Published in final edited form as:

Nano Lett. 2018 October 10; 18(10): 6195–6206. doi:10.1021/acs.nanolett.8b02179.

Personalized Tumor RNA Loaded Lipid-Nanoparticles Prime the Systemic and Intratumoral Milieu for Response to Cancer Immunotherapy

Elias J. Sayour^{*,†}, Adam Grippin[†], Gabriel De Leon[†], Brian Stover[†], Maryam Rahman[†], Aida Karachi[†], Brandon Wummer[†], Ginger Moore[†], Paul Castillo-Caro[†], Kristianna Fredenburg[‡], Matthew R. Sarkisian^{†,§}, Jianping Huang[†], Loic P. Deleyrolle[†], Bikash Sahay^{||}, Sheila Carrera-Justiz[⊥], Hector R. Mendez-Gomez[†], and Duane A. Mitchell[†]

[†]Preston A. Wells Jr. Center for Brain Tumor Therapy, UF Brain Tumor Immunotherapy Program, McKnight Brain Institute, Lillian S. Wells Department of Neurosurgery, College of Medicine, University of Florida, Gainesville, Florida 32611, United States

[‡]Department of Pathology, College of Medicine, University of Florida, Gainesville, Florida 32611, United States

[§]Department of Neuroscience, College of Medicine, University of Florida, Gainesville, Florida 32611, United States

^{||}Department of Infectious Disease, College of Veterinary Medicine, University of Florida, Gainesville, Florida 32611, United States

[⊥]Department of Small Animal Clinical Sciences, College of Veterinary Medicine, University of Florida, Gainesville, Florida 32611, United States

Abstract

Translation of nanoparticles (NPs) into human clinical trials for patients with refractory cancers has lagged due to unknown biologic reactivities of novel NP designs. To overcome these limitations, simple well-characterized mRNA lipid-NPs have been developed as cancer immunotherapeutic vaccines. While the preponderance of RNA lipid-NPs encoding for tumor-associated antigens or neopeptides have been designed to target lymphoid organs, they remain

*Corresponding Author Phone: (352) 273-9000. Fax: (352) 392-8413. elias.sayour@neurosurgery.ufl.edu.

Author Contributions

E. J. Sayour designed the research, contributed reagents, performed the experiments, analyzed the data, and wrote the paper. A. Grippin designed the research, performed the experiments, analyzed the data, and wrote the paper. G. De Leon performed the experiments and analyzed the data. B. Stover wrote the paper and performed the experiments. M. Rahman analyzed the data and contributed reagents. A. Karachi performed the experiments. B. Wummer performed the experiments. G. Moore performed the experiments. P. Castillo-Caro performed the experiments. K. Fredenburg wrote the paper and performed the experiments. M. R. Sarkisian wrote the paper, contributed reagents, analyzed the data and performed the experiments. J. Huang analyzed the data and contributed reagents. L. P. Deleyrolle analyzed the data and wrote the paper. B. Sahay analyzed the data and performed the experiments. S. Carrera-Justiz analyzed the data and performed the experiments. H. R. Mendez-Gomez designed the research, performed the experiments, analyzed the data, and wrote the paper. D. A. Mitchell designed the research, contributed reagents, analyzed the data, and wrote the paper.

ASSOCIATED CONTENT

The authors declare the following competing financial interest(s): Duane A. Mitchell holds ownership interest (including patents) in iOncologi, Inc. Duane A. Mitchell has patented immunotherapy-related technology that has been licensed by Annias Immunotherapeutics, Inc., Celldex Therapeutics, Inc., and Immunomic Therapeutics, Inc. The other authors declare no conflicts.

encumbered by the profound intratumoral and systemic immunosuppression that may stymie an activated T cell response. Herein, we show that systemic localization of untargeted tumor RNA (derived from whole transcriptome) encapsulated in lipid-NPs, with excess positive charge, primes the peripheral and intratumoral milieu for response to immunotherapy. In immunologically resistant tumor models, these RNA-NPs activate the preponderance of systemic and intratumoral myeloid cells (characterized by coexpression of PD-L1 and CD86). Addition of immune checkpoint inhibitors (ICIs) (to animals primed with RNA-NPs) augments peripheral/intratumoral PD-1⁺CD8⁺ cells and mediates synergistic antitumor efficacy in settings where ICIs alone do not confer therapeutic benefit. These synergistic effects are mediated by type I interferon released from plasmacytoid dendritic cells (pDCs). In translational studies, personalized mRNA-NPs were safe and active in a client-owned canine with a spontaneous malignant glioma. In summary, we demonstrate widespread immune activation from tumor loaded RNA-NPs concomitant with inducible PD-L1 expression that can be therapeutically exploited. While immunotherapy remains effective for only a subset of cancer patients, combination therapy with systemic immunomodulating RNA-NPs may broaden its therapeutic potency.

Graphical Abstract

Keywords

RNA nanoparticles; cancer vaccines; liposomes; immune checkpoint inhibitors; cancer immunotherapy; personalized therapy

Cancer immunotherapy with immune checkpoint inhibitors (ICIs) has shown significant promise against malignancies with immunologically active (“hot”) microenvironments, but has failed in clinical trials for patients with immunologically inactive (“cold”) tumors.^{1–4} Response to ICIs appears to be predicated on the presence of intratumoral CD8⁺PD-1⁺ cells^{5,6} and on activated PD-L1⁺ host-myeloid cells.⁷ These cell populations may be naturally increased in patients with high mutational burdens, but absent in those without response.^{5,6}

Cancer vaccines may be used to de novo activate these cell populations against immunologically inactive tumors, but have lagged in part by their inability to incite expeditious and robust antitumor immunity.^{8–12} RNA-lipid-nanoparticle (NP) vaccines have multiple advantages over traditional vaccines (i.e., bypassing MHC class restriction, targeted localization, and immunogenic potential of RNA as a toll-like receptor agonist), and have

shown promising preclinical results. By engineering RNA lipid-NPs to be anionic in composition, Kranz et al. and Broos et al. showed preferential localization to the spleen after intravenous administration.^{13,14} Similarly, Oberli et al. developed RNA lipid-NPs optimized with cholesterol and PEG for preferential localization to regional lymph nodes after subcutaneous administration.¹⁵ While these RNA lipid-NP vaccines have focused on targeting tumor-associated antigens or neoepitopes, and have been designed to target lymphoid organs of interest, they remain encumbered by the profound intratumoral and systemic immunosuppression that may stymie an activated T cell response.

To overcome these limitations, we sought to assess if systemic localization of untargeted tumor RNA (derived from whole transcriptome) encapsulated in lipid-NPs with excess positive charge could prime the peripheral and intratumoral milieu for response to immunotherapy. We developed a personalized RNA-nanoliposomal (tumor derived mRNA complexed into cationic DOTAP nanoliposomes with strongly positive ζ potentials) immunomodulating vaccine for diffuse in vivo localization and enhanced transfection efficiency (Supporting Information, Figure S1). From as few as 100–500 cells, we can manufacture personalized RNA-NP vaccines for patients soon after tumor biopsy. We hypothesized that systemic administration of these personalized RNA-NPs would prime the peripheral and intratumoral microenvironments with PD-L1⁺ host-myeloid cells sensitizing immunologically “cold” tumors for immunotherapeutic response.

