Preclinical evaluation of TriMix and antigen mRNA-based anti-tumor therapy

mRNA-based anti-tumor vaccination

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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 [DCs]. 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 situ modification and maturation of DCs hence priming of TAA-specific T-cells. We demonstrated selective uptake and translation of mRNA in vivo by lymph node resident CD11c⁺ cells. This process was hampered by co-delivery 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 cytotoxic T lymphocytes [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 immune modulating molecules is a promising vaccination strategy.
**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\(^+\) cytotoxic T lymphocytes [CTLs], can be enhanced or induced *de novo* by immunotherapeutic strategies using antigen-loaded dendritic cells [DCs] (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, demonstrating their ability to induce functional Th1 cells and CTLs (7-10). Moreover, clinical testing demonstrated 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 demonstrated that intranodal delivery of mRNA results in the engulfment of mRNA by DCs, as well as the activation of toll-like receptors [TLRs] (15-17). Nevertheless, it is suggested that naked mRNA is insufficient to fully harness the stimulatory potential of DCs (9, 18). Therefore, co-delivery of additional stimuli, such as lipopolysaccharide [LPS], CD40 ligand [CD40L], polyinosinic:polyctidylic 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 immune modulating proteins might be an attractive alternative to potentiate DCs *in situ*.
We previously demonstrated 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 co-stimulatory signal to CD27+ T-cells. We showed that DCs modified with this so-called TriMix induce tumor-specific T-cell responses \textit{in vitro} as well as in vaccinated melanoma patients (20-23).

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

Mice

Female, 6 to 12 weeks old C57BL/6, DBA/2 and BALB/c mice were purchased from Harlan. Transgenic mice were provided by B. Lambrecht [University of Ghent] 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, Université Libre de Bruxelles] 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.

Mouse cell lines and dendritic cells

The melanoma MO4, the T-cell lymphoma EG7-OVA, the mastocytoma P815 and the myeloid leukemia C1498-WT1 were obtained from the American Type Culture Collection [ATCC], C. Uytttenhove [Université Catholique de Louvain] and H.E. Kohrt [Stanford University Medical Centre], respectively. No full authentication was carried out. Cell lines were evaluated for the expression of MHC molecules and antigens [OVA: MO4 and EG7-OVA, P1A: P815 and WT1: C1498-WT1] by 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]. The vectors pGEM-II80tOVA, pST1-tyrosinase-DC-LAMP, pST1-sig-WT1-ΔNLS-DC-LAMP, pST1-caTLR4 and pGEM-tNGFR have been described (9, 21, 24, 25). The sequence encoding Firefly Luciferase [FLuc] was cloned into pST1 with minor modifications. The vector pGEM-II80P1A was cloned analogous to the cloning of pGEM-II80tOVA. The codon optimized cDNA encoding mouse CD40L or CD70 were obtained from Geneart and cloned as a SpeI-XhoI fragment in the pST1-vector. A fragment of the mouse Trp2 gene that encodes SVYDFFVWL was amplified with the following primers: 5’-GGGGATCCGGCCATCCTAAGACGG-3’ and 3’-GGGGGATCCGTGCACACGTCACACTCGTTC-5’ and cloned as a BamHI fragment in the BamHI linearized and shrimp alkaline phosphatase treated pST1-sig-DC-LAMP. The sequence encoding enhanced green fluorescent protein [eGFP] was isolated from p-eGFP-N1 as a HinDIII-NotI fragment and cloned into the HinDIII-NotI digested pST1-vector. All enzymes were purchased from Fermentas.

Prior to *in vitro* transcription, pGEM- and pST1-vectors were linearized with SpeI and Sapi, respectively. *In vitro* transcription was performed as described (9). The mRNA was dissolved in phosphate buffered saline [PBS], Ca²⁺-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 10⁶ DCs were pelleted and incubated for 15 minutes with 10 μg tNGFR or FLuc mRNA in 15 μl. Where indicated pulsing was performed in the presence of 1 ng/ml LPS from E. coli serotype 055:B5 [Sigma-Aldrich], 10 μg/ml polyI:C [Sigma] or 100 ng/ml monophosphoryl lipid A [MPL, GlaxoSmithKline]. DCs were cultured in RPMI 1640 medium supplemented with 5% FCI [Harlan], 50 μM β-mercaptoethanol and 20 ng/ml
mouse GM-CSF [prepared in-house] at a cell density of $10^6$ DCs/ml. Four hours later, DCs were lysed using the Reporter lysis buffer from Promega. D-Luciferin [Xenogen] was added, luminescence measured using the Glomax™ 96-luminometer and data analyzed with Glomax™ software [Promega]. Electroporation of DCs with mRNA was performed as described (9). Where indicated DCs were activated for 4 hours with 100 ng/ml LPS.

