Intratumoral Delivery of TriMix mRNA Results in T-cell Activation by Cross-Presenting Dendritic Cells

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Abstract

Modulating the activity of tumor-infiltrating dendritic cells (TiDC) provides opportunities for novel cancer interventions. In this article, we report on our study of the uptake of mRNA by CD88C cross-presenting TiDCs upon their intratumoral (i.t.) delivery. We exploited this property to deliver mRNA encoding the costimulatory molecule CD70, the activation stimuli CD40 ligand, and constitutively active Toll-like receptor 4, referred to as TriMix mRNA. We show that TiDCs are reprogrammed to mature antigen-presenting cells that migrate to tumor-draining lymph nodes (TDLN). TriMix stimulated antitumor T-cell responses to spontaneously engulfed cancer antigens, including a neoepitope. We show in various mouse cancer models that i.t. delivery of TriMix mRNA results in systemic therapeutic antitumor immunity. Finally, we show that the induction of antitumor responses critically depends on TiDCs, whereas it only partially depends on TDLNs. As such, we provide a platform and a mechanistic rationale for the clinical testing of i.t. administration of TriMix mRNA. Cancer Immunol Res, 4(2): 146–56. © 2015 AACR.

Introduction

The tumor microenvironment exerts suppressive influences on tumor-infiltrating dendritic cells (TiDC) and as such prevents induction of antitumor immunity (1, 2). It has been proposed that adjuvants like Toll-like receptor (TLR) ligands and agonistic antibodies to CD40 can restore the function of TiDCs (3–5). However, adjuvants are not TiDC-specific and could therefore evoke adverse effects when administered in an untargeted fashion. For example, intratumoral (i.t.) delivery of TLR4 ligands induces tumor cell–resistance to cytotoxic T lymphocytes (CTL), and CD40 stimulation induces neoangiogenesis of tumor blood vessels (6, 7). Consequently, strategies need to be developed that exclusively act on TiDCs, preferably on CD88C TiDCs, which are critical for the stimulation of antitumor immunity (8, 9).

Several strategies have been developed to target adjuvants like TLR ligands or IL12, together with antigens, to cross-priming DCs (10–15). Often these exploit antibodies or nanobodies to surface markers differentially expressed by DC subsets (13, 15). Such markers include C-type lectin receptors like DEC205 and DC-SIGN (11, 12). In other cases, like in the use of DC-specific nanobodies, the target is unknown (16). These targeting moieties selectively direct the adjuvant and antigen to DCs, resulting in induction of antigen-specific immunity. However, the adjuvant and antigen coupled to isotype-matched antibodies (conjugate or nanoparticle format) or irrelevant nanobodies (lentiviral vector) evoke T-cell stimulation, albeit to a lesser extent (11, 12, 16). This is attributed to the ability of DCs to ingest antigens through various mechanisms. Thus, overall these strategies are not as “targeted” as anticipated. Moreover, none of these studies addressed whether adjuvants could be selectively delivered to cross-priming TiDCs without codelivery of antigens.

We developed an mRNA-based adjuvant, consisting of three mRNA molecules encoding the costimulatory molecule CD70, the activation stimulus CD40 ligand (CD40L), and constitutively active TLR4 (cTLR4), referred to as TriMix mRNA (17). Delivery of tumor-associated antigen (TAA) and TriMix mRNA to DCs, ex vivo or in situ, reprograms them to mature antigen-presenting cells (18, 19). These DCs and the T cells they activate are protected from regulatory T cells (Treg; ref. 20). These observations, together with the fact that mRNA as a biopharmaceutical fulfills all requirements of an optimal adjuvant, prompted us to evaluate whether i.t. delivery of TriMix mRNA is a feasible strategy to activate TiDCs and as such induce antitumor immunity.

Materials and Methods

Mice

Female 6- to 12-week-old C57BL/6, DBA/2, BALB/c, and OT-1 mice were purchased from Charles River. B. Lambrecht (Ghent University, Belgium) provided CD11c-diphtheria toxin receptor (DTR) mice. V. Flamand (Université Libre de Bruxelles, Belgium) provided constitutively active TLR4 (caTLR4) mice.

