glomax 96-luminometer, and data analyzed with Glomax software (Promega). Electroporation of DCs with mRNA was carried out as described (9). Where indicated, DCs were activated for 4 hours with 100 ng/mL LPS.

**In situ delivery of mRNA**

To deliver mRNA, C57BL/6 mice were anesthetized with ketamine (70 mg/kg; Ceva) and xylazine (10 mg/kg; Bayer). The inguinal lymph node was surgically exposed and injected with the indicated amount of mRNA (and...
where indicated 1 ng LPS). Subsequently, the wound was closed. On 3 consecutive days before intradermal delivery of mRNA, mice were injected intradermally with PBS or 20 ng of mouse GM-CSF, after which the mRNA was administered.

RNA isolation, cDNA synthesis, and RT-PCR
RNA was extracted using the SV Total RNA Isolation System (Promega) and converted to cDNA by the RevertAid H-Minus First Strand cDNA Synthesis Kit (Fermentas). The sequence encoding Fluc was amplified with 5'-AAGGTGTGGCCCTTCC-3' and 5'-CCAGAATGAAAATAGGTTG-3', whereas the sequence encoding β-actin was amplified with 5'-TGCTATCCAGGCTGTGCTAT-3' and 5'-GATGGAATTTGAGTGT-3' using the following PCR program: 94°C 5', 45× (94°C 30', 52°C 30', 72°C 30'), 72°C 10', hold 4°C.

Immune array
RNA of lymph nodes injected with 0.8 RL, 10 μg antigen mRNA supplemented with 20 μg tNGFR mRNA or TriMix (10 μg per component) was extracted and converted to cDNA. Quantitative RT-PCR by the TaqMan mouse immune response array (Applied Biosystems) and analysis was conducted according to the manufacturer's instructions.

Flow cytometry
Allophycocyanin-conjugated anti-CD11c (HL3), -CCR7 (2H4), and phycoerythrin-conjugated anti-CD40L (MR1) and -CD70 (FR70) antibodies were purchased from Pharmingen. The antibodies against CD40 (FGK45), CD80 (16-10A1), and CD86 (GL-1) were prepared in-house. Nonreactive isotype matched antibodies served as controls (Pharmingen). Labeling of DCs was carried out as described (9). Data were collected using the FACSCanto Flow Cytometer (Becton Dickinson) and analyzed with FACS Diva or FlowJo software.

Allogeneic mixed lymphocyte reaction
The ability of electroporated DCs to stimulate allogeneic CD90 purified (Miltenyi Biotec) T cells was assessed in a mixed lymphocyte reaction (25).

ELISA
Supernatants were screened in a sandwich ELISA for the presence of interleukin (IL)-6, IL-12p70, TNF-α, or IFN-γ (eBioscience).

In vivo bioluminescence imaging
In vivo bioluminescence imaging was conducted as described (26).

Fluorescence microscopy
Lymph nodes were injected with 10 μg eGFP mRNA, 1 day before isolation. Single-cell suspensions were prepared and stained with a phycoerythrin-conjugated anti-CD11c antibody. Expression of CD11c and eGFP was evaluated with the Evos fluorescence microscope.

Immunization of mice
Mice were immunized intravenously with 5 × 10^5 antigen-presenting DCs activated with TriMix or LPS, or intranodally or intradermally with 10 μg antigen mRNA supplemented with 30 μg tNGFR mRNA or TriMix (10 μg per component). Immunization with DCs electroporated with tNGFR mRNA or with tNGFR mRNA as such served as a control. For assessment of therapeutic efficacy, 5 × 10^5 tumor cells were administered subcutaneously in the lower back, 7 days before immunization.

Intracytoplasmatic staining of IFN-γ
Spleen cells of immunized mice were stimulated for 24 hours with DCs pulsed for 2 hours with 5 μg/mL SIINFEKL peptide and matured with LPS. GolgiPlug was added 24 hours before intracytoplasmatic staining of IFN-γ.