**In situ delivery of mRNA**

For intranodal delivery of 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 prior to 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 using the RevertAid™ H-Minus First strand cDNA synthesis kit [Fermentas]. The sequence encoding FLuc was amplified with 5’-AAGGTGTGGCCCTTCC-3’ and 5’-CCAAGAATGAAAATAGGGTTG-3’, whereas the sequence encoding β-actin was amplified with 5’-TGCTATCCAGGCTGTGCTAT-3’ and 5’-GATGGAGTTGAAGGTAGTTT-3’ using the following PCR program: 94°C 5’, 45x [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 30 µg tNGFR mRNA or TriMix [10 µg/component] was extracted and converted to cDNA.
Quantitative RT-PCR using the TaqMan® mouse immune response array [Applied Biosystems] and analysis was performed 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. Non-reactive isotype matched antibodies served as controls [Pharmingen]. Labeling of DCs was performed as described (9). Data were collected using the FACSCanto flow cytometer [Becton Dickinson] and analyzed using FACSDiva™ 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 (26).

**Enzyme-linked immunosorbent assay**

Supernatants were screened in a sandwich enzyme-linked immunosorbent assay for the presence of IL-6, IL-12p70, TNF-α or IFN-γ [eBioscience].

**In vivo bioluminescence imaging**

*In vivo* bioluminescence imaging was performed as described (27).

**Fluorescence microscopy**

Lymph nodes were injected with 10 μg eGFP mRNA, one day prior to isolation. Single cell suspensions were prepared and stained with a phycoerythrin-conjugated anti-CD11c antibody. Expression of CD11c and eGFP was evaluated using the Evos® fluorescence microscope.
**Immunization of mice**

Mice were immunized intravenously with $5 \times 10^5$ 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/component]. Immunization with DCs electroporated with tNGFR mRNA or with tNGFR mRNA as such served as a control. For assessment of therapeutic efficacy, $5 \times 10^5$ tumor cells were administered subcutaneously in the lower back, 7 days prior to immunization.

**Intracytoplasmatic staining of IFN-γ**

Spleen cells of immunized mice were stimulated for 24 hours with DCs pulsed for 2 hours with 5 µM SIINFEKL peptide and matured with LPS. GolgiPlug was added 24 hours prior to intracytoplasmatic staining of IFN-γ.

**Pentamer staining**

The staining of CD8$^+$ T-cells with H2-K$^b$/SIINFEKL pentamers [Immunosource] was performed as described (26).

**In vivo cytotoxicity assay**

Spleen cells from syngeneic mice were labeled with 10 µM carboxyfluorescein diacetate succinimidine 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] or tyrosinase [EMC microcultures] at 5 µM for 2 hours. Peptide-pulsed cells were mixed at a 1:1
ratio with non-pulsed cells, labeled with 0.5 μM CFSE. Specific lysis of target cells was analyzed 18 hours later by flow cytometry. The % of killing was calculated as described (28).

In vivo proliferation assay

One day prior to 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 post-immunization, proliferation of T-cells was analyzed in peripheral blood, spleen and lymph nodes (28).

Statistical analyses

A one-way ANOVA followed by a Bonferroni’s multiple comparison test was performed. 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

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

We recently demonstrated that the T-cell stimulatory capacity of human DCs electroporated with TAA mRNA is considerably increased by simultaneous co-electroporation 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 demonstrated that TriMix electroporated DCs displayed a phenotype [Fig. 1a], cytokine secretion profile [Fig. 1b] and allogeneic T-cell stimulatory capacity [Fig. 1c] comparable to that of LPS activated DCs. Importantly, we demonstrated that TriMix matured DCs were superior to LPS matured DCs in stimulation of functional antigen-specific CD8+ T-cells in vivo. This was demonstrated for OVA [Figs. 1d-f] and the TAA Trp2 [Fig. 1g].

