Supporting information

of

Broadening the Message: A Nanovaccine Co-loaded with Messenger RNA and α-GalCer Induce Antitumor Immunity Through Conventional and Natural Killer T Cells

Rein Verbeke†, Ine Lentacker†, Karine Breckpot†, Jonas Janssens§, Serge Van Calenbergh§, Stefaan C. De Smedt**#, and Heleen Dewitte††#

† Ghent Research Group on Nanomedicines, Faculty of Pharmacy & Cancer Research Institute Ghent (CRIG), Ghent University Hospital, Ghent University, Ghent, Belgium

‡ Laboratory of Molecular and Cellular Therapy, Department of Biomedical Sciences, Vrije Universiteit Brussel (VUB), Jette, Belgium

§ Laboratory for Medicinal Chemistry, Faculty of Pharmacy, Ghent University, Ghent, Belgium

# Senior authors contributed equally to this work.

KEYWORDS: iNKT cells, T cell, mRNA vaccine, nanoparticle, checkpoint inhibition, α-Galactosylceramide, modified nucleotides
**Figure S1.** Intracellular uptake of α-GC by BM-DCs. Overlay of a transmission and confocal fluorescence image showing the nucleus in blue (DAPI) and BODIPY-labeled α-GC in green, 24h after addition of mRNA Galsomes (incorporating fLuc mRNA) or an equimolar amount of α-GC dissolved in DMSO (in the figure referred to as “unformulated α-GC”) to BM-DCs.

**Figure S2.** mRNA transfection efficiency of mRNA Galsomes in (A) murine BM-DCs and (B) human peripheral blood monocyte-derived DCs. Transfections were performed in serum-containing medium, using nucleoside-modified mRNA encoding eGFP. Panels show representative flow cytometry scatter plots of eGFP expression, along with the percentage of eGFP transfected DCs, 24h after the cells were incubated with eGFP-mRNA Galsomes (n=3). Mock-transfected DCs (fLuc mRNA, 5meC, ψ) served as controls.
Figure S3. Transfection experiments in C57BL/6 mice with DOTAP-cholesterol mRNA LNPs. Graph shows expression levels of nucleoside-modified fLuc mRNA in isolated lungs and spleen 6h after i.v. injection of mRNA LNPs at decreasing doses (i.d., mRNA LNPs corresponding to 10 µg down to 2.5 µg mRNA) (n=3).

Figure S4. Adjuvant effect of mRNA Galsomes on BM-DCs in vitro. Expression of the maturation markers CD40 (A) and CD80 (B) on BM-DCs was evaluated 24h after transfection with mRNA Galsomes encoding fLuc. Untreated cells and cells stimulated with E. coli-derived lipopolysaccharide (0.2 µg ml⁻¹ LPS), were used as negative and positive controls, respectively. Representative histograms of transfected cells shows that mRNA Galsomes had no direct adjuvant effect on DCs, and retain their capacity to acquire an activated phenotype, as they were able to mature upon LPS-stimulation to a similar extent as the positive control.
Figure S5. Toxicity evaluation of mRNA Galsomes in monotherapy and combined with anti-PD-L1 antibodies. (A) To evaluate liver toxicity, ALT enzyme activity was measured in serum, collected 6h after one administration of mRNA Galsomes encoding OVA (with or without anti-PD-L1 antibodies), or 72h after two combination therapies (n=4). (B) Animal weight was monitored as a means to evaluate treatment toxicity. Treatment with mRNA Galsomes resulted in a slight drop in weight (<5% of total body weight), which restored a few days after administration. Panel (C) shows organ sections of liver, lungs and spleen 24h after administration of mRNA Galsomes, which were stained with hematoxylin and eosin, and examined for tissue inflammatory reactions by a pathologist in a blinded manner. All organs showed normal tissue morphologies, without any signs of necrosis or apoptosis or induction of inflammation.
Figure S6. Nanoparticles with nucleoside-modified mRNA alone could not break the immune tolerance and inhibit the tumor growth in established E.G7-OVA tumors. Average tumor growth curve of E.G7-OVA tumor bearing mice after systemic administration of liposomes with OVA-encoding nucleoside-modified mRNA alone versus treatment with mRNA Galsomes. Mice were vaccinated on day 8 and day 12 after tumor inoculation. Untreated animals were used as control group (n=6).

