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Intranasal mRNA nanoparticle vaccination induces prophylactic and therapeutic anti-tumor immunity

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Direct *in vivo* administration of messenger RNA (mRNA) delivered in both naked and nanoparticle formats are actively investigated because the use of dendritic cells transfected *ex vivo* with mRNA for cancer therapy is expensive and needs significant infrastructure. Notably, intravenous and subcutaneous injections are the only routes of administration tested for mRNA nanoparticle tumor vaccination. In this report, we demonstrate that tumor immunity can be achieved via nasal administration of mRNA. Mice nasally immunized with mRNA delivered in nanoparticle format demonstrate delayed tumor progression in both prophylactic and therapeutic immunization models. The observed tumor immunity correlates with splenic antigen-specific CD8+ T cells and is achieved only when mRNA is delivered in nanoparticle but not in naked format. In conclusion, we demonstrate, as a proof-of-concept, a non-invasive approach to mRNA tumor vaccination, increasing its potential as a broadly applicable and off-the-shelf therapy for cancer treatment.

Tumor vaccination employing mRNA transfected dendritic cells (DCs) has been shown to be an effective strategy for treatment of cancer[1–6]. Promising results emerging from recent clinical trials[7–9] supports the notion that this is a strategy that can be translated to humans and is amenable to commercialization. However, this process involves harvesting cells from patients via leukapheresis, generating DCs *in vitro* from adherent monocytes, loading them with mRNA, maturing them *in vitro* and re-injecting these mRNA-loaded DCs back into the patient. This is a cost, labor and resource intensive procedure. Because of these reasons, researchers have explored alternative cell-based approaches[10,11], as well as direct *in vivo* injection of mRNA in naked[12,13] and nanoparticle formats[14–19]. However, due to rapid degradation of naked mRNA *in vivo*, direct injection of mRNA is effective only when it is injected directly into lymph nodes[12,13]. Intranodal injection is an extremely invasive procedure in mice and hence not amenable for repeated administrations. Moreover, although intranodal injection is performed using ultrasound-guidance in humans, it remains a technically challenging procedure that requires surgical expertise. While this approach is an improvement over the existing *ex vivo* DC-based approach, scale-up remains a significant challenge thus hindering its broad application.

A strategy that overcomes this problem is encapsulating mRNA in nanoparticles, which not only protects mRNA from nuclease degradation, but also facilitates its uptake by cells and endosome escape within cells leading to enhanced delivery efficiencies. This approach may obviate the need for intranodal administration, while still permitting direct *in vivo* application of an off-the-shelf mRNA vaccine formulation via conventional routes of administration. Indeed, mRNA nanoparticle delivery has attracted interest from many research groups in recent years[17,20–23]. In addition, therapeutic efficacy of mRNA encapsulated in nanoparticles for tumor vaccination has also been recently demonstrated[16,17]. Notably, tail vein and subcutaneous injections are the only routes evaluated in all of these studies. We have previously reported that primary DCs can be efficiently transfected by mRNA encapsulated in nanoparticles *in vitro*. These particles are about 180 nm and 300 nm in hydrodynamic diameter and have zeta potentials of +40 mV/−12 mV in water and 10% FBS supplemented media, respectively[24]. In the same study, we determined that luciferase expression mediated by nasally administered mRNA nanoparticles lasts for about 24 hours compared to naked mRNA, which is detectable only up to 4 hours post-administration[24]. The bioavailability of transgene product is clearly superior to nasally instilled soluble protein antigens, where >85% of the soluble antigen is cleared from the nasal site within 6 hours[25].

In this study, we hypothesize that nasal vaccination could be an effective strategy for mRNA tumor vaccination. Intranasal route of immunization is desirable because of its non-invasive nature, amenability for repeated administration and is associated with high patient compliance. It has been previously reported that intranasal immunization with naked mRNA can induce a moderate level of protection against tuberculosis in mice[26]. We reason that significantly higher nasal transfection efficiencies mediated by mRNA nanoparticles could translate to the induction of anti-tumor immunity. In addition, a previous study has reported that intranasal tumor vaccination with soluble OVA peptides can induce robust anti-tumor immunity[27]. Therefore we reason that the prolonged presence of antigen at the nasal site where the Nasal-Associated Lymphoid Tissues (NALT) are located[28] could translate to enhanced immune responses. Last but not least, we observe that nasally administered nanoparticles are taken up by CD11c^{high} cells isolated from NALT (Supplementary Figure S1), indicating that this route of administration could be used to directly target DCs.

