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

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ABSTRACT: Messenger RNA encoding tumor antigens has the potential to evoke effective antitumor immunity. This study reports on a nanoparticle platform, named mRNA Galsomes, that successfully co-delivers nucleoside-modified antigen-encoding mRNA and the glycolipid antigen and immunopotentiator α-Galactosylceramide (α-GC) to antigen-presenting cells after intravenous administration. By co-formulating low doses of α-GC, mRNA Galsomes induce a pluripotent innate and adaptive tumor-specific immune response in mice, with invariant natural killer T cells (iNKT) as a driving force. In comparison, mRNA Galsomes exhibit advantages over the state of the art cancer vaccines using unmodified ovalbumin (OVA)-encoding mRNA, as we observed up to seven times more tumor-infiltrating antigen-specific CTLs, combined with a strong iNKT cell and NK cell activation. In addition, the presence of suppressive myeloid cells (myeloid-derived suppressor cells and tumor-associated macrophages) in the tumor micro-environment was significantly lowered. Owing to these antitumor effects, OVA mRNA Galsomes significantly reduced tumor growth in established E.G7-OVA lymphoma, with a complete tumor rejection in 40% of the animals. Moreover, therapeutic vaccination with mRNA Galsomes enhanced the responsiveness to treatment with a PD-L1 checkpoint inhibitor in B16-OVA melanoma, as evidenced by a synergistic reduction of tumor outgrowth and a significantly prolonged median survival. Taken together, these data show that intravenously administered mRNA Galsomes can provide controllable, multi-faceted and effective antitumor immunity, especially when combined with checkpoint inhibition.

Where for long the use of mRNA was limited due to its perceived instability, it is nowadays possible to successfully deliver mRNA *in vivo*.[1] This is strongly supported by two recent breakthroughs: (i) the packaging of mRNA molecules inside nanoparticles, designed to improve the selective cell targeting and cytosolic delivery of mRNA [2-9] and (ii) the technical progress in the mRNA construct, including the incorporation of modified nucleotides, yielding more stable mRNA with an improved translation capacity.[10-13] Particularly in the field of vaccination, mRNA encoding antigens has emerged as a versatile and promising platform.[14] Preclinical studies and first clinical trials with mRNA-based vaccines have shown to afford protective humoral responses, as well as strong cellular responses against diverse diseases.[5, 15, 16]

In the field of cancer immunotherapy, Kranz *et al.* provided first-in-human proof that by targeting mRNA lipid nanoparticles (LNPs) to dendritic cells (DCs), cytotoxic T cell (CTL) responses were induced against the encoded tumor antigens.[5] They and others demonstrated that besides the successful mRNA expression by DCs, the mode of action of mRNA vaccines largely depends on the induction of type I interferon (IFN).[6, 17-19] More specifically, upon cellular entry, mRNA molecules trigger innate immune activation pathways, including the endosomal Toll-like receptor (TLR)-7 and cytosolic receptors MDA-5 (melanoma differentiation-associated protein 5) and RIG-I (retinoic acid-inducible gene I), which results in type I IFN signaling and the induction of an antiviral immune response. Importantly, virtually all mRNA vaccines in clinical development rely on this inherent self-adjuvant effect of mRNA. However, it was shown that type I IFN signaling acts as a double-edged sword as the evoked immune response prematurely stops mRNA-translation, thereby lowering antigen bioavailability.[20, 21] Moreover, it was suggested that type I IFN, depending on the relative timing to T cell priming, can either positively or negatively affect T cell responses, with pre-exposure to type I IFN resulting in T cell exhaustion and apoptosis.[22, 23] In addition, high

levels of IFN-α can induce adverse effects ranging from flu-like symptoms to autoimmune sequelae and even life-threatening events.[24, 25]

Besides issues related to vaccine-induced type I IFN secretion, the current mRNA vaccines often fall short to evoke durable antitumor immunity. Indeed, while mRNA-based cancer vaccines show optimal properties for the activation of cytotoxic T lymphocyte (CTL) responses, such as the preferential antigen presentation *via* MHC-I molecules and the activation of type I IFN-dependent immunity, the evoked immunity is generally counteracted by a plethora of tumor-mediated immunosuppressive mechanisms. First of all, tumor cells may have an impaired or deficit presentation of antigens, making them invisible to CTLs.[26] Additionally, during an immune attack and as a result of the production of interferons, immune checkpoint pathways are activated as mechanisms to resist adaptive immunity, such as the expression of programmed cell death 1 (PD-1) ligand (PD-L1) by tumor cells, antigen-presenting cells (APCs) and its receptor PD-1 on the effector cells.[27-29] Moreover, tumor-infiltrating CTLs have to deal with immune resistance mediated by various suppressive cells, including alternatively activated M2 macrophages, myeloid derived suppressor cells (MDSCs) and regulatory T cells.[30] Thus, ideally, mRNA vaccines should not solely focus on the activation of CTLs, but should more broadly harness the host's immune system to tackle these different suppressive mechanisms.

To address these issues, continued research is required to develop more safe mRNA vaccines, while further improving their effectiveness for cancer immunotherapy. Here, we propose an mRNA nanovaccine, which explores the combined delivery of immunosilent, nucleoside-modified mRNA and the glycolipid α-galactosylceramide (α-GC), to achieve broad and durable, but controllable and effective antitumor immunity.

By using mRNA containing pseudouridine (Ψ) and 5-methylcytidine (5meC), we choose to minimize the immune recognition of mRNA and associated anti-mRNA mechanisms and toxicity issues. As such, we have previously established a liposomal formulation for mRNA delivery, composed of DOTAP (1,2-dioleoyl-3-trimethylammonium-propane) and cholesterol, efficiently targeting DCs after intravenous (i.v.). administration. By using nucleoside-modified mRNA, we obtained highly improved mRNA expression levels, while strongly reducing the release of type I IFN.[31]

In addition, we hypothesized that instead of inducing type I IFN responses, pairing nucleoside-modified mRNA with α-GC could hold numerous appealing features for cancer immunotherapy. α-GC is a well-known glycolipid antigen that, when presented by the MHC-I-like molecule CD1d on APCs, leads to the potent activation of invariant natural killer T cells (iNKT). This subset of unconventional T cells contributes to innate and adaptive immunity, but can also exert direct and indirect antitumor effects.[32-34] Unlike the classical immune adjuvants that directly trigger danger pathways, α-GC exerts an indirect adjuvant effect through the bidirectional interaction between α-GC-presenting DCs and iNKT cells.[35] As such, iNKT cells can activate DCs through the interaction between CD40 and CD40 ligand, evoking the production of cytokines (*e.g.,* IL-12p70) and expression of co-stimulatory receptors (*e.g.,* CD80, CD86, and CD70). In turn, when activated, iNKT cells proliferate and secrete a wide range of cytokines (*e.g.,* IL-2, IFN-γ, IL-4 and TNF-α), which further promotes the recruitment and activation of DCs, B cells, and T cells. Furthermore, iNKT cells are known to induce the down-stream activation of NK cells, to exhibit direct tumor killing effects (*e.g., via* CD1d recognition) and have been shown to positively modulate tumor-associated macrophages (TAMs) and MDSCs in the tumor microenvironment.[36-39]

In order to efficiently co-deliver nucleoside-modified mRNA and the iNKT ligand α-GC, we developed a nanoformulation, which will be referred to as “mRNA Galsomes”. The potential

of this formulation was evaluated by investigating its adjuvancy, the activation of antigen-specific T cell responses, NK- and iNKT cells, and therapeutic effectiveness in two tumor models. Importantly, the i.v. delivery of mRNA Galsomes was compared to the current "gold standard" in mRNA vaccination, which is currently under clinical investigation and consists of LNPs loaded with immunogenic (unmodified) mRNA and therefore rely on the mRNA's self-adjuvanticity and corresponding type I IFN to drive immunity.[5] Finally, we evaluated if we could further improve the therapeutic outcome by co-administering checkpoint inhibitors (anti-PD-L1 antibody) to enhance CTL responses and prevent the induction of adaptive resistance (see **Graphical Table of Contents**).

RESULTS

mRNA Galsomes: DOTAP-cholesterol liposomes as delivery agent for nucleoside-modified mRNA and α-GC

Previously, we succeeded in developing a liposomal formulation for i.v. injection composed of DOTAP and cholesterol, that can be used to complex mRNA and retains it full transfection potential in serum.[31] DOTAP/cholesterol LNPs loaded with mRNA remain intact in biologically relevant media, as shown by a stable particle size when incubated in human serum for up to 24h, and ensure a durable protection of the mRNA cargo from degradation. A schematic representation of mRNA Galsomes is shown in **Figure 1A**. We demonstrate that the physicochemical properties of mRNA Galsomes containing 1 mol% of a-GC were unaltered compared to mRNA LNPs formulated without α-GC, with a mean size of 190 nm and a zeta potential of +47 mV (**Figure 1B**).

By co-formulating the α-GC and the antigen-encoding mRNA in a single nanoparticle system, it is possible to achieve simultaneous delivery of both components to the same DCs, thus ensuring optimal synchronization between the interactions of the particle-laden DCs with

$CD8^+$ T cells on one hand, and the iNKT cells on the other hand. In this regard, it is important to note that both nanoparticle cargos, mRNA and α-GC, have different requirements regarding their intracellular delivery. mRNA should escape from the endosomes to the cytoplasm to allow adequate antigen synthesis by the ribosomes. By contrast, α-GC must accumulate in late endosomes and lysosomes of DCs, where it is loaded on CD1d molecules and presented to iNKT cells. As such, we first estimated the delivery efficiency for both mRNA and α-GC, using DOTAP-cholesterol liposomes as carrier system, and assessed whether or not the immune response that occurs upon α-GC presentation to iNKT cells affects the mRNA expression levels.