We have previously shown that tumor RNA encapsulated into lipid DOTAP NPs of heterogeneous sizes (70–200 nm) can be used in place of dendritic cell vaccines.¹⁶ We sought to visualize RNA-NP transfected dendritic cells (DCs) in vitro by transmission electron microscopy (TEM) and assessed PD-L1 expression post-transfection by flow cytometry. RNA-NPs were visualized outside and inside DCs (after internalization) (Figure 1A, Supporting Information, Figure S2). Compared to cells treated with NPs alone, GFP RNA-NPs induced a >~4-fold increase in PD-L1 expression (Figure 1B). This was confirmed on primary human DCs (Figure 1C), matured ex vivo from source donor white blood cells, before in vitro transfection with RNA-NPs. These RNA-NPs were composed in excess of positively charged DOTAP liposomes at a concentration of 10¹² particles/mL (Figure 1D) with ζ potentials of 40–50 mV (Figure 1E) (at a ratio of 15 μ g of liposome to 1 μ g of mRNA). Unlike anionic RNA-NPs with an excess of RNA for enhanced localization to the spleen,¹³ these cationic RNA-NPs (with an excess of positively charged particles) localize nonspecifically to lung, heart, liver, and lymphoid organs (Figure 1F). We then assessed the effects of RNA-NPs on PD-L1⁺ activated myeloid cell expression throughout these organs. We vaccinated C57Bl/6 mice with iv RNA-NPs; spleens were harvested the next day for assessment of PD-L1 expression. We observed increased coexpression of CD11c and PD-L1 by immunofluorescent microscopy (Supporting Information, Figure S3). We then confirmed and quantitated these results by flow cytometry. Compared to ovalbumin (OVA) expressing RNA or NPs alone, RNA-NPs elicited a greater than 2-fold increase in the

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: [10.1021/acs.nanolett.8b02179](https://doi.org/10.1021/acs.nanolett.8b02179). Additional schematic and supporting data figures including TEM images, immunofluorescent analysis results, anti-tumor efficacy, toxicity studies, and MR imaging (PDF)

percentage of CD11c⁺ splenocytes expressing PD-L1 (Figure 1G). We uncovered that the increase in PD-L1 rose concomitantly with prototypical activation markers (i.e., CD86). In animals receiving RNA-NPs, there was a >~4- to 5-fold increase in the percentage and >~2-fold increase in the absolute number of CD11c⁺ splenocytes expressing both PD-L1 and CD86 (Figure 1H). To determine if the activated splenic phenotype (mediated by RNA-NPs) was present elsewhere, we analyzed CD11c⁺ cells from lungs, livers, lymph nodes, and bone marrow of C57Bl/6 mice (within 24 h of RNA-NPs). Compared to animals receiving NPs alone, mice receiving OVA RNA-NPs had a >~2-fold increase in PD-L1/CD86 on CD11c⁺ pulmonary cells, a >~3-fold increase on CD11c⁺ intrahepatic cells, a greater than 4-fold increase on CD11c⁺ lymph node cells, and a >~3-fold increase on CD11c⁺ bone marrow derived cells (Figure 1I). We then assessed if RNA-NPs could activate myeloid cells in the intratumoral microenvironment. In C57Bl/6 mice bearing established intracranial KR158-luc, RNA-NPs mediated increased colocalization of intratumoral PD-L1 and CD11c expression by immunofluorescent microscopy (Supporting Information, Figure S4A). We then evaluated if these intratumoral changes were present in immunologically “cold” B16-F10 tumors.¹ We implanted C57Bl/6 mice with flank B16-F10 tumors and evaluated intratumoral cells postvaccination for expression of PD-L1, CD86, and MHC class expression. We administered OVA RNA-NPs intravenously to animals bearing subcutaneous melanomas, and the next day, we harvested tumors for analysis of CD45⁺ and CD45⁺CD11c⁺ cells (after RBC lysis and live/dead exclusion, Figure 1J). There was a >~3-fold increase in PD-L1/CD86 on CD45⁺ intratumoral cells (Figure 1K,L), a >~50% increase in PD-L1 / CD86 on CD11c⁺ intratumoral cells (Supporting Information, Figure S4B), and a >~50% increase in MHC-I expression (by mean fluorescence intensity (MFI)) on CD45⁺ intratumoral cells (Figure 1M). Interestingly, after RNA-NPs, there was no change in PD-L1 expression on CD45⁻ tumor cells (Figure 1N) demonstrating these effects are enriched among CD45⁺ and CD11c⁺ tumor infiltrating cells.

Although increased PD-L1 expression on parenchymal cells is expected to protect normal cells from inflammatory damage, PD-L1 upregulation on myeloid APCs may predispose a regulatory phenotype that confounds the effector phase of the immune response. To determine if the increase in PD-L1 correlated with a suppressor phenotype, we vaccinated naïve C57Bl/6 mice with OVA RNA-NPs, FACS sorted PD-L1⁺ myeloid APCs within 24 h (sorted by expression of PD-L1⁺, CD11c⁺, MHCII⁺, and CD86⁺), and cocultured them in vitro with OVA-mRNA pulsed dendritic cells and splenocytes from OTI transgenic mice. In a coculture of DCs and OTI splenocytes, addition of PD-L1⁺ APCs from mice vaccinated with OVA RNA-NPs did not suppress interferon- γ (IFN- γ) secretion (Figure 2A), but rather mediated increased IFN- γ when cocultured alone with OTI splenocytes (Figure 2B). We show that these PD-L1⁺ APCs elicit IFN- γ production (Supporting Information, Figure S5A) not achieved by PD-L1⁺ APCs negative for CD86 expression, or PD-L1⁺ cells negative for CD11c and CD86 expression (Supporting Information, Figure S5B). Next, we administered these PD-L1⁺ APCs (from mice vaccinated with OVA RNA- NPs) to naïve mice receiving OTI splenocytes and found a modestly increased percentage of OVA-specific CD8⁺ splenocytes (Figure 2C). In a separate experiment, we show that PD-L1⁺ APCs sorted from tumors of mice vaccinated with OVA RNA-NPs lead to expansion of OVA-specific CD8⁺ cells in the tumor and draining lymph nodes of recipient animals (Supporting Information,

Figure S6). These intratumoral PD-L1⁺ APCs (sorted by expression of PD-L1⁺, CD11c⁺, MHCII⁺, and CD86⁺) could elicit similar effects to peripherally sorted PD-L1⁺ APCs. In an in vitro coculture of OVA-mRNA pulsed dendritic cells and OVA-specific T cells, in contrast to PD-L1 negative APCs, PD-L1⁺ intratumoral APCs (from mice bearing intracranial B16F10-OVA vaccinated with OVA RNA-NPs) did not suppress but enhanced IFN- γ release (Figure 2D,E). Thus, while RNA-NPs increase systemic and intratumoral expression of PD-L1 on myeloid APCs upon activation, these cells do not suppress but enhance antigen-specific T cell responses.

Since PD-L1 was markedly increased on activated CD11c⁺ myeloid cells, we assessed the effects of RNA-NPs and ICIs on antitumor immunity. To validate the activity of murine ICIs, GFP RNA-NPs were administered to naïve C57Bl/6 mice, with or without PD-L1 mAbs, and spleens were harvested the next day. RNA-NPs mediated an increase in PD-L1 on splenic (Figure 2F) CD11c⁺ cells, which was not observed following vaccination with peptide vaccines in complete Freund's adjuvant (CFA); however, these effects could be abrogated in animals receiving concomitant PD-L1 mAbs, but not isotype mAbs (Figure 2G). Next, we explored the immunologic effects of combination therapy; we vaccinated mice with pp65 loaded RNA-NPs once weekly ($\times 3$), harvested spleens 1 week after the last vaccine, and restimulated splenocytes with overlapping pp65 peptide pool for assessment of IFN- γ reactivity. While there were comparable levels of IFN- γ between RNA-NPs and RNA-NPs with PD-L1 mAbs, combination therapy elicited a greater than 2-fold increase in the percentage of CD8⁺ splenocytes expressing PD-1 (Figure 2H). We then assessed the effects of combination therapy on antitumor efficacy. C57Bl/6 mice were implanted with subcutaneous B16F10-OVA, and RNA-NPs were administered once weekly ($\times 3$). Compared with OVA RNA-NPs or PD-1/PD-L1 mAbs alone, animals receiving combination therapy with OVA RNA-NPs and PD-L1 mAbs had significantly prolonged median overall survival benefits (Figure 2I). Monotherapy (with either PD-1/PD-L1 mAbs or RNA-NPs) was insufficient to produce long-term survivors, but combination therapy of RNA-NPs and PD-L1 mAbs mediated long-term survivorship in over 60% of the animals (Figure 2I).