Pentamer staining
The staining of CD8+ T cells with H2-Kb/SIINFEKL pentamers (Immunosource) was carried out as described (25).

*In vivo* cytotoxicity assay
*Spleen* cells from syngeneic mice were labeled with 10 μmol/L carboxyfluorescein diacetate succinimidyl ester (CFSE) as described (9). These were pulsed with the peptide SIINFEKL (OVA) or SVYDFFVWL (Trp2; Thermo Electron Cooperation) or a set of overlapping peptides covering WT1 (kind gift from V. Van Tendeloo, University of Antwerp, Edegem, Belgium) or tyrosinase (EMC microcultures) at 5 μmol/L for 2 hours. Peptide-pulsed cells were mixed at a 1:1 ratio with nonpulsed cells, labeled with 0.5 μmol/L CFSE.

Table 1. Intronodal delivery of TriMix mRNA generates an immunostimulatory environment

<table>
<thead>
<tr>
<th>Antigen mRNA</th>
<th>TriMix mRNA</th>
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<tbody>
<tr>
<td>Antigen-presenting molecules</td>
<td>Anti-MHC II</td>
</tr>
<tr>
<td>Proinflammatory cytokines</td>
<td>IL-6</td>
</tr>
<tr>
<td></td>
<td>IL-15</td>
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<td></td>
<td>IFN-γ</td>
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<tr>
<td>T-cell–attracting molecules</td>
<td>MCP-1</td>
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<td></td>
<td>IP-10</td>
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<tr>
<td>Signaling molecules</td>
<td>SOCS1</td>
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<tr>
<td></td>
<td>STAT1</td>
</tr>
<tr>
<td>Others</td>
<td>Granzyme B</td>
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</tbody>
</table>

NOTE: Mice received an intranodal injection of 0.8 RL, antigen mRNA combined with tNGFR mRNA, or with TriMix. Lymph nodes were removed 8 hours later, RNA extracted, cDNA synthesized, and quantitative RT-PCR carried out. It summarizes the molecules of which the upregulation was at least 2-fold higher when TriMix was coadministered when compared with antigen mRNA alone. The data show the relative upregulation compared with injection with 0.8 RL alone. The results are shown as mean ± SEM of 3 experiments.
Specific lysis of target cells was analyzed 18 hours later by flow cytometry. The percentage of killing was calculated as described (27).

**In vivo proliferation assay**

One day before immunization, 10^6 purified and CFSE-labeled CD8^- OT-I or CD4^- OT-II spleen cells were transferred to mice by intravenous injection. Five days postimmunization, proliferation of T cells was analyzed in peripheral blood, spleen, and lymph nodes (27).

**Statistical analyses**

A one-way ANOVA followed by the Bonferroni multiple comparison test was conducted. Sample sizes and number of times experiments were repeated are indicated in the figure legends. Number of asterisks in the figures indicates the level of statistical significance as follows: *, P < 0.05; **, P < 0.01; ***, P < 0.001. The results are shown in a scatter plot in which each mouse is depicted as a dot and the mean as a horizontal line or in a column graph or table as the mean ± SEM. Survival was visualized in a Kaplan–Meier plot. Differences in survival were analyzed by the log-rank test.

**Results**

DCs matured through electroporation with TriMix mRNA efficiently stimulate antigen-specific T cells

We recently showed that the T-cell stimulatory capacity of human DCs electroporated with TAA mRNA is