Based on above rationale, we set up an immunization scheme to investigate the therapeutic efficacy (Figure 1) of chicken ovalbumin (OVA) encoding mRNA nanoparticle vaccination in prophylactic and therapeutic immunotherapy models with E.G7-OVA tumor cells. The immunization schemes are based on published prime-boost protocol that entails weekly nasal administration for three weeks[29] (prophylactic) and four injections every other day[12] (therapeutic). Because we use cholera toxin (CT) as an adjuvant, there is a possibility that tumor immunity is induced by CT and not the OVA mRNA nanoparticle. To rule out this possibility, we immunize mice with green fluorescent protein (GFP) mRNA nanoparticles as controls.

Results

Prophylactic immunization with nasally administered mRNA vaccine. We tested intranasal immunization using a prophylactic tumor model, where mice were challenged with 4×10^5 E.G7-OVA cells injected into the left flank 7 days after the last immunization (Figure 1a). Mice intranasally immunized with OVA mRNA nanoparticles (mOVA-NP) demonstrated tumor inhibition ($p < 0.01$) and overall survival efficacy ($p < 0.01$) compared to mice immunized with GFP mRNA nanoparticles (mGFP-NP) or naked OVA mRNA (mOVA-N) (Figures 2a and b). The median tumor free and overall survival duration for the mOVA-NP group were 14.5 and 23 days, which were significantly longer compared to control groups (6 and 14 days for mGFP-NP group; 7 and 13 days for mOVA-N group, respectively). 2 out of 10 mice in the mOVA-NP group remained tumor free for the duration of the study (40 days).

Notably, the tumor growth kinetics of mOVA-N group overlapped completely with negative control (mGFP-NP group, Figure 2c), indicating that intranasal administration with naked mRNA did not induce prophylactic tumor immunity. Hence, we concluded that intranasal vaccination with mRNA encapsulated in nanoparticle has an anti-tumor effect in the murine prophylactic E.G7-OVA tumor model.

Therapeutic immunization with nasally administered mRNA vaccine. We further evaluated the potential of intranasal mRNA tumor vaccination using a therapeutic tumor model, where mice were injected with 2×10^5 E.G7-OVA cells into the left flank 2 days before the first vaccine dose (Figure 1b). The number of tumor cells used in the therapeutic tumor model is half that used in the prophylactic model because of the increased stringency of a therapeutic protocol wherein tumor cells are implanted prior to start of immunizations. This provided a treatment window of 7–10 days to determine if the test vaccination regimen could have a potential therapeutic effect. The therapeutic immunization scheme (Figure 1b) was based on a similar protocol used for intranodal naked mRNA vaccination that entailed four immunizations performed every other day[12]. The median tumor free duration for mOVA-NP group (15 days) was statistically significant compared to the control mGFP-NP group (9 days, $p < 0.01$) but not mOVA-N group (11 days, $p = 0.067$) (Figure 3a). However, the median overall survival for mOVA-NP group (23.5 days) was significant when compared to both mGFP-NP group (15 days, $p < 0.001$) and mOVA-N group (17.5 days, $p < 0.05$). Two out of eight mice in the mOVA-NP group remained tumor free (Figure 3b) for the duration of the study (40 days).