ICIs appear to elicit their activity in immunologically active cancers but, as of yet, have not been fully unlocked in poorly immunogenic malignancies. To determine if RNA-NPs targeting physiologically relevant tumor antigens could sensitize immunologically "cold" tumors to effects of ICIs, we administered RNA-NPs with PD-L1 mAbs to poorly immunogenic murine models (i.e., B16F0, B16-F10, and Lewis lung carcinoma (LLC)) with downregulated expression of immune-related genes.¹⁷ C57Bl/6 mice were implanted with B16F0 tumors and treated with weekly total tumor derived RNA-NPs (TTRNA-NPs) ($\times 3$) with twice weekly anti-PD-L1 mAbs (until last TTRNA-NP vaccine). While monotherapy with PD-L1 (or PD-1 mAbs) did not confer benefit, in combination with TTRNA-NPs, there was synergistic antitumor efficacy (Figure 3A,B). On day 27, spleens and tumors were harvested from surviving animals. Compared to RNA-NPs alone, animals receiving TTRNA-NPs with PD-L1 mAbs had increased PD-1⁺ splenocytes, and increased CD45, CD4 and a trend toward increased CD8⁺ tumor infiltrating lymphocytes (TILs) (Figure 3C). These synergistic effects and sensitization to PD-L1 inhibition from TTRNA-NPs were also shown in other poorly immunologic murine tumor models. TTRNA-NPs sensitized response to PD-L1 mAbs in a B16-F10 model (Figure 3D), and in C57Bl/6 mice implanted with LLC,

TTRNA-NPs elicited a moderately enhanced response to ICIs (Supporting Information, Figure S7), with strong biologic activity (Figure 3E). In animals implanted with LLC, only combination therapy elicited a significant increase (>4 -fold) in intratumoral PD-1⁺CD8⁺ cells (Figure 3E).

We have previously shown that antitumor activity from RNA-NPs is dependent on type I IFN.¹⁶ In animals receiving concomitant RNA-NPs with IFN- α blocking antibodies that disrupt receptor signaling (IFNAR1 mAbs), antitumor efficacy was abrogated.¹⁶ We sought to assess if type I IFN was also responsible for increased PD-L1 expression and synergistic effects from RNA-NPs and ICIs. We administered RNA-NPs with IFNAR1 mAbs to tumor-bearing and non-tumor-bearing C57Bl/6 mice, and harvested spleens and tumors within 24 h for assessment of CD11c, CD86, and PD-L1. RNA-NPs mediated an increase in CD86/PD-L1 on CD11c⁺ splenocytes that was significantly decreased by coadministration of IFNAR1 mAbs (Figure 4A, Supporting Information, Figure S8A). However, these effects were not limited to CD11c⁺ cells; after RNA-NPs, there was a >20 -fold increase in the percentage of PD-L1⁺CD86⁺ splenocytes that was nearly abrogated after coadministration of IFNAR1 mAbs (Figure 4B). Subsequently, we assessed other myeloid cell populations including macrophages and plasmacytoid DCs (pDCs). While macrophages were not increased (Figure 4C), the percentage of MHCII⁺PDCA-1⁺ cells were considerably increased (Figure 4D). These cells were nearly uniformly positive for coexpression of PD-L1/CD86, and could be significantly decreased by coadministration of PDCA-1 mAbs (Figure 4D). Administration of PDCA-1 depleting mAbs (prior to RNA-NPs) decreased the percentage of PD-L1⁺CD86⁺ splenocytes and nearly abrogated the surge in IFN- α in non-tumor-bearing and tumor-bearing mice (Figure 4E, Supporting Information, Figure S8B), suggesting pDCs to be the predominant cell type responsible for the type I IFN response elicited by RNA-NPs. In C57Bl/6 mice bearing subcutaneous B16F10-OVA, concomitant administration of IFNAR1 mAbs abrogated the antitumor efficacy (Figure 4F) and the PD-1⁺CD8⁺ signature (Figure 4G) from both PD-L1 mAbs alone and combination RNA-NPs with PD-L1 mAbs. In summary, systemic RNA-NPs localize to reticuloendothelial organs activating CD11c⁺ myeloid cells. Type I IFN release from pDCs mediates increased PD-L1 expression, and in the presence of ICIs, leads to enhanced T cell activity (characterized by CD8⁺PD-1⁺ expression) and synergistic antitumor efficacy.

Since RNA-NPs mediate response through type I IFNs, and systemic IFN- α can be toxic in human trials, we evaluated the safety of RNA-NPs in acute and chronic toxicity studies in C57Bl/6 mice (Supporting Information, Figures S9 and S10). In these studies, RNA-NPs appeared safe based on CBCs/ differentials, chemistries, organ function tests, and end-organ histology (Supporting Information, Figures S9 and S10). We then evaluated the safety and activity of personalized tumor mRNA-NPs in a client-owned canine. A 9 year old spayed female boxer was diagnosed with a malignant glioma by MRI (Figure 5A) and subsequently by histopathology after presenting with seizure activity; she subsequently enrolled on trial to receive RNA-NPs per UFIACUC#201609430. The tumor was biopsied, and total RNA was extracted for generation of a cDNA library. We amplified mRNA from this library and complexed it with nanoliposomes. The nanoparticles (1.375 mg of RNA encapsulated in 20.6 mg of DOTAP liposomes) were administered intravenously by iv push over 5 min (Figure 5B). These RNA-NPs were composed in excess of positively charged DOTAP

liposomes at a concentration of $>10^{12}$ particles/mL (Figure 5C) with ζ potentials of >40 – 50 mV (Figure 5D). The patient received RNA-NPs beginning 2 weeks after her biopsy. Aside from low-grade fevers (normal temperature range for canines: 100 – 102 °F) that spiked 6 h postvaccination on the initial day (Figure 5E), personalized mRNA-NPs were well-tolerated with stable blood counts, differentials, and renal and liver function tests (Supporting Information, Figure S11). RNA-NPs elicited an increase in serum IFN- μ that peaked at 2 h (Figure 5F). RNA-NPs also elicited an increase in PD-L1, CD80, CD86, and MHCII on CD11c $^{+}$ peripheral blood cells in the first 6 h postvaccination (Figure 5G,H, gating strategy shown in Figure 5G), demonstrating activation of peripheral myeloid cells with concomitant PD-L1 expression. We then assessed CD3 $^{+}$ lymphoid populations. While percentages of CD4 and CD8 $^{+}$ cells remained relatively unchanged, IFN γ $^{+}$ CD8 $^{+}$ cells increased, and regulatory FOXP3 $^{+}$ CD4 $^{+}$ cells decreased in the first few hours postvaccination (Figure 5I). After receiving weekly RNA-NPs ($\times 3$), the canine had a steady course. Postvaccination MRI was consistent with either progression or pseudoprogression (Supporting Information, Figure S12); however, the canine was stable at the time, which may be more consistent with pseudoprogression from an immunotherapeutic response. Ultimately, the canine progressed over time as would be expected for a malignant glioma without definitive surgical resection or radiotherapy. Although these results need to be validated in larger canine data sets, these data suggest safety and activity of personalized mRNA-NPs in a spontaneous canine disease model in a patient that did not receive any other antitumoral therapeutic interventions.

Cancer immunotherapy holds the promise of targeting tumors with exquisite specificity, but remains limited by the local and systemic regulatory microenvironments pervasive in refractory malignancies.^{2–4,18–20} Reshaping the systemic/intratumoral immunologic microenvironments to maximize effective immunotherapy hinges on breaking peripheral tolerance and overcoming immunosuppression.^{12,21,22} We have developed a new personalized RNA-NP technology that simultaneously functions as both a vaccine and immunomodulating agent. This platform can be generated from few tumor cells where total RNA can be extracted for generation of a cDNA library and amplification of personalized tumor-specific mRNA. Through electrostatic interactions, this personalized tumor-specific mRNA complexes with positively charged lipid-NPs forming RNA-NPs. After systemic administration, RNA-NPs mediate release of type I IFN from pDCs into the regional milieu activating APCs therein. Upon activation, these APCs express copious amounts of PD-L1. Although PD-L1 expression is prototypically associated with a regulatory cell phenotype, we demonstrate that these PD-L1 $^{+}$ myeloid cells enhance antigen-specific immune responses.