To evaluate α-GC delivery, bone marrow-derived DCs (BM-DCs) were incubated either with α-GC alone (dissolved in DMSO) or mRNA Galsomes (containing equal doses of α-GC). When using BODIPY-labeled α-GC, it was clear that the intracellular delivery of α-GC was up to ten timer higher by the delivery *via* mRNA Galsomes (**Figure 1C**). This was confirmed by representative confocal microscopy images of BM-DCs after uptake of BODIPY-labeled α-GC, either by incubation of the cells with α-GC alone or α-GC formulated in mRNA Galsomes (**Supplementary Figure S1**). This also translated into an enhanced presentation of α-GC by CD1d: we observed a threefold increase in the presentation of α-GC *via* CD1d complexes compared to α-GC alone (**Figure 1D**).

To determine the potency of mRNA Galsomes to initiate an immune response, we performed a dose-response study by injecting mice i.v. with mRNA Galsomes containing a fixed dose of 10 µg mRNA and decreasing doses of α-GC (**Figure 1E**). Twelve hours post-injection, iNKT cell activation was measured indirectly by quantifying the levels of IFN-γ in serum. When injecting mRNA Galsomes containing 1.4 µg α-GC, a dose which corresponds to the amount of α-GC that is typically administered systemically in mice, levels of IFN-γ up to 25 000 pg ml^{-1} could be detected in serum.[35] Although this indicates that mRNA Galsomes are very

potent to induce immune responses, this coincided with splenomegaly in all of the animals. However, the levels of IFN-γ could easily be refined by dose adjustments of α-GC. Importantly, when drastically lower doses were used, down to 20 ng α-GC per mouse (or 0.015 mol% of the total amount of lipids in the nanoparticle), this still resulted in high levels of IFN-γ (~4000 pg ml^{-1}), but without any signs of acute toxicity. Therefore, we used mRNA Galsomes packaging 20 ng of α-GC for further experiments.

Previous attempts where mRNA was combined with other adjuvants have raised compatibility issues, since DC maturation can prematurely abrogate cellular uptake mechanisms (*e.g.,* macropinocytosis) or potentially induce anti-mRNA defense mechanisms (*e.g.,* type I IFN signaling) leading to fast mRNA degradation.[40, 41] For these reasons we investigated the impact of α-GC inclusion on mRNA translation. Firstly, *in vitro* experiments in murine BM-DCs as well as human monocyte-derived DCs demonstrated that mRNA Galsomes could efficiently transfect both cell types with eGFP-encoding mRNA in serum-containing medium (**Supplementary Figure S2**). In addition, we performed transfection experiments in mice, using mRNA encoding firefly luciferase (fLuc) as reporter gene. Mice were injected i.v. with nanoparticles encapsulating different cargos; (i) unmodified mRNA (unmod. mRNA LNPs), (ii) nucleoside-modified mRNA alone (mod. mRNA LNPs), or (iii) nucleoside-modified mRNA combined with α-GC (mRNA Galsomes). Bioluminescence was evaluated 6h later. **Figures 1F-G** demonstrate that incorporation of α-GC did not interfere with the translation of mRNA in lungs and spleen, and that increasing mRNA doses up to 10 μg per animal, resulted in a higher protein expression in the lungs and spleen (**Supplementary Figure S3**).

As expected, nanoparticles containing unmodified fLuc mRNA display significantly lower expression levels, which results from their lower intracellular stability, as well as from type I IFN-mediated antiviral pathways programmed to degrade and avoid the translation of mRNA.[41] Together, these results indicate that co-packaging of nucleoside-modified mRNA

and α-GC improves α-GC delivery and presentation, without affecting the mRNA expression levels.

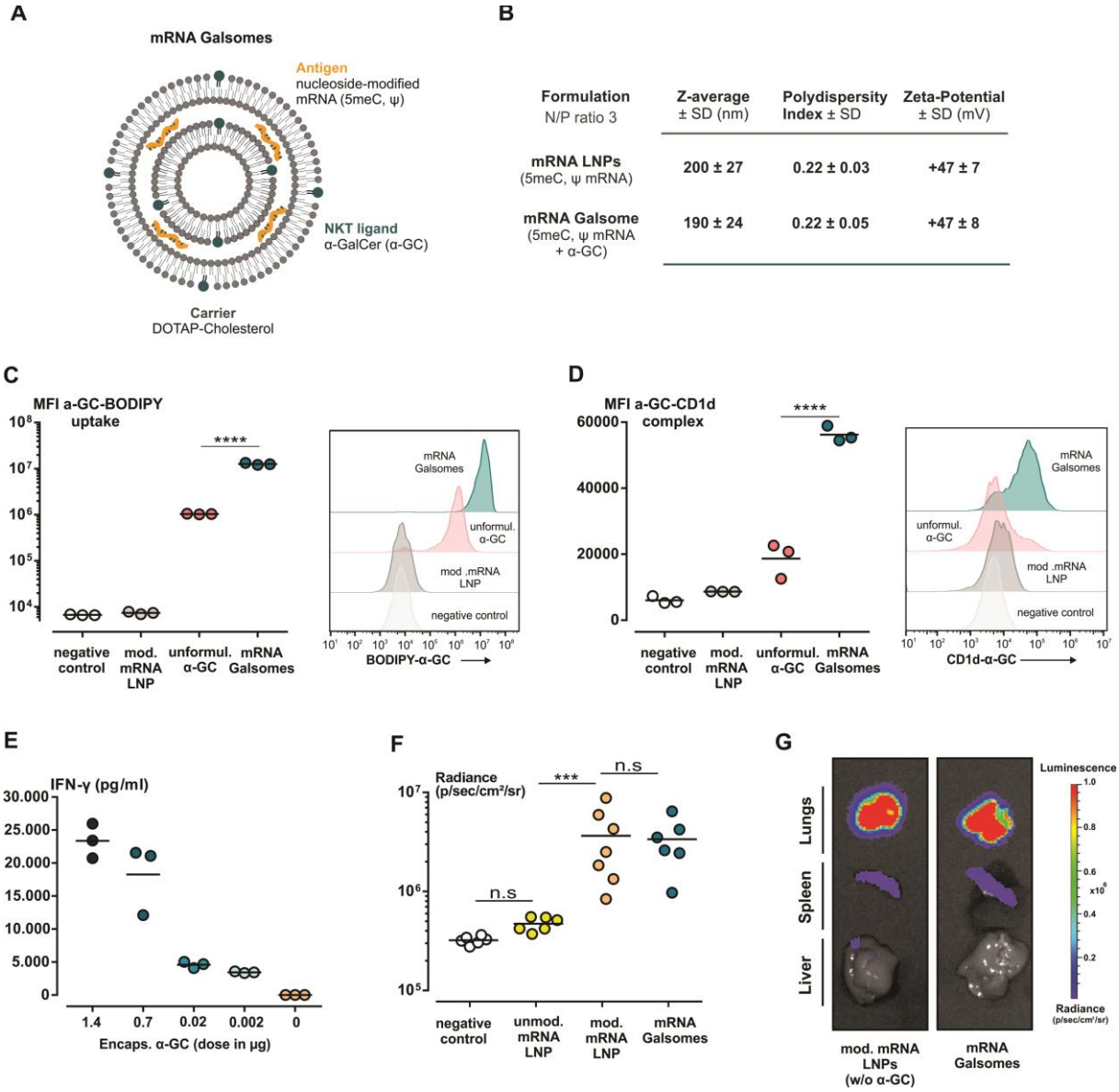

Figure 1. mRNA Galsomes promote the delivery of α-GC to DCs without affecting mRNA transfection. **(A)** Schematic representation of a nanoparticle consisting of DOTAP-cholesterol LNPs, nucleoside-modified mRNA (5meC, ψ) and the NKT ligand α-GC. (B) Size (Z average), polydispersity index (PdI) and zeta potential of mRNA LNPs dispersed in HEPES buffer, formulated with or without α-GC (*i.d.* 1 mol% of total lipids, n=3). A quantitative flow cytometric analysis of the increase in intracellular delivery of BODIPY-labeled α-GC

formulated in mRNA Galsomes is shown in **(C)**, where untreated cells served as negative controls. **(D)** Enhanced surface presentation of α-GC in CD1d complexes by BM-DCs 24h after incubation with α-GC in mRNA Galsomes. (panels C-D show representative data of three independent experiments, untreated cells serve as negative controls). **(E)** Dose-response experiment: IFN-γ levels in serum of C57BL/6 mice 12h after i.v. injection of mRNA Galsomes containing decreasing doses of α-GC. **(F)** Graph summarizing whole-body expression levels of fLuc mRNA in C57Bl/6 mice 6h after i.v. injection of nanoparticles containing different cargos, or PBS (negative control); liposomes containing unmodified mRNA, nucleoside-modified- or nucleoside-modified mRNA nanoparticles formulated with α-GC (*n*=6-7, pooled from two independent experiments) **(G)** Representative bioluminescence images of isolated organs (lungs, spleen and liver). For all these experiments, mRNA encoding fLuc was used. Statistical analyses on datasets were performed by One-Way ANOVA followed by Turkey's post hoc test. Asterisks indicate statistical significance (n.s., $p > 0.05$; ***, $p < 0.001$; ****, $p < 0.0001$).

mRNA Galsomes mediate strong adjuvant effects and activate dendritic cells *in vivo*

Immune activation by α-GC is an indirect phenomenon: DCs that present α-GC by CD1d will stimulate iNKT cells, which, in turn, cause phenotypic maturation of DCs by CD40-CD40-ligand interaction. To assess if this is also the case for mRNA Galsomes, especially with the low (20 ng) α-GC doses used, we investigated the maturation status of splenic DCs 24h after particle injection (**Figure 2A-B**). We could observe a strong and significant up-regulation of the activation markers CD40 and CD86 on splenic DCs of mice treated with mRNA Galsomes, relative to untreated mice and mice injected with LNPs containing nucleoside-modified mRNA without α-GC. Importantly, no DC maturation was observed when mRNA Galsomes were added to BM-DC cultures *in vitro*, in the absence of iNKT cells. This

indicates that this *in vivo* maturation effect was mediated by the ligation with iNKT cells (**Supplementary Figure S4**).