Formulation and pharmacokinetics of mRNA for vaccination purposes

It was previously demonstrated that cellular uptake of mRNA can be influenced by the composition of the injection solution (29). Therefore, we evaluated which buffer is best suited to deliver mRNA to DCs. FLuc mRNA was dissolved in PBS, Ca2+-containing HBSS or 0.8 RL. Luminescence analysis of passively pulsed DCs demonstrated 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 demonstrated short-term FLuc expression when mRNA was formulated in PBS when compared to 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 (30). To analyze the stability of mRNA in vivo upon delivery in 0.8 RL, we resected lymph nodes injected with FLuc mRNA 6, 12 and 24 hours after injection. RT-PCR demonstrated 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 prior to 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+, demonstrating 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 demonstrated the absence of FLuc expression in mice that were treated with DT prior to 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 the feasibility of delivering mRNA intradermally. Since we demonstrated in the former experiment that CD11c+ cells are responsible for the DC uptake, we pre-treated the mice with an intradermal injection of PBS or GM-CSF on 3 consecutive days prior to the intradermal injection of Fluc mRNA. In vivo bioluminescence imaging, performed 6 hours later, demonstrated Fluc expression only in mice pre-treated with GM-CSF [Fig. 2f].

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

Induction of anti-tumor 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, demonstrating a reduction in FLuc expression after pulsing of DCs with FLuc mRNA in the presence of LPS, MPL or polyI:C. This reduction in protein expression was less pronounced when TriMix was co-delivered [Fig. 3a]. In addition, DCs pulsed with TriMix mRNA demonstrated a higher expression of CD40,
CD70, CD80 and CD86 compared to DCs pulsed with MPL [data not shown], LPS or polyI:C [Fig. 3b].
Next we evaluated the uptake of FLuc mRNA when delivered as such or together with LPS or TriMix in vivo. We demonstrated that co-delivery of TriMix had a lesser impact on the uptake of mRNA than its co-delivery with LPS [Fig. 3c]. To increase the number of DCs that can be recovered from the injected lymph node for analysis, we pre-treated the mice with a hydrodynamic injection of a plasmid encoding Flt3-ligand. In analogy with the data described by Kreiter et al (31), FLuc mRNA injected into these mice resulted in increased luminescence reflecting the specific uptake by the DCs [data not shown]. Flow cytometry demonstrated that DCs [CD11c+] from lymph nodes co-injected with TriMix displayed the highest expression of CD40, CD80 and CD86 when compared to DCs isolated from lymph nodes injected with FLuc mRNA alone or combined with LPS [Fig. 3d].
These findings prompted us to analyze, whether co-delivery 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 up-regulation of several markers in lymph nodes injected with FLuc and tNGFR mRNA when compared to lymph nodes injected with 0.8 RL. Importantly, the up-regulation of the following markers MHC II, IL-6, IL-15, IFN-γ, MCP-1, IP-10, granzyme B, SOCS1 and STAT1 was at least two-fold higher when TriMix was co-delivered [Table 1].

Intranodal 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 anti-tumor immunity (32). Therefore, we evaluated the expansion of OVA-specific CD4+ T-cells upon intranodal 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, demonstrating enhanced
proliferation of OT-II cells in mice receiving OVA and TriMix mRNA. Of note, transferred T-cells hardly proliferated when LPS was co-injected 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 one day after adoptive transfer of CD8\(^+\) OT-I cells. Five days post-immunization, we performed an H2-k\(^b\)/SIINFEKL pentamer staining or an \textit{in vivo} cytotoxicity assay. Both assays demonstrated the enhanced stimulation of OVA-specific CD8\(^+\) T-cells when mice were immunized with OVA mRNA and TriMix when compared to mice immunized with OVA mRNA alone or combined with LPS [Fig. 4b-c].

Using the model antigen OVA, we finally compared intradermal delivery of OVA and TriMix mRNA in mice pre-treated with GM-CSF to its intranodal delivery. Using the \textit{in vivo} cytotoxicity assay we demonstrated that the lysis of target cells was the highest when the mRNA was delivered intranodally [Fig. 4d].

\textbf{Inclusion of TriMix in the mRNA-based anti-tumor 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 \textit{in vivo} cytotoxicity assay demonstrated enhanced lysis of target cells when TriMix was included in the immunization regimen [Fig. 5a-c].

\textbf{Immunization with antigen mRNA and TriMix is as efficient in stimulating cytotoxic T-cells and in therapy as immunization with \textit{ex vivo} modified dendritic cells}