Recently, the work by Tang et al. demonstrated that PD-L1 expression on myeloid cells, and not tumor cells, were critical for effects of ICIs.⁷ To identify which myeloid populations were requisite for mediating responsiveness to PD-L1 mAbs, macrophages, myeloid-derived suppressor cells (MDSCs), and phagocytes were selectively depleted, but neither population was essential; alternatively, PD-L1 blockade on APCs potentiated IFN- γ release from T cells.⁷ These results suggest that PD-L1 blockade on APCs enhances their ability to activate T cells.⁷ Although PD-L1 is a regulatory ligand expressed on inhibitory immune cell populations, PD-L1 upregulation on APCs may represent an early activation marker that can

be preferentially exploited with ICIs.^{5,21,23} PD-L1 blockade may allow APCs (i.e., dendritic cells) to survive longer with enhanced intrinsic functions perpetuating their role as activators of antigen-specific T cell immunity. In this context, early PD-L1 blockade may enhance survival of RNA-NP induced PD-L1⁺ APCs, increasing presentation to naïve T cells, and augmenting numbers of activated TILs. Alternatively, PD-L1 blockade may enhance intrinsic functions of PD-L1⁺ APCs through increased cytokine secretion (i.e., IL-12) polarizing Th1 type cells and augmenting CD4 help for antigen-specific CD8⁺ T cells. These effects may be distinct from other synergistic immunotherapeutic combinations (i.e., CAR-T cells and ICIs), which are predicated on maintaining the survival/cytotoxicity of activated T cells (through either receptor PD-1 or ligand PD-L1 inhibition).

While ICIs targeting exhaustion markers (i.e., PD-1) can increase persistence and therapeutic efficacy of chimeric antigen receptor modified (CAR) modified T cells, CAR-T technologies remain limited by (1) the complexity of cell therapy, (2) off-target effects from surface antigen targeting (i.e., CD19, HER2, IL-13 α , EGFR), (3) antigen loss (i.e., CD19 loss in B cell malignancies), and (4) profound intratumoral immune suppression. Personalized mRNA-NPs overcome these challenges. They bypass the need for leukapheresis/complex cellular processing and can be expeditiously generated after a patient's biopsy. Through MHC class presentation of whole tumor transcriptome, RNA-NPs allow APCs to activate TCRs against a cohort of tumor-specific epitopes for maximal tumor-specific immunity and minimal off-target effects. Unlike CAR-T cells, they recruit multiple arms of the immune system (i.e., innate and adaptive), remodeling the systemic and intratumoral immune milieu, which remains a potent barrier for vaccine, cellular, and checkpoint inhibiting immunotherapies.

In summary, RNA-NPs break tolerance to induce a myeloid and lymphoid phenotype requisite for ICI activity. They activate systemic and intratumoral immunity within 24 h eliciting significant increases in the percentage of PD-L1⁺ CD86⁺ myeloid cells in systemic organs (i.e., spleen, bone marrow, liver) and within the tumor microenvironment. Concomitant administration of anti-PD-L1 mAbs with RNA-NPs increases the percentage of peripheral/intratumoral PD-1⁺CD8⁺ cells and elicits synergistic antitumor activity in immunologically "cold" tumor models resistant to ICI monotherapy. These results underscore that RNA-NPs may reprogram systemic immunity in part by unmasking the PD-L1 axis on activated myeloid cells for synergy with immune checkpoint blockade. While activity of ICIs appears dependent on activated PD-L1⁺ myeloid cells, limiting effectiveness of ICIs to malignancies enriched in these populations, we show that RNA-NPs increase PD-L1 upon activation of murine and canine myeloid cells priming systemic and intratumoral immunity for response to immunotherapy. This evidence establishes a role for RNA-NPs as both potent antigen-specific vaccines and immune modulators of host response.

Materials and Methods.

Experimental Design.

Since systemic delivery of RNA-NPs has been previously shown to induce activation of APCs, we sought to (1) assess these cells for expression of regulatory checkpoint ligands, (2) explore the functional capacity of these cells in vitro and in vivo, (3) elucidate

immunologic and antitumor effects of combination therapy with RNA-NPs and ICIs, (4) identify mechanistic interactions from RNA-NPs and ICIs, and (5) model these effects in a spontaneous large animal canine model. Mice were controlled for age and sex across experiments. Tumor-bearing mice were randomized prior to receiving interventional treatments. Timing of treatments and end-points were determined based on prespecified conditions. Animals were vaccinated within 48 h post-tumor implantation to mimic treatment effects of a minimal residual disease state. For analysis of APCs, mice were euthanized within 24 h to assess activating effects of RNA-NPs. For analysis of immunologic T cell responses, animals were euthanized after 7–12 days following the last RNA-NP vaccine to allow for T cell priming and trafficking. Animals reaching humane end-points (i.e., tumor volume size, tumor ulceration) prior to meeting a prespecified condition were euthanized.

Isolation of RNA.

RNA was isolated as previously described.¹⁶ Briefly, total tumor derived RNA from tumor cells (B16F0, B16-F10, LLC, KR158-luc) was isolated from commercially available RNeasy minikits (Qiagen) per manufacturer's instructions. To generate model RNA templates (GFP and OVA) for in vitro transcription (IVT), plasmids were linearized and purified with Qiagen PCR MiniElute kit before linearized DNA was transcribed using mMessenger RNA IVT kit (Life technologies, Invitrogen). Final RNA product was cleaned up using RNA Maxi kits (Qiagen). Cre mRNA (and in some experiments, GFP, luciferase, and OVA-mRNA) was purchased from Trilink Biotechnologies. For generation of personalized mRNA, total RNA was isolated from RNeasy kits (Qiagen), and cDNA libraries were generated by RT-PCR. Using a SMARTScribe Reverse Transcriptase kit (Takara), a reverse transcriptase reaction by PCR was performed on the total tumor RNA in order to generate cDNA libraries. The resulting cDNA was then amplified using Takara Advantage 2 Polymerase mix with T7/SMART and CDS III primers, with the total number of amplification cycles determined by gel electrophoresis. Purification of the cDNA was performed using a Qiagen PCR purification kit per manufacturer's instructions. In order to isolate sufficient mRNA for use in each RNA-nanoparticle vaccine, mMESAGE mMACHINE (Invitrogen) kits with T7 enzyme mix were used to perform overnight in vitro transcription on the cDNA libraries. The resulting mRNA was then purified with a Qiagen RNeasy Maxi kit to obtain the final mRNA product.

Nanoparticles.

Nanoparticles were generated as previously described.¹⁶ The cationic lipid DOTAP (powder form) was acquired from Avanti, Polar Lipids Inc. (Alabaster, AL). For preparation, chloroform was added to resuspend 25–100 mg; chloroform was evaporated off until a thin lipid layer remained. The mixture was resuspended in 5–20 mL of PBS before being placed in 50 °C water bath for 1 —2 h with intermittent vortexing. Within 24 h, 5—20 mL of PBS was added to the mixture, vortexed, and placed in a bath sonicator for 5 min before passage through a 0.43 μm and a 0.22 μm syringe filter (PALL Acrodisc syringe filter with Supor membrane). The final NP solution (2.5 $\mu\text{g}/\mu\text{L}$) was based on a prefiltration DOTAP concentration (2.5 $\mu\text{g}/\mu\text{L}$).

RNA-NP Complex Preparation.

RNA-NPs complexes were prepared as previously described.¹⁶ In vivo, 25 μg of RNA was added to 375 μg of DOTAP (per mouse) in PBS buffer; 200 μL of the RNA-NPs was injected into the tail vein of C57Bl/6 mice. In vitro, 1.67 μg of RNA was added to 25 μg of DOTAP (per 1×10^5 cells) in PBS buffer. In both cases, the mixture was kept at room temperature (~ 15 –20 min) to facilitate complex formation.

Canine mRNA-NPs.

After consent was obtained from the canine's owner, a study-enrolled dog with spontaneously occurring high-grade glioma underwent CT guided core needle biopsy under sedation per UFIACUC#201609430. The total tumor RNA from this biopsy was extracted using a Qiagen RNeasy minikit per manufacturer's instructions and manufactured into a personalized mRNA product as indicated above. Personalized tumor mRNA (0.05 mg/kg) was encapsulated into DOTAP nanoliposomes (0.75 mg/kg) and administered once weekly for 3 weeks. Blood was sequentially drawn for analysis of hematology, serum chemistry, and immunologic response.

ζ Potential and Concentration of Nanoparticles.