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provided Batf-3−/− mice. Animals were treated according to the European guidelines. The institute’s ethical committee for use of laboratory animals approved the experiments.

**Mouse cell lines, DCs, and peptides**

The E.G7-OVA T-cell lymphoma, the A20 B-cell lymphoma, and the P815 mastocyteoma cell lines were obtained from the ATCC in 2013. The lung epithelial cell line TC-1 was provided by T.C. Wu (John Hopkins Medical Institution, Baltimore, MD) in 2012 and authenticated by RT-PCR for the expression of the HPV E7 antigen. Mouse DCs were generated as described (21). Cell lines were thawed within 1 week after arrival, and 10 aliquots of 5 × 10⁶ cells were frozen as soon as feasible. Cells were passaged for less than 3 weeks after thawing. The peptides SIINFEKL (ovalbumin, OVA) and SYVYDFFWLVF (T2p2) were purchased from Eurogentec (Belgium), whereas P. Coulie (Université Catholique de Louvain, Belgium) provided LPYLGLWFV (P1A) and GYCGILGTMVG (P1E).

**mRNA**

The vectors pAT1-FLuc (Firefly luciferase), pGEM-tNGFR (truncated nerve growth factor receptor), pST1-eGFP (enhanced green fluorescent protein), pST1-mouse-CD40L-OPT, pST1-mouse-CD70-OPT, and pST1-catTLR4-OPT have been described (19). A codon-optimized version of the Mus musculus Thy1.1 gene was purchased from GeneArt (Life Technologies) and cloned as an NcoI-Xhol fragment in the vector pEtherRNA-v2. Prior to in vitro transcription, plasmids pAT1, pGEM, pST1, and pEtherRNA-v2 were linearized with AciI, SpeI, SapI, and BfuAI, respectively (Fermentas). In vitro mRNA transcription and quality control were performed as described (22).

**Tumor cell inoculation and i.t. delivery of mRNA**

Mice were injected subcutaneously (50 μL) with 2 × 10⁴ TC-1 cells or 5 × 10⁵ E.G7-OVA, P815, or A20 cells at the lower back or both flanks. Tumors that reached a volume of ≥100 mm³ as measured by caliper and determined by the formula for a prolate ellipsoid were injected with mRNA resuspended in 50 μL 0.8% Hartmann solution (23).

**In vivo bioluminescence imaging**

To assess i.t. delivery of mRNA and the cells involved in its uptake, tumors grown in wild-type, Batf-3−/−, or CD11c-DTR mice were injected with 10 μg Fluc mRNA. CD11c-DTR mice were treated a day before mRNA delivery with PBS or 4 ng DT/g body weight (Sigma-Aldrich). In vivo bioluminescence imaging (BLI) was performed at the indicated time points (24).

**Tracking of in vivo mRNA-transfected cells**

Twenty-four hours before their isolation, tumors were injected with 10 μg Thy1.1 mRNA. Single cells were stained with antibodies specific for Thy1.1 coupled to phycoerythrin (PE; clone OX7; Becton Dickinson, BD), CD11c-AlexaFluor647 (clone N418; Biolegend), CD11b-FITC (clone M1/70; BD), CD90.2-FITC (clone 30H-12; BD), and F4/80-biotin (prepared in-house and detected with streptavidin-FITC; BD). Staining was performed in the presence of antibodies to CD16/CD32 (BD). Data were collected on the BD LSR Fortessa flow cytometer and analyzed using FACS Diva software (BD). Samples stained with isotype-matched antibodies were used to delineate Thy1.1+ cells.