We observed that following tumor onset, it took a relatively long time for tumors to grow in the mOVA-NP group. The effect of immunization in controlling tumor growth rate was consistently observed in every animal in the mOVA-NP group (Figure 3c). To gain further insight, we analyzed specific tumor growth rates[30] (Equation 2) of each tumor bearing animal every 48 hours for 16 days (Figure 3d). In the mGFP-NP and mOVA-N immunized mice, tumor volumes increased aggressively as soon as nascent tumors appeared. Specific growth rates were arrested rapidly from 0.9 day^{-1} (on day 2) to about 0.3 day^{-1} (day 6 and onwards) for reasons we speculated were related to tumor size (inefficient nutritional transport and onset of necrosis at later time points). In mOVA-NP immunized mice, specific growth rates did not progress at early time points, and this translated into the observed growth delay (Figure 3c). Amongst the six tumor-bearing mice within the mOVA-NP group (two were tumor free), three showed negligible growth during the first 48 hours. Hence, we concluded that intranasal vaccination with
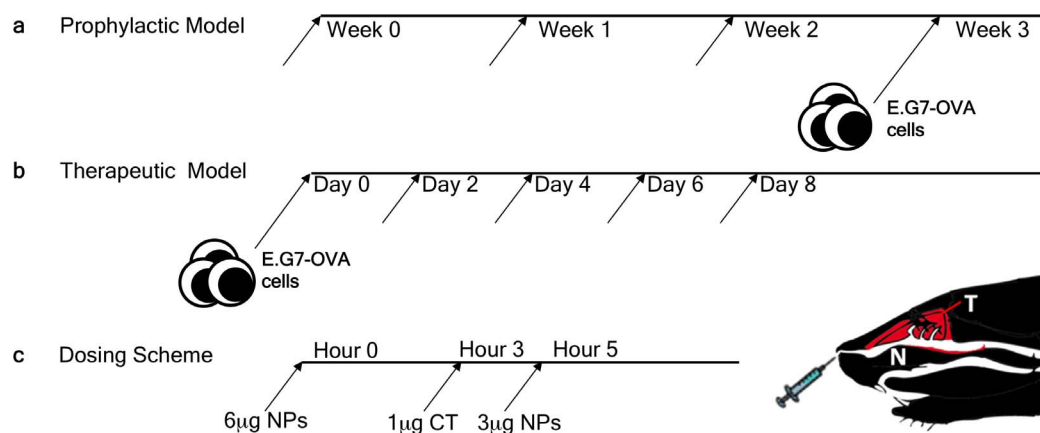
Figure 1 | Immunization scheme for intranasal mRNA tumor vaccine. (a) Prophylactic immunization. (b) Therapeutic immunization. (c) Dosing Scheme. (NP: mRNA nanoparticles, CT: Cholera Toxin, N: NALT, T: Turbinates). Additional details are provided in Materials and Methods.
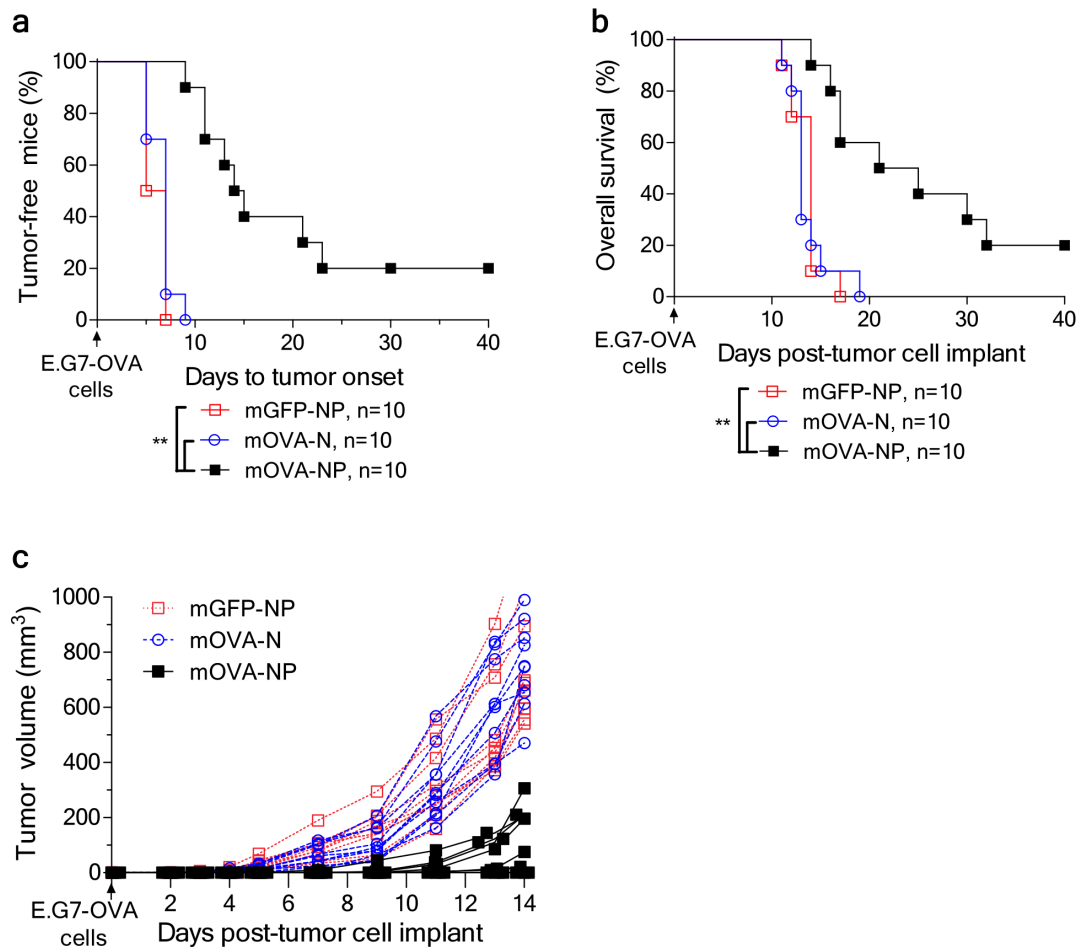
Figure 2 | Prophylactic immunization with OVA mRNA nanoparticles, but not GFP mRNA nanoparticles or naked OVA mRNA induces anti-tumor immunity. Female C57Bl/6 mice were immunized as shown in Figure 1. 4×10^5 E.G7-OVA tumor cells were injected subcutaneously 7 days later. Mice were sacrificed once tumor volume reached 500 mm^3. This experiment was conducted two times with similar results. Data from the second experiment are depicted. (a) Onset of palpable tumors. (b) Overall survival. (c) Tumor growth over 14 days. **p < 0.01.