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 \textit{in vivo}. We
demonstrated 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 TAAas Trp2 and WT1 [Fig. 6a-c]. We next evaluated the therapeutic efficacy of such vaccines. Firstly, 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 intranodal 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 (33). It is not clear, however, whether the intrinsic adjuvant effect of mRNA is sufficient to fully exploit the immunostimulatory capacity of DCs (34). Therefore, we evaluated the local delivery of mRNA encoding CD40L, CD70 and caTLR4 [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 demonstrated 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 (35). First, we demonstrated 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 demonstrated that several adjuvants hamper mRNA uptake, as it is critically dependent on macropinocytosis, a process that is rapidly down-regulated upon DC activation (36). Therefore, we next evaluated the engulfment of antigen mRNA when co-delivered with TriMix or LPS. We confirmed the severe reduction in antigen expression when LPS was co-administered. However, this phenomenon was less pronounced when TriMix was co-delivered 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 et al (36) hypothesized that simultaneous delivery of classical activation stimuli might result in imperilment of the induction of an immune response. We now demonstrate that the co-delivery of LPS but not TriMix indeed completely abrogates the
stimulation of antigen-specific T-cells. In contrast, we demonstrated that the co-delivery 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 when compared to the use of antigen mRNA alone. However, this cannot be the only explanation as co-delivery 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 demonstrated that a certain threshold antigen dose is required for T-cells to decide to participate in immune responses (37). We hypothesize that this threshold is not met when classical adjuvants are co-delivered with antigen mRNA, as these almost completely abrogate the engulfment of mRNA. We demonstrated that co-delivery of TriMix mRNA with antigen mRNA resulted in a two-fold higher antigen expression when compared to 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 co-delivery of TriMix resulted in enhanced T-cell responses. The latter might be partially explained by the observation that lower antigen doses that exceed the above-mentioned threshold are correlated with enhanced T-cell activation and functionality (37, 38).

Since we demonstrated that co-delivery 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. Since we described the induction of antigen-specific T-cells both in vitro (20, 21) and in vaccinated melanoma patients (22) by TAA and TriMix mRNA electroporated human DCs, we decided to compare DC- to mRNA-immunization. We demonstrated that the therapeutic efficacy of antigen and TriMix mRNA is comparable to 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 (31). 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 co-delivery 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.
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References


Legends

Figure 1: Dendritic cells matured through electroporation of TriMix efficiently stimulate antigen-specific T-cells. The histogram overlays in [a] show the phenotype of DCs electroporated with tNGFR mRNA and left immature or matured by co-electroporation 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 $^3$H thymidine by allogeneic spleen cells cultured with these DCs [n = 3]. [d-f] Mice were immunized intravenously with $5 \times 10^5$ DCs electroporated with OVA mRNA and matured by co-electroporation of TriMix mRNA or addition of LPS. Five days later the expansion of functional OVA-specific CD$^8^+$ T-cells was assessed. The results of [d] the pentamer staining, [e] the in vivo cytotoxicity assay and [f] the intracytoplasmatic staining of IFN-$\gamma$ on spleen cells restimulated with SIINFEKL-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 CD$^8^+$ 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-c] Mice were injected intranodally with FLuc mRNA. [b] In vivo bioluminescence imaging was performed 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 performed 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 photo obtained by fluorescence microscopy shows eGFP [green] expression by CD11c$^+$ cells [red, n = 4]. [e] Transgenic CD11c-DTR mice, which were pre-treated with PBS or DT, received an intranodal injection with FLuc mRNA. In vivo bioluminescence imaging was performed 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 pre-treated with PBS or GM-CSF, were injected intradermally with FLuc mRNA. In vivo bioluminescence imaging was performed 6 hours later [n = 3].

Figure 3: Intranodal delivery of TriMix generates an immune stimulatory environment. [a-b] DCs were pulsed with FLuc mRNA in the presence of activation stimuli after which uptake of mRNA and the DCs’ phenotype was analyzed [n = 4]. The graph in [a] shows the photon emission as mean ± SEM of 4 experiments. The histogram overlays in [b] show the expression of CD70, CD40, CD80 and CD86 by DCs pulsed in the absence of a maturation stimulus, in the presence of LPS, poly[I:C] or TriMix. [c] Mice were injected intranodally with FLuc mRNA alone or combined with TriMix or LPS after which in vivo bioluminescence imaging was performed [n = 5]. [d] Activation of DCs in mice pre-treated with Flt3-L and injected with FLuc mRNA alone or combined with LPS or TriMix was evaluated by flow cytometry. The histograms depict the expression of CD40, CD80 and CD86 by CD11c⁺ cells obtained from lymph nodes injected with FLuc mRNA alone or the latter together with TriMix mRNA or LPS [n = 3].

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 one day prior to 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 post-immunization, 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-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 pre-treated with GM-CSF or intranodally was analyzed by \textit{in vivo} cytotoxicity assay [n = 2].