**In vivo proliferation assay**

A day before treatment, 1 × 10⁶ MACS-sorted (Miltenyi Biotec) and 0.5 μmol/L carboxyfluorescein diacetate succinimidyl ester (CFSE)-labeled (Life Technologies) CD8+ OT-1 T cells were injected intravenously (200 μL PBS). Tumors were treated with 40 μg tNGFR mRNA, 10 μg tNGFR, and 30 μg TriMix mRNA (10 μg/component), 10 μg OVA and 30 μg TriMix mRNA, or 10 μg OVA and 30 μg tNGFR mRNA. The presence and phenotype of CFSElow T cells in tumor-draining lymph nodes (TDLN) and tumors were analyzed 5 days later by flow cytometry. Cells were stained with peridinin-chlorophyll proteins (PerCP-Cy5.5)–conjugated antibodies to CD8 (clone 53–6.7; BD) together with antibodies to CD62L-PE-Cy7 (clone MEL-14; BD) and CD44-AF647 (clone IM7; BioLegend), antibodies to CD25-PE (clone 3C7; BD) and CD69-allophycocyanin (APC; clone H1.2F3; BD), or antibodies to CD27-APC (clone LG.7F9; eBioscience), and PD-1-PE (clone BMP1; BioLegend). Perforin was stained intracellularly using antibodies to perforin–PE (clone eBOMAK-D; eBioscience). Isotype-matched antibodies (BD) were used to verify staining specificity and to delineate positive populations. Data were collected using the BD LSR Fortessa flow cytometer. Analyses were performed with the FACS Diva software (BD). A light scatter gate excluding death lymphocytes and additional gating on CD8+ CFSElow cells was used.

**IFNγ ELISPOT assay**

Tumors were injected with 30 μg tNGFR or TriMix mRNA (10 μg/component). Five days later, CD8+ T cells were MACS-sorted from TDLNs. CD8+ T cells (2 × 10⁵) were stimulated in duplicate for 3 days with 5 μmol/L of the appropriate peptide in a 96-well multiscreen PVDF-membrane plate (Millipore). Control peptides and CD8+ T cells obtained from LNs of naïve mice were used for comparison. As a positive control, CD8+ T cells were stimulated with mouse CD3/CD28 antibody–coated beads (1/100; Invitrogen). The ELISPOT was performed following the manufacturer’s instructions (Cell Sciences).

**In vivo cytotoxicity assay**

Tumors were injected with 30 μg tNGFR or TriMix mRNA (10 μg/component), or 20 μg tNGFR and 10 μg catTLR4 mRNA. When indicated, tumors were further injected with 10 μg tNGFR or OVA mRNA. CD11c-DTR mice were treated intraperitoneally with PBS or 4 ng DT/g body weight a day before treatment. When indicated, mice received 25 μg FYT-720 (Enzo Life Sciences) by oral gavage (200 μL) 4 hours before treatment. The in vivo cytotoxicity assay was performed as described (19).

**Therapy experiments**

Tumors were injected with 30 μg tNGFR or TriMix mRNA (10 μg/mRNA), or 20 μg tNGFR and 10 μg catTLR4 mRNA. When indicated, mice received 25 μg FYT-720 as described above. Tumor length and width were measured using a caliper, and volumes were calculated using the formula for a prolate ellipsoid. Mice were killed when tumors exceeded 1,500 mm³.

**In vivo migration of DCs**

Bone marrow–derived DCs were electroporated with 10 μg Fluc or eGFP mRNA (25). Four hours before injecting 30 μg tNGFR or TriMix mRNA, 2 × 10⁶ DCs (50 μL) were administered to tumors. Migration of Fluc+ or eGFP+ DCs to TDLNs...
was evaluated 24 hours later using ex vivo BLI on TDLNs or flow cytometry analysis on cell suspensions of TDLNs, respectively (26). Staining was performed after blocking Fc receptors with CD16/CD32 antibodies, using anti–CD11c-AF647, biotinylated anti-CD40 (clone FGK45), and anti-CD86 (clone GL-1). Biotinylated antibodies were prepared in-house and detected with SA-PerCP-Cy5.5 (BioLegend) or SA-eFluor450 (eBioScience). Isotype-matched antibodies served as controls (BD). Data were collected and analyzed as described in Supplementary Fig. S1.

**In vitro proliferation assay**

Tumors were injected with 30 µg tNGFR or TriMix mRNA (10 µg/component). Three days later, CD11c⁺ cells were MACS-enriched from TDLNs. These were cocultured in duplicate at a 1:10 ratio with 2 × 10⁵ MACS-sorted and 0.5 µmol/L CellTrace Violet–labeled CD8⁺ OT-1 cells (Life Technologies). Cell proliferation was analyzed 3 days later by flow cytometry. Supernatants were screened in a sandwich ELISA for the presence of IFNγ (eBioscience).

**Statistical analyses**

A nonparametric Mann–Whitney U test or one-way ANOVA followed by Bonferroni correction was performed to compare two or multiple datasets, respectively. Sample sizes are indicated in the figure legends and represent the summary of at least two independent experiments. Numbers of asterisks in the figures indicate the level of statistical significance: *, P < 0.05; **, P < 0.01; ***, P < 0.001. Results are shown in a column graph as mean ± SEM. Survival was visualized in a Kaplan–Meier plot. Differences in survival were analyzed by the log-rank (Mantel Cox) test.

**Results**

**CD8α⁺ TiDCs engulf mRNA**

To address whether cells in the tumor microenvironment engulf mRNA, we injected tumors of different histologic origin in genetically distinct immunocompetent hosts with FLuc mRNA. In vivo BLI was performed 24 hours later, showing FLuc expression in E.G7-OVA, P815, TC-1, and A20 tumors (Fig. 1A). Fluorescence...
at the tumor site was readily detectable in the E.G7-OVA model for about 10 hours (Fig. 1B). Flow cytometry was performed on cell suspensions of P815 tumors injected with Thy1.1 mRNA, showing that a small percentage of mainly CD11c+ cells were Thy1.1+ (data not shown). Mice bearing E.G7-OVA–CD11c-DTR tumors were treated with DT to deplete CD11c+ cells. Uptake of mRNA, as assessed by BLI, was inhibited, which confirmed the role of CD11c+ cells (Fig. 1C). In vivo BLI performed on E.G7-OVA–bearing Batf-3−/− mice, which lack CD8α+ DCs, showed significantly reduced radiance compared with wild-type mice, showing that these cross-presenting DCs are mainly responsible for mRNA uptake (Fig. 1D).

TriMix stimulates functional tumor-specific T cells

As cross-presenting CD8α+ TiDCs are at least partially responsible for mRNA uptake, we evaluated whether i.t. delivery of TriMix mRNA results in stimulation of tumor-specific T cells (Fig. 2). We first assessed proliferation by transferring CFSE-labeled CD8+ OT-1 cells into E.G7-OVA–bearing mice and studying their expansion. The OT-1 T cells in the TDLNs of mice treated with TriMix mRNA expanded significantly more than OT-1 cells in mice treated with tNGFR mRNA. Immunization with antigen mRNA alone did not result in a significant change in proliferation compared with immunization with the tNGFR mRNA, hinting that TiDCs have access to TAAs but are dysfunctional. Moreover, coadministration of OVA and TriMix mRNA did not boost OT-1 expansion compared with TriMix delivery alone (Fig. 2A). Nonetheless, antigen presentation is critical, as i.t. injection of TriMix mRNA in mice bearing EL4 tumors (OVA−) did not induce significant proliferation of OT-1 cells (Supplementary Fig. S2A).