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**Figure 4.** Intranodal delivery of TriMix but not LPS together with OVA mRNA results in stimulation of OVA-specific CD4^- and CD8^- T cells. CFSE-labeled CD4^- OT-II or CD8^- OT-I cells were adoptively transferred 1 day before immunization of mice with tNGFR mRNA, OVA mRNA alone, or combined with TriMix or LPS. The amount of mRNA was kept constant by addition of tNGFR mRNA. Five days postimmunization, stimulation of T cells within the lymph node was analyzed. A, proliferation of CD4^- OT-II cells was analyzed by flow cytometry (n = 3). B and C, stimulation of CD8^- OT-I cells was analyzed by (B) pentamer staining (n = 5) and (C) in vivo cytotoxicity assay (n = 3). D, stimulation of CTLs after immunization with OVA and TriMix mRNA either delivered intradermally in mice pretreated with GM-CSF or intranodally was analyzed by in vivo cytotoxicity assay (n = 2).
considerably increased by simultaneous coelectroporation with TriMix (20). As we wanted to investigate the use of TriMix for the in situ modification of mouse DCs, we evaluated whether electroporation of mouse DCs with TriMix results in immunogenic DCs. We showed that TriMix-electroporated DCs displayed a phenotype (Fig. 1A), cytokine secretion profile (Fig. 1B), and allogeneic T-cell stimulatory capacity (Fig. 1C) comparable with that of LPS-activated DCs. Importantly, we showed that TriMix-matured DCs were superior to LPS-matured DCs in stimulation of functional antigen-specific CD8\(^+\) T cells in vivo. This was shown for OVA (Fig. 1D–F) and the TAA Trp2 (Fig. 1G).

**Formulation and pharmacokinetics of mRNA for vaccination purposes**

It was previously shown that cellular uptake of mRNA can be influenced by the composition of the injection solution (28). Therefore, we evaluated which buffer is best suited to deliver mRNA to DCs. FLuc mRNA was dissolved in PBS, Ca\(^{2+}\)-containing HBSS, or 0.8 RL. Luminescence analysis of passively pulsed DCs showed high FLuc expression when the mRNA was dissolved in 0.8 RL or HBSS (Fig. 2A). Next, we administered FLuc mRNA intranodally. In vivo bioluminescence imaging showed short-term FLuc expression when mRNA was formulated in PBS when compared with high and long FLuc expression when mRNA was formulated in HBSS or 0.8 RL (Fig. 2B). The latter was unexpected as naked mRNA is believed to have a short extracellular half-life (29). To analyze the stability of mRNA in vivo upon delivery in 0.8 RL or HBSS, we resected lymph nodes injected with FLuc mRNA 6, 12, and 24 hours after injection. RT-PCR showed the presence of FLuc mRNA up to 12 hours after injection. No FLuc mRNA was detectable at later time points (Fig. 2C).

Next, we evaluated the role of DCs in the uptake of mRNA in vivo. Lymph nodes were injected with eGFP mRNA 24 hours before their isolation. Single-cell suspensions were prepared and stained for CD11c. Fluorescence microscopy showed a small number of eGFP\(^+\) cells. Importantly, all eGFP\(^+\) cells were CD11c\(^+\), showing uptake and translation of mRNA by DCs (Fig. 2D). To further evidence a role for DCs, we used CD11c-DTR transgenic mice in which administration of DT results in the depletion of CD11c\(^+\) cells. In vivo bioluminescence imaging showed the absence of FLuc expression in mice that were treated with DT before intranodal administration of FLuc mRNA. Mice treated with PBS served as a control (Fig. 2E). Flow cytometric analysis of the lymph nodes of these mice confirmed that the absence of luminescence was correlated with the depletion of DCs (Fig. 2E). As delivery of mRNA into the inguinal lymph node is technically challenging, we finally examined