To investigate the immune response in more detail, a broad screening of inflammatory cytokines was performed in blood of animals 6h after vaccination (**Figure 2C**). As expected, nanoparticles containing unmodified mRNA induced a strong release of IFN-α, while this was not the case with nucleoside-modified mRNA nanoparticles (with or without α-GC). By contrast, mRNA Galsomes induced a pronounced production of IFN-γ and IL-4. What is more, in the group receiving mRNA Galsomes, we could also detect the presence of T cell-stimulating cytokines, such as IL-12p70 and IL-27, and elevated levels of IL-6, TNFα and IL-17. Importantly, this broad spectrum of cytokines did not induce visible toxicity symptoms (changes in body condition score, behavior, global appearance or posture) and no pathological changes were identified in H&E stained organ sections of lungs, spleen and liver, while normal levels of ALT activity were measured (**Supplementary Figure S5**).

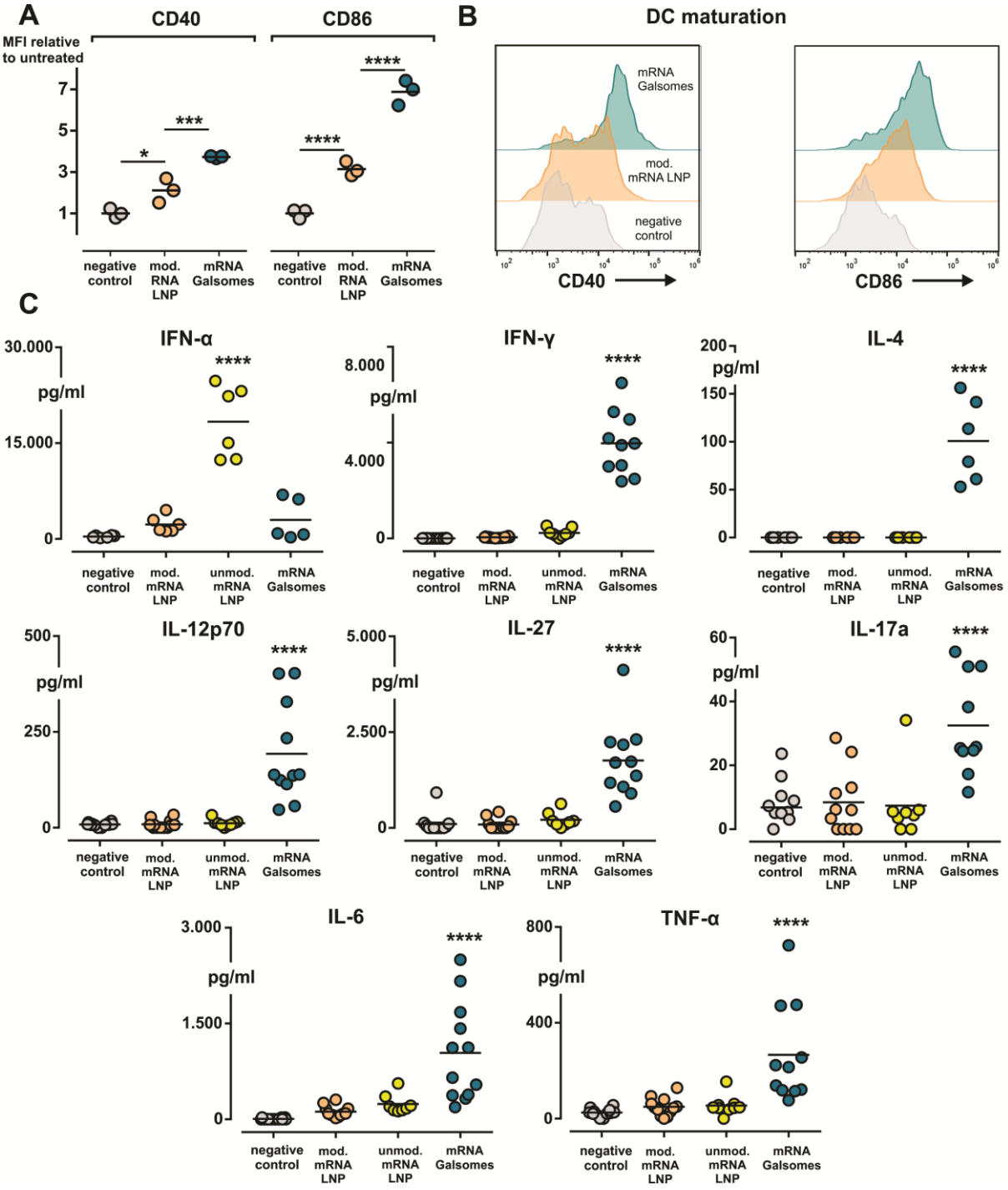

Figure 2. mRNA Galsomes containing low doses of α-GC (20 ng) induce the maturation of dendritic cells *in vivo* and stimulate the release of immunostimulatory cytokines. **(A)** Splenic DCs ($CD11c^+$) showed an increase in the expression of the activation markers CD40 and CD86 (expressed as a fold change in MFI) 24h after administration of mRNA Galsomes, while incomplete maturation was observed with nanoparticles containing nucleoside-modified

mRNA alone (*n*=4). **(B)** Representative histograms of CD40 and CD86 expression by $CD11c^+$ cells. **(C)** Serum samples were collected at 6h post-injection and screened for the release of inflammatory cytokines: while nanoparticles containing unmodified mRNA trigger the production of IFN-α, mRNA Galsomes induce distinct cytokine responses, including IFN-γ, IL-4, IL-12p70, IL-27, IL-17a, IL-6 and TNF-α. For all experiments in this figure, fLuc mRNA was used. Data are pooled from at least two independent experiments. Mice injected with PBS serve as negative controls. Statistical analyses on datasets were performed by One-Way ANOVA followed by Turkey's post hoc test. Asterisks indicate statistical significance compared to negative control (****, $p < 0.0001$).

mRNA Galsomes as pluripotent inducers of immunity

One could expect that the increased mRNA expression levels combined with strong DC maturation, and the production of CTL-inducing cytokines such as IL-12p70, hold potential for mRNA Galsomes to compete with type I IFN-dependent mRNA vaccines. In addition to T cell-mediated immunity, the combination with α-GC could also offer the advantage of activating both iNKT- and NK cells shaping a broader and potentially synergistic antitumor immunity.

To evaluate these multiple effector responses, animals were immunized with mRNA encoding chicken ovalbumin (OVA) as a model antigen. Six days after immunization, cell numbers of OVA-specific CTLs were measured in isolated spleens (**Figure 3A-B**). Interestingly, we observed that mRNA Galsomes generated four to five times higher levels of OVA-specific CTLs compared to mice treated with nanoparticles containing unmodified OVA mRNA.

To evaluate the proliferation of iNKT and down-stream NK cell responses, spleen and liver were isolated three days after vaccination. Corresponding to the cytokine responses (**Figure 2B**), we observed an increased proliferation of iNKT cells, from 0.8 to 2.3% in spleen and 4.4

to 14% in liver (**Figure 3C-D**). Accordingly, mRNA Galsomes also mediated the proliferation of NK cells as their levels increased from 2.2 to 3.8 % and 4.7 to 12%, in spleen and liver, respectively. (**Figure 3E**).

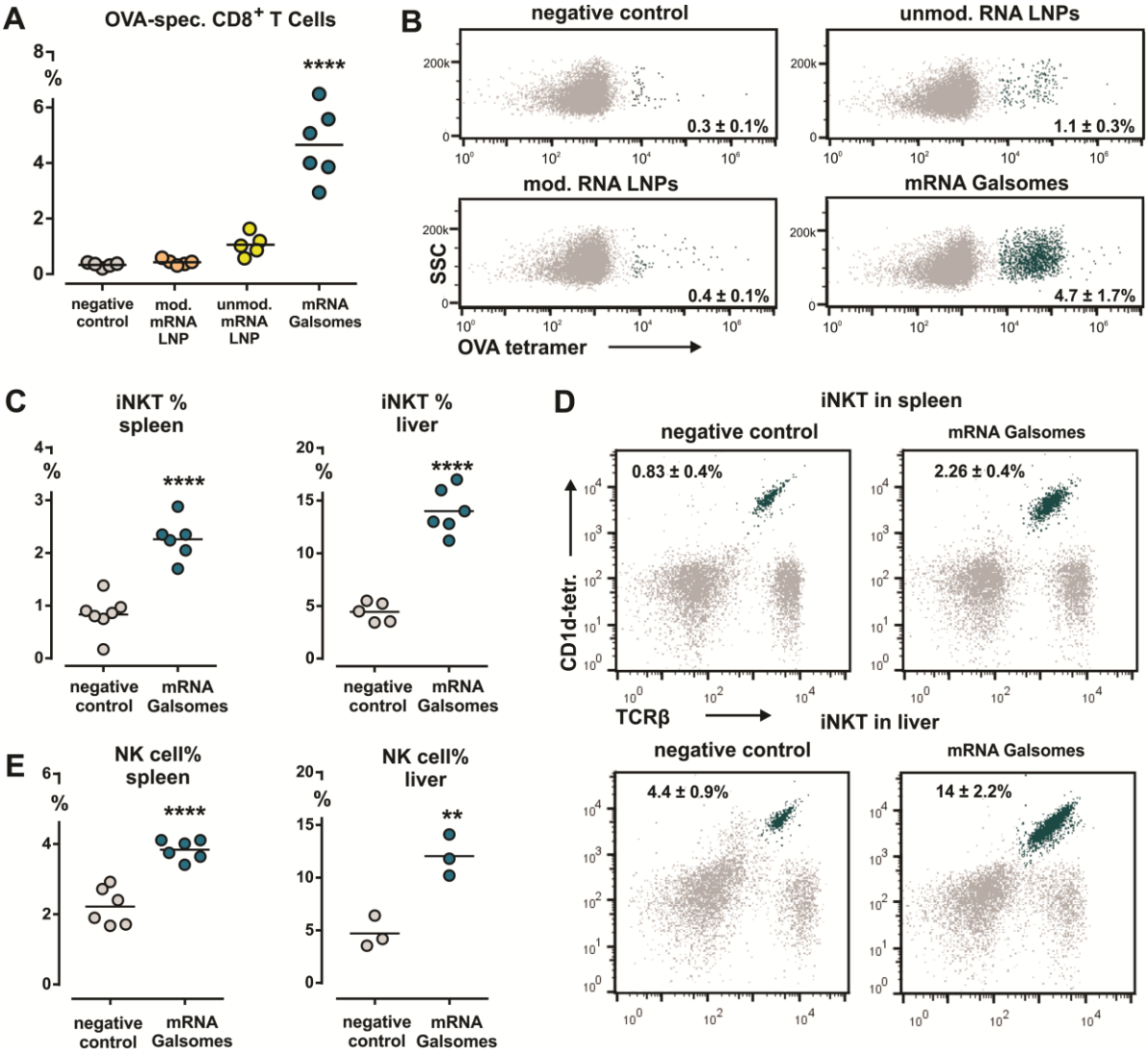

Figure 3. mRNA Galsomes mediate pluripotent innate and adaptive immune responses**. (A)** mRNA Galsomes strongly mediate the induction of antigen-specific $CD8^+$ T cell responses. Mice were immunized with OVA mRNA LNPs. Six days later, percentages of OVA-specific splenic $CD8^+$ T cells were measured using an H-2kb OVA tetramer staining. **(B)** Representative flow cytometry scatter plots of OVA-specific $CD8^+$ T cells. Blue events represent tetramer-stained $CD8^+$ cells. **(C)** Three days post-injection of mRNA Galsomes,