mRNA encapsulated in nanoparticle format could also be effective for therapeutic tumor vaccination.

Tumor immunity requires mRNA to be delivered in nanoparticle but not in naked format. Because T cells are the major cell type involved in tumor clearance, we hypothesized that anti-tumor immunity observed in mOVA-NP treated mice (Figures 2 and 3) would correlate with the presence of OVA-specific T cells. Indeed, consistent with this hypothesis, we observed the presence of H-2Kb OVA tetramer+ CD8+ T cells in splenocytes isolated from mice immunized with mOVA-NP but not mGFP-NP or mOVA-N (Figure 4). Anti-tumor immunity was only observed in mice immunized with OVA mRNA delivered in nanoparticle format in both the prophylactic and therapeutic tumor model. These data suggests that the use of mRNA for intranasal vaccination applications will require delivery in nanoparticle format.

Discussion

In this proof-of-concept study, we demonstrate for the first time that intranasally administered mRNA encoding a tumor antigen can induce tumor immunity for the treatment of cancer. Our hypothesis is based on higher nasal transfection efficiencies and longer transgene expression kinetics achieved by mRNA nanoparticles as compared to mRNA delivered in the naked format. Mice treated with OVA mRNA encapsulated in nanoparticles, demonstrated delay in both tumor onset and overall survival compared to controls in prophylactic and therapeutic E.G7-OVA tumor model.

The overall survival and tumor onset of mOVA-NP group in the prophylactic model are clearly superior to mOVA-N group (Figure 2, Table 1). However, in therapeutic model the improvement is less distinct. When tumor growth kinetics between mOVA-N and mGFP-NP groups are compared, we observe that growth curves in the mOVA-N group completely overlapped with mGFP-NP group in the prophylactic model (Figure 2c), but a minor difference is seen in the therapeutic model (Figure 3c). This suggests that naked mRNA immunization had a slight effect in the latter. However, the difference in the latter is not statistically significant. The reason for this could be that the robust innate immune response mediated by cholera toxin facilitated the induction of an adaptive immune response following naked mRNA immunization in the nasal cavity. This corroborates data from another study demonstrating that nasally administered naked mRNA induces immune responses for the treatment of tuberculosis[26].

In the therapeutic setting, we also observe that nascent tumors in all tumor-bearing mice treated with OVA mRNA nanoparticles do not proliferate aggressively. This could be attributed to immune response generated from intranasal immunization. Since tumor cells can escape immune surveillance[31–34] through immune suppression[35,36], altered expression of MHC class I[37,38], as well as generation of immune escape tumor variants[39,40], specific growth rates eventually
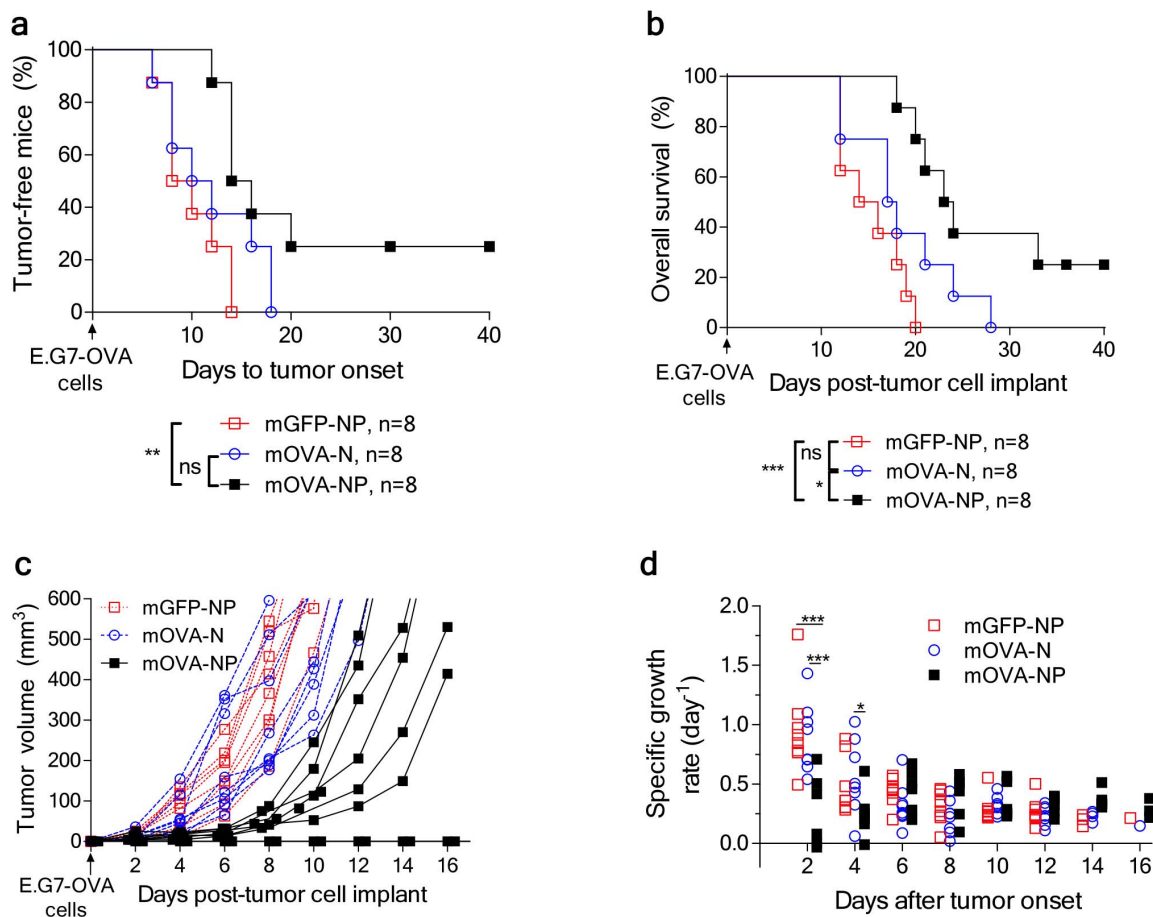
Figure 3 | Therapeutic immunization with OVA mRNA nanoparticles, but not GFP mRNA nanoparticles or naked OVA mRNA induces anti-tumor immunity. Female C57Bl/6 mice were injected subcutaneously with 2×10^5 E.G7-OVA tumor cells. 2 days later mice were immunized as shown in Figure 1. Mice were sacrificed once tumor volume reached 500 mm³. This experiment was conducted two times with similar results. Data from the second experiment are depicted. (a) Onset of palpable tumors. (b) Overall survival. (c) Tumor growth over 16 days. (d) Specific growth rate of tumors over time. *p < 0.05; **p < 0.01; ***p < 0.001.

caught up with that of control groups (Figures 3c and d). Lastly, through tetramer analysis we demonstrate that to induce anti-tumor immunity via intranasal route, it is necessary that mRNA is delivered in a nanoparticle.

Because intranasal delivery is a desirable route for vaccination, it has been extensively studied in the past decade. In particular, micro- and nano-particle delivery systems that encapsulate protein antigens or DNA that encode for antigens have been evaluated. However, the focus of intranasal vaccination has often been on the treatment of infectious diseases[29,41–47]. Nonetheless, a recent study has demonstrated that nanoparticles composed of modified γ-polyglutamic acid (γ-PGA) encapsulating full length OVA protein instilled intranasally induced anti-tumor immunity against melanoma[48]. In addition, a recent study also investigated the use of mannosylated chitosan-DNA (CS-DNA) nanoparticle vaccine for the prophylactic treatment of prostate carcinoma via the intranasal route. Anti-tumor response was only observed in the group that received targeted CS-DNA nanoparticles, but not in the group that received non-targeted nanoparticles. However, the therapeutic efficacy of the targeted CS-DNA nanoparticles was relatively similar to intramuscular vaccination using soluble antigen[49].

Our results contribute to a relatively small number of studies published on mRNA nanoparticle mediated tumor vaccination where overall survival is one of the endpoints[14,16,17,19]. In addition, results from our current study also support the concept of nasal vaccination as an option for mRNA cancer immunotherapy.

However, the therapeutic efficacy achieved in our current study is relatively moderate and we are uncertain how it compares with other administration routes or other established methods of mRNA vaccination. Therefore, future studies will focus on comparing this approach with other RNA-based methods and optimization of the current protocol to improve therapeutic efficacy.

For mRNA tumor vaccination to be clinically useful and broadly applicable, it is important that it is an off-the-shelf therapy that can be administered directly *in vivo*. In this report, we show that a convenient, non-invasive method can be used for direct *in vivo* administration of mRNA encoding tumor antigen, however it has to be delivered in nanoparticle format. This is an attractive prospect for the broad application of mRNA vaccines and reveals a major gap in the development of mRNA gene carriers for cancer immunotherapy.

Methods

Cloning of pGEM4Z/GFP/A64 and pGEM4Z/OVA/A64. The cDNA for green fluorescent protein (GFP) was derived from pEGFP-N1 (Clontech, Palo Alto, California) and inserted into pGEM4Z/A64[50]. Chicken ovalbumin cDNA in pUC18 was kindly provided by Dr. Barry T. Rouse, University of Tennessee, Knoxville. The 1.9 kb EcoR1 fragment containing the coding region and 3′ untranslated region was cloned into the EcoR1 site of pGEM4Z/A64 to generate plasmid pGEM4Z/OVA/A64[51].

***In vitro* transcription of mRNA.** Each plasmid of interest was digested with the restriction enzyme SpeI to linearize the DNA. After purification, DNA was used as template for *in vitro* transcription using T7 High Yield RNA Synthesis Kit (New England Biolabs, NEB) in the presence of anti-reverse cap analogue (ARCA, NEB)
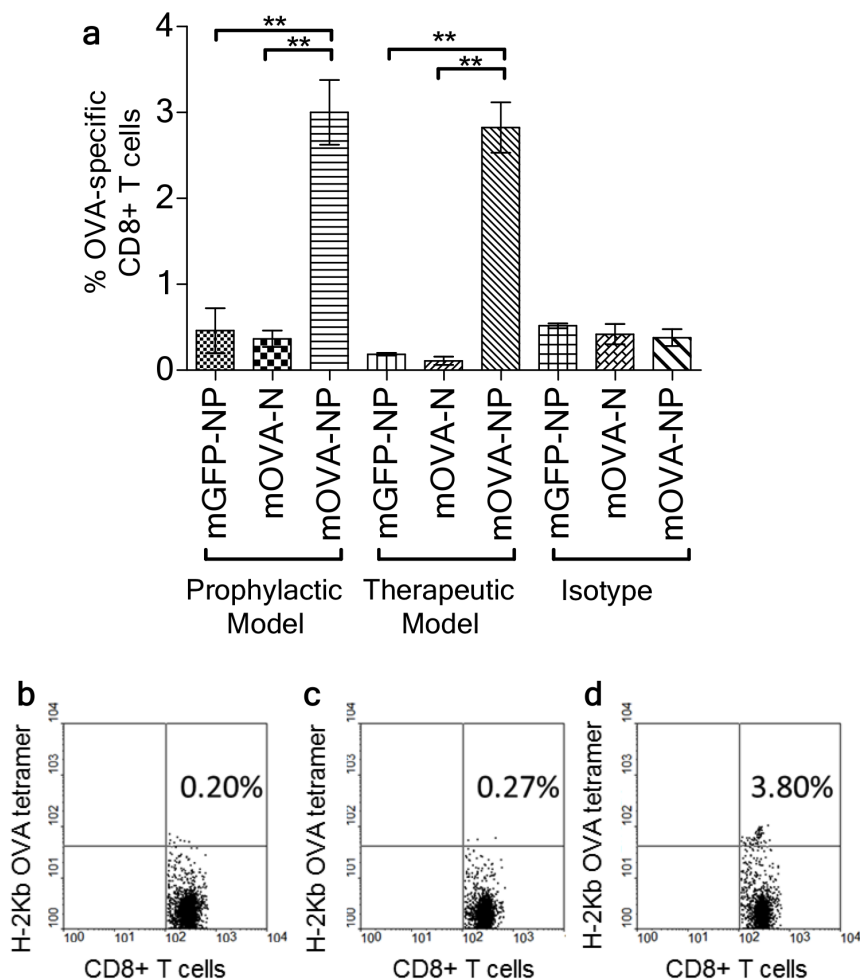
Figure 4 | Induction of antigen-specific T cells following intranasal immunization with OVA mRNA nanoparticles. (a) OVA-specific splenic CD8+ T cells stained with H-2Kb OVA tetramer. Mice (n = 2 per group) were immunized as shown in Figure 1 and described in Methods. Groups are mGFP-NP: mice immunized with GFP mRNA nanoparticles, mOVA-N: mice immunized with naked OVA mRNA and mOVA-NP: mice immunized with OVA mRNA nanoparticles. **p < 0.01. Results are presented as an average of 2 independent experiments. Representative data depicts analysis of cells harvested from mice immunized using the prophylactic model regimen with (b) mGFP-NP, (c) mOVA-N and (d) mOVA-NP. % H-2Kb OVA tetramer+ CD8+ T cells represents the percent of OVA tetramer positive cells within the CD8+ T cell population. Analysis of cells harvested from mice immunized using the therapeutic model regimen is shown in Supplementary Figure S3.

according to manufacturer's protocol. We routinely obtain 40–50 μg of OVA mRNA from a 20 μl reaction (1 : 3 GTP : ARCA mole ratio). *In vitro* transcribed (IVT) mRNA was purified with RNEasykit (Qiagen), quantified by spectrophotometry, and analyzed by agarose gel electrophoresis to confirm the synthesis of full-length mRNA. GFP mRNA was labeled with Cy5 labeling kit (Mirusbio) according to manufacturer's protocol.

Nanoparticle formulation. mRNA nanoparticles were formulated (as previously described[24]) by adding 8 μl ethanol reagent (mRNA Transfection Reagent, Stemgent) to 10 μl of mRNA (0.2 μg/μl) suspended in Stemfect buffer under gentle vortexing for 10 seconds. The mixture was incubated at room temperature (RT) for 12 minutes under vacuum to completely remove ethanol. Size and zeta potential of nanoparticles were confirmed using NanoZS (Malvern) in both DI water (180 nm/+40 mV) and

10% FBS-supplemented media (300 nm, −12 mV), consistent with what we have previously reported[24].

Ethics statement. In conducting the research described in this paper, the investigators adhered to the "Guide for the Care and Use of Laboratory Animals" as proposed by the committee on care of Laboratory Animal Resources Commission on Life Sciences, National Research Council. The facilities at the Duke University are fully accredited by the American Association for Accreditation of Laboratory Animal Care (AAALAC), and all studies were conducted using a protocol approved by the Duke University IACUC.

Intranasal vaccination. 6 to 7-week old female C57Bl/6 mice were obtained from Jackson Laboratories. Intranasal immunization with mRNA encoding chicken

Table 1 | Summary of anti-tumor effect of intranasal vaccination in prophylactic and therapeutic E.G7-OVA tumor model

Groups	Prophylactic Model		Therapeutic Model	
	Median Tumor Onset (days)	Median Survival (days ± SD*)	Median Tumor Onset (days)	Median Survival (days ± SD)
OVA mRNA nanoparticle (mOVA-NP)	14.5	23 ± 1.4	15	23.5 ± 0.4
Naked OVA mRNA (mOVA-N)	7	13 ± 2.1	11	17.5 ± 0.4
GFP mRNA nanoparticle (mGFP-NP)	6	14 ± 0.7	9	15 ± 0.7
*SD: standard deviation.				