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Figure 5. Inclusion of TriMix in the mRNA vaccine enhances the induction of TAA-specific CTLs. An in vivo cytotoxicity assay was conducted to evaluate the induction of CTLs in mice immunized intranodally with TAA mRNA alone or combined with TriMix. The graphs depict the specific lysis of target cells upon immunization against (A) Trp2 (n = 2), (B) WT1 (n = 3), and (C) tyrosinase (n = 2).
Figure 6. Immunization with antigen mRNA and TriMix is as efficient in stimulation of CTLs and in therapy as immunization with ex vivo–modified DCs. A–C, C57BL/6 mice were immunized intravenously with antigen and TriMix mRNA–modified DCs or intranodally with antigen and TriMix mRNA. The in vivo cytotoxicity assay was conducted 5 days later. The graphs show the specific lysis of target cells in peripheral blood upon immunization against (A) OVA (n = 2), (B) Trp2 (n = 2), or (C) WT1 (n = 2). D–H, mice bearing palpable tumors (10 mice per group) were immunized by intravenous injection of antigen and TriMix mRNA–electroporated DCs or by intranodal injection with antigen and TriMix mRNA. The graphs show the tumor growth (left) and survival (right) in the MO4 model after immunization with the antigen OVA (D) or the TAA Trp2 (E), in the EG7-OVA model after immunization with OVA (F), in the C1498-WT1 model after immunization with the TAA WT1 (G) all in C57BL/6 mice, and in the P815 model after immunization with the TAA P1A (H) in DBA-2 mice.
the feasibility of delivering mRNA intradermally. Because we showed in the former experiment that CD11c\(^+\) cells are responsible for the DC uptake, we pretreated the mice with an intraderal injection of PBS or GM-CSF on 3 consecutive days before the intraderal injection of FLuc mRNA. In vivo bioluminescence imaging, conducted 6 hours later, showed FLuc expression only in mice pretreated with GM-CSF (Fig. 2F).

**Intronal delivery of TriMix generates an immune stimulatory environment**

Induction of antitumor immune responses requires antigen-presentation by mature DCs (1–3). To evaluate the effect of TriMix and classical maturation stimuli on the engulfment of mRNA and the induction of an immune stimulatory environment, we first passively pulsed DCs in vitro with FLuc mRNA and these maturation stimuli, showing a reduction in FLuc expression after pulsing of DCs with FLuc mRNA in the presence of LPS, MPL, or poly(I:C). This reduction in protein expression was less pronounced when TriMix was codelivered (Fig. 3A). In addition, DCs pulsed with TriMix mRNA showed a higher expression of CD40, CD70, CD80, and CD86 than the DCs pulsed with MPL (data not shown), LPS, or poly(I:C) (Fig. 3B).

Next, we evaluated the uptake of FLuc mRNA when delivered as such or together with LPS or TriMix in vivo. We showed that codelivery of TriMix had a lesser impact on the uptake of mRNA than its codelivery with LPS (Fig. 3C). To increase the number of DCs that can be recovered from the injected lymph node for analysis, we pretreated the mice with a hydrodynamic injection of a plasmid encoding Flt3 ligand. In analogy with the data described by Kreiter and colleagues (30), FLuc mRNA injected into these mice resulted in increased luminescence reflecting the specific uptake by the DCs (data not shown). Flow cytometry showed that DCs (CD11c\(^+\)) from lymph nodes coinjected with TriMix displayed the highest expression of CD40, CD80, and CD86 than DCs isolated from lymph nodes injected with FLuc mRNA alone or combined with LPS (Fig. 3D).

These findings prompted us to analyze, whether codelivery of TriMix promotes a T-cell-attracting and activating environment, by profiling the expression levels of maturation-associated markers by quantitative RT-PCR. We observed upregulation of several markers in lymph nodes injected with FLuc and tNGFR mRNA when compared with lymph nodes injected with 0.8 RL. Importantly, the upregulation of the following markers: MHC II, IL-6, IL-15, IFN-\(\gamma\), MCP-1, IP-10, granzyme B, SOCS1, and STAT1 was at least 2-fold higher when TriMix was codelivered (Table 1).

