The potential of antigen and TriMix sonoporation using mRNA-loaded microbubbles for ultrasound-triggered cancer immunotherapy

Heleen Dewitte[a], Sandra Van Lint[b], Carlo Heirman[b], Kris Thielemans[b], Stefaan C. De Smedt[a,*], Karine Breckpot[b,1] and Ine Lentacker[a,1]

[1]Both senior authors contributed equally to this work

[a]Lab for General Biochemistry and Physical Pharmacy, Faculty of Pharmacy, Ghent University, Ottergemsesteenweg 460, 9000 Ghent, Belgium. Email addresses: Heleen.Dewitte@UGent.be, Stefaan.DeSmedt@UGent.be, Ine.Lentacker@UGent.be

[b]Laboratory of Molecular and Cellular Therapy, Department Immunology-Physiology, Medical School of the Vrije Universiteit Brussel (VUB), Laarbeeklaan 103, 1050 Jette, Belgium. Email addresses: sandra.van.lint@vub.ac.be, carlo.heirman@vub.ac.be, Kris.Thielemans@vub.ac.be, karine.breckpot@vub.ac.be

*Corresponding author: Tel +32 9 264 80 76; Fax +32 9 264 81 89; Email address: Stefaan.DeSmedt@UGent.be

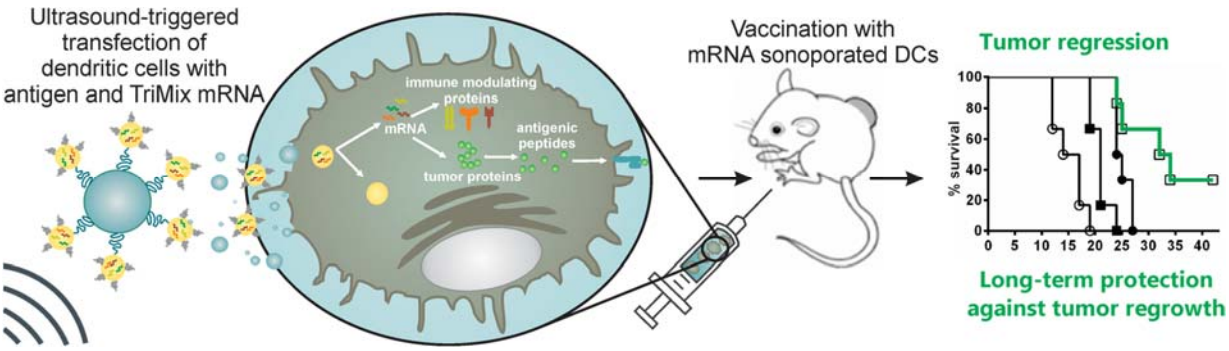
ABSTRACT

Dendritic cell (DC)-based cancer vaccines, where the patient's own immune system is harnessed to target and destroy tumor tissue, has emerged as a potent therapeutic strategy. In the development of such DC vaccines, it is crucial to load the DCs with tumor antigens, and to simultaneously activate them to become more potent antigen-presenting cells. For this, we report on microbubbles, loaded with both antigen mRNA as well as immunomodulating TriMix mRNA, which can be used for the ultrasound-triggered transfection of DCs. *In vivo* experiments with *in vitro* sonoporated DCs, show the effective induction of antigen-specific T cells, resulting in specific lysis of antigen-expressing cells. Especially in a therapeutic setting, sonoporation with TriMix has a significant added value, resulting in a significant reduction of tumor outgrowth and a marked increase in overall survival. What is more, complete tumor regression was observed in 30% of the antigen+TriMix DC vaccinated animals, which also displayed long-term antigen-specific immunological memory. As a result, DC sonoporation using microbubbles loaded with a combination of antigen and TriMix mRNA can elicit powerful immune responses *in vivo*, and might serve as a potential tool for further *in vivo* DC vaccination applications.

KEYWORDS: mRNA, microbubble, dendritic cell, cancer vaccination, sonoporation, immune modulation

CHEMICAL COMPOUNDS: DOTAP (PubChem CID: 6437371); DOPE (PubChem CID: 9546757); CFSE (PubChem CID: 16211581); SIINFEKL (PubChem CID: 71311993);

GRAPHICAL ABSTRACT:



INTRODUCTION

Since its discovery in the early '70s by Noble Prize winner Ralph M. Steinman, the dendritic cell (DC) came to be known as nature's adjuvant [1]. Its unique capacity to present antigens and initiate antigen-specific T cell responses, makes the DC an interesting target in cancer immunotherapy. By modifying DCs to present tumor-derived antigens to T cells, the patient's own immune system can be harnessed in the battle against the tumor [2]. For such a DC-based vaccine to be effective, two major requirements need to be satisfied. Firstly, the immune system should be able to discriminate between healthy tissue and cancer cells. For this, it is crucial to load DCs with cancer-specific tumor associated antigens (TAAs) [3, 4]. Upon presentation of these TAAs in major histocompatibility complexes (MHC) on the DC surface, TAA-specific immune responses can be mounted. Secondly, DCs should receive additional stimulation to become fully mature antigen-presenting cells that can provide the necessary co-stimulatory signals to activate antigen-specific T cells.

TAA-loading of DCs can be achieved in various ways: whole-tumor vaccines have been produced, as well as strategies based on isolated or recombinant TAA protein, antigenic peptides, and nucleic acid sequences encoding the antigens. Especially the use of TAA mRNA is appealing, as it offers several advantages [5]. First of all, using mRNA encoding full-length TAAs allows the DCs to present multiple epitopes to T cells, thus broadening the immune responses. Secondly, mRNA translation into protein occurs within the cytosol, resulting in preferential presentation of antigenic peptides via the MHC class I pathway. This will allow the presented antigens to be recognized by $CD8^+$ T cells, resulting in the induction of cytotoxic T lymphocytes (CTL) that can cause TAA-specific lysis of tumor cells. In addition, the fact that mRNA does not need to cross the nuclear membrane represents an important advantage over plasmid DNA. Transfection with mRNA can therefore be performed in non-dividing cells (such as DCs) leading to fast and transient protein expression [6]. Moreover, by using mRNA, the risks of inducing insertional mutagenesis, which is considered a major drawback of DNA-based gene therapy, can be avoided.

After antigen introduction, it is essential that the DCs undergo a process called "maturation" in order for antigen-presentation to effectively result in T cell activation. Maturation is initiated when DCs encounter "danger" signals, such as toll like receptor (TLR) agonists, causing them to shift in phenotype and function from antigen-capturing to antigen-presenting cells [7]. This is an absolute necessity for a DC-based cancer vaccine to be successful, as it is now clear that mature DCs are capable of inducing immunity against the presented antigens (i.e. stimulation of effector T cells), whereas their immature counterparts will rather induce tolerance (i.e. T cell anergy and regulatory T cells) [8]. The main reason for this is that immature DCs lack co-stimulatory molecules required for effective T cell activation [9]. Therefore, it is crucial to not only deliver antigens to DCs, but also maturation stimuli to ensure optimal immune activation by the DC vaccine.

To respond to these requirements, we established a novel transfection method that offers possibilities for image-guided loading of DCs with antigen mRNA *in vivo*: mRNA-sonoporation. This technique makes use of microbubbles, that consist of a gas core which is surrounded by a stabilizing lipid or polymer shell, and are known in the clinic as contrast

agents for ultrasound imaging [10]. Besides imaging, they are currently under investigation for the ultrasound-triggered delivery of drugs and genes: when ultrasound is applied to microbubbles, their gas core starts to oscillate, and can eventually implode when the ultrasound intensities are sufficiently high. Both the stable oscillations but especially microbubble implosion can be used to temporarily disrupt neighboring cell membranes in a process called sonoporation. Through these membrane pores, macromolecules and nanoparticles can be delivered directly into the cell's cytoplasm [11, 12]. We previously showed that by using microbubbles that are loaded with mRNA-lipoplexes (i.e. complexes of mRNA and cationic liposomes), we could induce fast and efficient, ultrasound-triggered mRNA-loading of murine bone marrow-derived DCs *in vitro*, without important negative repercussions on the DC viability and capacity to respond to maturation stimuli [13]. Interestingly, Sever and colleagues reported that upon intradermal injection of SonoVue® microbubbles around the lesions of breast cancer patients, they drain to the lymphatics and appear in the tumor-draining lymph nodes [14, 15]. This not only makes them useful for the non-invasive identification of the sentinel lymph node, but could also pave the way for intranodal sonoporations using our mRNA-loaded microbubbles. In this way, mRNA sonoporation could be performed on intranodal DCs under image-guidance, and an off-the-shelf cancer vaccine could be produced.

In this study, we aimed to evaluate the capacity of mRNA sonoporated DCs to induce immune responses *in vivo*. Moreover, to address the second DC vaccine requirement, we aimed to obtain both antigen-loading and stimulation of maturation by a single step sonoporation. This is important, as we previously reported that mRNA sonoporation without inclusion of additional immune stimulants merely induces partial maturation of the DCs. Therefore, we performed sonoporations with antigen mRNA and TriMix mRNA. TriMix is a mixture of 3 mRNAs, encoding CD40-ligand, a constitutively active form of TLR4 and CD70 (a co-stimulatory molecule required for effective $CD8^+$ T cell priming) [16]. Co-delivery of these 3 nucleic acid sequences was already shown to modulate the DCs' functionality, resulting in APCs that display a more mature, T cell activating phenotype [17]. Previous studies have demonstrated the superiority of TriMix over other, more conventional, maturation stimuli after intranodal injection of TAA and TriMix mRNA in tumor-bearing mice [18]. Also in a clinical setting, vaccination with DCs electroporated with TAA and TriMix mRNA lead to the induction of durable antitumor responses in a chemorefractory melanoma patient [19, 20]. On the basis of these results, we evaluated the potential of simultaneous delivery of TAA mRNA and TriMix via microbubbles and ultrasound to induce potent antitumor immune responses in mice, as schematically depicted in **Figure 1A**.

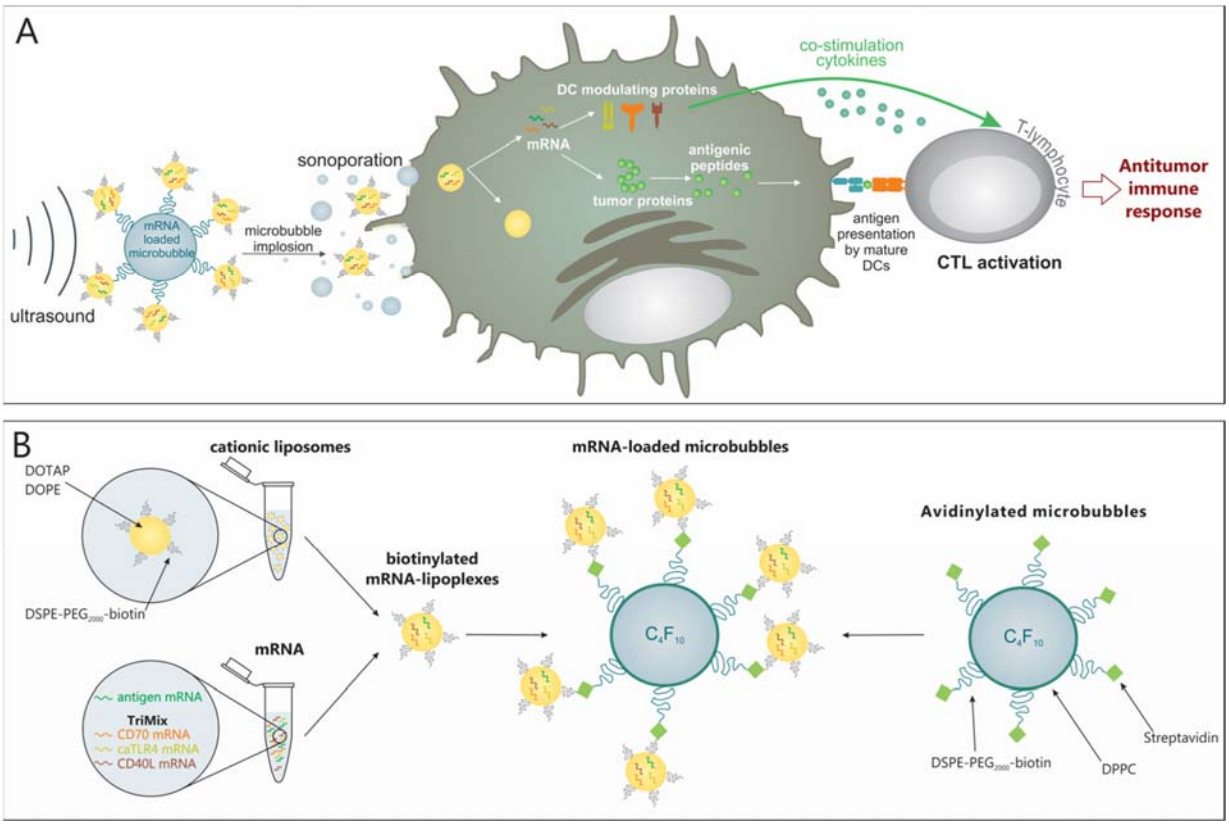


Figure 1. mRNA sonoporation of DCs using mRNA-loaded microbubbles and ultrasound. (A) Schematic representation of the use of mRNA-loaded microbubbles, which implode upon exposure to ultrasound and sonoporate the DCs. As a result, both antigen and DC modulating proteins are produced by the DC, which can lead to antigen presentation and T cell activation. (B) Schematic representation of the production of mRNA-loaded microbubbles. Antigen and TriMix mRNA are first mixed and complexed to biotinylated cationic liposomes. The resulting mRNA-lipoplexes can then be attached to the surface of avidinylated lipid microbubbles.

MATERIALS AND METHODS

Cell culture and mice

Primary murine bone marrow-derived DC (BM-DC) cultures were generated from C57BL/6 mice. Female C57BL/6 mice were purchased from Harlan (Gannat, France) and housed in an SPF facility according to the regulations of the Belgian law and the local Ethical Committee. Mice were sacrificed and bone marrow was flushed from the hind limbs. After red blood cell lysis (Pharm Lyse Buffer, BD Biosciences, Erembodegem, Belgium), the collected cells were seeded in 100 mm not TC-treated polystyrene culture dishes (Corning®, Amsterdam, The Netherlands) at $2x10^6$ cells ml^{-1} in 15 ml. The cell culture medium used was RPMI 1640 (Gibco-Invitrogen, Merelbeke, Belgium) supplemented with penicillin/streptomycin/L-glutamine (1%, Gibco-Invitrogen), β-mercaptoethanol (50 µM, Gibco-Invitrogen), 5% FetalClone™ I (FCI, 5%, HyClone™) and recombinant murine GM-CSF (20 ng ml^{-1}, Peprotech, Rock Hill, NJ). On day 3 of the culture, an additional 15 ml complete culture medium containing GM-CSF (40 ng ml^{-1}) was added. On day 5, all cells were collected by centrifugation (5 min at 300 *g*),

resuspended in the appropriate culture medium at $5x10^5$ cells ml^{-1} and seeded per 10 ml in OptiCells™ (Nunc, Thermo Scientific, Aalst, Belgium) for sonoporation the next day.

The mouse melanoma cell line MO4 (kindly provided by K. Rock, University of Massachusetts Medical Center) and the T cell lymphoma E.G7-OVA (obtained from the American Type Culture Collection, Rockville, MD, USA) were cultured at 37°C in a humidified 5% CO_2 atmosphere in RPMI 1640 medium (Sigma-Aldrich, Diegem, Belgium) supplemented with 5% FCI, 100 U ml^{-1} penicillin, 100 µg ml^{-1} streptomycin, 2 mM L-glutamine, 1 mM sodium pyruvate and nonessential amino acids (Sigma-Aldrich).

C57BL/6 and OT-I mice for *in vivo* experiments were ordered from Charles River (L'Arbresle, France) and housed in SPF facilities according to the regulations of the Belgian law and the local Ethical Committee.

Messenger RNA

The vector, pST1 was provided by U. Sahin (Johannes- Gutenberg University, Mainz, Germany). The vectors pGEM-li80tOVA, pST1-eGFP-bis, pST1-caTLR4, pST1-mouse CD40-L and pST1-mouse CD70 were previously described [16, 18, 21]. Before *in vitro* transcription, pGEM and pST1 vectors were linearized with the restriction enzymes *Spe* I and *Sap* I, respectively. All enzymes were purchased from Fermentas (Vilnius, Lithuania). The *in vitro* transcription of mRNA and its subsequent quality control were performed as previously described [21].

***In vitro* mRNA sonoporation**

Lipid microbubbles loaded with mRNA-lipoplexes were prepared as described previously [13]. A schematic representation of the material production is shown in **Figure 1B**. Briefly, perfluorobutane (F2 chemicals, Preston, UK) microbubbles stabilized by a lipid coat consisting of DPPC (1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine) (Lipoid, Ludwigshafen, Germany) and DSPE-PEG-biotin [1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-N-[biotin(polyethylene glycol)-2000] (Avanti Polar Lipids, Alabaster, AL) in a 85:15 molar ratio were prepared. These biotinylated microbubbles were subsequently coated with avidin (Cell Sciences, Canton, USA), to allow coupling to biotin-containing mRNA lipoplexes. The mRNA lipoplexes were prepared by mixing mRNA with cationic liposomes consisting of 48.75% DOTAP (1,2-dioleoyl-3-trimethylammonium-propane), 48.75% DOPE (1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine) and 2.5% DSPE-PEG-2000-biotin [1,2-distearoyl-*sn*-glycero-3-phosphoethanol-amine-N-[biotinyl(polyethylene glycol)-2000] (all Avanti Polar Lipids), at a cationic lipid-to-mRNA charge (N/P) ratio of 8 in OptiMem® (Gibco Invitrogen). When multiple mRNAs were used, these were first pre-mixed in a 1:1 ratio prior to complexation to the cationic liposomes. The resulting mRNA-lipoplexes (corresponding to 10 µg mRNA when GFP or OVA were used alone, or 40 µg when co-sonoporations with TriMix were performed) were incubated with the microbubbles for 5 min prior to use. After injection of mRNA lipoplex-loaded microbubbles in the OptiCells™, ultrasound was applied using a Sonitron 2000 (Artison, Inola, USA) (1 MHz, 2 W cm^{-2} corresponding to a peak-negative-pressure of 800 kPa, 20% duty cycle (2 ms on, 8 ms off), 30 s total insonation time per OptiCell™).