A Brookhaven ZetaPlus instrument (Brookhaven Instruments Corporation, Holtsville, NY) was used to measure nanoparticle ζ potential by phase analysis light scattering (or PALS), using Smoluchowski model, 5 runs per sample, and 25 cycles per run. A Malvern Panalytical NanoSight NS300 instrument (Malvern Panalytical Ltd., Malvern, UK) was used to calculate particle concentration, using 5 acquisitions per sample and 60 s per acquisition.

Electron Microscopy.

DC2.4s were grown on a sterilized Aclar sheet (Electron Microscopy Sciences, Hatfield, PA) before transfection with RNA-NPs. Within 24 h, Aclar sheets were removed from culture, washed, and fixed in a mix of 3% paraformaldehyde and 2% glutaraldehyde in 0.1 M phosphate buffer for 1 h. Cells were washed, postfixed in 1% osmium, dehydrated in ethanol, and embedded in resin. Ultrathin sections (70 nm) stained with uranyl acetate and lead citrate were analyzed using a Hitachi H-7600 transmission electron microscope at 80 kV.

Mice.

C57Bl/6 mice, B6-GFP mice, tdTomato reporter A14 mice, and OTI transgenic mice on the C57Bl/6 background were purchased from Jackson Laboratories. Animal procedures were approved by the Institutional Animal Care and Use Committee at the University of Florida.

OTI Transfer.

In some experiments, mice receiving OVA RNA-NPs were “spiked” with a responder T cell population from OTI mice (C57Bl/6-Tg(Tcr α Tcr β)1100Mjb/J, Jackson Laboratory) containing transgenic T cell receptors recognizing OVA peptide epitopes 257–264. Spleens were harvested from OTI transgenic mice, and underwent RBC lysis before transfer of 0.5 – 1×10^7 cells.

Coculture Assays.

Cells were harvested from spleens or tumors of RNA-NP treated mice, stained and sorted through a BD Aria II cell sorter into a 50–100% FBS solution. These cells were then cultured with antigen-specific T cells for 48 h at a ratio of 40 000 APCs:400 000 T cells in a 96 well plate.

Pp65 Restimulation Assay and ELISAs.

Splenocytes from mice vaccinated with RNA-NPs were resuspended in T cell media with IL-2 (1 $\mu\text{g}/\text{mL}$) at a concentration of 4×10^6 per mL and were cocultured with $>1 \mu\text{g}$ (per peptide) of overlapping pp65 peptide mix (JPT Peptides) for 48 h. Soups were harvested at 48 h for assessment of IFN- γ by ELISA (ebioscience). In separate experiments, IFN- α detection from serum was also performed via ELISA in mouse (ebioscience) and canine (neoscientific).

CFA Vaccination.

Complete Freund's adjuvant (CFA) was emulsified with an equal volume of 100 μg of OVA class I peptide (SIINFEKL; American Peptide Company, Inc.) in 10% DMSO and administered intradermally (100 $\mu\text{L}/\text{mouse}$).

Dendritic Cells and T Cells.

T cells were ex vivo expanded and activated based on previously published methods.²⁴ Bone marrow was harvested from naïve C57Bl/6 mice, and dendritic cells were matured, electroporated and activated ex vivo based on previously published methods.²⁴ Human DCs were matured from source donor white blood cells from LifeSouth Community Blood Center (Gainesville, FL) and cultured in AIM V (2% HABS) with GM-CSF and IL-4.

Flow Cytometric Analysis.

Organs (i.e., spleen, liver, bone marrow, lymph nodes) or subcutaneous tumors were harvested from mice within 24 h of RNA-NPs injection. Prior to FACS sorting, intracranial tumors were harvested from mice 17 days after treatment with RNA-NPs, washed with PBS, and digested in collagenase for 30 min at 37 °C before staining. Peripheral organs underwent RBC lysis before being washed and stained for surface expression (i.e., CD45, CD11c, CD86, MHC-I, MHCII, F4/80, PDCA-1, CD4, CD8, PD-1, and PD-L1 (Affymetrix, BDBioscience, and Biolegend); LIVE/DEAD fixable near-IR dead cell stain kit (Invitrogen)). PD-L1 detecting antibody (clone: MIH5) was distinct from PD-L1 blocking antibody (clone: 10F.9G2). Counting beads (Life Technologies) were added to samples to assess absolute counts. Afterwards the cells were sorted with a BD Aria II FACS sorter or fixed and analyzed using a BD Bioscience FACS Calibur, Canto, or LSR II.

Immunofluorescence.

Organs were suspended overnight in 4% buffered paraformaldehyde solution at 4 °C. Tissues were washed with PBS before overnight immersion in 15% sucrose at 4 °C, followed by overnight immersion in 30% sucrose at 4 °C. Tissues were then embedded in Tissue-Tek OCT (Electron Microscopy Sciences, Hatfield, PA), frozen and stored at –80 °C

until sectioning. Sections of 20 μm thickness were cut on a Leica CM1850 cryostat (Leica Microsystems, Wetzlar, Germany) and stored at $-20\text{ }^{\circ}\text{C}$ until immunohistochemistry (IHC) processing. Immunofluorescence staining was performed as follows: incubation with 1% normal goat serum (NGS) (cat. 01–6201, ThermoFisher Scientific, Waltham, MA) in PBS for 1 h at room temperature (RT); overnight incubation ($4\text{ }^{\circ}\text{C}$) in 1% NGS and PBS with the primary antibodies rat anti-PD-L1 (clone MIH6, 1:1000 dilution, cat. ab80276, Abcam, Cambridge, UK), and Armenian hamster antimouse CD11c (clone N418, 1:1000 dilution, cat. 14–0114-82, ThermoFisher Scientific). Subsequently, samples were washed ($\times 3$) with PBS before incubation (diluted 1:500 for 2 h at RT) with secondary antibodies: Alexa Fluor 488 goat antirat (ThermoFisher Scientific), and Alexa Fluor 647 goat antihamster (ThermoFisher Scientific). Samples were incubated with 4,6-diamidino-2-phenylindole dihydrochloride (or DAPI, Sigma-Aldrich, St. Louis, MO) for 10 min before being washed ($\times 3$) with PBS and mounted with Mowiol 4–88 (Sigma-Aldrich). Sections were evaluated using an Olympus DSU-IX81 confocal microscope (Olympus, Tokyo, Japan).

Hematology Analysis.

Blood was obtained from mice and submitted for comprehensive chemistry and CBC analysis (cat. 61331, IDEXX, North Grafton, MA). EDTA-anticoagulated whole blood samples were analyzed using a flow-based Sysmex XT-2000iV Automated Hematology Analyzer (Sysmex, Kobe, Japan).

Hematoxylin and Eosin (H&E) Histochemistry.

Mouse organs were collected and immersed in 10% formalin-buffered solution overnight at RT. Samples were paraffin processed by a Tissue Tek VIP automatic tissue processor (Sakura Finetek, Torrance, CA), sectioned at 4 μm using a Microm microtome, placed in Superfrost Plus glass slides and H&E (hematoxylin and eosin) stained by UF Molecular Pathology Core (UF, Gainesville, FL). Slides were analyzed by a trained in-house pathologist and by HistoWiz (Histopathology Site, HistoWiz Inc.).

Tumor Cells.

Tumor cell lines B16-F10, B16F10-OVA, and KR158B-luc were obtained as previously described.¹⁶ B16F0 and LLC were purchased from ATCC. CT-2A tumors were originally generated as previously described²⁵ and maintained in culture. Culture media utilized to grow tumor cells consisted of DMEM with sodium pyruvate (KR158B-luc and CT-2A were grown without sodium pyruvate), supplemented with 10% fetal bovine serum (FBS), and 5.5 mL of penicillin/streptomycin. Cells were cultured in an incubator at a temperature of $37\text{ }^{\circ}\text{C}$ (5% CO_2 levels).

Tumor Implantations.