spleen and liver displayed expanded iNKT cell numbers compared to untreated mice. **(D)** Representative flow plots of iNKT cells in spleen and liver (Blue events indicate $TCR\beta^+$, mCD1d PBS-57^+ cells). **(E)** Down-stream activation of NK cells ($CD3^-$, $NK1.1^+$ cells). The data in this figure (n=6) are pooled from two independent experiments. PBS-injected mice serve as negative controls. Statistical analyses on datasets were performed by One-Way ANOVA followed by Turkey's post hoc test for (A). Pairwise comparisons in sections (C) and (E) were performed *via* a student's t test. Asterisks indicate statistical significance (**, $p < 0.01$; ****, $p < 0.0001$).

Therapeutic efficacy of mRNA Galsomes in E.G7-OVA lymphoma- and B16-OVA melanoma

To assess the potential of mRNA Galsomes in a therapeutic vaccination study, mice were subcutaneously inoculated with OVA-expressing E.G7 lymphoma cells or B16-OVA melanoma cells and vaccinated when tumors were palpable at day 8 with mRNA encoding OVA.

E.G7-OVA bearing mice were treated with a single administration of either mRNA Galsomes or nanoparticles containing unmodified mRNA, in order to differentiate between the therapeutic potential of nanoparticles that evoke immunity based on iNKT cell activation or a type I IFN response, respectively. First of all, both therapies resulted in a significant reduction in tumor growth and prolonged survival, relative to untreated mice (**Figure 4A-B**). Overall, mice treated with nanoparticles containing unmodified mRNA, showed complete tumor regression in 4/6 animals, and treatment with mRNA Galsomes resulted in complete tumor regression in 3/7 animals, and no significant differences could be observed in tumor growth between both treatment groups. It should be noted that nucleoside-modified mRNA

nanoparticles without α-GC did not have significant effects on tumor growth (**Supplementary Figure S6**).

In a more aggressive B16-OVA melanoma model, animals received three administrations of either mRNA Galsomes or umodified mRNA nanoparticles on day 8, day 12 and day 16 after tumor inoculation. Although we observed a delay of tumor outgrowth, there was only a modest prolongation of survival for mice treated with mRNA Galsomes or nanoparticles containing unmodified mRNA, with median survival of 28 days and 29 days, respectively, compared to 22.5 days for untreated animals (**Figure 4C-D**). In addition, we noticed that multiple doses could not efficiently boost or prolong the antitumor responses. This also occurred in the E.G7-OVA model, where a second (boost) vaccination did not result in better control of tumor outgrowth (**Supplementary Figure S7**).

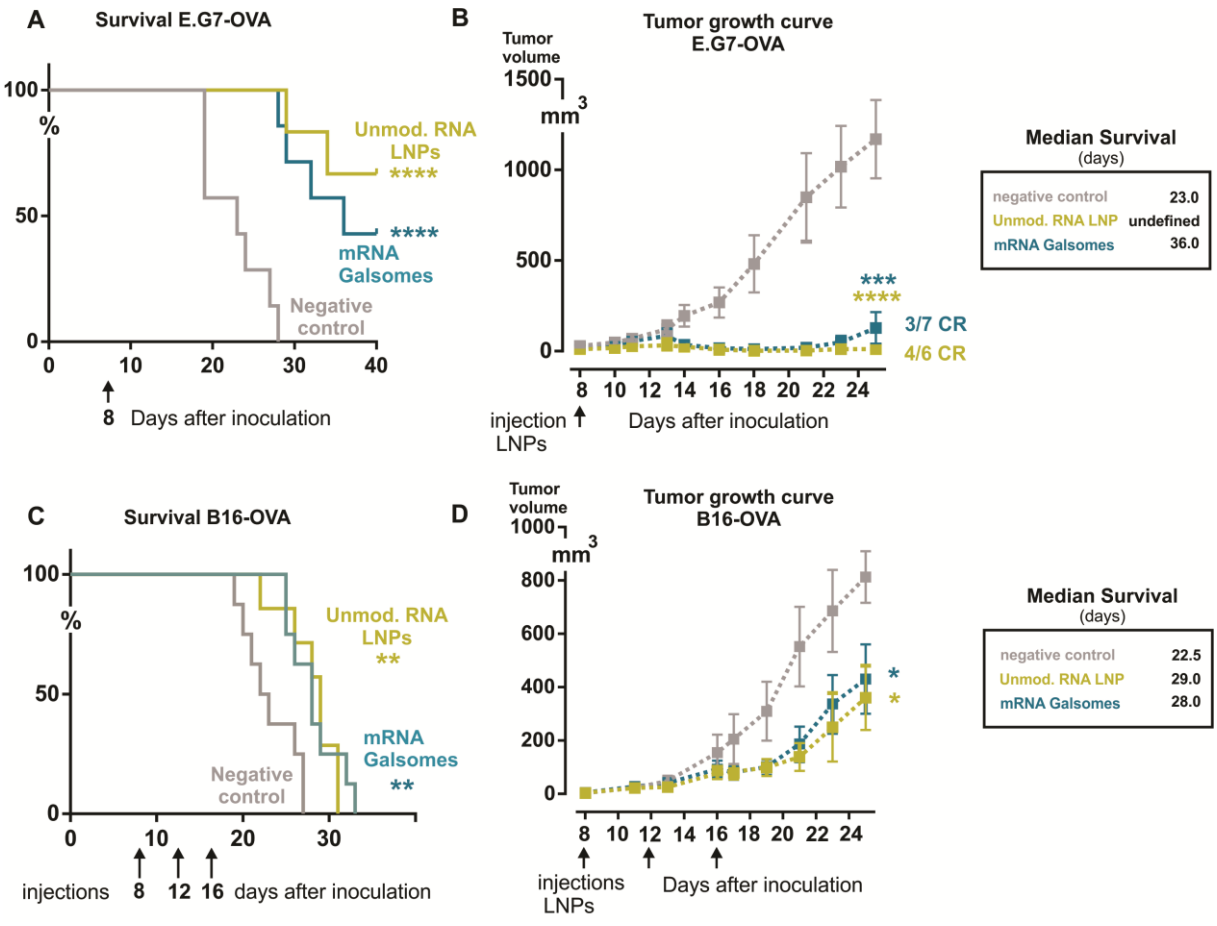

Figure 4. Therapeutic vaccination with OVA mRNA Galsomes or nanoparticles containing unmodified OVA mRNA in E.G7-OVA lymphoma and B16-OVA melanoma model. Mice were subcutaneously inoculated with E.G7-OVA cells or B16-OVA cells ($3x10^5$ cells). E.G7-OVA bearing mice were vaccinated on day 8 when tumors were clearly visible. B16-OVA bearing mice received three vaccinations on day 8, day 12 and day 16. Graphs show Kaplan–Meier survival curves in E.G7-OVA model **(A)** and B16-OVA model **(C),** and the respective tumor growth curves **(B and D)** as a function of time for an untreated control group (negative control), and for mice treated with OVA mRNA Galsomes or nanoparticles containing unmodified OVA mRNA (*n*=7-8). Statistical analysis on the survival curves (A) and (C) was performed using a log-rank (Mantel–Cox) test. In (B) and (D), tumor volumes measured at day 25 were compared by a One-way ANOVA followed by Tukey's post hoc test. Asterisks indicate statistical significance compared to the untreated group (* $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; **** $p < 0.0001$)

mRNA Galsomes promote the tumor infiltration of CTLs, iNKT cells and NK cells, but immune surveillance is hampered by the PD-1/PD-L1 axis

Since complete control of tumor outgrowth was not achieved, we aimed to investigate which immune suppressive mechanisms might be at play to counteract the evoked antitumor immunity by mRNA Galsomes or nanoparticles containing unmodified mRNA. Therefore, experiments were performed where B16-OVA bearing mice were euthanized two days after a second nanoparticle administration (day 14), and a detailed analysis of the tumor immune microenvironment was performed. Tumor and spleen were screened for effector responses and/or suppressive mechanisms that could impact the therapeutic outcome. The most important findings are shown in **Figure 5**. The flow cytometry gating strategy for each cell type can be found in **Supplementary Figure S8**.