BM-DC antibody staining and flow cytometric analysis

To evaluate the impact of mRNA sonoporation on the transfection efficiency and DC phenotype, the mRNA sonoporated cells were collected 24 h or 48 h after ultrasound application. The cells were washed and surface-stained with anti-CD11c-APC (BM-DC marker, Affymetrix eBioscience, Vienna, Austria) and anti-CD40-PE (Affymetrix), anti-CD86-PE (BD biosciences) or anti-CD70-PE (Affymetrix) for 45 min at 4°C in the dark. After washing, the cells were resuspended in FACS buffer (phosphate buffered saline (PBS, Gibco-invitrogen), supplemented with 5% bovine serum albumin (BSA, Sigma-Aldrich)) and analyzed using a FACSCalibur™ (BD) equipped with CellQuest™ software.

Vaccination of mice with mRNA sonoporated DCs

After *in vitro* mRNA sonoporation, the DCs were incubated for 2 h at 37°C in the OptiCells™. Then, all the cells (both the cells in suspension as well as the cells that grow adherent to the OptiCell™ membranes) were collected, and washed twice with PBS. Finally, the cells were resuspended at $2x10^6$ cells ml^{-1} in PBS, of which 50 µl was injected s.c. into the flank of the mice.

In vivo T cell proliferation assay

In order to assess the potential of mRNA sonoporated DCs to efficiently induce proliferation of antigen-specific T cells, an *in vivo* OT-I proliferation assay was performed. This assay uses OVA as a model antigen, and is based on adoptive transfer of OT-I cells, which carry a transgenic CD8 T cell receptor which specifically recognizes the MHC I restricted OVA peptide SIINFEKL. For this, spleens from OT-I mice were processed into a single-cell suspension. After red blood cell lysis, $CD8^+$ T cells were isolated via magnetically activated cell sorting, using a MACS CD8a T cell isolation kit (MACS Miltenyi, Leiden, The Netherlands) according to the manufacturer's instructions. The collected $CD8^+$ T cells were fluorescently labeled using carboxyfluorescein diacetate succinimidyl ester (CFSE, Molecular probes, 10 µM in PBS supplemented with 0.1% BSA) for 15 min at 37°C. After washing the cells with RPMI medium, the cells were resuspended at $1x10^8$ cells ml^{-1} in PBS. All mice received 200 µl of this CFSE-labeled OT-I cell suspension i.v. via tailvein injection. The next day, the mice received s.c. injections with PBS (negative control) or mRNA sonoporated DCs, as described previously. 5 days after vaccination, the mice were sacrificed and the inguinal lymph nodes were collected. After 30 min digestion of the nodes in liberase TL (100 µl, Roche, Vilvoorde, Belgium), a single-cell suspension was prepared. Half of the cells were used for flow cytometric analysis of proliferation (using a FACSCanto flow cytometer with FACSDiva™ software, BD Biosciences). On the other half of the suspension, a $CD8^+$ T cell sort was performed. The resulting cells were resuspended at $2x10^6$ cells ml^{-1} in RPMI medium and seeded per 100 µl in a round-bottom 96 well plate for restimulation. To this extent, day 6 BM-DCs were loaded with 1 µg ml^{-1} SIINFEKL peptide (Eurogentec, Seraing, Belgium) for 30 min at 37°C. After washing, 100 µl SIINFEKL-pulsed DCs, suspended at $2x10^5$ cells ml^{-1} were added to the OT-I cells in the 96 well plates for 24 h at 37°C.

In vivo cytotoxic T lymphocyte assay

To investigate the potential of mRNA sonoporated DCs to induce antigen-specific CTL responses, an *in vivo* CTL assay was performed using an OVA model. For this, 5 days after vaccination with mRNA sonoporated DCs, the vaccinated mice were challenged i.v. with both target cells and control cells in a 1:1 ratio. The injected cells were splenocytes of untreated C57BL/6 mice that were either labeled with CFSE at low intensity ($CFSE^{lo}$ control cells, $5x10^6$ cells per injection), or labeled with CFSE at high intensity and additionally pulsed with the peptide SIINFEKL ($CFSE^{hi}$ target cells, $5x10^6$ cells per injection). The next day, the mice were sacrificed, inguinal lymph nodes were collected, processed into a single-cell suspension, and analyzed for CFSE staining via flow cytometry using a BD FACSCanto. Mice injected s.c. with PBS served as non-immunized controls. The percentage specific lysis of target cells was calculated as $(1–((\%CFSE^{hi}/\%CFSE^{lo})_{immunized}/(\%CFSE^{hi}/\%CFSE^{lo})_{non\text{-}immunized})x100\%$.

In vivo therapeutic vaccination experiments

The therapeutic potential of mRNA sonoporated DCs to reduce tumor growth was evaluated by performing therapeutic vaccinations in tumor-bearing mice. For this, C57BL/6 received a s.c. injection of $3x10^5$ MO4 or E.G7-OVA tumor cells (suspended in PBS) in the flank. 10 days after tumor inoculation, when the lesions were palpable, the mice were randomized in different treatment groups based on tumor volume, and vaccinated with mRNA sonoporated DCs. A second therapeutic vaccination was performed at day 13 or 14 after tumor cell injection. Tumor growth was measured every other day using a caliper. When the tumor volume exceeded 1500 mm^3, the mice were euthanized via cervical dislocation. Animals that completely rejected their tumors were rechallenged with $3x10^5$ E.G7-OVA tumor cells, and subsequently with $3x10^5$ MO4 cells. As controls for the rechallenge study, naïve (non-vaccinated) C57BL/6 were used.

ELISA measurements

Supernatants of mRNA sonoporated DCs were screened for the presence of IL-10 and IL-12p70. Supernatants of DC and T cell co-cultures were assayed for IFNγ. Cytokine measurements were performed via ELISA (all Ready-SET-Go!® ELISA kits, Affymetrix) according to the manufacturer's instructions.

Statistical analysis

A one-way ANOVA followed by a Bonferroni multiple comparison test, was carried out (GraphPad software, la Jolla, CA, USA). Sample sizes and number of times experiments were repeated are indicated in the figure legends. The results are shown in column graphs as mean ± standard deviation. The number of asterisks in the figures indicates the statistical significance as follows: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. Survival was visualized in a Kaplan-Meier plot. Differences in survival were analyzed by the log-rank (Mantel-Cox) test.

RESULTS

Transfection efficiency and phenotype of mRNA sonoporated DCs

In our previous report, we demonstrated that sonoporation could be used for ultrasound-triggered transfection of primary DCs with mRNA *in vitro*. In addition, the sonoporation procedure as such, without the use of additional maturation stimuli, was shown to readily induce a small but significant shift in the expression of the DC maturation markers CD40 and CD86 [13]. However, compared to a positive control where the DCs' TLR4 was stimulated by incubation with bacteria-derived lipopolysaccharide (LPS), this maturation was merely partial. As complete DC maturation is warranted for effective immunostimulation, this prompted us to include additional stimuli to further increase the expression of co-stimulatory markers. Therefore, we included TriMix mRNA into the antigen mRNA-containing lipoplexes. We evaluated the effect of TriMix inclusion on the transfection efficiency as well as the DC phenotype, by analyzing the expression of 3 molecules involved in T cell activation by DCs 24 h after sonoporation with either reporter mRNA alone (GFP) or in combination with TriMix (GFP+TriMix).

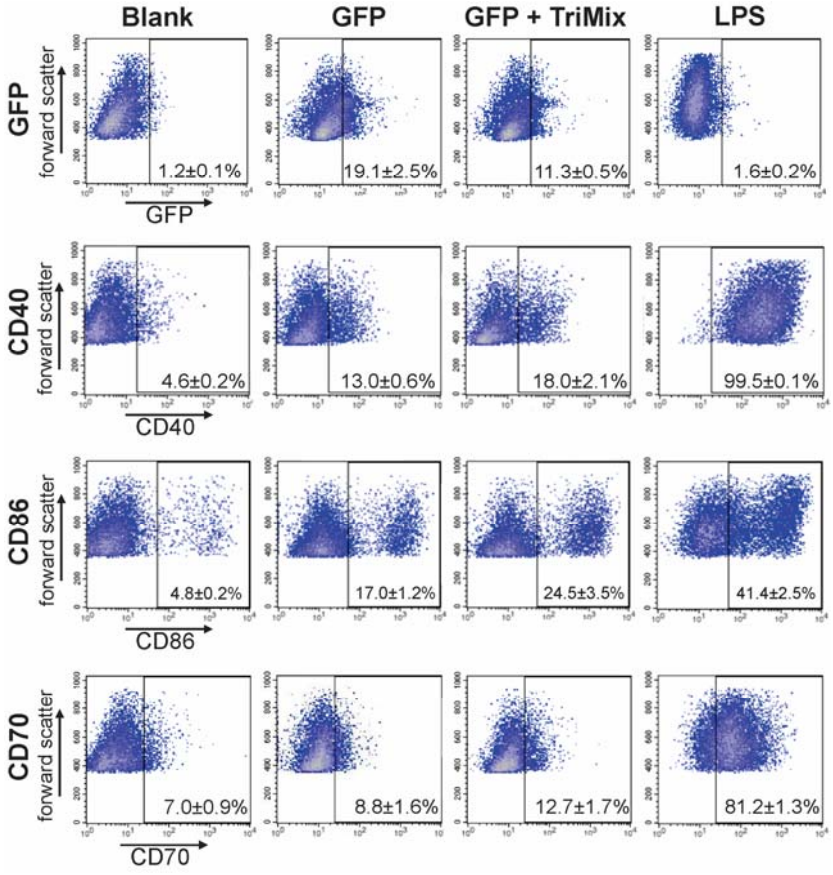


Figure 2. Transfection efficiency and phenotypical analysis of DCs after mRNA sonoporation. 24 h after treatment of the DCs by sonoporation with GFP mRNA alone (GFP) or in combination with TriMix (GFP+TriMix), the cells were analyzed for the expression of GFP and different maturation markers (CD40, CD86 and CD70). DCs were gated based on CD11c staining. Untreated cells and LPS-stimulated cells served as negative and positive controls, respectively (*n*=3).

In accordance with our previous report, the results in **Figure 2** indicate efficient transfection with GFP mRNA, resulting in 19% GFP-expressing DCs 24 h after sonoporation. Inclusion of TriMix results in a decrease in transfection efficiency to 11%. With regards to the DC phenotype, we observed a significant up-regulation of CD86 and CD40 expression in the GFP group compared to blank. The effects on CD70 expression are less pronounced. Sonoporation with GFP+TriMix, lead to a slight but significant additional increase in the expression of CD40, CD86 and CD70 over sonoporation with GFP mRNA alone. However, the expression levels of these surface molecules after GFP+TriMix sonoporation are still significantly lower in comparison to an LPS-stimulated positive control.

Similar observations can be made when looking at the levels of immune stimulating (IL-12p70) and immune suppressive (IL-10) cytokines that are produced by the mRNA sonoporated DCs 24 h after treatment (**Figure 3**). Sonoporation with GFP mRNA alone resulted in a significant increase in IL-10, but not IL-12p70 secretion. For both cytokines, inclusion of TriMix into the mRNA lipoplexes surrounding the microbubbles results in higher cytokine production. But still, the concentrations are significantly lower than when LPS was applied to induce maturation of the DCs.

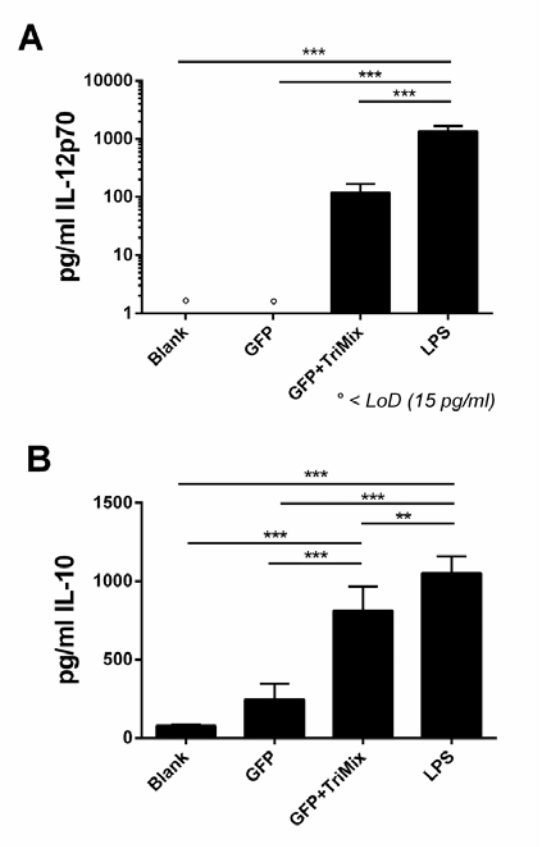


Figure 3. Cytokine production by mRNA-sonoporated DCs. The amounts of cytokines produced by untreated DCs (blank), DCs sonoporated with GFP mRNA alone, or in combination with TriMix (GFP+TriMix) and DCs that were matured with LPS were measured 24 h after cell

treatment. Figures show graphical representations of the levels of (A) IL-12p70 and (B) IL-10. Graphs are summaries of 2 independent experiments at n=3, results were analyzed via a one-way ANOVA followed by a Bonferroni multiple comparison test. **p<0.01, ***p<0.001.

In vivo induction of antigen-specific T cell proliferation by mRNA sonoporated DCs

To assess the impact of mRNA sonoporation on the capacity of DCs to prime antigen-specific $CD8^+$ T cells, we performed an *in vivo* T cell proliferation study using chicken ovalbumin (OVA) as a model antigen. This makes use of an adoptive transfer of OT-I cells, which are transgenic T cells that carry a CD8 T cell receptor that specifically recognizes the OVA-derived peptide SIINFEKL when presented in MHC I. 24 h after adoptive OT-I cell transfer, tumor-free mice were vaccinated with mRNA sonoporated DCs, and the percentage proliferating OT-I cells was evaluated 5 days later. Results were normalized to the background signal in mice that received PBS instead of DCs. As demonstrated in **Figure 4A**, no aspecific OT-I proliferation was observed in the negative control group that received DCs sonoporated with GFP mRNA (3 ± 3%). When vaccination was performed with DCs sonoporated with antigen mRNA alone (OVA), this resulted in 31 ± 16% proliferation of OVA-specific $CD8^+$ T cells, indicating efficient intracellular processing and presentation of OVA by the mRNA sonoporated DCs without additional stimulation of maturation. When OVA sonoporation was combined with phenotype-modulating stimuli, either by 2 h incubation of the sonoporated DCs with LPS (OVA/LPS as a positive control) or by sonoporation with OVA and TriMix mRNA (TriO), antigen-specific T cell proliferation increased to respectively 41 ± 15% and 48 ± 19%. Although there is a trend towards more extensive proliferation in the OVA/LPS and TriO groups, the observed differences were not statistically significant (p>0.05).

In addition, we evaluated the functionality of the proliferating T cells, by performing a re-stimulation with peptide. For this, a fraction of the proliferated OT-I cells were co-cultured in a 10:1 ratio with DCs that were pulsed with SIINFEKL peptide. 24 h later, the response of these restimulated T cells to the antigen-presenting DCs was evaluated by measuring the levels of IFNγ, an important mediator of CTL responses, in the co-culture supernatant. The results in **Figure 4B** display a similar trend to the proliferation results: T cells activated by DCs that received OVA mRNA via sonoporation produce significantly larger amounts of IFNγ compared to those stimulated with DCs sonoporated with GFP mRNA. After additional maturation by LPS incubation or sonoporation with TriMix, a trend towards higher IFNγ levels was observed.

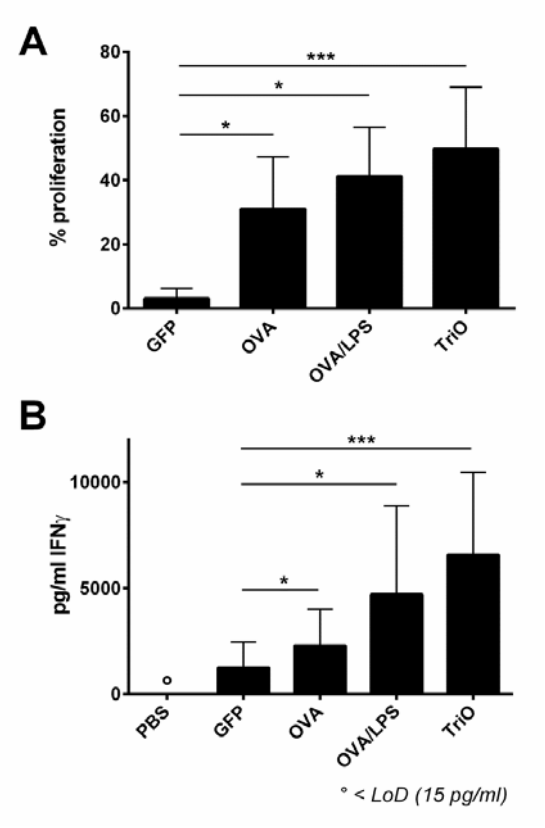


Figure 4. *In vivo* proliferation of antigen-specific T cells. 1 day after adoptive OT-I transfer, mice were vaccinated with DCs sonoporated with GFP mRNA (negative control), OVA mRNA alone (OVA) or in combination with TriMix (TriO), or OVA-sonoporated DCs that were matured with LPS (OVA/LPS). Read-out was performed 24h later. Graphs show (A) percentages proliferating OT-I cells normalized to the background that was detected in mice that received a PBS injection. (B) levels of IFNγ measured 24h after restimulation of proliferated OT-I cells with peptide-pulsed DCs. Graphs are summaries of 2 independent experiments at n=3. Results were analyzed via a one-way ANOVA followed by a Bonferroni multiple comparison test. *p<0.05, ***p<0.001.

In vivo cytotoxic T cell response induction by mRNA sonoporated DCs

Besides the capacity to stimulate antigen-specific T cells to proliferate, mRNA sonoporated DCs should be able to activate $CD8^+$ T cells to cause antigen-specific lysis of target cells. To evaluate this, an *in vivo* CTL assay was performed, again using OVA as a model antigen. For this, tumor-free mice were vaccinated with mRNA sonoporated DCs and 5 days later, the animals were challenged with allogenic splenocytes that were either untreated (non-target cells) or pulsed with SIINFEKL peptide (target cells). The next day, the ratio of target cells versus non-target cells was studied as a measure for antigen-specific lysis. The results, as represented in **Figure 5**, show 41 ± 31% OVA-specific lysis of target cells when mice were vaccinated with DCs sonoporated with OVA mRNA. LPS-induced maturation of the DCs after sonoporation (OVA/LPS), could not further increase this percentage (39 ± 23%). Unexpectedly, DCs sonoporated with TriO were markedly less efficient in inducing antigen-specific lysis (25 ± 18%). Based on the proliferative

potential of TriO DCs as well as on their capacity to stimulate IFNγ-producing $CD8^+$ T cells, more extensive CTL responses were expected.