**Intronal delivery of TriMix but not LPS together with OVA mRNA results in expansion of OVA-specific CD4\(^+\) and CD8\(^+\) T cells with potent effector function**

Activation of CD4\(^+\) T cells is critical for the induction of long-lasting antitumor immunity (31). Therefore, we evaluated the expansion of OVA-specific CD4\(^+\) T cells upon intranal delivery of tNGFR mRNA, OVA mRNA, or combined with TriMix or LPS. Proliferation of CFSE-labeled CD4\(^+\) OT-II cells was evaluated by flow cytometry, showing enhanced proliferation of OT-II cells in mice receiving OVA and TriMix mRNA. Of note, transferred T cells hardly proliferated when LPS was coinjected with OVA mRNA (Fig. 4A). Similar results were obtained with CD8\(^+\) OT-I cells (data not shown). To further evaluate the expansion and function of OVA-specific CD8\(^+\) T cells, mice were immunized 1 day after adoptive transfer of CD8\(^+\) OT-I cells. Five days postimmunization, we carried out an H2-K\(^b\)/SIINFEKTL pentamer staining or an in vivo cytotoxicity assay. Both assays showed the enhanced stimulation of OVA-specific CD8\(^+\) T cells when mice were immunized with OVA mRNA and TriMix when compared with mice immunized with OVA mRNA alone or combined with LPS (Fig. 4B and C).

Using the model antigen OVA, we finally compared intranal delivery of OVA and TriMix mRNA in mice pretreated with GM-CSF to its intranal delivery. Using the in vivo cytotoxicity assay we showed that the lysis of target cells was the highest when the mRNA was delivered intranodally (Fig. 4D).

**Inclusion of TriMix in the mRNA-based antitumor vaccine enhances the induction of TAA-specific cytotoxic T cells**

Next, we assessed whether the results obtained with the antigen OVA are representative for other TAAs. Mice were immunized with Trp2, WT1, or tyrosinase mRNA alone or combined with TriMix. The in vivo cytotoxicity assay showed enhanced lysis of target cells when TriMix was included in the immunization regimen (Fig. 5A–C).

**Immunization with antigen mRNA and TriMix is as efficient in stimulating cytotoxic T cells and in therapy as immunization with ex vivo–modified DCs**

Therapeutic immunization with human DCs electroporated with TAA and TriMix mRNA has shown promise in clinical evaluation (23). Therefore, we compared the efficacy of DC- to mRNA-based immunization, evaluating the induction of antigen-specific CTLs in vivo. We showed that immunization with antigen and TriMix mRNA was as efficient as immunization with antigen and TriMix mRNA–electroporated DCs for the antigen OVA and the TAAs, Trp2, and WT1 (Fig. 6A–C). We next evaluated the therapeutic efficacy of such vaccines. First, mice bearing MO4 tumors were treated with antigen and TriMix mRNA–modified DCs or antigen and TriMix mRNA as such. Similar results were obtained upon immunization with OVA (Fig. 6D) or Trp2 (Fig. 6E) as an antigen. Mice treated with tNGFR-electroporated DCs or tNGFR mRNA as such served as controls. Mice from control groups showed rapid tumor growth, whereas mice immunized with a single intravenous injection of DCs electroporated with antigen and TriMix mRNA or an intranal injection of antigen and TriMix mRNA showed a reduced tumor growth, hence, prolonged survival. These data were extended to the mouse T-cell lymphoma EG7-OVA, the myeloid leukaemia C1498-WT1 in
C57BL/6 mice, and the mastocytoma P815 in DBA-2 mice using OVA, WT1, and P1A as the antigen applied for immunization, respectively (Fig. 6F–H).

Discussion

Delivery of TAA mRNA to DCs for cancer therapy offers many advantages, which can be fully exploited when the mRNA is administered intranodally (15). It is proposed that mRNA functions as a template for translation as well as a ligand for TLRs (32). It is not clear, however, whether the intrinsic adjuvant effect of mRNA is sufficient to fully exploit the immunostimulatory capacity of DCs (33).

Therefore, we evaluated the local delivery of mRNA encoding CD40L, CD70, and cTLR4 (referred to as TriMix) as an adjuvant in conjunction with intranodal TAA RNA vaccination. We show that TriMix but not classical maturation stimuli potentiates the immunogenicity of intranodal mRNA vaccination. We moreover showed that the strength of TriMix is dual: low impact on antigen mRNA immunobioavailability and simultaneous delivery of stimuli that act synergistic in terms of activation of T-cell responses.