Second, we analyzed the phenotype of proliferating OT-1 cells in E.G7-OVA–bearing mice treated with tNGFR or TriMix mRNA. These were CD25−CD44hiCD69−CD62Llow in both TDLNs and tumors irrespective of the treatment. However, CFSElow OT-1 cells in tumors were CD27−PD-1−, which is in contrast with those in TDLNs. CFSElow OT-1 cells in TriMix-treated mice showed a higher expression of perforin compared with those in mice treated with tNGFR mRNA (81.2% ± 1.2% and 44.8% ± 2.8%,
respectively, P < 0.001; Supplementary Fig. S2B and S2C). Therefore, we next evaluated the ability of i.t. TriMix mRNA delivery to induce tumor-specific CTLs and compared it with CTL induction after delivery of caTLR4 or tNGFR mRNA (Fig. 2B). Whereas caTLR4 or tNGFR mRNA injection induced specific lysis of target cells of 8.2% ± 2.0% and 3.7% ± 0.9%, respectively, we found that i.t. injection of TriMix mRNA led to 69.0% ± 1.2% of specific lysis, indicating induction of more CTLs. We also evaluated whether addition of OVA mRNA to TriMix would lead to a higher induction of CTLs, but found this was not the case. We confirmed these results in the P815 model (Fig. 2C), where we found that we could induce CTLs against P1A, a cancer-testis antigen, and P1E, a tumor-specific antigen resulting from a mutation in the methionine sulfoxide reductase gene.

Finally, we evaluated the capacity of the stimulated T cells to produce IFNγ upon restimulation using ELISPOT and found that there was a significantly higher number of IFNγ-producing CD8+ T cells in TriMix mRNA-treated mice, compared with tNGFR mRNA-treated mice in both the P815 (Fig. 2D) and E.G7-OVA (Supplementary Fig. S2D) models.

**Intratumoral delivery of TriMix mRNA significantly delays tumor growth**

We next assessed the therapeutic potential of i.t. TriMix mRNA delivery. We showed prolonged survival in mice treated with TriMix mRNA compared with mice treated with tNGFR mRNA in various tumor models (Fig. 3A–D). When we evaluated the effect of i.t. injection of caTLR4 mRNA in the P815 model, we observed that these tumors followed the same growth curve as tumors treated with tNGFR mRNA (data not shown). Encouraged by the results obtained with TriMix mRNA, we next used a two-sided tumor model. Herein, only the left tumor was treated while the contralateral tumor (the "control" in Fig. 4) was used to evaluate the induction of systemic antitumor immunity. Treatment with TriMix mRNA resulted in a reduced growth of both A20 and P815 tumors, consequently prolonging survival (Fig. 4A–D).

**Antitumor immunity upon i.t. delivery of TriMix mRNA depends on DCs**

To study the role of TiDCs in the outcome of i.t. delivery of TriMix mRNA, we used E.G7-OVA-bearing Batf-3−/− mice, which lack splenic CD8α+ DCs. The therapeutic effect of i.t. injection of TriMix mRNA in these mice was dampened (Fig. 5A and B). The lower therapeutic benefit in mice lacking CD8α+ DCs was explained by their lower CTL stimulation, as shown in an in vivo cytotoxicity assay (Fig. 5C). Similarly, CTL induction upon i.t. delivery of TriMix mRNA was abrogated when mice lacked CD11c+ cells (Fig. 5D).

**Stimulation of T cells upon i.t. delivery of TriMix mRNA occurs in TDLNs**

We next addressed whether CTLs are activated in the tumor and/or in the TDLNs. First, we set up a model to evaluate migration of DCs to TDLNs upon i.t. delivery of TriMix mRNA.
Tumors were injected with FLuc⁺ DCs before treatment. Upon i.t. delivery of TriMix mRNA, FLuc⁺ DCs migrated into the TDLNs in the E.G7-OVA (Fig. 6A) and P815 (data not shown) models, as measured by ex vivo BLI. To characterize DCs that entered TDLNs, we used eGFP⁺ DCs and flow cytometry. The TDLNs of mice treated with TriMix mRNA had a higher percentage of CD40- and CD86-expressing eGFP⁺ DCs when compared with mice treated with tNGFR mRNA (Fig. 6B and C; Supplementary Fig. S2). This supports the hypothesis that TriMix mRNA induces in situ activation and migration of TiDCs. We further stimulated OVA-specific CD8⁺ T cells in vitro with CD11c⁺ cells sorted from TDLNs of E.G7-OVA–bearing mice treated with tNGFR or TriMix mRNA. We demonstrated enhanced T-cell proliferation (Fig. 6D) and IFNγ secretion (data not shown), indicating that T-cell stimulation could occur in TDLNs.

These data, however, do not exclude the possibility that T cells are activated at the tumor site as well. Therefore, we evaluated the induction of CTLs in E.G7-OVA–bearing mice treated with tNGFR or TriMix mRNA from which the TDLN had been resected 3 days before treatment. CTL induction in these mice was dramatically decreased (Fig. 7A). Nonetheless, mice from which the TDLNs were removed and that were treated with TriMix mRNA showed a prolonged survival (Fig. 7B), suggesting that tumor-infiltrating T lymphocytes (TIL) could be reactivated upon i.t. delivery of TriMix mRNA. To study this, we pretreated tumor-bearing mice with FTY-720, an agonist of the sphingosine 1-phosphate receptor that abrogates the egress of T cells (27). In these mice, treatment of tumors with TriMix mRNA did not stimulate tumor-specific CTLs (Fig. 7C). FTY-720 pretreatment only marginally affected the therapeutic potential of i.t. treatment with TriMix mRNA, supporting the hypothesis that i.t. delivery of TriMix mRNA results in de novo activation of tumor-specific T cells as well as reactivation of TILs (Fig. 7D).

**Discussion**

In most human and mouse solid cancers, the tumor microenvironment is infiltrated with DCs. These TiDCs acquire TAA; however, they are unable to present them to and properly activate CTLs. This dysfunction is due to immunosuppressive factors present in the tumor microenvironment (28). Several strategies aimed at improving the function of TiDCs have been developed. Examples are the use of TLR agonists and agonistic CD40 antibodies. However, TLRs and CD40 are not exclusively expressed on DCs, and negative signaling resulting in tumor immune escape and tumor cell dissemination has been described (3, 6, 7). Consequently, strategies need to be developed that mediate selective activation of TiDCs. Here, we show that TriMix mRNA reprograms...
CD8α− TiDCs in vivo into stimulatory cells that efficiently process spontaneously engulfed TAAs, upregulate costimulatory molecules, and migrate to TDLNs to activate CTLs. Moreover, we provide evidence that i.t. delivery of TriMix mRNA results in reactivation of TILs. Together, this results in a delay in growth of established tumors.

We showed that upon i.t. delivery of mRNA, the encoded antigen is mostly but not exclusively expressed in Batf-3−/− mice, which lack CD8α− cross-presenting DCs, fail to clear highly immunogenic tumors (8, 9). Our study confirms that CD8α− TiDCs have spontaneously acquired TAAs but fail to induce antitumor immunity, unless activated with TriMix mRNA. We furthermore show that induction of CTLs and control of tumor growth are exerted by Batf-3−/−-dependent CD8α+ DCs as well as CD11c+ Batf-3−/− cells. Based on the current knowledge on DC subsets (15), we contend that CD11b+ CD11c+ DCs could play a role, despite their weak in vitro-stimulatory properties (29).

Our work further suggests that i.t. delivery of TriMix mRNA results in de novo activation of tumor-specific CTLs and reactivation of TILs. Broz and colleagues (29) also showed that Batf-3−/−dependent cells play a key role in reactivating TILs. Whereas that study shifts the emphasis for T-cell control from TDLNs to tumors, our data show that both the tumor and TDLNs are critical for CTL induction (30–32). However, we cannot exclude that de novo T cells trigger a cascade of events at the tumor, resulting in revival of TILs, as previously proposed (33). Therefore, we suggest that i.t. delivery of TriMix mRNA activates tumor-specific T cells de novo and results in reactivation of TILs, probably because of their contact with TriMix-modified TiDCs as well as a cascade of TIL-reviving events instigated by de novo tumor-specific T cells.

A key point is that the activation of CTLs upon i.t. delivery of TriMix mRNA depends on the spontaneous acquisition of TAAs by TiDCs, avoiding the need to prime TiDCs with exogenous TAAs. Intratumoral delivery of TriMix eliminates the challenge of trying to define the best-suitied TAA for vaccination. In this regard, cancer-testis and differentiation antigens are often used. These are self-proteins and are thus subjected to tolerance mechanisms. In contrast, mutated antigens are not in the cross-line of tolerance mechanisms (34–36). Therefore, the use...
of the tumor’s antigenic repertoire, including neoepitopes, is an attractive approach. We provide evidence that i.t. delivery of TriMix mRNA induces CTLs specific for cancer-testis antigens as well as for a neoepitope. TriMix-activated TiDCs thus have the potential to present a variety of antigens that they acquire at the tumor site.

Large numbers of tumor-specific T cells expanded in mice treated with TriMix mRNA, relative to the expansion in mice treated with tNGFR mRNA. The T cells had a comparable phenotype, characterized by high expression of CD25, CD44, and CD69, and low expression of CD62L and CD27. It is suggested that CD27+ T cells represent a memory subset with cytolytic capacity (37). In this regard, we observed that perforin expression was significantly higher in mice treated with TriMix mRNA. Therefore, it is not surprising that tumor-bearing mice treated with TriMix mRNA were able to delay tumor growth, whereas mice treated with tNGFR mRNA were not. This could be attributed to the lower expansion of perforin-positive tumor-specific T cells. Alternatively, one could argue that CTLs stimulated after i.t. delivery of TriMix mRNA are (partially) protected from suppressive mechanisms exerted in the tumor microenvironment. This hypothesis is supported by a previous study showing that CD8+ T cells activated by TriMix mRNA-modified DCs were protected from Tregs (20). Although we did not study T-cell trafficking in detail, our data on the outgrowth of tumors in the two-sided tumor model further suggest that tumor-specific CTLs efficiently screen the body for tumors, irrespective of whether the primary tumor was cured.

The promise of many cancer immunotherapies is often hampered by the difficulties regarding its targeted in situ delivery. In our approach, however, CD8α+ TiDCs are the target, and these cells were shown to engulf and translate mRNA dissolved in Hartmann solution. Most likely, the mRNA uptake is mediated by macropinocytosis as described for the mRNA uptake by intranodal DCs (38). Consequently, when targeting DCs in situ, mRNA is an attractive vector as it enables selective delivery without prior manipulation. The mRNA itself can trigger several pattern recognition receptors (PRR; ref. 39), whose activation...
negatively affects its translation, implying that stronger TiDC activation could be obtained using "modified" mRNA that does not trigger PRRs (40–42). If PRRs are triggered, the effects observed in this study may be due to the delivery of mRNA rather than the delivery of TriMix mRNA. However, when using equal amounts of tNGFR or caTLR4 mRNA, no strong induction of CTLs or therapeutic benefit was seen. These data highlight the added benefit of TriMix mRNA, even if mRNA were to trigger PRRs.

In conclusion, we provide proof-of-concept for the use of TriMix mRNA in i.t. delivery and as such propose a novel cancer immunotherapy that exploits cross-presenting DCs and the tumor's antigenic repertoire to stimulate effective antitumor immune responses.

Disclosure of Potential Conflicts of Interest
C. Heirman is an employee at eTheRNA. K. Thielemans is the holder of a patent for dendritic cells electroporated with tumor antigen mRNA and TriMix (WO2009/034172). No other potential conflicts of interest were disclosed by the other authors.

Figure 7.
T-cell stimulation upon i.t. delivery of TriMix mRNA occurs in TDLNs. A, 24 hours before i.t. treatment with 30 μg tNGFR or TriMix mRNA, TDLNs of E.G7-OVA-bearing mice were removed. An in vivo cytotoxicity assay was performed. The graph shows the specific lysis as mean ± SEM (n = 12). B, mice were treated as described in A, after which tumor growth was monitored. The graph shows the survival of mice (n = 6). C, 4 hours before i.t. treatment with 30 μg tNGFR of TriMix mRNA, E.G7-OVA-bearing mice were treated with FTY-720 via oral gavage. An in vivo cytotoxicity assay was performed. The graph shows the specific lysis as mean ± SEM (n = 6). D, mice were treated as described in C, after which tumor growth was monitored. The graph shows the survival of mice (n = 6).

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