Figure 5: Inclusion of TriMix in the mRNA-vaccine enhances the induction of TAA-specific CTLs. An \textit{in vivo} cytotoxicity assay was performed 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 \textit{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 \textit{in vivo} cytotoxicity assay was performed 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/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 panel] and survival [right panel] 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.
Table

Table 1: Intranodal delivery of TriMix mRNA generates an immune stimulatory environment. 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 performed. Table 1 summarizes the molecules of which the up-regulation was at least two-fold higher when TriMix was co-administered when compared to antigen mRNA alone. The data show the relative up-regulation compared to injection with 0.8 RL alone. The results are shown as mean ± SEM of 3 experiments.

<table>
<thead>
<tr>
<th></th>
<th>antigen mRNA</th>
<th>TriMix mRNA</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>antigen-presenting molecules</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MHC II</td>
<td>6.2 ± 2.3</td>
<td>27.9 ± 6.5</td>
</tr>
<tr>
<td><strong>pro-inflammatory cytokines</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-6</td>
<td>3.7 ± 1.3</td>
<td>9.0 ± 3.0</td>
</tr>
<tr>
<td>IL-15</td>
<td>5.9 ± 0.8</td>
<td>16.1 ± 1.5</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>2.3 ± 0.1</td>
<td>5.1 ± 0.1</td>
</tr>
<tr>
<td><strong>T cell attracting molecules</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MCP-1</td>
<td>1.9 ± 0.2</td>
<td>6.1 ± 1.1</td>
</tr>
<tr>
<td>IP-10</td>
<td>10.3 ± 2.3</td>
<td>35.9 ± 5.1</td>
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<tr>
<td><strong>signalling molecules</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SOCS1</td>
<td>2.5 ± 0.6</td>
<td>7.1 ± 1.9</td>
</tr>
<tr>
<td>STAT1</td>
<td>2.8 ± 0.7</td>
<td>4.3 ± 0.1</td>
</tr>
<tr>
<td><strong>others</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Granzyme B</td>
<td>9.2 ± 1.7</td>
<td>24.4 ± 1.8</td>
</tr>
</tbody>
</table>
Fig. 1

a) 

b) 

CD70  CD40  CD86

---

IL-6  TNF-α

---

CD8

pentamer

---

CFSE

---

CD8

IFN-γ

---

CFSE

---
Fig. 2

a) Bar chart showing photon emission counts for PBS, HBSS, and 0.8 RL treatments. The p-value is 0.2058.

b) Heat maps showing the distribution of photon counts over time (24, 72, 144, 216, 240 hours) for PBS, HBSS, and 0.8 RL samples.

c) Gel images comparing β-actin and FLuc mRNA expression levels under different conditions (6, 12, 24 hours).

d) Fluorescence microscopy images with scale bar indicating 10 μm.

e) Table showing the effects of PBS, Diphteria toxin, and FLuc mRNA on FLuc counts.

f) Scatter plots showing the CD11c expression levels with 2.3% and 0.2% counts.

g) Diagram illustrating the effects of PBS, GM-CSF, and FLuc mRNA on FLuc counts.
Fig. 3

(a) Graph showing mRNA levels of various molecules under different conditions.

(b) Graphs comparing mRNA levels of different molecules at various time points.

(c) Heatmap showing expression levels of different molecules.

(d) Graphs showing changes in mRNA levels over time.

Legend:
- Fluc
- LPS
- PolyI:C
- TriMix
Fig. 5

a

\[
\text{tNGFR mRNA} \quad \text{Trp2 mRNA} \quad \text{TriMix mRNA}
\]

\[
\begin{array}{c}
\text{+} \\
\text{+} \\
\text{-} \\
\text{-} \\
\text{+} \\
\text{+}
\end{array}
\]

\[
\text{p < 0.0001}
\]

\[
\text{CFSE}^-
\]

b

\[
\text{tNGFR mRNA} \quad \text{WT1 mRNA} \quad \text{TriMix mRNA}
\]

\[
\begin{array}{c}
\text{+} \\
\text{+} \\
\text{-} \\
\text{-} \\
\text{+} \\
\text{+}
\end{array}
\]

\[
\text{p < 0.0001}
\]

\[
\text{CFSE}^-
\]

c

\[
\text{tNGFR mRNA} \quad \text{Tyrosinase mRNA} \quad \text{TriMix mRNA}
\]

\[
\begin{array}{c}
\text{+} \\
\text{+} \\
\text{-} \\
\text{-} \\
\text{+} \\
\text{+}
\end{array}
\]

\[
\text{p < 0.0001}
\]

\[
\text{CFSE}^-
\]
Preclinical evaluation of TriMix and antigen mRNA-based anti-tumour therapy

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