First of all, animals treated with mRNA Galsomes exhibited up to five times higher levels of tumor infiltrating OVA-specific CTLs, whereas CTL presence at the tumor site merely doubled after vaccination with nanoparticles containing unmodified mRNA compared to the untreated group (**Figure 5A**). Likewise, vaccinations with mRNA Galsomes resulted in six to seven times higher numbers of CTLs specific for OVA (10% of viable cells in tumor, as determined by SIINFEKL-H2Kb tetramer staining) compared to animals treated with nanoparticles containing unmodified mRNA, while almost no (< 0.04 %) OVA-specific CTLs were detected in the tumors of the untreated animals (**Figure 5B**). In addition, we detected a fourfold increase in iNKT cell numbers in the tumors of mice treated with mRNA Galsomes, compared to the other groups (**Figure 5C**). For both treatments, increased levels of tumor-infiltrating NK cells were observed, with ± 13% NK cells for mRNA Galsomes and ± 11% NK cells for nanoparticles containing unmodified mRNA compared to untreated mice with only ± 5% NK cells (**Figure 5D**). By analyzing the tumor site for suppressive immune cells, we found that the delivery of unmodified mRNA resulted in a twofold increase in MDSCs ($CD11b^+$, $GR1^+$, MHC-II^- cells) compared to untreated controls. Interestingly, this rise in MDSC levels was not observed in animals treated with mRNA Galsomes (**Figure 5E**). Furthermore, we noticed that almost all TAMs ($CD11b^+$, $F4/80^+$ cells) in the mRNA Galsome-group displayed a pro-inflammatory M1-like phenotype, marked by increased levels of MHC-II (**Figure 5F**).

While vaccination with mRNA Galsomes resulted in "*hot*" T cell-infiltrated tumors, we investigated whether immune suppression *via* the PD-1/PD-L1 axis could be involved in countering the vaccine-induced immune response.[29] As shown in **Figure 5G**, LNP vaccination caused a significant up-regulation of PD-1 on the activated OVA-specific splenic CTLs compared to CTLs of untreated mice. These increased PD-1 levels were comparable for mice injected with mRNA Galsomes or LNPs containing unmodified mRNA. Within the

tumor, PD-1 levels on intratumoral CTLs are up to 10 times higher compared to CTLs in the spleen for untreated animals. Although PD-1 expression levels on intratumoral CTLs are lower in vaccinated animals, it remains obvious that in this microenvironment, the influence of the PD-1/PD-L1 axis is much more pronounced compared to the spleen. A different situation is observed for iNKT cells. As shown in **Figure 5H**, mRNA Galsomes not only trigger the proliferation of splenic iNKT cells, but this coincides with a doubling in their PD-1 expression levels. LNPs containing unmodified mRNA without α-GC, did not have this effect. Within the tumor, the crosstalk between iNKT cells and the tumor microenvironment once more results in stronger PD-1 expression in all treatment groups. This PD-1 up-regulation on previously activated iNKT cells matches previous reports where inhibitory signals *via* the PD-1/PDL-1 pathway were suggested to play a role in the loss of responsiveness to subsequent α-GC stimulations after (over-) stimulation. Moreover, this is in line with the limited boost-effect we observed upon multiple injections of the mRNA Galsomes (**Figure 4C-D**).

In addition to expression levels of PD-1, we measured the presence of its ligand on DCs (in spleen and tumor), as well as on tumor cells, specifically for mRNA Galsomes (**Supplementary Figure S9**). Similar to other reports, we observed that 37.5 ± 4.9 % of splenic DCs up-regulated the expression of PD-L1 after vaccination with mRNA Galsomes. Similar to what we observed for intratumoral PD-1 levels, DCs within the tumor exhibit much higher basal levels of PD-L1, which are further increased upon mRNA Galsome vaccination. When evaluating the tumor cells' PD-L1 levels, a fourfold increase in expression could be observed in animals treated with mRNA Galsomes compared to untreated controls.

Taken together, these findings demonstrate that the inhibitory PD-1/PD-L1 immune checkpoint axis is involved in the suppression of CTLs, and that it could potentially explain

the limited responsiveness of iNKT cells to a second (boost) vaccination, thus limiting antitumor immunity.

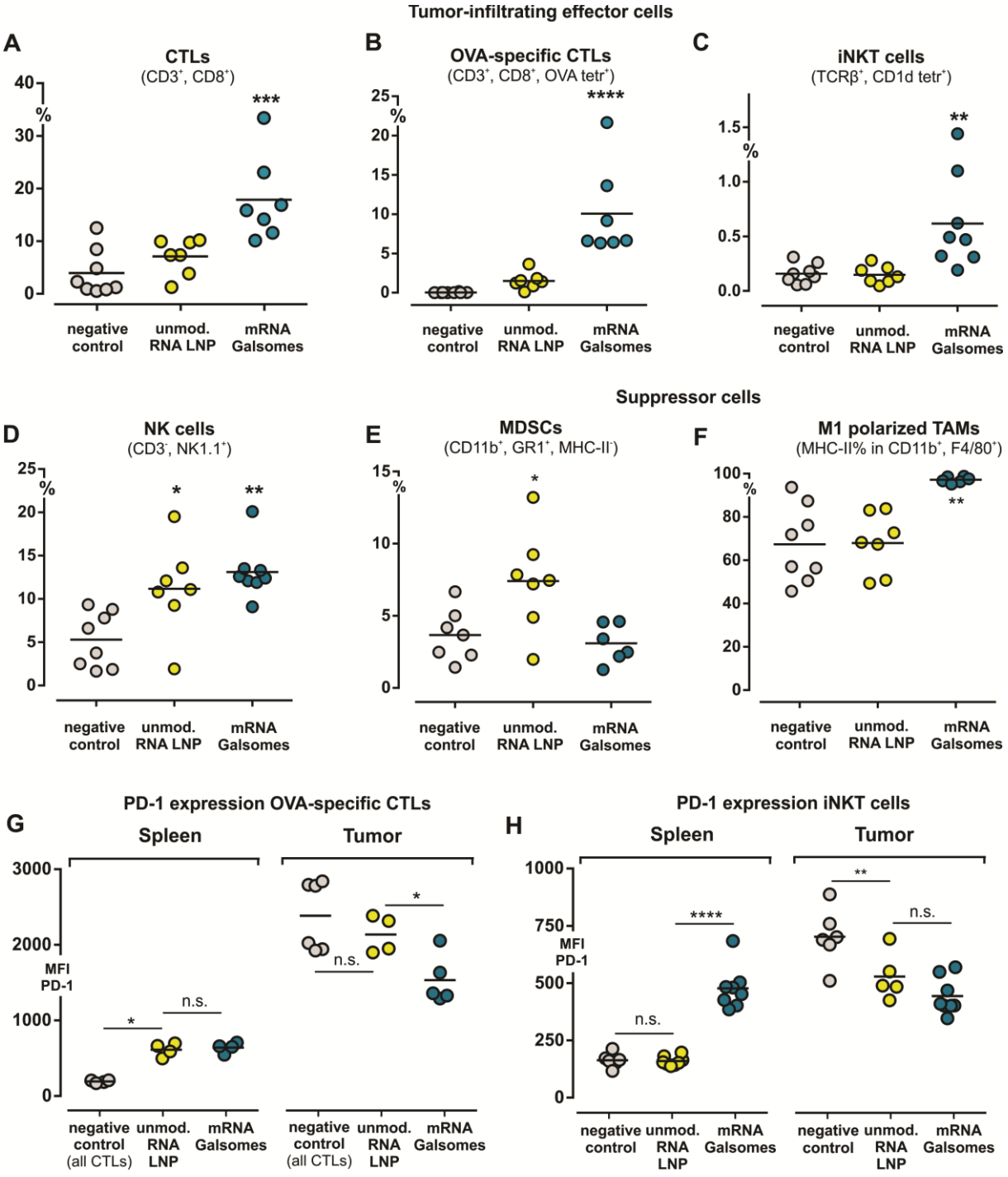

Figure 5. Therapeutic vaccination with mRNA Galsomes attracts immune effector cells to the tumor and positively affects suppressive myeloid cells in B16-OVA melanoma. B16-OVA bearing mice which received two vaccinations with OVA mRNA Galsomes were euthanized

on day 14 and evaluated for infiltration $CD8^+$ T cells **(A)** and OVA-specific $CD8^+$ T cells **(B)**, iNKT cells **(C)** and NK cells **(D). (E)** Presence of MDSCs ($CD11b^+$, $GR1^+$, MHC-II^-) and **(F)** M1 polarized TAMs ($CD11b^+$, $F4/80^+$, MHC-II^+) in tumor site (n=8, pooled from two independent experiments). **(G)** PD-1 expression on splenic and tumor-infiltrating OVA-specific $CD8^+$ T cells at day 14 (two days after boost) (n=4-6). **(H)** PD-1 expression levels on (activated) iNKT cells in spleen and B16-OVA tumors at day 14 (n=5-8, pooled from two independent experiments). Statistical analyses on datasets were performed by One-Way ANOVA followed by Turkey's post hoc test. Asterisks indicate statistical significance compared to the untreated group (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$).

Anti-PD-L1 checkpoint blockade synergizes with the antitumor effects of mRNA Galsomes

To first investigate the problem of iNKT anergy in more detail, naive mice were vaccinated twice with mRNA Galsomes with a five day interval. At respectively 6h and three days after each administration, we evaluated the cytokine release and iNKT activation. As shown in **Figure 6A**, IFN-γ levels measured after the second vaccination, were only half of the levels measured after the first administration. In addition, we could observe a shift towards the production of IL-4 and IL-10, which counteract Th1 and therefore CTL responses (**Supplementary Figure S10**). Finally, we noticed that a boost-vaccination with mRNA Galsomes did not further augment iNKT cell numbers, confirming the induction of a hypo-responsive state of iNKT cells. In shear contrast, when mice were simultaneously vaccinated and injected with an anti-PD-L1 antibody, IFN-γ production rose up to four times higher after the second vaccination, compared to that after the initial challenge. Along the same line, the combination with an anti-PD-L1 antibody further boosted the expansion of iNKT cells, as a second administration doubled the number of splenic iNKT cells (2.25 ± 0.7 *versus* 4.73 ± 1.2 %, **Figure 6B**). Evaluation of PD-L1 on splenic DCs showed that the previously observed up-

regulation of this molecule upon mRNA Galsome vaccination could be fully eliminated by the concomitant delivery of an anti-PD-L1 antibody (**Figure 6E**).[42, 43] In addition, these data show that repeated activation of iNKT cells by the mRNA Galsomes/checkpoint combination strategy is feasible, as the PD-1 levels on iNKT cells were halved compared to levels in mice that merely received a single vaccination with mRNA Galsomes alone (**Figure 6C**).

To evaluate if the above-mentioned effects of the combination therapy could also be translated into an improved therapeutic outcome, B16-OVA bearing mice were vaccinated with mRNA Galsomes combined with intraperitoneal (i.p.) administrations of either an anti-PD-L1 antibody or an isotype control antibody. Monotherapy of an anti-PD-L1 or isotype control antibody was used as additional controls. The results in **Figure 6D** show that by the time of the last vaccination (day 22), the average tumor volume of mice treated with a combination of mRNA Galsome and a checkpoint blocking antibody, remained limited to 59 ± 46 mm^3. By contrast, in all other groups, tumors had already grown up to 10 times larger. This also translated into a significant increase in median survival. Mice receiving monotherapy of an anti-PD-L1 antibody or mRNA Galsomes had reached their median survival at day 21 and 22.5, respectively, which is not significantly later than animals in the control group where only an isotype control antibody was injected (median survival of 19 days). The combination treatment significantly prolonged median survival to 30 days, indicating a synergistic effect between both treatment strategies.

Overall, we clearly show that the therapeutic potential of mRNA Galsomes can be strengthened by rationally combining with PD-L1 checkpoint inhibition. This checkpoint blockage (1) prevents the induction of iNKT anergy allowing multiple vaccination rounds, and (2) avoids adaptive resistance mechanisms at the tumor site prolonging antitumor effects and promoting the survival of B16-OVA tumor-bearing mice.

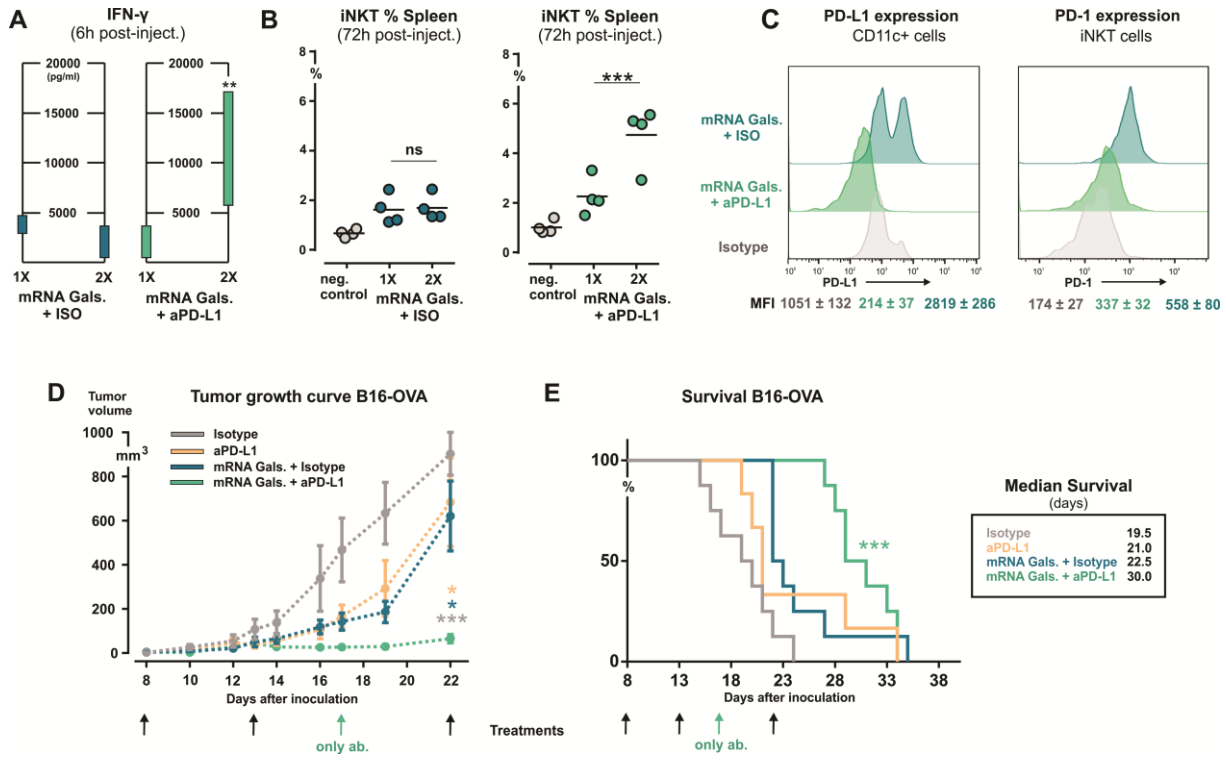

Figure 6. Checkpoint inhibition with an anti-PD-L1 antibody prevents the induction of iNKT anergy and avoids adaptive resistance to antitumor effects of mRNA Galsomes. For the evaluation of iNKT anergy, naive mice received two subsequent injections with OVA mRNA Galsomes at a five day interval. **(A)** Graph showing IFN-γ levels in serum, collected 6h after the first and second administration of mRNA Galsomes combined with either isotype or anti-PD-L1 antibody. (*n*=4). **(B)** Percentages of iNKT cells (TCRβ$^+$, mCD1d PBS-57$^+$ cells) among splenocytes, measured three days after each vaccination. **(C)** PD-L1 expression on splenic DCs (CD11c$^+$ cells) and PD-1 expression on proliferated iNKT cells, measured 6h after the first and three days after the second vaccination, respectively (*n*=3-4). B16-OVA bearing mice were i.p. administered with 100 µg of a rat IgG2b antibody (isotype control) or anti-PD-L1 antibody in monotherapy, or in combination therapy with mRNA Galsomes. Graph **(D)** shows average tumor growth curves and **(E)** Kaplan–Meier survival curves for respective treatments, demonstrating synergistic effects between the anti-PD-L1 antibody and mRNA Galsomes (*n*=6-8). Black arrows indicate the days of treatment with both i.v. mRNA

Galsomes and i.p. antibody. Blue arrows indicate when only antibody was injected (i.p.). Pairwise statistical analysis in (A) and (B) were performed using a student's t test. For (D), tumor volumes at day 22 after tumor inoculation were compared by a One-way ANOVA followed by Tukey's post hoc test. Asterisks indicate statistical significance compared to the untreated group (n.s., $p > 0.05$; * $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$).

DISCUSSION

In this study, we report on the use of an mRNA nanovaccine that is capable of inducing a broad set of antitumor effector cells, including CTLs, iNKT cells and NK cells, while reducing local immune suppression at the tumor site. This was achieved by co-packaging nucleoside-modified mRNA encoding tumor antigens and the glycolipid α-GC, which functions as an iNKT cell agonist and broadly acting immune adjuvant.

In the specific case of cancer immunotherapy, it was proven essential to evoke a systemic immune response to obtain antitumor immunity, which makes i.v. delivery the preferred administration route for mRNA-based cancer vaccines.[5, 7, 44] In our previous research, we showed that DOTAP-cholesterol nanoparticles could efficiently deliver mRNA to DCs and macrophages in lungs and spleen after systemic delivery.[31] By introducing modified nucleotides in the mRNA construct, the stability and translation are enhanced, resulting in higher and more sustainable mRNA expression levels. This enhanced mRNA expression is advantageous in the development of vaccines, since the resulting increased antigen presentation is shown to be beneficial for the induction of long-lived antibody and helper T cell responses, including the formation of follicular T cells.[45] However, nucleoside-modified mRNA largely loses its self-adjuvancy, resulting in reduced type I IFN levels, and a limited

capacity to evoke CTL immunity. To compensate for this, vaccines based on immunosilent mRNA require additional immune stimulation, which we provided by including α-GC.[6, 31]

We demonstrated that by embedding the glycolipid α-GC within mRNA DOTAP-cholesterol nanoparticles, the delivery of α-GC and its subsequent CD1d-mediated presentation were enhanced, while the mRNA transfection remained equally high (**Figure 1**). In all likelihood, this can be attributed to a difference in cellular uptake, which largely determines the trafficking and loading into CD1d. Exogenous glycolipid antigens, can either directly associate with cell surface-bound CD1d or, when these lipids are present in the endolysosomal compartments, they can be complexed with CD1d, which is being recycled from the plasma membrane through the endosomes and lysosomes.[46] Especially this last form of α-GC-CD1d complexation is interesting, as Torreno-Pina demonstrated that this process results in the formation of nanoclusters of CD1d-loaded α-GC at the APC's surface, a process referred to as "lipid-raft mediated CD1d presentation".[47] Since the occurrence of larger nanoclusters is correlated with enhanced iNKT cell stimulation, superior DC licensing and improved transactivation of bystander NK cells, this pathway will likely proved the strongest immune adjuvant effect.[48, 49] Taken together, it is likely that by particulating α-GC, it is preferentially trafficked through the endolysosomes, not only enabling a higher intracellular presence, but also resulting in superior lipid antigen presentation.

As a result of this enhanced α-GC presentation capacity, mRNA Galsomes containing low doses of α-GC were able to trigger the stimulation of iNKT cells *in vivo*, which rapidly induced the maturation of transfected APCs and coincided with the release of a plethora of cytokines (**Figure 2**). Shortly after the systemic delivery of mRNA Galsomes, we detected high levels of the Th1 cytokines IFN-γ, TNF-α and IL-12p70, as well as of the Th2 cytokines IL-6 and IL-4. Similar to type I IFN, IL-12p70 acts as a "third signal cytokine", promoting the activation and survival of T cells [50]. We also detected high levels of IL-27, a stimulatory

cytokine that plays a pivotal role in the expansion and programming of T cells, and is involved in the regulation of NK and iNKT cells and their recruitment to the tumor site.[51-53] In connection herewith, Semmling *et al.* demonstrated that DCs licensed by iNKT cells recruited CTLs *via* a particular chemokine mechanism, comprising the production of CCL17 and the expression of CCR4.[54, 55] They showed that CCL17 acted synergistically with other chemokine pathways, resulting in more rapid and improved CTL responses. Indeed, we showed that vaccination with mRNA Galsomes resulted in approximately five times more antigen-specific CTLs compared to LNPs loaded with unmodified mRNA (**Figure 3**).