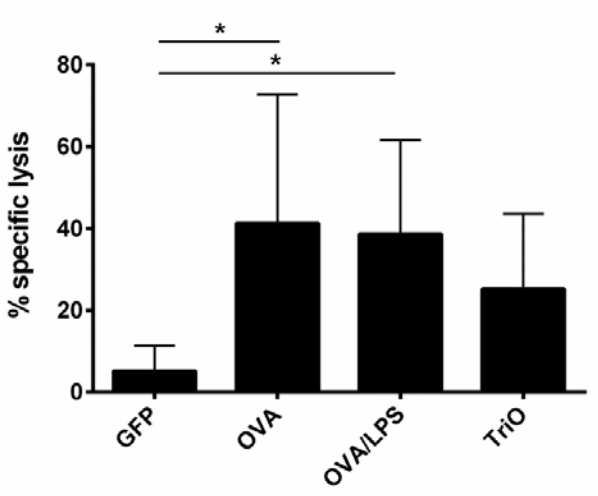


Figure 5. *In vivo* cytotoxic T cell assay. 5 days after vaccination of mice with DCs sonoporated with GFP mRNA (negative control), OVA mRNA alone (OVA) or in combination with TriMix (TriO), or OVA-sonoporated DCs that were matured with LPS (OVA/LPS), mice were challenged with target cells and non-target cells in a 1:1 ratio. The next day, the percentage antigens-specific lysis of target cells compared to non-target cells was measured. Graphs are summaries of 3 independent experiments at n=3. Results were analyzed via a one-way ANOVA followed by a Bonferroni multiple comparison test. *p<0.05.

Therapeutic vaccinations with mRNA-sonoporated DCs

Immune induction is a complex orchestrated response that relies on numberous synergistic components of both cellular and humoral immunity. Moreover, tumor-mediated suppression of immune responses, regulated by various immunosuppressive cell types such as myeloid-derived suppressor cells and regulatory T cells (T_{regs}) that reside in the tumor microenvironment, could potentially limit the therapeutic benefits of DC-based immunotherapy [22, 23]. Therefore, the impact of cancer vaccines can only truly be evaluated when all these different players are taken into account. For that reason, we assessed the potential of DCs sonoporated with antigen and TriMix mRNA in a therapeutic setting. For this, we examined the effect of vaccination with *ex vivo* mRNA sonoporated DCs in mice with pre-existing tumors. We initially opted for an OVA-expressing B16 melanoma model (MO4). 10 days after tumor inoculation in the flank, tumors were palpable and the animals were randomized into 3 treatment groups based on the tumor volume (as shown in Supplemetary Data, **Figure S1**). Subsequently, the animals were given a s.c. injection with either DCs sonoporated with GFP mRNA (DC GFP, negative control, n=9), OVA mRNA (DC OVA, n=9), or sonoporated with TriMix and OVA mRNA (DC TriO, n=8). A second vaccination was performed 3 days later. When measuring tumor growth as a function of time, we observed a significant slow-down of tumor outgrowth in the DC OVA, and especially in the DC TriO group compared to the DC GFP group (**Figure 6**). This translated in a prolongation of median survival of 41% and 82% for DC OVA and

DC TriO respectively. This indicates that antigen mRNA sonoporated DCs can induce potent antitumor immune responses *in vivo*, which can further be boosted by sonoporation with DC-modulating TriMix mRNA.

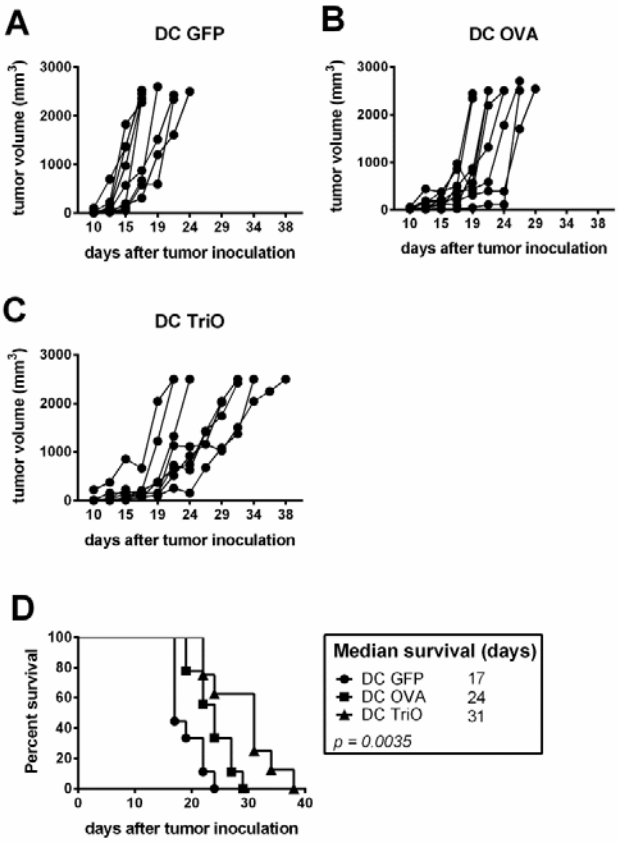


Figure 6. Therapeutic vaccination of MO4-bearing mice with mRNA-sonoporated DCs. 10 and 13 days after inoculation of mice with MO4 melanoma cells, therapeutic vaccinations with mRNA sonoporated DCs were performed. Graphs show tumor growth as a function of time for mice vaccinated with DCs sonoporated with (A) GFP mRNA (control), (B) OVA mRNA and (C) OVA mRNA and TriMix (DC TriO). A Kaplan-Meier survival curve is shown in (D).

These therapeutic vaccination experiments were repeated in a different tumor model, expressing the same antigen. For this, mice were inoculated with E.G7-OVA, which are OVA-expressing lymphoma cells. Randomization was done based on tumor volume at day 10 after tumor inoculation (as shown in Supplemetary Data, **Figure S2**). Once more, two therapeutic vaccinations were performed at 10 and 14 days after tumor inoculation. In addition to the 3 treatment groups described in the previous experiment, we included a group that received OVA mRNA sonoporated DCs that were additionally matured with LPS for 2 h prior to injection (DC OVA/LPS) (*n*=6 in all groups). This way, we aimed to compare the therapeutic effects of TriMix sonoporation to the use of a known and potent maturation inducer. In accordance to the previous experiment, the tumor growth curves in **Figure 7** indicate that sonoporation with antigen results in a significant delay of tumor outgrowth, resulting in a 58% increase in median survival. Interestingly, the slow-down of

tumor growth was markedly shorter-lived in the DC OVA/LPS group compared to their unstimulated counterparts (DC OVA). This resulted in merely 35% prolongation of median survival of animals in the DC OVA/LPS group compared to the DC GFP group. In contrast, stimulation of antigen presentation by sonoporation with OVA and TriMix mRNA, resulted in a pronounced effect on tumor growth: median survival was more than doubled (212% increase), and complete tumor regression was observed in 2/6 animals in the DC TriO group.

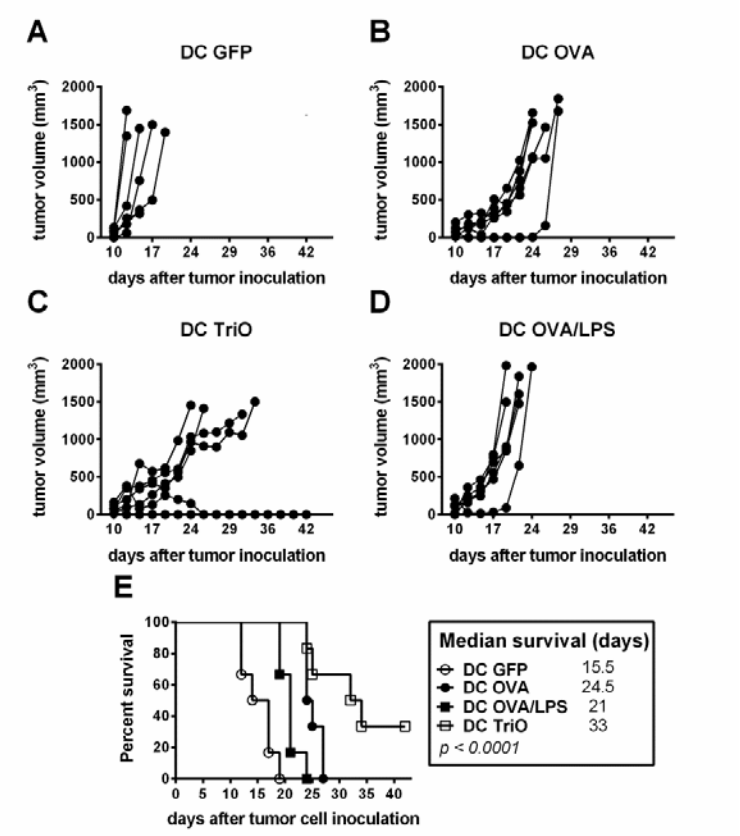


Figure 7. Therapeutic vaccination of E.G7-OVA-bearing mice with mRNA-sonoporated DCs. 10 and 14 days after inoculation of mice with E.G7-OVA lymphoma cells, therapeutic vaccinations with mRNA sonoporated DCs were performed. Graphs show tumor growth as a function of time for mice vaccinated with DCs sonoporated with (A) GFP mRNA (control), (B) OVA mRNA, (C) OVA mRNA and TriMix (DC TriO) and (D) OVA mRNA followed by a 2h maturation with LPS (DC OVA/LPS). A Kaplan-Meier survival curve is shown in (E).

These surviving animals, as well as two naïve control mice, were rechallenged with E.G7-OVA tumor cells 42 days after the first tumor inoculation. This to evaluate whether durable antitumor immunity was induced by the vaccinations. Indeed, in comparison to the control mice where rapid tumor growth was observed, the DC TriO vaccinated mice were protected against a second injection with the same tumor (**Figure 8**). To prove that this protective immunity is antigen-specific, we challenged these animals with a different tumor (MO4 melanoma instead of the initial lymphoma) that expresses the same antigen. Once more, no tumor growth could be detected in the DC TriO vaccinated animals in contrast

to 3 naïve control mice. Thus, vaccination with DC TriO can induce antigen-specific immunological memory, that is capable of protecting tumor-free survivors against tumor recurrence.

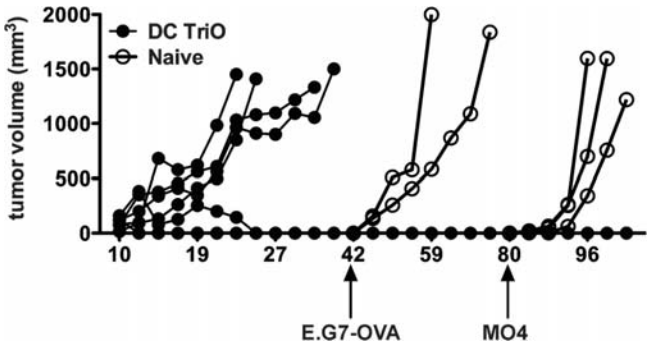


Figure 8. Rechallenge of tumor-free DC TriO treated mice. The two E.G7-OVA challenged mice that showed complete tumor regression after two vaccinations with DC TriO , were rechallenged with E.G7-OVA and MO4 tumor cells on day 42 and day 80 after initial tumor inoculation, respectively. As controls, naïve, non-vaccinated mice were injected with the same tumor load.

DISCUSSION

In continuation of our previous report on the use of mRNA-loaded microbubbles and ultrasound for the transfection of DCs *in vitro* [13], we further investigated if antigen transfer into DCs via mRNA sonoporation could result in the induction of antitumor immune responses. For this, it was crucial to not only deliver antigen mRNA to the DCs, but at the same time to induce a shift in activity of these cells, in order to make them more effective in antigen presentation to naïve T cells. This should in turn result in protection against tumor growth. To allow this antigen-loading and maturation to occur simultaneously, we included TriMix into the mRNA-lipoplexes that surround the microbubbles, and performed sonoporations with 4 mRNA sequences in total.

First of all, we were able to show that sonoporation with multiple mRNAs is feasible. Upon sonoporation with GFP mRNA and TriMix, both GFP expression as well as a slight but significant up-regulation of maturation marker expression by DCs was observed. However, sonoporation with TriMix lead to a reduction in the percentage of GFP-expressing cells, which is probably due to limitations in the amount of mRNA that can be loaded onto the microbubble surface. For sonoporation with TriMix, a four-fold increase in total amount of mRNA, and therefore in amount of mRNA-lipoplexes is required. However, the number of binding spaces on the bubble surface is limited [24]. Therefore, it is likely that in the TriMix conditions, complete microbubble saturation occurs, resulting in the presence of a substantial portion of free mRNA-lipoplexes in the medium. In our previous reports, we demonstrated that the highest transfection efficiencies could be reached when the lipoplexes are physically attached to the microbubbles and not just co-delivered [12, 25]. For that reason, sonoporation with multiple mRNAs could lead to a lower protein expression. In addition, there is the possibility that the co-transfection with multiple RNAs results in competition for mRNA translation. This theory is supported by the observations

of Chen et al., who also reported a drop in transfection efficiency 2 mRNAs were co-electroporated in DCs compared to electroporation of each of these mRNAs alone [26]. This was also reported by Bonehill and colleagues who reported that upon electroporation of DCs with TriMix the number of mRNA-expressing DCs was always slightly decreased when two or three mRNAs were co-electroporated, in comparison to the electroporation of each mRNA alone [16]. This might indicate that there is a limit to the amount of mRNA that can be transferred into the cells. This raises the question whether this reduced transfection efficiency could limit the use of antigen mRNA and TriMix sonoporation. To answer this, it is important to take the therapeutic studies performed with TriO sonoporated DCs into account. These confirm that a higher transfection efficiency does not necessarily result in more potent immune responses. Indeed, DCs sonoporated with antigen mRNA alone, which allows higher antigen expression, result in less powerful immune responses, even after additional LPS-maturation, compared to TriO sonoporated DCs, where the amount of antigen that is produced by the DCs is lower. This discrepancy between transfection efficiency and immunogenicity was previously reported by Grünebach et al., who postulated that it is important to realize that T cell receptors can detect antigens at much higher sensitivity than any routinely used analytical instrument in the lab [27]. In addition, T cell activation does not only depend on the presence of the antigen. Only when the antigens are presented to T cells by fully functional and mature DCs, can this result in effective T cell activation [8].

Thus, it is crucial to obtain antigen-loaded DCs with a mature phenotype. In accordance to our previous reports, mRNA sonoporation as such, without incorporation of additional immune stimulants, readily results in a distinct up-regulation of maturation marker expression at the DC surface. This in contrast to mRNA electroporation, which is considered the golden standard for mRNA transfer into DCs *in vitro*, where no effects on the DC maturation status were observed [16, 21]. Two possible mechanisms might explain this sonoporation-induced maturation. First of all, sonoporation is a physical transfection method, where pores are created as a result from shear forces and violent microbubble movements [28]. DCs are known to be sensitive to shear forces, and can already up-regulate maturation markers upon stress due to pipetting and centrifugation [29]. Thus, the shear stress induced by sonoporation could contribute to the observed phenotypical changes. In addition, sonoporation was reported to influence Ca^{2+} signaling within cells [28, 30]. Importantly, increased intracellular Ca^{2+} concentrations were also reported as one of the early events after LPS stimulation of mouse bone marrow-derived DCs [31]. Another possibility is direct triggering of innate immune responses by the cationic lipids (in this case DOTAP) within the mRNA lipoplexes, via TLR4 ligation. This, charge-related TLR activation was previously reported for DOTAP-containing lipoplexes as well as for various cationic polymers that are routinely used as transfection reagents [32, 33]. Likely, the observed effects are caused by a combination of these different mechanisms.

Much to our surprise, this limited maturation induced by antigen sonoporation alone already rendered the cells capable of inducing potent T cell proliferation, CTL responses and antitumor immune effects in tumor-free animals *in vivo*. Therefore, it was crucial to evaluate whether or not sonoporation with TriMix had an added value on the induced immune responses. By looking at the relative maturation marker expression and cytokine production by mRNA sonoporated DCs *in vitro*, it would appear that the contribution of

TriMix is limited, especially in comparison to maturation by LPS as a positive control. A possible explanation for this could be the transfection efficiency that can be obtained with mRNA sonoporation. Although this technique allows substantial transfection of DCs, its efficiency is still relatively low in comparison to electroporation, where over 90% of the DCs can be transfected with mRNA. Indeed, with mRNA electroporation, TriMix does result in a significant up-regulation of maturation markers and cytokine expression, as previously reported by Bonehill et al. [17]. In addition, based on the results obtained in the OT-I proliferation assay and the *in vivo* CTL assay, it would appear that TriO sonoporation works fine to enhance the quantity of antigen-specific $CD8^+$ T lymphocytes (i.e. numbers of proliferating cells), but at a first glance, their quality (i.e. cytolytic activity) is not as good as when OVA or OVA/LPS are used. However, it should be noted that these experiments were performed in naïve, healthy mice and not in tumor-bearing animals. In the latter, it was obvious that TriO sonoporation was superior, and resulted in the most pronounced slowdown of tumor outgrowth and prolongation of median survival. Importantly, TriO DCs could even induce complete regression in 30% of the vaccinated animals, resulting in long-lasting protection against (re)challenges with tumor cells that express the vaccine antigen (in this case OVA).

A possible explanation for this discrepancy between immune responses observed in healthy versus tumor-bearing animals, is the existence of pre-vaccination antitumor immune responses. It was shown by Germeau and colleagues that large numbers of antitumor T cells are already present within melanoma patients prior to vaccination with tumor antigens. However, these natural pre-vaccine immune responses are often too weak to result in tumor rejection or even a reduction in tumor growth, due to the various immune suppressive mechanisms that occur within the tumor microenvironment [34]. Vaccination can then aid in boosting these spontaneous immune responses, either by aiding to overcome tumor-induced immune suppression, by re-activating anergic tumor-reactive T cells at the tumor site, or by inducing new antitumor CTL clonotypes (probably due to additional antigen release from attacked tumor cells) [3, 35]. This priming of the immune system by a growing tumor could explain why the immunogenicity of the mRNA sonoporated DCs was more pronounced in a therapeutic setting.

As to the question what could explain the superiority of TriMix over LPS in the therapeutic vaccination experiment, there are a number of possible causes. In essence, it is important to always keep in mind that immune responses are regulated in a complex manner, involving numerous different cell types. As discussed earlier, the effectiveness of a DC vaccine is not only determined by its capacity to induce effector $CD8^+$ T cells. Major factors that limit the potency of cancer vaccines are the hostile tumor microenvironment and the presence of T_{regs} that counteract CTL activity [36]. Therefore, undermining these immunosuppressive mechanisms might be the key to successful cancer vaccines. Interestingly, previous research by Pen et al. exposed TriMix as a potential tool in subverting T_{reg} effects. They demonstrated that $CD8^+$ T cells that were preactivated by TriMix electroporated DCs were protected against T_{reg} suppression. What was more, TriMix electroporated DCs could reprogram T_{regs} towards a Th1 phenotype, thus reinforcing cellular immunity against the tumor [37]. Based on these observations, it is quite likely that in the therapeutic vaccination setting, the superior antitumor effects of TriO DCs could be, at least partially, attributed to a reduction of T_{reg}-mediated immune

suppression. Of course, many other cell types are involved in the regulation of antitumor immunity, and the effects of TriMix on these different cells is not yet known. For instance, it might be possible that TriMix transfected DCs could stimulate natural killer (NK) cells, which act synergistically with CTLs and were recently reported to mount antigen-specific responses that can lead to long-term memory [38-41].

We reported that the transfection efficiencies that can be reached with mRNA sonoporation are significantly lower than when mRNA is electroporated into DCs, however it should be noted that the importance of mRNA sonoporation as a transfection technique lies within its possible *in vivo* applicability [42, 43]. Commercially available microbubble contrast agents were shown to migrate to the tumor-draining lymph nodes upon intradermal injection around the tumor of breast cancer patients [15]. This makes them useful to identify and localize sentinel lymph nodes in a non-invasive manner, using contrast-enhanced ultrasound imaging (CEUS). In addition, their lymphatic uptake is particularly interesting, since lymph nodes harbor large numbers of immune cells, including a substantial fraction of DCs [44, 45]. Therefore, microbubbles could be used for image-guided *in vivo* vaccination. The immunotherapy could then consist of a subcutaneous injection of mRNA-loaded microbubbles, of which the migration to the draining lymph nodes could be visualized using CEUS. Once the microbubbles and hence the mRNA are localized within the lymph nodes, higher intensity ultrasound pulses could induce localized microbubble implosion, resulting in spatiotemporally controlled delivery of both antigen mRNA and immunomodulating TriMix to intranodal DCs. This could allow a minimally invasive vaccination procedure that could substantially reduce the costs and laborious procedures that are currently associated with the production of *ex vivo* generated DC vaccines. Of course, this will require a thorough optimization of microbubble and mRNA dosages and ultrasound parameters, as these greatly influence the transfection efficiency with microbubbles and ultrasound *in vivo* [46].

CONCLUSIONS

Taken together, DCs that were sonoporated with antigen mRNA via mRNA loaded microbubbles and ultrasound can induce potent antigen-specific immune responses *in vivo*. Sonoporation with TriMix, to further modulate the DC's antigen-presenting functionality, could be used to further augment immunity. Especially in a therapeutic setting, vaccination with antigen and TriMix mRNA sonoporated DCs resulted in a significant reduction in tumor growth, leading to a marked increase in overall survival and long-lasting antigen-specific protection against tumor recurrence. Therefore, sonoporation could be a useful tool in the future development of *in vivo* DC vaccines.

ACKNOWLEDGEMENTS

The authors would like to thank Elsy Vaeremans and Petra Roman for their help with the mRNA production. Heleen Dewitte is a doctoral fellow of the Institute for the Promotion of Innovation through Science and Technology in Flanders, Belgium (IWT-Vlaanderen). Ine Lentacker and Karine Breckpot are postdoctoral fellows of the Research Foundation-Flanders, Belgium (FWO-Vlaanderen). This project was funded through the FWO grant G016513N.

REFERENCES

[1] R.M. Steinman, Z.A. Cohn, Identification of a Novel Cell Type in Peripheral Lymphoid Organs of Mice, J Exp Med, 137 (1973) 1142-1162.
[2] K. Palucka, J. Banchereau, Dendritic-Cell-Based Therapeutic Cancer Vaccines, Immunity, 39 (2013) 38-48.
[3] P.G. Coulie, B.J. Van den Eynde, P. van der Bruggen, T. Boon, Tumour antigens recognized by T lymphocytes: at the core of cancer immunotherapy, Nature reviews. Cancer, 14 (2014) 135-146.
[4] L. Wayteck, K. Breckpot, J. Demeester, S.C. De Smedt, K. Raemdonck, A personalized view on cancer immunotherapy, Cancer Lett, (2013).
[5] S. Van Lint, C. Heirman, K. Thielemans, K. Breckpot, mRNA: From a chemical blueprint for protein production to an off-the-shelf therapeutic, Hum Vaccin Immunother, 9 (2013).
[6] G. Tavernier, O. Andries, J. Demeester, N.N. Sanders, S.C. De Smedt, J. Rejman, mRNA as gene therapeutic: how to control protein expression, Journal of controlled release : official journal of the Controlled Release Society, 150 (2011) 238-247.
[7] C. Reis e Sousa, Dendritic cells in a mature age, Nature reviews. Immunology, 6 (2006) 476-483.
[8] M.B. Lutz, G. Schuler, Immature, semi-mature and fully mature dendritic cells: which signals induce tolerance or immunity?, Trends Immunol, 23 (2002) 445-449.
[9] R.M. Steinman, D. Hawiger, M.C. Nussenzweig, Tolerogenic dendritic cells, Annual Review of Immunology, 21 (2003) 685-711.
[10] J.R. Lindner, Microbubbles in medical imaging: current applications and future directions, Nat Rev Drug Discov, 3 (2004) 527-532.
[11] I. Lentacker, R.E. Vandenbroucke, B. Lucas, J. Demeester, S.C. De Smedt, N.N. Sanders, New strategies for nucleic acid delivery to conquer cellular and nuclear membranes, J Control Release, 132 (2008) 279-288.
[12] I. Lentacker, N. Wang, R.E. Vandenbroucke, J. Demeester, S.C. De Smedt, N.N. Sander, Ultrasound Exposure of Lipoplex Loaded Microbubbles Facilitates Direct Cytoplasmic Entry of the Lipoplexes, Mol Pharmaceut, 6 (2009) 457-467.
[13] M.L. De Temmerman, H. Dewitte, R.E. Vandenbroucke, B. Lucas, C. Libert, J. Demeester, S.C. De Smedt, I. Lentacker, J. Rejman, mRNA-Lipoplex loaded microbubble contrast agents for ultrasound-assisted transfection of dendritic cells, Biomaterials, 32 (2011) 9128-9135.
[14] A.R. Sever, P. Mills, S.E. Jones, W. Mali, P.A. Jones, Sentinel node identification using microbubbles and contrast-enhanced ultrasonography, Clin Radiol, 67 (2012) 687-694.
[15] A.R. Sever, P. Mills, S.E. Jones, K. Cox, J. Weeks, D. Fish, P.A. Jones, Preoperative Sentinel Node Identification With Ultrasound Using Microbubbles in Patients With Breast Cancer, Am J Roentgenol, 196 (2011) 251-256.
[16] A. Bonehill, S. Tuyaerts, A.M. Van Nuffel, C. Heirman, T.J. Bos, K. Fostier, B. Neyns, K. Thielemans, Enhancing the T-cell stimulatory capacity of human dendritic cells by co-electroporation with CD40L, CD70 and constitutively active TLR4 encoding mRNA, Molecular therapy : the journal of the American Society of Gene Therapy, 16 (2008) 1170-1180.
[17] A. Bonehill, A.M.T. Van Nuffel, J. Corthals, S. Tuyaerts, C. Heirman, V. Francois, D. Colau, P. van der Bruggen, B. Neyns, K. Thielemans, Single-Step Antigen Loading and

Activation of Dendritic Cells by mRNA Electroporation for the Purpose of Therapeutic Vaccination in Melanoma Patients, Clin Cancer Res, 15 (2009) 3366-3375.
[18] S. Van Lint, C. Goyvaerts, S. Maenhout, L. Goethals, A. Disy, D. Benteyn, J. Pen, A. Bonehill, C. Heirman, K. Breckpot, K. Thielemans, Preclinical Evaluation of TriMix and Antigen mRNA-Based Antitumor Therapy, Cancer Res, 72 (2012) 1661-1671.
[19] A.M.T. Van Nuffel, D. Benteyn, S. Wilgenhof, J. Corthals, C. Heirman, B. Neyns, K. Thielemans, A. Bonehill, Intravenous and intradermal TriMix-dendritic cell therapy results in a broad T-cell response and durable tumor response in a chemorefractory stage IV-M1c melanoma patient, Cancer Immunol Immun, 61 (2012) 1033-1043.
[20] S. Wilgenhof, A.M.T. Van Nuffel, D. Benteyn, J. Corthals, C. Aerts, C. Heirman, I. Van Riet, A. Bonehill, K. Thielemans, B. Neyns, A phase IB study on intravenous synthetic mRNA electroporated dendritic cell immunotherapy in pretreated advanced melanoma patients, Ann Oncol, 24 (2013) 2686-2693.
[21] S. Van Meirvenne, L. Straetman, C. Heirman, M. Dullaers, C. De Greef, V. Van Tendeloo, K. Thielemans, Efficient genetic modification of murine dendritic cells by electroporation with mRNA, Cancer Gene Ther, 9 (2002) 787-797.
[22] G.T. Motz, G. Coukos, Deciphering and Reversing Tumor Immune Suppression, Immunity, 39 (2013) 61-73.
[23] D.I. Gabrilovich, S. Nagaraj, Myeloid-derived suppressor cells as regulators of the immune system, Nat Rev Immunol, 9 (2009) 162-174.
[24] B. Geers, I. Lentacker, N.N. Sanders, J. Demeester, S. Meairs, S.C. De Smedt, Self-assembled liposome-loaded microbubbles: The missing link for safe and efficient ultrasound triggered drug-delivery, Journal of controlled release : official journal of the Controlled Release Society, 152 (2011) 249-256.
[25] I. Lentacker, B. Geers, J. Demeester, S.C. De Smedt, N.N. Sanders, Design and Evaluation of Doxorubicin-containing Microbubbles for Ultrasound-triggered Doxorubicin Delivery: Cytotoxicity and Mechanisms Involved, Mol Ther, 18 (2010) 101-108.
[26] J. Chen, X.Z. Guo, H.Y. Li, X. Liu, L.N. Ren, D. Wang, J.J. Zhao, Generation of CTL responses against pancreatic cancer in vitro using dendritic cells co-transfected with MUC4 and survivin RNA, Vaccine, 31 (2013) 4585-4590.
[27] F. Grunebach, M.R. Muller, A. Nencioni, P. Brossart, Delivery of tumor-derived RNA for the induction of cytotoxic T-lymphocytes, Gene Ther, 10 (2003) 367-374.
[28] I. Lentacker, I. De Cock, R. Deckers, S.C. De Smedt, C.T. Moonen, Understanding ultrasound induced sonoporation: Definitions and underlying mechanisms, Adv Drug Deliver Rev, (2013).
[29] M.B. Lutz, S. Rossner, Factors influencing the generation of murine dendritic cells from bone marrow: The special role of fetal calf serum, Immunobiology, 212 (2007) 855-862.
[30] Z. Fan, R.E. Kumon, J. Park, C.X. Deng, Intracellular delivery and calcium transients generated in sonoporation facilitated by microbubbles, J Control Release, 142 (2010) 31-39.
[31] E. Shumilina, S.M. Huber, F. Lang, Ca2+ signaling in the regulation of dendritic cell functions, Am J Physiol-Cell Ph, 300 (2011) C1205-C1214.
[32] E.A. Vasievich, W. Chen, L. Huang, Enantiospecific adjuvant activity of cationic lipid DOTAP in cancer vaccine, Cancer Immunol Immun, 60 (2011) 629-638.

[33] H. Chen, P. Li, Y. Yin, X. Cai, Z. Huang, J. Chen, L. Dong, J. Zhang, The promotion of type 1 T helper cell responses to cationic polymers in vivo via toll-like receptor-4 mediated IL-12 secretion, Biomaterials, 31 (2010) 8172-8180.
[34] C. Germeau, W. Ma, F. Schiavetti, C. Lurquin, E. Henry, N. Vigneron, F. Brasseur, B. Lethe, E. De Plaen, T. Velu, T. Boon, P.G. Coulie, High frequency of antitumor T cells in the blood of melanoma patients before and after vaccination with tumor antigens, The Journal of experimental medicine, 201 (2005) 241-248.
[35] C. Lurquin, B. Lethe, E. De Plaen, V. Corbiere, I. Theate, N. van Baren, P.G. Coulie, T. Boon, Contrasting frequencies of antitumor and anti-vaccine T cells in metastases of a melanoma patient vaccinated with a MAGE tumor antigen, The Journal of experimental medicine, 201 (2005) 249-257.
[36] A. Bonertz, J. Weitz, D.H.K. Pietsch, N.N. Rahbari, C. Schlude, Y.Z. Ge, S. Juenger, I. Vlodavsky, K. Khazaie, D. Jaeger, C. Reissfelder, D. Antolovic, M. Aigner, M. Koch, P. Beckhove, Antigen-specific Tregs control T cell responses against a limited repertoire of tumor antigens in patients with colorectal carcinoma, J Clin Invest, 119 (2009) 3311-3321.
[37] J.J. Pen, B. De Keersmaecker, S.K. Maenhout, A.M. Van Nuffel, C. Heirman, J. Corthals, D. Escors, A. Bonehill, K. Thielemans, K. Breckpot, J.L. Aerts, Modulation of regulatory T cell function by monocyte-derived dendritic cells matured through electroporation with mRNA encoding CD40 ligand, constitutively active TLR4, and CD70, J Immunol, 191 (2013) 1976-1983.
[38] K. Karimi, J.E. Boudreau, K. Fraser, H. Liu, J. Delanghe, J. Gauldie, Z. Xing, J.L. Bramson, Y. Wan, Enhanced antitumor immunity elicited by dendritic cell vaccines is a result of their ability to engage both CTL and IFN gamma-producing NK cells, Mol Ther, 16 (2008) 411-418.
[39] C.H.M.J. Van Elssen, T. Oth, W.T.V. Germeraad, G.M.J. Bos, J. Vanderlocht, Natural Killer Cells: The Secret Weapon in Dendritic Cell Vaccination Strategies, Clin Cancer Res, 20 (2014) 1095-1103.
[40] A. Marcus, D.H. Raulet, Evidence for Natural Killer Cell Memory, Current Biology, 23 (2013) R817-R820.
[41] S. Paust, U.H. von Andrian, Natural killer cell memory, Nat Immunol, 12 (2011) 500-508.
[42] A. Xie, T. Belcik, Y. Qi, T.K. Morgan, S.A. Champaneri, S. Taylor, B.P. Davidson, Y. Zhao, A.L. Klibanov, M.A. Kuliszewski, H. Leong-Poi, A. Ammi, J.R. Lindner, Ultrasound-mediated vascular gene transfection by cavitation of endothelial-targeted cationic microbubbles, JACC Cardiovasc Imaging, 5 (2012) 1253-1262.
[43] N. Nomikou, P. Tiwari, T. Trehan, K. Gulati, A.P. McHale, Studies on neutral, cationic and biotinylated cationic microbubbles in enhancing ultrasound-mediated gene delivery in vitro and in vivo, Acta Biomater, 8 (2012) 1273-1280.
[44] G.J. Randolph, V. Angeli, M.A. Swartz, Dendritic-cell trafficking to lymph nodes through lymphatic vessels, Nat Rev Immunol, 5 (2005) 617-628.
[45] S. Henri, D. Vremec, A. Kamath, J. Waithman, S. Williams, C. Benoist, K. Burnham, S. Saeland, E. Handman, K. Shortman, The dendritic cell populations of mouse lymph nodes, J Immunol, 167 (2001) 741-748.
[46] C.M. Newman, T. Bettinger, Gene therapy progress and prospects: ultrasound for gene transfer, Gene Ther, 14 (2007) 465-475.

SUPPLEMENTARY DATA

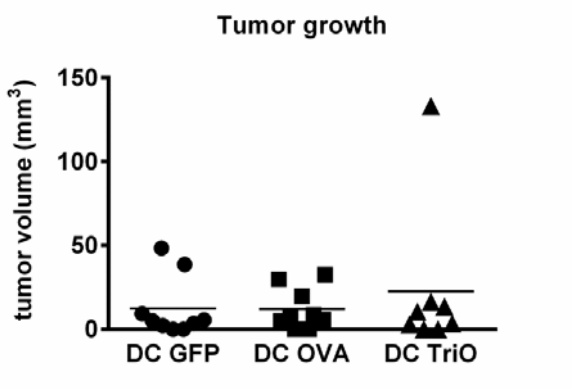


Figure S1. Randomization for the MO4 therapeutic vaccination experiment. 10 days after MO4 tumor inoculation, mice were randomized in three treatment groups based on tumor volume. Tumor volumes were analyzed via a one-way ANOVA followed by a Bonferroni multiple comparison test. No significant differences were observed at the 5% significance level.

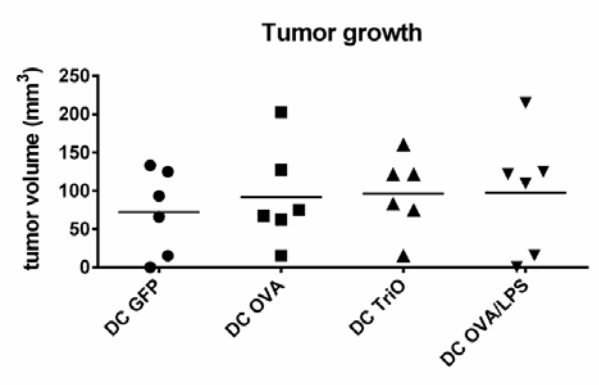


Figure S2. Randomization for the E.G7-OVA therapeutic vaccination experiment. 10 days after E.G7-OVA tumor inoculation, mice were randomized in four treatment groups based on tumor volume. Tumor volumes were analyzed via a one-way ANOVA followed by a Bonferroni multiple comparison test. No significant differences were observed at the 5% significance level.