Tumor cells were harvested with trypsin (Gibco) and washed in serum-containing media, before being washed in phosphate-buffered saline (PBS). For subcutaneous tumors, cell pellets were resuspended in the appropriate PBS concentration; viable tumor cells as determined by differential trypan blue dye uptake were counted. Tumors were injected subcutaneously in the flank of C57Bl/6 mice anesthetized with isoflurane. Tumor volumes

were measured using calipers assessing the greatest longitudinal (length) and transverse (width) diameters and calculated by the modified ellipsoidal formula ($0.5(\text{length} \times \text{width}^2)$).²⁶ Mice that did not develop non-OVA transfected B16 tumors were removed from analysis across all experimental groups. Animals were sacrificed for significant tumor ulcerations, or when the tumor grew larger than 2 cm in any one dimension. Animals reaching humane endpoints were dropped/removed from analysis of tumor-volume measurements at subsequent time-points.

Intracranial Tumors.

Cell pellets were resuspended in a mixture of PBS:methyl cellulose mixture (at a 1:1 ratio) at 4 million cells/mL before stereotactic intracranial implantation (3 mm deep into the brain at a site 2 mm to the right of the bregma).

Monoclonal Antibodies.

IFN- α blocking monoclonal antibodies against the IFNAR1 receptor (clone: MAR1-5A3) were purchased from BioXcell, and 250–500 μg (loading dose 500 μg followed by 250 μg twice weekly) was administered in the peritoneum (ip). Anti-PD-L1 (clone: 10F.9G2) and anti-PD-1 monoclonal antibodies (clone: RMP1-14) were purchased from BioXcell, and 200–400 μg /dose (loading dose 400 μg followed by 200 μg twice weekly) was administered in the peritoneum of C57Bl/6 mice. PDCA-1 depleting mAbs (clone: 120G8.04) were purchased from Novus Biologicals, and 200–250 μg was administered in the peritoneum of C57Bl/6 mice (1 day before RNA-NP administration).

Statistical Analysis.

Mann-Whitney nonparametric tests were used to analyze data from in vivo experiments; however, in experiments with three animals per group, Student t tests were used for analysis of closely clustered data points. Unless otherwise specified in figure legends, mean values in figures were plotted with standard deviations (SD). Mann-Whitney tests were used to determine statistical significance between group means from subcutaneous tumor growth volumes; tumor volumes were plotted with standard error of the mean (SEM). Survival data from our murine tumor efficacy studies were analyzed by the Gehan-Breslow-Wilcoxon test. In all experiments, statistical significance was set at p values < 0.05 . Sample sizes are included in figures/legends. Data analyses were conducted in GraphPad Prism.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGMENTS

The authors would like to acknowledge Daniel Valladares for his assistance with immunofluorescence, Jeffrey Drake for his molecular biology assistance in generating in vitro transcribed mRNAs, Jill Verlander and Sharon Matthews in the UF College of Medicine EM Facility for technical assistance, and Anjelika Dechkovskaia for her assistance with preparation of human DCs. The authors would also like to acknowledge Vrunda Trivedi and Catherine Flores for their assistance in setting up and analyzing our cell therapy and ex vivo coculture systems, and Lana Fagman for her role in supporting our canine trial.

Funding:

This work was supported by the National Institutes of Health-R01CA195563 (to D. A. Mitchell), TL1TR001428, and K08CA199224 (to E. J. Sayour). This work is also supported by the U.S. Department of Defense-W81XWH-17-1-0510 (to E. J. Sayour), Hyundai Hope on Wheels (Hope Award to E. J. Sayour), St. Baldricks Foundation (Hannah's Heroes Scholar Award to E. J. Sayour), Ocala Royal Dames Cancer Research Award (to E. J. Sayour), B*Cured Foundation (Research Investigator Grant to E. J. Sayour), American Cancer Society Research Scholar Grant (#RSG-13-031-01-DDC) (to M. R. Sarkisian), and the Thomas H. Maren Junior Investigator's Fund (to E. J. Sayour).

REFERENCES

- (1). Filley AC; Henriquez M; Dey M *Oncotarget* 2017, 8 (53), 91779–91794. [PubMed: 29207684]
- (2). Brahmer JR; Tykodi SS; Chow LQ; Hwu WJ; Topalian SL; Hwu P; Drake CG; Camacho LH; Kauh J; Odunsi K; Pitot HC; Hamid O; Bhatia S; Martins R; Eaton K; Chen S; Salay TM; Alaparthi S; Grosso JF; Korman AJ; Parker SM; Agrawal S; Goldberg SM; Pardoll DM; Gupta A; Wigginton JM *N. Engl. J. Med.* 2012, 366 (26), 2455–65. [PubMed: 22658128]
- (3). Borghaei H; Paz-Ares L; Horn L; Spigel DR; Steins M; Ready NE; Chow LQ; Vokes EE; Felip E; Holgado E; Barlesi F; Kohlhaufl M; Arrieta O; Burgio MA; Fayette J; Lena H; Poddubskaya E; Gerber DE; Gettinger SN; Rudin CM; Rizvi N; Crino L; Blumenschein GR Jr.; Antonia SJ; Dorange C; HARBISON CT; Graf Finckenstein F; Brahmer JR *N. Engl. J. Med.* 2015, 373 (17), 1627–39. [PubMed: 26412456]
- (4). Topalian SL; Hodi FS; Brahmer JR; Gettinger SN; Smith DC; McDermott DF; Powderly JD; Carvajal RD; Sosman JA; Atkins MB; Leming PD; Spigel DR; Antonia SJ; Horn L; Drake CG; Pardoll DM; Chen L; Sharfman WH; Anders RA; Taube JM; McMiller TL; Xu H; Korman AJ; Jure-Kunkel M; Agrawal S; McDonald D; Kollia GD; Gupta A; Wigginton JM; Sznol M *N. Engl. J. Med.* 2012, 366 (26), 2443–54. [PubMed: 22658127]
- (5). Gros A; Robbins PF; Yao X; Li YF; Turcotte S; Tran E; Wunderlich JR; Mixon A; Farid S; Dudley ME; Hanada K; Almeida JR; Darko S; Douek DC; Yang JC; Rosenberg SA *J. Clin. Invest.* 2014, 124 (5), 2246–59. [PubMed: 24667641]
- (6). Badoual C; Hans S; Merillon N; Van Ryswick C; Ravel P; Benhamouda N; Levionnois E; Nizard M; Si-Mohamed A; Besnier N; Gey A; Rotem-Yehudar R; Pere H; Tran T; Guerin CL; Chauvat A; Dransart E; Alanio C; Albert S; Barry B; Sandoval F; Quintin-Colonna F; Bruneval P; Fridman WH; Lemoine FM; Oudard S; Johannes L; Olive D; Brasnu D; Tartour E *Cancer Res.* 2013, 73 (1), 128–38. [PubMed: 23135914]
- (7). Tang H; Liang Y; Anders RA; Taube JM; Qiu X; Mulgaonkar A; Liu X; Harrington SM; Guo J; Xin Y; Xiong Y; Nham K; Silvers W; Hao G; Sun X; Chen M; Hannan R; Qiao J; Dong H; Peng H; Fu YX *J. Clin. Invest.* 2018, 128 (2), 580–588. [PubMed: 29337303]
- (8). Rosenberg SA; Dudley ME *Proc. Natl. Acad. Sci. U. S. A.* 2004, 101, 14639–14645. [PubMed: 15381769]
- (9). Hinrichs CS; Rosenberg SA *Immunological reviews* 2014, 257 (1), 56–71. [PubMed: 24329789]
- (10). Rosenberg SA *Sci. Transl. Med.* 2012, 4 (127), 127ps8.
- (11). Weller M; Butowski N; Tran DD; Recht LD; Lim M; Hirte H; Ashby L; Mechtler L; Goldlust SA; Iwamoto F; Drappatz J; O'Rourke DM; Wong M; Hamilton MG; Finocchiaro G; Perry J; Wick W; Green J; He Y; Turner CD; Yellin MJ; Keler T; Davis TA; Stupp R; Sampson JH; et al. *Lancet Oncol.* 2017, 18 (10), 1373–1385. [PubMed: 28844499]
- (12). van der Burg SH; Arens R; Ossendorp F; van Hall T; Melief CJ *Nat. Rev. Cancer* 2016, 16 (4), 219–33. [PubMed: 26965076]
- (13). Kranz LM; Diken M; Haas H; Kreiter S; Loquai C; Reuter KC; Meng M; Fritz D; Vascotto F; Hefesha H; Grunwitz C; Vormehr M; Husemann Y; Selmi A; Kuhn AN; Buck J; Derhovanessian E; Rae R; Attig S; Diekmann J; Jabulowsky RA; Heesch S; Hassel J; Langguth P; Grabbe S; Huber C; Tureci O; Sahin U *Nature* 2016, 534, 396. [PubMed: 27281205]
- (14). Broos K; Van der Jeught K; Puttemans J; Goyvaerts C; Heirman C; Dewitte H; Verbeke R; Lentacker I; Thielemans K; Breckpot K *Mol. Ther.-Nucleic Acids* 2016, 5 (6), e326. [PubMed: 27327138]
- (15). Oberli MA; Reichmuth AM; Dorkin JR; Mitchell MJ; Fenton OS; Jaklenec A; Anderson DG; Langer R; Blankschtein D *Nano Lett.* 2017, 17 (3), 1326–1335. [PubMed: 28273716]