It has been suggested that the immunobioavailability of antigen mRNA is a critical success-limiting factor in view of cancer therapy (34). First, we showed high antigen expression when mRNA was delivered in Ca\(^{2+}\)-containing HBSS or the clinically applied 0.8 RL, confirming the Ca\(^{2+}\) dependency for efficient uptake of mRNA (29). It was previously shown that several adjuvants hamper mRNA uptake, as it is critically dependent on macrophocytosis, a process that is rapidly downregulated upon DC activation (35). Therefore, we next evaluated the engulfment of antigen mRNA when codelivered with TriMix or LPS. We confirmed the severe reduction in antigen expression when LPS was coadministered. However, this phenomenon was less pronounced when TriMix was codelivered and might be explained by the timing of DC activation, which most likely is initiated after the uptake and translation of the TriMix mRNA.

Recently, Diken and colleagues (35) hypothesized that simultaneous delivery of classical activation stimuli might result in imperilment of the induction of an immune response. We now show that the codelivery of LPS but not TriMix indeed completely abrogates the stimulation of antigen-specific T cells. In contrast, we showed that the codelivery of TriMix mRNA significantly enhanced the induction of antigen-specific T cells. The latter can be explained in part by the fact that the intranodal delivery of TriMix mRNA resulted in phenotypically more mature DCs and created an environment that is even better suited to recruit and activate T cells than with the use of antigen mRNA alone. However, this cannot be the only explanation as codelivery of LPS also resulted in highly mature DCs. The explanation for the differences in T-cell stimulation in mice immunized with antigen mRNA or the latter combined with TriMix or LPS might be found in the levels of MHC/peptide complexes on DCs, which are determined by the availability of the antigen. It was shown that a certain threshold antigen dose is required for T cells to decide to participate in immune responses (36). We hypothesize that this threshold is not met when classical adjuvants are codelivered with antigen mRNA, as these almost completely abrogate the engulfment of mRNA. We showed that codelivery of TriMix mRNA with antigen mRNA resulted in a 2-fold higher antigen expression than with the delivery in the presence of LPS. This amount might surpass the required threshold for T-cell recognition and engagement. Although the delivery of mRNA alone resulted in the highest availability of the antigen mRNA and activated the DCs to a certain extent, we observed that the codelivery of TriMix resulted in enhanced T-cell responses. The latter might be partially explained by the observation that antigen doses that exceed the above-mentioned threshold are correlated with enhanced T-cell activation and functionality (36, 37).

Because we showed that codelivery of TriMix allows the uptake of antigen mRNA and has an added benefit in terms of activation of adaptive T-cell responses, we next evaluated its therapeutic efficacy. Because we described the induction of antigen-specific T cells both in vitro (20, 21) and in vaccinated patients with melanoma (22) by TAA and TriMix mRNA–electroporated human DCs, we decided to compare DC to mRNA immunization. We showed that the therapeutic efficacy of antigen and TriMix mRNA is comparable with that of DCs electroporated with this mRNA. As such we here highlight the feasibility and potency of the TriMix and antigen mRNA-based immunization strategy.

It was recently implied that an adjuvant should be chosen on the basis of complementarity of its mode of action with that of the vaccine format it will be combined with (30). In that regard, the efficacy of mRNA administered into lymph nodes depends on its uptake and its ability to create a CTL inducing milieu. We conclude that these prerequisites are met through the codelivery of TriMix mRNA, as it allows antigen mRNA uptake, confers a high T-cell stimulatory capacity to DCs, and as such enhances their ability to stimulate antigen-specific immunity.

Disclosure of Potential Conflicts of Interest

The use of DCs electroporated with TAA mRNA and TriMix is the topic of a patent application (WO2009/034172) on which A. Bonehill and K. Thielemans are listed as inventors. None of the authors receive any support or remuneration related to this platform. No potential conflicts of interest were disclosed.

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