ovalbumin (mOVA) or green fluorescent protein (mGFP) encapsulated in nanoparticles was performed according to Figure 1. Each nasal administration was done in 3 steps as detailed in Figure 1C. Mice were anesthetized with isofluorane in a gas chamber and queued for nasal administration. Each time a single mouse was taken out of the chamber, held in supine position, nasally administered with 15 µl of mRNA nanoparticles (3 µg) using a P20 pipette (fitted with a gel loading tip) and laid back inside the gas chamber in supine position. This procedure was repeated for the next animal in sequence. Consequently, each mouse was handled twice at an interval of approximately 5 minutes between each 15 µl dose for a total of 30 µl (6 µg) of mRNA nanoparticles. Mice were rested for 4 hours to allow gene expression to peak and subsequently administered with 1 µg cholera toxin (CT, List Biologicals) in 10 µl PBS. Mice were rested for another 2 hours to allow early immune response at the nasal site and subsequently administered with an additional 15 µl (3 µg) of mRNA nanoparticles. The procedure for naked OVA mRNA administration was identical to that used for OVA mRNA nanoparticles. In summary, each mouse received a total of 9 µg of OVA (or GFP) mRNA nanoparticles and 1 µg of CT per vaccination.

Tumor immunotherapy models. For prophylactic immunization, 4×10^5 E.G7-OVA tumor cells (in 100 µl PBS) were injected subcutaneously into the left flanks of immunized mice 7 days after the last immunization (Figure 1). For therapeutic immunization, 2×10^5 E.G7-OVA tumor cells (in 100 µl PBS) were injected subcutaneously into the left flanks of naïve mice 2 days before the first vaccine dose. Tumors were monitored every other day for tumor onset and measured with vernier calipers. Mice with tumors greater or equal to 500 mm³ were sacrificed. Tumor volume was calculated using Equation 1, where length is the longer of the 2 orthogonal measurements. Specific growth rate was calculated using Equation 2[30].

$$Tumor\ Size = \frac{Length \times Width^2}{2} \qquad (1)$$

$$Specific\ Growth\ Rate = \frac{\ln(V_2/V_1)}{D_2 - D_1} \qquad (2)$$

Where V_2/V_1 is the numerical ratio of tumor size measured from the same animal on respective days (D_2 and D_1).

Tetramer staining. Female C57Bl/6 mice were immunized using the prophylactic or therapeutic regimens as detailed above. 7 days after the last immunization, spleens were isolated and crushed through a 70 micron filter. Splenocytes were depleted of erythrocytes with ammonium chloride/Tris and re-suspended in PBS/10%FBS at a concentration of 10^7 cells/ml. Cells were blocked on ice with CD16/32 (Fc-block, BioLegend) for 15 minutes and subsequently stained with CD8-APC, isotype-PE antibodies (BioLegend) and PE-iTag-MHCI-OVA tetramer (Beckman Coulter) for 30 minutes on ice. Antibody staining was carried out per manufacturer's protocol. For tetramer staining, 2 µl of CD8-APC antibody and 5 µl of MHC class I H-2Kb OVA tetramer (amino acids 257–264, SIINFEKL) were added to 10^6 cells (in 100 µl) and incubated for 30 minutes at room temperature. Cells were washed, fixed with PBS/1% paraformaldehyde, data were acquired using flow cytometry (FACSCaliber, BD Biosciences) and analyzed using WinMDI 2.9 (freeware). Gating strategy for analysis of % H-2Kb OVA tetramer+ CD8+ T cells is described in Supplementary Figure S2.

Statistical analysis. For tumor studies, comparison between two groups was performed using the log-rank test (Mantel-Cox test). Additional comparisons between groups were done by determining the median survival for each group. Tumor growth curves over time were compared using two-way ANOVA with Bonferroni multiple comparison post-test. Statistical significance in tetramer staining comparing two groups was done using paired two-tailed Student's *t* test. A probability of less than 0.05 (p < 0.05) was considered statistically significant. Calculations were performed using GraphPad Prism.

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Author contributions

K.K.L.P. conceived the study. K.K.L.P. and H.F.S. designed the experiment. K.K.L.P. performed the experiment. K.K.L.P., S.K.N., K.W.L. analyzed, interpreted the data and wrote the manuscript.

Additional information

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