- (16). Sayour EJ; De Leon G; Pham C; Grippin A; Kemeny H; Chua J; Huang J; Sampson JH; Sanchez-Perez L; Flores C; Mitchell DA *Oncoimmunology* 2016, 6, e1256527.
- (17). Lechner MG; Karimi SS; Barry-Holson K; Angell TE; Murphy KA; Church CH; Ohlfest JR; Hu P; Epstein AL *J. Immunother.* 2013, 36 (9), 477–89. [PubMed: 24145359]
- (18). Sonabend AM; Ogden AT; Maier LM; Anderson DE; Canoll P; Bruce JN; Anderson RC *J. Neuro-Oncol* 2012, 108 (1), 1–10.
- (19). Ansell SM; Lesokhin AM; Borrello I; Halwani A; Scott EC; Gutierrez M; Schuster SJ; Millenson MM; Cattray D; Freeman GJ; Rodig SJ; Chapuy B; Ligon AH; Zhu L; Grosso JF; Kim SY; Timmerman JM; Shipp MA; Armand P *N. Engl. J. Med.* 2015, 372 (4), 311–9. [PubMed: 25482239]
- (20). Zaretsky JM; Garcia-Diaz A; Shin DS; Escuin-Ordinas H; Hugo W; Hu-Lieskovan S; Torrejon DY; Abril-Rodriguez G; Sandoval S; Barthly L; Saco J; Homet Moreno B; Mezzadra R; Chmielowski B; Ruchalski K; Shintaku IP; Sanchez PJ; Puig-Saus C; Cherry G; Seja E; Kong X; Pang J; Berent-Maoz B; Comin-Anduix B; Graeber TG; Tumei PC; Schumacher TN; Lo RS; Ribas A *N. Engl. J. Med.* 2016, 375 (9), 819–29. [PubMed: 27433843]
- (21). Bloch O; Crane CA; Kaur R; Safaei M; Rutkowski MJ; Parsa AT *Clin. Cancer Res.* 2013, 19 (12), 3165–75.
- (22). Zitvogel L; Tesniere A; Kroemer G *Nat. Rev. Immunol.* 2006, 6 (10), 715–27. [PubMed: 16977338]
- (23). Gros A; Parkhurst MR; Tran E; Pasetto A; Robbins PF; Ilyas S; Prickett TD; Gartner JJ; Crystal JS; Roberts IM; Trebska-McGowan K; Wunderlich JR; Yang JC; Rosenberg SA *Nat. Med.* 2016, 22 (4), 433–8. [PubMed: 26901407]
- (24). Flores C; Pham C; Snyder D; Yang S; Sanchez-Perez L; Sayour E; Cui X; Kemeny H; Friedman H; Bigner DD; Sampson J; Mitchell DA *Oncoimmunology* 2015, 4 (3), e994374. [PubMed: 25949916]
- (25). Ecsedy JA; Manfredi MG; Yohe HC; Seyfried TN *Cancer research* 1997, 57 (8), 1580–1583. [PubMed: 9108463]
- (26). Jensen MM; Jorgensen JT; Binderup T; Kjaer A *BMC Med. Imaging* 2008, 8, 16.

Figure 1.

RNA-NPs mediate increased PD-L1 upon activation of myeloid APCs. (A) A cell line of dendritic cells (DC2.4s) was transfected with RNA-NPs. Cells were harvested within 24 h and imaged by TEM (scale bars = 500 nm). (B) DC2.4s were left untransfected or transfected with NPs alone or GFP RNA-NPs and harvested within 24 h for assessment of PD-L1 expression (** $p < 0.01$, unpaired t test). (C) Human DCs were matured from source donor white blood cells, and were left untransfected or transfected with NPs alone or GFP RNA-NPs. Cells were harvested within 24 h for assessment of surface PD-L1 expression by

flow cytometry ($***p < 0.001$, unpaired t test). (D, E) Three different batches of nanoliposomes were generated and assessed for concentration and ζ potential over time with serial measurements (mean $\pm$ SEM). (F) Cre encoding mRNA-NPs were administered to Ai14 transgenic mice (which carry a loxP-flanked STOP cassette impeding tdTomato transcription until Cre-recombinase expression), and 1 week later, lungs, hearts, livers, spleen, and lymph nodes were harvested, sectioned, and stained with DAPI before analysis of tdTomato expression by fluorescent microscopy. (G) FACS plots are displayed for PD-L1 (PE, phycoerythrin) and CD86 (APC, allophycocyanins) expression on CD11c⁺ splenocytes. (H, I) NPs were complexed with OVA-mRNA and injected iv into C57Bl/6 mice ($n = 3-4$ /group) bearing subcutaneous B16-F10 tumors. Spleens, lungs, livers, lymph nodes, and bone marrow were harvested the next day for assessment of CD11c cells expressing CD86/PD-L1 ($*p < 0.05$, $**p < 0.01$, $****p < 0.0001$, unpaired t test). (J) Gating strategy for CD45⁺ intratumoral and CD45⁻ tumor cells: cells are shown by forward and side scatter (left panel); among these, live cells are selected based on live/dead exclusion (middle panel), and CD45⁺ intratumoral or CD45⁻ tumor cells are selected for analysis (right panel). (K) FACS plots for PD-L1⁺CD86⁺ intratumoral cells are shown from mice receiving either NPs alone (left) or RNA-NPs (right). (L-N) Within 3 weeks of subcutaneous B16-F10 tumor cell (500 000 cells) implantation, NPs were complexed with OVA-mRNA and injected iv into C57Bl/6 mice ($n = 3$ /group); tumors were harvested the next day for flow cytometric analysis of CD11c, MHC-I, CD45, CD86, and PD-L1 expression ($**p < 0.01$, $***p < 0.001$, unpaired t test).

Figure 2.

PD-L1⁺ APCs do not suppress, but mediate enhancement of antigen-specific T cell immunity. (A, B) OVA RNA-NPs were injected iv into C57Bl/6 mice, and spleens were harvested the next day; spleens were FACS sorted for CD11c⁺MHCII⁺CD86⁺PD-L1⁺ cells (PD-L1⁺ APCs) (40 000 cells) which were added to a standard coculture assay (3 replicates/group) of OVA-mRNA electroporated primary murine bone marrow derived DCs (40 000 cells) and/or OTI derived splenocytes (400 000 cells) followed by serum assessment of IFN- γ by ELISA (***p* < 0.01, unpaired *t* test). (C) OVA RNA-NPs were injected iv into C57Bl/6

mice, and spleens were harvested the next day; spleens were FACS sorted for CD11c⁺MHCII⁺CD86⁺PD-L1⁺ (PD-L1⁺ APCs) cells which were adoptively transferred to naïve C57Bl/6 mice ($n = 5/\text{group}$) “spiked” with a responder T cell population from OTI transgenic mice (1×10^7 OTI splenocytes per mouse) ($*p < 0.05$, Mann–Whitney test). (D, E) OVA RNA-NPs were injected iv into tumor-bearing C57Bl/6 mice. Tumors were harvested within 3 weeks, FACS sorted for CD11c⁺MHCII⁺CD86⁺PD-L1⁺ cells (PD-L1⁺ intratumoral APCs) or CD 11 c⁺MHCII⁺CD86⁺PDL1⁻ cells (PD-L1⁻ intratumoral APCs) (40 000 cells) which were added to a standard coculture assay ($n = 3$ replicates/group) of OVA-mRNA electroporated primary murine bone marrow derived DCs (40 000 cells) and OVA mRNA activated T cells (400 000 cells). T cell activation was assessed by ELISA for IFN- γ production after 48 h ($*p < 0.05$, $**p < 0.01$, unpaired t test). (F, G) PD-L1 expression by flow cytometry from splenocytes of C57Bl/6 mice ($n = 3\text{--}5/\text{group}$) vaccinated with iv GFP RNA-NPs, CFA-OVA peptide (administered intradermal), NPs alone, or iv GFP RNA-NPs with anti-PD-L1 mAbs or isotype mAbs (administered intraperitoneal (ip) 24 h prior to RNA-NPs) ($*p < 0.05$, $**p < 0.01$, Mann–Whitney test). (H) C57Bl/6 mice ($n = 4\text{--}5/\text{group}$) were vaccinated with pp65 RNA-NPs iv once weekly ($\times 3$) before spleens were harvested a week later for analysis of IFN- γ by ELISA (from soups of restimulated splenocytes with overlapping pp65 peptide pool) and by flow cytometric analysis of percent PD-1⁺ CD8⁺ cells ($**p < 0.01$, Mann–Whitney test). (I) B16F10-OVA (1 000 000 cells) was implanted subcutaneously in the flanks of C57Bl/6 mice ($n = 5\text{--}9/\text{group}$), and PD-1 mAbs, PD-L1 mAbs, OVA RNA-NPs, or OVA RNA-NP⁺ PD-L1 mAbs were administered the following day. OVA RNA-NPs were injected iv once weekly ($\times 3$), and PD-1/PD-L1 mAbs were injected ip twice weekly ($\times 3$ weeks). Survival is plotted on a Kaplan–Meier curve ($*p < 0.05$, $**p < 0.01$, Gehan–Breslow–Wilcoxon test).

Figure 3.

RNA-NPs sensitize poorly immunogenic murine tumor models to ICIs. (A) B16F0 melanomas (50 000 cells) were implanted in the flanks of C57Bl/6 mice ($n = 5-7/\text{group}$), and the following day, PD-L1 mAbs, TTRNA-NPs, or TTRNA-NP+PD-L1 mAbs were administered. TTRNA-NPs were injected iv once weekly ($\times 3$); PD-L1 mAbs were injected ip twice weekly (until last RNA-NP vaccine). Early time-point measurements of tumor volumes were plotted (day 22 tumor volume: RNA-NP versus RNA-NP + PD L1 mAb, $*p < 0.05$, Mann-Whitney test). (B) B16F0 melanomas were implanted in the flanks of C57Bl/6

mice ($n = 5\text{--}7/\text{group}$), and the following day, PD-1 mAbs, TTRNA-NPs, or TTRNA-NP with PD-L1 mAbs were administered. TTRNA-NPs were injected iv once weekly ($\times 3$); PD-1 and PD-L1 mAbs were injected ip twice weekly (until last RNA-NP vaccine). Early time-point measurements of tumor volumes were plotted (day 21 and day 24 tumor volumes: PD-1 mAb versus RNA-NP + PD-L1 mAb, $*p < 0.05$, $**p < 0.01$, Mann–Whitney test), and (C) on day 27, spleens were harvested for assessment of peripheral PD-1 expression, and intratumoral CD45, CD4, and CD8 expression ($*p < 0.05$, Mann–Whitney test). (D) B16–F10 tumors (150 000 cells) were implanted in the flanks of C57Bl/6 mice ($n = 5$), and in the following days, PD-L1 mAbs, TTRNA-NPs, or TTRNA-NP+PD-L1 mAbs were administered. TTRNA-NPs were injected iv once weekly ($\times 3$); PD-L1 mAbs were injected ip twice weekly (until last RNA-NP vaccine). Tumor volume measurements were plotted over time ($*p < 0.05$, $**p < 0.01$, Mann–Whitney test, mean $\pm$ SEM). Animals receiving PD-L1 mAbs alone were removed from analysis on days 16–19 after $>50\%$ of animals were euthanized. (E) LLC (300 000 cells) was implanted in the flanks of C57Bl/6 mice ($n = 7\text{--}8/\text{group}$), and the next day, PD-L1 mAbs, TTRNA-NPs, or TTRNA-NP +PD-L1 mAbs were administered. TTRNA-NPs were injected iv once weekly ($\times 3$); PD-L1 mAbs were injected ip twice weekly (until last RNA-NP vaccine). Spleens and tumors were harvested on day 20 for assessment of PD-1 and CD8 expression ($*p < 0.05$, $**p < 0.01$, Mann–Whitney test).

Figure 4.

Type I IFN drives synergistic activity from RNA-NP and ICIs. (A, B) NPs alone, GFP RNA-NPs, or GFP RNA-NPs with IFNAR1 blocking antibodies (500 μ g administered ip) were administered systemically into naïve C57Bl/6 mice ($n = 4-5$ /group), and spleens were harvested the next day for flow cytometric analysis of CD86, PD-L1, and CD11c expression (** $p < 0.01$, Mann-Whitney test). (C) RNA-NPs were administered iv to naïve C57Bl/6 mice ($n = 4-5$ /group), and spleens were harvested the next day for assessment of F4/80 expression. (D, E) NPs alone, luciferase RNA-NPs, or luciferase RNA-NPs with PDCA-1

blocking antibodies were administered systemically into naïve C57Bl/6 mice ($n = 4/\text{group}$). Within 24 h, serum was collected for IFN- α detection by ELISA (overflow values assigned concentration of 2,000 pg/mL), and spleens were harvested for flow cytometric analysis of PDCA-1, MHCII, CD86, and PD-L1 expression ($*p < 0.05$, Mann–Whitney test). (F) B16F10-OVA melanomas (1 000 000 cells) were implanted in the flanks of C57Bl/6 mice ($n = 7\text{--}8$), and the following day, all mice received 1×10^7 splenocytes iv obtained from OTI mice. Animals were left unvaccinated or received treatment with PD-L1 mAbs, OVA RNA-NPs, or OVA RNA-NP+PD-L1 mAbs with or without concomitant IFNAR1 mAbs. PD-L1 and IFNAR1 mAbs were administered ip twice weekly, and RNA-NPs were administered iv on day 1. Early time-point measurements of tumor volumes were plotted (day 22 tumor volumes: RNA-NP+PD-L1 mAb versus RNA-NP+ PD-L1 mAb + IFNAR1 mAb, $**p < 0.01$, Mann–Whitney test). (G) Spleens were harvested from all groups on day 25 for assessment of PD-1 expression on CD8⁺ splenocytes ($**p < 0.01$, Mann–Whitney test).

Figure 5.

Personalized mRNA-NPs are safe and active in a translational disease model for spontaneous canine glioma. (A) A 9-year old spayed female Boxer was diagnosed with a malignant glioma on MRI (confirmed by histopathology) and enrolled to receive personalized mRNA-NPs per UFIACUC#201609430. (B) mRNA was amplified from a cDNA library prepared from extracted total RNA material from the patient's biopsy. mRNA was sterilely complexed with DOTAP nanoliposomes, and drawn up from a vacutainer with a needle syringe. The RNA-NPs (>~12 mL) were then administered via iv push over 5 min

through an intravenous catheter placed in the patient's right front leg. (C, D) Nanoliposomes were generated for the canine patient and assessed for concentration and ζ potential over time with serial measurements. (E) Temperatures were plotted over time during the patient's initial 6 h observation period postvaccination. (F) IFN- α ELISA was performed in duplicate on serum from the canine at baseline, 2 and 6 h postvaccination. (G) Blood was drawn at baseline, 2 and 6 h postvaccination for assessment of PD-L1, MHCII, CD80, and CD86 on CD11c⁺ cells. Gating strategy is shown for canine CD11c⁺ cells and expression of PD-L1, CD80, CD86, and MHCII. (H) CD11c expression of PD-L1, MHC-II, PD-L1/CD80, and PD-L1/CD86 is plotted over time during the canine's initial observation period. (I) CD3⁺ cells were analyzed over time during the canine's initial observation period for percent CD4 and CD8, and these subsets were assessed for expression of FoxP3 and IFN- γ , respectively.