With respect to the therapeutic effects of mRNA Galsomes, we could determine a switch from a tumor-promoting towards a more antitumoral immune infiltrate within the tumor. First of all, we observed significantly higher levels of CTL, iNKT and NK cell levels after vaccination with mRNA Galsomes, compared to vaccination with gold standard particles containing unmodified mRNA (**Figure 3**). In this regard, it is important to consider that even without specifically targeting NK and iNKT cells in the vaccine design, it is known that these cells contribute to the outcome of therapeutic vaccines. As such, Fotin-Mleczek *et al.* showed that depletion of NK and iNKT cells *via* NK1.1 antibodies, partially reduced the antitumor effects of an mRNA vaccine encoding OVA in E.G7-OVA lymphoma implanted mice.[56] Therefore, the specific stimulation of iNKT responses by including glycolipid antigens can be expected to enhance the vaccine effect. Indeed, we observed a more favorable, antitumoral immune infiltrate in B16-OVA tumors treated with mRNA Galsomes compared to nanoparticles containing unmodified mRNA (**Figure 5**). mRNA Galsomes evoked a stronger infiltration of effector immune cells, with seven times more OVA-specific CTLs and approximately four times more iNKT cells. For NK cells, we found that the cell numbers in mRNA Galsomes-treated tumors had more than doubled compared to untreated controls, which matched levels evoked by vaccination with unmodified mRNA.

A second observation was the reduced presence of immunosuppressive tumor-infiltrating cells (Figure 5). To exemplify, mRNA Galsomes positively modulated suppressive myeloid cells in B16-OVA tumors, as demonstrated by the reduced infiltration of MDSCs and TAMs and their increased expression levels of MHC-II. These observations can be further explained by the research of Cortesi *et al.*, who evidenced that iNKT cells could, *via* CD1d and CD40/Fas-dependent cell interactions, selectively kill M2-like macrophages, while promoting the survival of M1-like macrophages.[37] Moreover, it was shown that iNKTs can mediate the conversion of immunosuppressive MDSCs into immunogenic APCs in a CD1d-dependent manner.[36] In addition, multiple studies provided evidence that the cytokine responses to α-GC have the capacity to restore the functionality of tumor-suppressed DCs and exhausted CTLs, breaking local tolerance in the tumor microenvironment.[53, 57]

Despite these promising changes in the tumor immune infiltrate, mRNA Galsomes merely had a limited impact on tumor progression and survival of B16-OVA-bearing mice, which was comparable to the effects of gold-standard nanoparticles containing unmodified mRNA (**Figure 4**). In part, this could be linked to the observation that repeated vaccinations with mRNA Galsomes did not exert a boost effect on tumor reduction, nor did it evoke further expansion of iNKT cells. This strongly suggested the induction of a hypo-responsive or anergic state, which was previously reported to occur after strong iNKT activation by α-GC.[58-60] This anergy is generally characterized by the impaired production of Th1 cytokines and reduction in iNKT cell numbers upon re-stimulation. While literature contains conflicting data about iNKT anergy, multiple studies reported that the PD-1/PD-L1 checkpoint pathway plays a critical role in regulation of iNKT cell activity.[42, 57, 61] When investigating the involvement of this checkpoint axis in the present study, we confirmed the up-regulation of PD-1 in activated NKT cells, as well as its ligand PD-L1 in APCs in the systemic organs (**Figure 5** and **Figure S9**). We could also detect a pronounced PD-L1 expression in tumors of

nanoparticle-treated animals. This coincided with high PD-1 expression levels on OVA-specific tumor infiltrating CTLs, which was both the case for animals treated with mRNA Galsomes and LNPs containing unmodified mRNA. This is consistent with recent research by Sayour *et al*., who reported that i.v. administered DOTAP LNPs containing unmodified mRNA increased the levels of PD-L1 on myeloid cells in the systemic organs and in the tumor micro-environment.[62]

The identification of PD-1/PD-L1-mediated adaptive resistance as one of the culprit mechanisms that hamper the antitumoral effects of T cells and iNKT cells, also offers an additional therapeutic point of engagement.[62-64] Consequently, we tested the combination of mRNA Galsomes with i.p. administration of an anti-PD-L1 antibody (**Figure 6**). Indeed, the combination therapy showed clear synergistic antitumor effects in every treated mouse, while mRNA Galsomes and the anti-PD-L1 antibody in monotherapy only exerted antitumor effects in a small portion of animals. Additionally, we could demonstrate that the administration of an anti-PD-L1 antibody at the same time as the mRNA Galsome vaccine, prevented the induction of iNKT anergy, and allowed to boost the stimulation of iNKT cells after successive administrations. These findings confirm previous observations made by Parekh *et al.* combining unformulated α-GC with antibodies directed against PD-L1 and PD-L2.[42] Importantly, one should bear in mind that recent studies suggest that the up-regulation of PD-1 by iNKT cells is only a small part of a complex immune cascade, involving different signalling pathways.[65-68] For instance, some reports showed associations between Wnt/β-catenin signalling and the regulation of cytokine responses by iNKT cells. More specifically, Kling *et al.* showed that upon challenge with α-GC, β-catenin activity rapidly suppresses the production of IFN-γ by iNKT cells in liver, while IL-4 production is promoted.[67] In addition, Berga-Bolaños *et al.* demonstrated that β-catenin expression promotes the development of iNKT2 and iNKT17 subsets in the thymus, at the expense of iNKT1 cells, potentially

explaining the polarization towards the production of Th2-biased cytokines upon sequential α-GC stimulations.[68] Taken together, it is likely that by also counteracting these other suppressive mechanisms, iNKT cell anergy and the resistance to the therapeutic effects of mRNA Galsomes, could be further tuned down.

Finally, it is important to consider that B16-OVA tumor cells are notorious for their low expression of CD1d, which makes them poor targets for direct killing by iNKT cells. The mode of action of the mRNA Galsomes in this model is therefore expected to be almost exclusively determined by the indirect effects of iNKT activation: (i) the licensing of DCs by iNKT cells, (ii) the promotion of CTL responses and NK cell responses, and (iii) the beneficial interactions between iNKT cells and suppressive myeloid cells in the tumor micro-environment.[32-34] Therefore, we would like to stress that it is likely that the true value of the mRNA Galsomes will probably lie in the treatment of tumors where direct iNKT cell-mediated killing effects can occur. For example, in most blood cancers, such as lymphomas, tumor cells express CD1d, which makes them vulnerable to direct killing effects of iNKT cells.[69-71] Also, renal cell carcinoma, colorectal carcinoma and some brain cancers (*e.g.,* malignant glioma and in some cases of medullablastoma) were shown to be CD1d positive, with iNKT cell infiltration being a marker of favorable outcome in patients.[69]

CONCLUSION

Taken together, we have developed a flexible and versatile mRNA nanoparticle platform that presents an attractive way of initiating tumor immunity by targeting and activating conventional T cells and iNKT cells directly *in vivo*. Importantly, by combining immune-silent mRNA with the iNKT ligand α-GC in a single particle, a broad, yet safe, effective and controllable immune response can be evoked in different tumor models. In combination

therapy with PD-1/PD-L1 checkpoint inhibition, mRNA Galsomes hold potential to achieve effective antitumor immunity, this by preventing the induction of iNKT anergy, as well as by overcoming adaptive resistance at the tumor site.

EXPERIMENTAL

Cell culture and mice

Female C57BL/6 mice (6 weeks old) were purchased from Envigo (Gannat, France) and housed in an SPF facility. All animal experiments were conducted according to the regulations of the Belgian law and approved by the local Ethical Committee. Primary murine bone marrow-derived DC (BM-DC) cultures were generated as previously described.[31] Briefly, bone marrow was isolated from the femurs and tibias and processed into a single cell suspension. After red blood cell lysis, cells were cultured in complete medium (RPMI containing 5% FetalClone^{TM} I (HyClone^{TM}), 1% penicillin/streptomycin/L-glutamin (Gibco-Invitrogen), β-mercaptoethanol (5 μM, Gibco-Invitrogen) and 40 ng ml^{-1} GM-CSF (Peprotech) in low-adherence culture dishes. At day 5, all cells were collected by centrifugation, resuspended in the complete medium and seeded for experiments at 5x10^5 cells per well in a 24 well plate.

The mouse melanoma cell line B16-OVA (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% CO2 atmosphere in RPMI 1640 medium (Sigma-Aldrich, Diegem, Belgium) supplemented with 10% FBS, 100 U ml^{-1} penicillin, 100 μg ml^{-1} streptomycin, 2 mM l-glutamine and 0.4 mg ml^{-1} of the selection agent G418 (Thermo-Scientific, Aalst, Belgium).

mRNA constructs

Unmodified and nucleoside-modified (5meC, Ψ) mRNA encoding firefly luciferase (fLuc) and the enhanced green fluorescent protein (eGFP) were purchased from TriLink (San Diego, CA). For the immunization studies, a truncated form of ovalbumin (tOVA) fused to the first 80 amino acids of the invariant chain (Ii80) was produced by *in vitro* mRNA transcription from pGEM-Ii80tOVA plasmids.[72] The *in vitro* transcription of mRNA and its subsequent quality control were performed as previously described.[31] For the transcription of nucleoside-modified mRNA, cytidine and uridine nucleotides were 100% replaced by 5-methylcytidine and pseudouridine (TriLink).

mRNA lipid nanoparticle preparation

DOTAP (1,2-dioleoyl-3-trimethylammonium-propane) and cholesterol were purchased from Avanti Polar Lipids (Alabaster, USA). Alpha-Galactosylceramide (α-GC) and 6"-BODIPY-analogue of α-GC were synthesized in house by S. Van Calenbergh. The synthesis of α-GC was performed as previously described.[73] A detailed description of the preparation of BODIPY-labelled α-GC can be found in **Supplementary Information**. Cationic liposomes of DOTAP-cholesterol (2:3 molar ratio) were prepared by transferring the appropriate amounts of lipids, dissolved in chloroform into a round-bottom flask. For liposomes formulated with α-GC, 0.5, 0.15, 0.015 or 0.0015 mol% of the total lipid amount was replaced by α-GC. The chloroform was evaporated under nitrogen, after which the lipid film was rehydrated in HEPES buffer (20 mM, pH 7.4, Sigma-Aldrich) to obtain a final lipid concentration of 12.5 mM. The resulting cationic liposomes were sonicated until the dispersion became clear in a bath sonicator (Branson Ultrasonics, Dansbury, USA). Then, they were mixed with mRNA to obtain mRNA nanoparticles at a cationic lipid-to-mRNA (N/P) ratio of 3. mRNA

nanoparticles for *in vivo* use were prepared in an isotonic HEPES buffer containing 5% glucose (Sigma-Aldrich).

In vitro evaluation of α-GC delivery and presentation by BM-DCs

The *in vitro* experiments were performed on BM-DCs at day 6 of cell culture. The day before transfection, cells were seeded in 24 well plates at $5x10^5$ cells per well, and grown in the cell culture medium with 5% FCI serum. mRNA nanoparticles containing 0.5 mol% of α-GC were added directly to the cells in the complete cell culture medium (1 µg mRNA per $5x10^5$ cells). As a control, an equimolar dose of α-GC alone was added, dissolved in DMSO. To evaluate the presentation of α-GC in cell-surface CD1d complexes, BM-DCs cells were surface-stained with a monoclonal antibody specific for α-GC-CD1d complexes (clone L363, eBioscience). Flow analysis was performed 24h after the addition of mRNA nanoparticles (fLuc mRNA). Cells were collected and washed with PBS, stained with a fixable viability dye eFluor® 450 (eBioscience) according to the manufacturer's instructions, incubated with Fc block (CD16/32) to block non-specific FcR binding (BD Biosciences, Erembodegem, Belgium), and surface stained for CD11c-APC (clone N418) and α-GC:CD1d complex-PE for 30 min at 4°C. Mouse IgG2a kappa PE antibody was used as isotype control for the presentation of α-GC:CD1d. After additional washing steps, the cells were analysed by flow cytometry using a CytoFLEX (Beckman Coulter, Krefeld, Germany) and analysis was performed using FlowJo software (FlowJo, OR, USA). Confocal microscopy images of the cells were recorded using a Nikon C1si confocal laser scanning module attached to a motorized Nikon TE2000-E inverted microscope (Nikon Benelux, Brussels, Belgium), equipped with a Plan Apo 60x 1.0 NA oil immersion objective lens (Nikon).

Administration of mRNA nanoparticles and an anti-PD-L1 antibody

Mice were anesthetized in a ventilated anesthesia chamber with 3% isoflurane in oxygen. Prior to injection, a catheter of polyethylene tubing (Intramedic PE10, BD) containing sterile 0.9% NaCl solution was inserted in the tail vein. After correct placement, nanoparticles with the indicated cargo diluted in sterile 5% glucose HEPES buffer were slowly injected (200 µl). A dose of 10 µg mRNA per mouse was chosen based on previous mRNA dose-optimization studies. The optimized dose of nanoparticle-encapsulated α-GC was 20 ng per mouse (0.015 mol%), determined based on cytokine production and iNKT activation. Anti-PD-L1 antibody (10F.9G2, Bio X cell, West Lebanon, USA) or rat IgG2b isotype control antibody (LTF-2, Bio X cell) was administered i.p. at a dose of 100 µg, immediately after the administration of mRNA nanoparticles.

Bioluminescence imaging

Six hours after the injection of nanoparticles containing fLuc mRNA (TriLink), mice were anesthetized and abdomen and chest were depilated with hair removal cream. Subsequently, VivoGlo™ Luciferin (Promega) was administered i.p. in a volume of 100 µl (33 mg ml^{-1}) per mouse. After 10 min, bioluminescence images were acquired by the IVIS lumina II system (PerkinElmer, Waltham, MA), and quantitative analysis of the images was performed using the Living Image software (PerkinElmer).

Therapeutic vaccination experiments

The therapeutic potential of mRNA nanoparticles (containing different cargo, or in combination with an anti-PD-L1 antibody) was evaluated by performing therapeutic vaccinations in tumor-bearing mice. For this, C57BL/6 received a s.c. injection of $3x10^5$ B16-OVA or E.G7-OVA tumor cells (suspended in PBS) in the flank. Eight 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 nanoparticles. In some

experiments, animals received a second and third therapeutic vaccination, every four or five days. Tumor growth was measured every other day or two days using a digital caliper. When the tumor volume exceeded 1000 mm^3 (B16-OVA) or 1500 mm^3 (E.G7-OVA), the mice were euthanized *via* cervical dislocation.

Flow cytometric analysis on single cell suspensions

At different time points after immunization, mice were euthanized and spleen, lungs, liver and tumors were harvested and processed into single cell suspensions as previously described.[31] Single cell suspensions were stained with either a fixable viability dye eFluor® 450 (Thermo Scientific) or Zombie Yellow™ (Biolegend, San Diego, CA) according to the manufacturer's instructions to exclude dead cells from analysis, incubated with Fc block (CD16/32) to block non-specific FcR binding (BD Biosciences, Erembodegem, Belgium), and surface stained with the indicated antibodies during 30 min at 4°C (all Thermo-Scientific). After additional washing steps, the cells were analyzed by flow cytometry. Compensation for spectral overlap was calculated using UltraComp eBeads™ Compensation Beads (Thermo-Scientific) stained with individual fluorochrome-conjugated antibodies.

The activation state of DC positive for CD11c-(APC or FITC) in the spleen was analysed by measuring the up-regulation of the co-stimulatory molecules CD40-FITC (HM40-3), CD86-FITC (CL1), CD80-PE/Cy7 (16-10A1), and the inhibitory molecule PD-L1-Super Bright 436 (MIH5). T cells were stained with monoclonal antibodies, including CD3e-PE (145-2C11), CD4-FITC (GK1.5), CD8a-(APC or AF488) (53-6.7) and PD-1-(efl450 or FITC) (RMP1-30). To stain OVA-specific T cells, an BV450-conjugated H-2Kb/SIINFEKL tetramer (OVA-tetramer) was used, obtained from the National Institutes of Health (NIH) Tetramer Core Facility. iNKT cells were stained with TCRβ-APC (H57-597), PD1-efl450 and mCD1d PBS-57 PE tetramer obtained from the NIH tetramer Core Facility. NK cells were detected using

CD3e-PE (negative gating) and NK1.1-APC (PK136) staining. In addition, myeloid derived suppressor cells (MDSCs) and tumor-associated macrophages (TAMs) were stained with antibodies including, CD11b-PE/Dazzle^{TM} 594 (Biolegend), MHC-II-efl450 (M5/114.15.2), F4/80-(FITC or AF700) (6F12), Ly-6G/Ly-6C-FITC (RB6-8C5) and CD206-APC (C068C2). DCs ($CD11c^{+}$) and tumor cells (CD45-PerCP-Cy5.5 negative cells) were evaluated for the expression of PD-L1.

Cytokine measurements and alanine transaminase (ALT) activity

Serum was collected 6h after i.v. injection of the mRNA nanoparticles and samples were stored at -80°C. Mouse Platinum IFN alpha ELISA kit , IFN-γ and IL-4 ELISA kits (Ready-SET-Go!®) were purchased from Thermo-Scientific. A panel of 13 other cytokines, including IL-1α, IL-1β, IL-6, IL-10, IL-12p70, IL-17A, IL-23, IL-27, MCP-1, IFN-β, IFN-γ, TNF-α, and GM-CSF, was quantified using a multiplex assay (LEGENDplex™ Mouse Inflammation Panel, Biolegend). ALT enzyme activity was measured using a colometric assay kit (MaxDiscovery™, Bioo Scientific Corporation, Austin, USA). All assays were performed according to the manufacturer's instructions.

Statistical analysis

All data are presented as mean ± standard deviation. Presented data of the *in vitro* experiments are representative for at least 3 independent experiments performed on three different days. The *in vivo* experiments contain data of at least two experiments merged into a single graph, this is explicitly mentioned in the figure caption. All statistical analysis were performed using GraphPad Prism6 (La Jolla, CA, USA). Information on the analysis per (sub)figure, is specified in the figure caption.

ASSOCIATED CONTENT

Supporting Information.

The following files are available free of charge. Supplementary figures showing the uptake of BODIPY-a-GC by BM-DCs, when formulated in mRNA Galsomes or dissolved in DMSO, the transfection capacity of mRNA Galsomes in BM-DCs and human monocyted-derived DCs, the adjuvant effect of mRNA Galsomes on BM-DCs *in vitro*, the toxicity evaluation of mRNA Galsomes in monotherapy and combined with an anti-PD-L1 antibody, the tumor growth in established E.G7-OVA tumors after vaccination with nanoparticles packaging nucleoside-modified mRNA alone, the comparison between one or two administrations of mRNA Galsomes to achieve antitumor immunity in E.G7-OVA bearing mice, the flow cytometric analysis of the immune filtrate of isolated tumors, the PD-L1 expression on splenic DCs, intratumoral DCs, and tumor cells after vaccination with mRNA Galsomes, the comparison between the cytokine responses after a first and second exposure to mRNA Galsomes (PDF).

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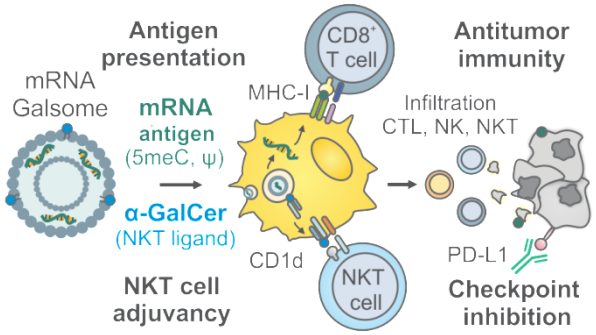

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