Figure S7. Comparison between one or two administrations of mRNA Galsomes to achieve antitumor immunity in E.G7-OVA bearing mice. Graphs show the individual tumor growth curves of E.G7-OVA bearing mice treated with one (day 8) or two administrations (day 8 and day 12) of mRNA Galsomes (incorporating OVA-encoding mRNA), showing no additional boost-effects. Data are obtained from two independent experiment (n=7 for single administration, n=6 for repeated administration).
Figure S8. Flow cytometric analysis of the immune filtrate of isolated tumors. Figure shows representative flow plots of tumors of mRNA Galsome-treated mice, isolated two days after the second administration (day 14). (A) Gating strategy to select viable cells (Zombie Yellow live/dead Stain). Additional antibody panels were used to evaluate (B) T cell infiltration with PD-1 expression and OVA specificity, (C) presence of iNKT cells and (D) NK cells, (E) accumulation of MDSCs and (F) TAMs, with their expression of MHC-II. (G) PD-L1 surface expression on tumor cells (CD45− fraction) and DCs (CD45+ CD11c+).
**Figure S9.** Vaccination with OVA-encoding mRNA Galsomes induces increased PD-L1 expression levels on systemic and intratumoral APCs, and on tumor cells. (A) Representative histograms are shown of the PD-L1 expression on APCs (CD11c+) in the spleen, which was measured 6h after vaccination and compared to untreated mice (n=3). (B) Representative histograms of PD-L1 expression on intratumoral APCs (CD45+, CD11c+) and (C) tumor cells (CD45), measured in single cell suspensions of isolated B16-OVA tumors at day 14 (two days after a second administration of mRNA Galsomes, n=5).

**Figure S10.** Comparison between the cytokine responses after a first and second exposure to mRNA Galsomes. Cytokine responses were shifted towards Th2 polarization after a second exposure of mRNA Galsomes (6h post-injection), with higher levels of IL-4 and IL-10, at cost of the production of IFN-γ and IL-12p70 compared to the first administration. mRNA encoding OVA was used for these experiments.
Preparation of the 6″-BODIPY-analogue of α-GalCer (3)

Compound 1 (0.054 g, 0.061 mmol, 1 equiv.) was taken up in water (1 ml) and DMF (1 ml), followed by the addition of green BOPIDY-alkyne 2 (0.046 g, 0.140 mmol, 2.6 equiv.), copper(II) sulfate (0.048 ml of a 0.5 M solution in water, 0.024 mmol, 0.4 equiv.), sodium ascorbate (0.244 ml of a 0.5 M solution in water, 0.122 mmol, 2.0 equiv.) and tris[(1-benzyl-1H-1,2,3-triazol-4-yl)methyl]amine (TBTA; 0.003 g, 0.006 mmol, 0.1 equiv.). After 24 h of stirring at room temperature, TLC-analysis (CH₂Cl₂/MeOH 90:10) showed near complete conversion of the starting material. The solvent was removed under reduced pressure and the crude material was co-evaporated with toluene (5 × 4 ml) to remove any traces of water. The product was purified by preparative TLC (silica), using CH₂Cl₂/MeOH 90:10 as the eluent, affording the title compound 3 (0.029 g, 39% yield) as an orange powder. The compound is unstable and should be stored at -30°C or lower to prevent degradation.

¹H NMR (300 MHz, CD₃OD): δ = 7.76 (s, 1H), 6.11 (s, 2H), 4.82 (d, J = 3.5 Hz, 1H), 4.62–4.55 (m, 2H), 4.21 (t, J = 6.1 Hz, 1H), 4.09 (dd, J = 4.4, 10.4 Hz, 1H), 3.81 (dd, J = 1.5, 2.7 Hz, 1H), 3.78 (dd, J = 3.3, 8.5 Hz, 1H), 3.72 (dd, J = 3.1, 10.3 Hz, 1H), 3.55–3.44 (m, 3H), 3.41 (dd, J = 4.4, 10.1 Hz, 1H), 3.04–3.00 (m, 2H), 2.79 (t, J = 7.5 Hz, 2H), 2.43 (s, 6H), 2.42 (s, 6H), 2.15 (t, J = 7.5 Hz, 2H), 1.92 (t, J = 7.5 Hz, 2H), 1.85–1.18 (m, 74H), 0.92–0.89 (m, 6H).

HRMS (ESI-MS) m/z: calcd for C₆⁹H₁₂₂BF₂N₆O₈ [M+H]⁺ 1211.9380, found 1211.9369

Rf(CH₂Cl₂/MeOH 90:10): 0.37

The preparation of this precursor has been previously reported:
