1 **Lipid-encapsulated mRNAs encoding complex fusion proteins potentiate anti-tumor immune**
2 **responses**

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9 **Running Title:** mRNA-encoded fusion proteins induce anti-tumor immunity

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12 **Abstract (249 words)**

13 Lipid nanoparticle (LNP)-encapsulated mRNA has been used for *in vivo* production of several secreted
14 protein classes, such as IgG, and has enabled the development of personalized vaccines in oncology.
15 Establishing the feasibility of delivering complex multi-specific modalities that require higher-order
16 structures important for their function could help expand the use of mRNA/LNP biologic formulations.
17 Here, we evaluated whether *in vivo* administration of mRNA/LNP formulations of SIRPα-Fc-CD40L and
18 TIGIT-Fc-LIGHT could achieve oligomerization and extend exposure, on-target activity, and anti-tumor
19 responses comparable to that of the corresponding recombinant fusion proteins. Intravenous infusion of
20 the formulated LNP-encapsulated mRNAs led to rapid and sustained production of functional hexameric
21 proteins *in vivo*, which increased the overall exposure relative to the recombinant protein controls by
22 ~28-140 fold over 96 hours. High concentrations of the mRNA-encoded proteins were also observed in
23 secondary lymphoid organs and within implanted tumors, with protein concentrations in tumors up to
24 134-fold greater than with the recombinant protein controls 24 hours after treatment. In addition,
25 SIRPα-Fc-CD40L and TIGIT-Fc-LIGHT mRNAs induced a greater increase in antigen-specific CD8$^+$ T cells in
26 the tumors. These mRNA/LNP formulations were well tolerated and led to a rapid increase in serum and
27 intratumoral IL-2, delayed tumor growth, extended survival, and outperformed the activities of
28 benchmark monoclonal antibody controls. Furthermore, the mRNA/LNPs demonstrated improved
29 efficacy in combination with anti-PD-L1 relative to the recombinant fusion proteins. These data support
30 the delivery of complex oligomeric biologics as mRNA/LNP formulations, where high therapeutic
31 expression and exposure could translate into improved patient outcomes.

32 **Statement of Significance:** Lipid nanoparticle-encapsulated mRNA can efficiently encode complex fusion
33 proteins encompassing immune checkpoint blockers and co-stimulators that functionally oligomerize *in*
34 *vivo* with extended pharmacokinetics and durable exposure to induce potent anti-tumor immunity.

35 **Conflict of Interest Disclosure Statement:** Casey Shuptrine, Yuhui Chen, Jayalakshmi Miriyala, Karen Lenz, Jenn
36 Michaux, Danielle Moffett, Kristen Campbell, Connor Smith, Marc Morra, Thuy-Ai Nguyen, Yisel Rivera-Molina,
37 Noah Murr, Sarah Cooper, Ashlyn McGuire, Vishruti Makani, Nathan Oien, Jeffery T. Zugates, Suresh de Silva,
38 Taylor Schreiber, and George Fromm are employees and shareholders of Shattuck Labs, Inc.. Seymour de Picciotto
39 is an employee and shareholder of Moderna, Inc..

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Introduction

At the core of the successful global response to the SARS-CoV-2 pandemic lies decades of research dedicated to the synthesis of mRNAs incorporating unique sequence elements and modifications. These modifications enhance their stability and translation efficiency. To enable their effective delivery, lipid nanoparticles (LNPs) have been developed to encapsulate the mRNA cargo, providing protection and facilitating transport into the cytosol of target cells (1,2). mRNA/LNP technology has been applied to a wide variety of protein formats, including the expression of a full-length neutralizing antibody to Chikungunya virus (3-6). Application of mRNA/LNP technology to antibodies requires the separate expression and pairing of both immunoglobulin heavy and light chains into the appropriate quaternary structure for secretion, stability and function *in vivo*. Other advances have enabled the development of personalized vaccines in oncology, including mRNA-4157, wherein patient-specific tumor antigens are rapidly identified and assembled as concatemers into a continuous mRNA capable of stimulating anti-tumor immune responses. In patients with high-risk melanoma, mRNA-4157 in combination with pembrolizumab, resulted in recurrence free survival at 18 months in 78.6% of patients, compared to 62.2% of patients with pembrolizumab alone (7,8). Current efforts aimed at enhancing the systemic safety of mRNA/LNP formulations, and reducing immunogenicity risks following repeated administration, are beginning to show promise in clinical studies, and may open the door for broader proliferation of mRNA/LNP formulations of biologics as an economical alternative to mass-produced recombinant proteins (9). Thus, establishing feasibility for the expression and assembly of complex biologics when delivered as mRNA/LNP formulations, and comparing the biologic properties of *in vivo* expressed biologics to bolus-injected recombinant proteins, are important goals.

SIRPα-Fc-CD40L and TIGIT-Fc-LIGHT are two such biologics that have been developed as recombinant Fc-fusion proteins in humans and non-human primates, respectively (Figure 1A)(10-12). Each of these recombinant proteins are expressed from single continuous open reading frames (ORFs) by CHO cells. Following the expression of a protein monomer, covalent dimers form through interchain disulfide bonds in the Fc region of the protein. Dimers then assemble first into tetramers and ultimately hexamers due to non-covalent trimerization by the TNF ligand domains (10,11,13,14)(Figure 1B-C). This oligomerization is crucial for the activity of TNF-ligands and their cognate TNF receptors (like CD40, 4-1BB, OX40, and others), which require trimerization in the cell membrane to effectively signal and induce immune cell agonism (Figure 1D)(14-16).

The goals of the current study were to determine the efficiency of expression of *SIRPα-Fc-CD40L* and *TIGIT-Fc-LIGHT* as mRNA/LNP formulations, assess whether the mRNA-encoded proteins attained functional hexameric structures *in vivo*, and to compare the biological activity of the mRNA/LNP expressed constructs to the corresponding recombinant fusion protein reference material (Figure 1B)(FP ref.). We found that intravenous infusion of mRNA/LNPs was well tolerated, and concentrations of the mRNA-encoded protein exceeded that of the FP ref. protein within two hours of delivery in serum and other tissues. The primary structure of the mRNA-encoded protein in the serum of treated animals *in vivo* was confirmed to be a hexamer, as the high serum concentrations facilitated purification and SEC analysis of the oligomer composition. To further confirm the potency of hexameric *SIRPα-Fc-CD40L* and *TIGIT-Fc-LIGHT* as lipid encapsulated mRNAs, the pharmacodynamic and anti-tumor activity of these formulations was compared to the well characterized recombinant protein equivalents using a large established CT26 colorectal carcinoma tumor model. In total, these studies confirmed that lipid-

83 encapsulated mRNA encoding *SIRPα-Fc-CD40L* and *TIGIT-Fc-LIGHT* dramatically extended the *in vivo*

84 exposure of predominantly hexameric fusion proteins, with improved pharmacodynamic and anti-tumor

85 effects when compared head-to-head against the corresponding recombinant protein. Taken together,

86 these results support the evaluation of complex secreted biologics by mRNA/LNP delivery.

87

Materials and Methods

Material Generation and Characterization

90 The generation of FP ref. material was accomplished as previously described, through transient

91 production in CHO cells followed by affinity capture of the mouse constructs using protein A and human

92 constructs using FcXL, followed by elution and buffer exchange(10,11). mRNA constructs were

93 synthesized by *in vitro* transcription (IVT) followed by DNase digestion. A plasmid encoding the T7 RNA

94 polymerase promoter followed by 5'untranslated region (UTR), open reading frame (ORF), 3'UTR, and

95 polyA tail was overexpressed in E. coli, linearized, and purified to homogeneity. The mRNA was

96 synthesized by using a modified T7 RNA polymerase IVT, where uridine triphosphate was substituted

97 completely with N1-methyl-pseudouridine triphosphate. Cap 1 was utilized to improve translation

98 efficiency(17). After the transcription reaction, mRNAs were purified, buffer exchanged into sodium

99 citrate, and stored at−20 °C until use. Formulation of mRNA were performed as described

100 previously(18). Analytical characterization assays included particle size and polydispersity,

101 encapsulation, and endotoxin content which had to meet predefined specifications before the material

102 was deemed acceptable for *in vivo* study. The mRNA was encapsulated in a lipid nanoparticle through a

103 modified ethanol-drop nanoprecipitation process described previously (18). Briefly, ionizable, structural,

104 helper, and PEG lipids were mixed with mRNA in acetate buffer, pH 5.0, at a ratio of 3:1 (lipids:mRNA).

105 The mixture was neutralized with Tris-Cl, pH 7.5, sucrose was added as a cryoprotectant, and the final

106 solution was sterile filtered. Vials were filled with formulated LNP and stored frozen at −70 °C until

107 further use. The final drug product underwent analytical characterization, which included the

108 determination of particle size and polydispersity, encapsulation, mRNA purity, and endotoxin. Final

109 particle size and encapsulation were <100 nm and >80%, respectively, with endotoxin below 10 EU/mL.

110 Both FP ref. material and protein encoded by mRNA contain a wild-type mouse IgG1 central domain.

In vitro Functional Activity

112 *HEK293 Transfection:* 1 μg of naked mRNA with Lipo2000 was used to transfect HEK293T cells

113 (RRID:CVCL_0063). After 24 or 48 hours, cell culture supernatant was collected, and mouse and human

114 mRNA-encoded proteins were purified as described above. SDS-PAGE was used to characterize the

115 purified FP using antibodies targeting mouse and human SIRPα, CD40L, TIGIT, LIGHT, or Fc (R&D Systems

116 and The Jackson Laboratory). mRNA-encoded FP were quantitated using MSD (Meso Scale Discovery) as

117 described using the corresponding recombinant FP control as a standard(12).

118 *MSD Potency:* Dual target binding MSD assays were developed for each construct to ensure full-length

119 protein was capable of engaging its intended targets at the same time, as previously described(10,11).

120 Here, FP ref. and mRNA-encoded proteins were assessed using capture/detect reagents to mouse and

121 human CD47/CD40 or PVR/HVEM.

122 *Cell-Based Potency Assays:* CHOK1-CD40/NFκB-luciferase cells were previously generated (10) and used
123 to assess luciferase dose responses of FP ref. and mRNA-encoded material. Macrophage/tumor
124 phagocytosis assays were performed as previously described(10). RAW264.7-Lucia-ISG-reporter cells
125 (InvivoGen, RRID:CVCL_X596) were used according to manufacturer recommendations to assess CD40
126 activation of a type I interferon stimulatory reporter in the presence of mouse SIRPα-Fc-CD40L FP ref. or
127 the mRNA-encoded material. PathHunter U2OS/LTβR/NIK/NFκB reporter cells (DiscoverX) were used
128 according to manufacturer recommendations to assess the activity of TIGIT-Fc-LIGHT FP ref. and mRNA
129 material. LTβR+ A375 (RRID:CVCL_0132) or CT26 (RRID:CVCL_7254) tumor cell lines were cultured with
130 test agents for 3 hours before reverse transcribing cDNA to assess the expression of ACTB, CXCL8, CCL2,
131 and CXCL5 using qPCR with SYBR green reagents, validated primer sequences from Origene, and the Bio-
132 Rad CFX96 Touch real-time PCR detection system. Fold change in gene expression was determined using
133 the ΔΔCt method relative to the housekeeping gene ACTB (11).

134 Reporter cell lines were obtained from the indicated vendors. Parental cell lines were purchased from
135 ATCC. Cells in active culture were passaged 2-3 times per week, kept in culture for a maximum of two
136 months, and tested monthly using the Venor GeM Mycoplasma Detection Kit (Sigma). All transfected
137 cell lines were tested an additional two times post transfection, separated by at least 2 weeks, and
138 confirmed to remain negative for mycoplasma.

139 *In vivo PK, PD, and anti-Tumor Activity*

140 *Pharmacokinetics:* BALB/c (8-12 week old female, The Jackson Laboratory, RRID:MGI:2161072) mice
141 were given a single intravenous tail vein injection of vehicle (PBS), mRNA/LNP constructs (0.5 mg/kg or
142 12.5 µg in total), or FP reference material (8 mg/kg or 200 µg in total); 3 mice per group per time point.
143 Tissues and serum were isolated and cell lysates were prepared at the indicated timepoints to
144 determine FP concentrations using a MSD-dual binding assay, and normalized as pg or ng per gram of
145 starting tissue. For SEC, serum samples were diluted with PBS and loaded onto an AKTA Start protein
146 purification system at a flow rate of 0.11 mL/min, using a 1 mL HiTrap column and Amsphere A3 protein
147 A resin. Eluted material was assessed by SEC using a Thermo Fisher Scientific Vanquish system with a Bio
148 SEC-5 (Agilent, 500Å, 5µm, 7.8 ID x 300 m).

149 *Pharmacodynamics:* Serum chemistries were evaluated by the Pathology Service Core and Animal
150 Clinical Laboratory Medicine Core at the University of North Carolina at Chapel Hill, using the Vet Axcel
151 Chemistry Analyzer (Alfa Wassermann). Serum and tumor lysate cytokines were assessed using a custom
152 MSD multiplex plate according to manufacturer instructions. Resulting data was plotted in heatmap
153 format depicting row min/max values across samples. Hierarchical clustering (1- Spearman correlation)
154 was used to demonstrate group and cytokine associations.

155 *Tumor Efficacy:* BALB/c mice (8 to 12 weeks old) were subcutaneously implanted with $5x10^5$ CT26 tumor
156 cells into the hind right flank. When tumors were approximately 90 mm^3 (8 days after inoculation on
157 average) mice were randomized across treatment groups and treatment was initiated (indicating day 0).
158 Tumor-bearing mice were treated via intraperitoneal (IP) injections with recombinant fusion proteins
159 (200 µg per dose) and antibodies (100 µg per dose for anti-CD47 (clone MIAP410 BioXCell,
160 RRID:AB_2687806) and anti-TIGIT (clone 1G9 BioXCell, RRID:AB_2687797) or 200 µg per dose for anti-
161 PDL1 (clone 10F.9G2, RRID:AB_10949073) or via IV injection for the mRNA/LNP constructs (0.5 mg/kg
162 per dose) on Days 0, 3, 7, 10, 14, and 17 of a 30-day time course. The anti-tumor efficacy benchmark for

163   these fusion protein constructs and antibodies has previously been described using IP administration,
164   and we and others have shown that IP and IV administration in mice results in similar drug exposure
165   levels (Supplemental Figure 1A)(10,11,19). Tumor volume (mm^3) and overall survival were assessed
166   throughout the time-course. Animals were humanely euthanized when total tumor volume reached
167   >1800 mm^3 or there were signs of significant tumor ulceration. Individual animal tumor growth curves,
168   the average time in which each treatment group reached full tumor burden, the number of mice that
169   rejected the primary tumor (tumor volume ≤0.5 mm^3), and overall survival were assessed over a 30-day
170   time-course. On Day 8 of the study, a group of treated animals was euthanized, and necropsy was
171   performed to collect serum and tumor tissue for PK, cytokine, and flow cytometry analysis.

172   *Flow Cytometry:* Tumor tissue was dissociated using an enzymatic tumor dissociation kit (Miltenyi
173   Biotec), and the resulting cells were exposed to a viability dye and then Fc receptors were blocked (both
174   BioLegend). Cell staining was performed for 30 minutes at 4°C in the dark. For FoxP3 staining, the FoxP3
175   fix/perm buffer kit (BioLegend) was used according to manufacturer instructions. After antibody
176   incubations, cells were washed 2x in 500 μL FACS buffer (1x DPBS, 1%BSA, 2 mM EDTA, and 0.02%
177   NaN_3), and then analyzed on the BD LSR Fortessa Cell Analyzer.

178   *Experimental Animal Guidelines*

179   All experimental mice used were female inbreed BALB/c co-housed during the course of an experiment
180   (The Jackson Laboratory). All murine animal studies have been conducted in accordance with, and with
181   the approval of an Institutional Animal Care and Use Committee (IACUC); and reviewed and approved by
182   a licensed veterinarian.

183   *Statistical Analysis*

184   Unless noted otherwise, values plotted represent the mean and error is SEM. Statistical significance (p-
185   value) was determined using One-Way ANOVA with multiple comparisons. Significant p-values are
186   labeled with one or more '*', denoting *p<.05, **p<.01, ***p<.001 and ****p<.0001. Mantel-Cox
187   statistical tests were used to determine the significance between the survival curves.
188

189   *Data Availability*

190   Data were generated by the authors and included in the article. All raw data are available upon request
191   from the corresponding author.

192

193   **Results**

194   mRNA encoding mouse and human *SIRPα-Fc-CD40L* and *TIGIT-Fc-LIGHT* were transiently transfected
195   into HEK293T cells and after 24 or 48 hours, supernatant was collected and affinity purified. The
196   resulting protein was assessed using SDS-PAGE and were detected with three separate domain-specific
197   antibodies. All three detection methods indicated full length proteins migrating at the expected
198   monomeric molecular weights of 88.1 kDa for SIRPα-Fc-CD40L and 59.3 kDa for TIGIT-Fc-LIGHT (Figure
199   2A-B and Supplemental Figure 1B-C)(10,11). Secreted protein concentrations were evaluated using a
200   dual-binding MSD method, normalized on a per-cell-basis and found to reach 7.76 pg/cell at 24 hours
201   and 27.4 pg/cell at 48 hours for human *SIRPα-Fc-CD40L*, and 4.46 pg/cell at 24 hours and 8.98 pg/cell at

202 48 hours for human *TIGIT-Fc-LIGHT*; which are on par with the productivity achieved from commercial
203 CHO and 293 based biomanufacturing processes (Figure 2C-D) (20). Parallel studies using the mouse-
204 equivalent mRNA constructs showed similar results (Supplemental Figure 1D-E).

205 The potency of the mRNA-encoded proteins was assessed using (1) dual-target MSD potency, (2) cell-
206 based CD40/NFκB-luciferase reporter, and (3) *in vitro* species-specific macrophage phagocytosis assays
207 for *SIRPα-Fc-CD40L* (Figure 2C). Additionally, a RAW264.7/ISG (interferon stimulatory gene) reporter cell
208 line was used to inform on the activation of interferon response pathways downstream of CD40 ligation
209 (Supplemental Figure 1D, parts 1-4). Human mRNA-encoded *TIGIT-Fc-LIGHT* was assessed using (1) dual-
210 target MSD potency, (2) cell-based U2OS/LTβR/NFκB/Nik-luciferase reporter, and (3) through the
211 activation of CCL2 and CXCL8 expression in LTβR+ A375 tumor cells (Figure 2D). Mouse TIGIT-Fc-LIGHT
212 activity was assessed using a (1) dual-target MSD potency, and (2) through the activation of Ccl2 and
213 Cxcl5 expression in LTβR+ CT26 tumor cells (Figure 2D). The recombinant fusion protein material (FP
214 ref.) was used as a reference standard to calculate the concentration of the mRNA supernatants. In all
215 cases, the mRNA-encoded fusion proteins demonstrated equivalent potency to that of the FP ref.
216 material. Taken together, the *in vitro* results suggested that both *SIRPα-Fc-CD40L* and *TIGIT-Fc-LIGHT*
217 were efficiently produced from mRNA/LNPs and assembled into functional higher-order quaternary
218 structures capable of agonizing trimeric TNF receptors in various cell-based potency assays.

219 To determine whether expression and bioactivity was achievable *in vivo,* BALB/c mice were given a
220 single tail vein injection of 0.5 mg/kg (12.5 μg) of the LNP formulated mRNA or 8 mg/kg (200 μg) of the
221 FP ref. After 0.5, 1, 2, 6, 24, 48, 72, and 96 hours, serum, liver, spleen, and iLN were isolated from
222 treated animals for the analysis of serum chemistries and cytokines, and serum and tissue quantification
223 of mRNA encoded protein (Figure 3A). All test articles were well tolerated and no significant elevations
224 of ALT, AST, LDH, creatinine, or total bilirubin were observed over that of the vehicle treated group
225 (Supplemental Figure 1F). Dual MSD PK assays (capture with anti-mouse CD40L and detect with anti-
226 mouse SIRPα or capture with anti-mouse LIGHT and detect with anti-mouse TIGIT) were used to assess
227 serum concentrations of the fusion proteins delivered as recombinant protein or mRNA/LNP
228 formulations. As expected, the serum concentration for bolus injected recombinant SIRPα-Fc-CD40L and
229 TIGIT-Fc-LIGHT was highest at the first post-dose timepoint (0.5 hours) and decreased rapidly thereafter
230 (Figure 3B). The targets for SIRPα-Fc-CD40L (CD47 and CD40) are more abundant than the targets for
231 TIGIT-Fc-LIGHT (PVR, HVEM, LTβR), and accordingly both the C_{max} (maximum concentration) and AUC
232 (area under the curve) were higher for TIGIT-Fc-LIGHT as compared to SIRPα-Fc-CD40L (Figure 3B)(10-
233 12). Remarkably, serum *SIRPα-Fc-CD40L* and *TIGIT-Fc-LIGHT* were detectable at the earliest timepoint in
234 both mRNA/LNP treated groups, and the concentration of protein remained consistently high across the
235 entire time course. Further, the concentration of mRNA/LNP expressed proteins remained higher than
236 the C_{max} in the recombinant protein treated groups through 48 hours for *SIRPα-Fc-CD40L* and through
237 the entire 96-hour time course for *TIGIT-Fc-LIGHT*. The overall protein C_{max} from *SIRPα-Fc-CD40L* mRNA
238 treatment was 1.8-fold higher than the FP ref. and was not achieved until 12 hours post-dose, compared
239 to 0.5 hours for the FP ref. control. The AUC from *SIRPα-Fc-CD40L* mRNA was 140-fold greater than that
240 achieved with the FP ref. (Figure 3B). Similar kinetics were observed with *TIGIT-Fc-LIGHT* mRNA, with a
241 C_{max} 6.1-fold higher, and an AUC 28.7-fold greater than that seen with the FP ref. material. The
242 maximum serum concentration for *TIGIT-Fc-LIGHT* was not reached until 32 hours post-dose (t_{max})
243 compared to 0.5 hours for the FP ref.. In addition to achieving elevated concentrations in the serum,

244 both mRNA/LNP formulations also translated to higher concentrations with more sustained exposures in
245 the liver, spleen, and inguinal lymph nodes (Figure 3C).

246 To assess the oligomeric structure of the fusion proteins, we took advantage of the high serum
247 concentrations of mRNA-encoded fusion proteins which facilitated affinity purification of SIRPα-Fc-
248 CD40L directly from the serum of treated animals, followed by size exclusion chromatography (SEC).
249 When the absorbance spectrum from the mRNA/LNP serum sample was overlayed with a known and
250 well-characterized human SIRPα-Fc-CD40L reference standard, the serum isolated mRNA-encoded
251 material was found to closely share the oligomeric profile and existed primarily as a hexamer (Figure
252 3D).

253 The administration of both *SIRPα-Fc-CD40L* and *TIGIT-Fc-LIGHT* mRNA/LNPs led to significantly elevated
254 serum concentrations of IL-2, which persisted for 48-96 hours following a single dose (Figure 3E). With
255 the FP reference material, we have previously observed transient increases in IL-2 and other innate and
256 adaptive immune cytokines, including IL-2, MCP5, IP10, and MIP1β, and observed similar results here
257 (Figure 3F)(10,11).

258 We next evaluated the pharmacodynamic and anti-tumor responses in mice bearing CT26 colorectal
259 tumors. Some mice were euthanized 24 hours post-dose and tumor tissue was dissociated and fusion
260 protein concentrations were assessed. In the *SIRPα-Fc-CD40L* group, 122-fold more protein was
261 detected in the tumors of mRNA/LNP treated animals compared to that of the FP ref. (220 pg/gram of
262 tissue vs. 1.8 pg/g). In the *TIGIT-Fc-LIGHT* group, 134.8-fold more protein was detected in the tumors of
263 mRNA/LNP treated animals compared to that of the FP ref. (13.48 ng/gram of tissue vs. 0.1 ng/g)(Figure
264 4A). The increased serum and tissue exposures (including in the tumor) mediated by the mRNA/LNP
265 delivery of *SIRPα-Fc-CD40L* and *TIGIT-Fc-LIGHT*, resulted in higher levels of serum and intra-tumor
266 expressed cytokines and chemokines (Figure 4B). In both the serum and tumor, unbiased hierarchical
267 clustering associated the SIRPα-Fc-CD40L and TIGIT-Fc-LIGHT treatment groups (both mRNA/LNP and FP
268 ref.) in proximity, suggesting that both therapeutic delivery methods achieved similar on-target
269 pharmacodynamic activity. In the tumor, the mRNA/LNP and FP ref. groups clustered a single branch
270 apart and appeared to differ by the overall magnitude of response, which was greater and broader in
271 the mRNA/LNP groups than what was observed with the FP reference material. Here, increased tumor
272 levels of IL2, IFNγ, IL-12p70, MDC, TNFα, MIP1α, MIP1β, MIP3α, and MCP1, were observed in all
273 mRNA/LNP and FP ref. treatment groups compared to vehicle or benchmark checkpoint inhibitor
274 antibody reference controls (anti-CD47 or anti-TIGIT)(Figure 4B). These proinflammatory cytokines have
275 been associated with the activation of myeloid cells through both CD40 and LTβR(11,21). However,
276 interesting construct-dependent differences in the magnitude of cytokine expression were seen in the
277 serum and tumors of treated animals, including for MIP1β, MCP1, MIP2, and MDC (Figure 3E-F, Figure
278 4B, and Supplemental Figure 1G)(10-12).

279 We next assessed whether mRNA/LNP formulations of *SIRPα-Fc-CD40L* or *TIGIT-Fc-LIGHT* could delay
280 the growth of established CT26 tumors and extend survival. Once tumors reached an average starting
281 tumor volume of ~90mm^3, mice were randomized and treated with either vehicle, anti-CD47, anti-TIGIT,
282 an irrelevant mRNA/LNP negative control, *SIRPα-Fc-CD40L* mRNA/LNP, SIRPα-Fc-CD40L FP ref., *TIGIT-Fc-*
283 *LIGHT* mRNA/LNP, or TIGIT-Fc-LIGHT FP ref. (Figure 4C). In the control groups, the average time it took
284 for mice to reach tumor burden was approximately 13-14 days following the first treatment. SIRPα-Fc-
285 CD40L FP ref. extended the time until reaching tumor burden to 18.80 days and the mRNA/LNP

286   extended this time to 23 days. The TIGIT-Fc-LIGHT FP ref. extended the time until reaching tumor
287   burden to 17.50 days and the mRNA/LNP extended this time to 23 days (Figure 4C). Several of the
288   treated mice completely rejected the established tumors, including with each of the mRNA/LNP groups.
289   The delay in tumor growth induced by the mRNA/LNP and FP ref. constructs also translated into
290   significant survival benefits over the vehicle control (Figure 4D).

291   Given the anti-tumor effects and observed changes in both serum and tumor cytokines and
292   chemokines, an analysis of tumor infiltrating lymphocytes was performed. Regulatory T cells (Treg;
293   CD4+CD25+Foxp3+) were not elevated in treatment groups, and instead the median percentage of Tregs
294   in the *TIGIT-Fc-LIGHT* mRNA group was 33.9% less than that of the vehicle control. All fusion protein and
295   mRNA treatments increased the infiltration of activated antigen-specific CD8+ T cells into the tumor
296   microenvironment (TME)(Figure 4E and Supplemental Figure 1H). *SIRPα-Fc-CD40L* and *TIGIT-Fc-LIGHT*
297   mRNAs induced the greatest increases in antigen-specific CD8+ T cells into the tumors (AH1 tetramer+),
298   with median values 1.62 and 2.29-fold greater than the vehicle group, respectively.

299   The efficacy of both SIRPα-Fc-CD40L and TIGIT-Fc-LIGHT were enhanced in combination with tumor
300   targeting antibodies that promote ADCC/ADCP and block checkpoint pathways(10,11). To further
301   characterize this combination activity with the mRNA-encoding constructs, anti-tumor activity and the
302   tumor infiltration of antigen-specific CD8+ T cells was evaluated in CT26 tumor bearing animals treated
303   with SIRPα-Fc-CD40L and TIGIT-Fc-LIGHT FP or mRNA/LNP in combination with anti-PDL1 (Figure 4F-G).
304   Consistent with previous findings, the combination of all constructs with anti-PDL1, significantly
305   improved tumor growth inhibition compared to anti-PDL1 on its own, with the mRNA/LNP combinations
306   demonstrating improved efficacy relative to the FP combinations (Figure 4G)(10,11). The mRNA/LNP +
307   anti-PDL1 combination groups also increased intratumoral percentages of CD8+CD69+ and CD8+AH1-
308   tetramer+ T cells compared to the corresponding monotherapy groups. *SIRPα-Fc-CD40L* mRNA + anti-
309   PDL1 increased CD8+CD69+ T cells 1.93-fold and CD8+AH1-tetramer+ T cells 2.75-fold greater than anti-
310   PDL1 monotherapy, and *TIGIT-Fc-LIGHT* mRNA + anti-PDL1 increased CD8+CD69+ T cells 1.72-fold and
311   CD8+AH1-tetramer+ T cells 2.33-fold greater than anti-PDL1 monotherapy (Figure 4G). These results
312   provide confirmation that *SIRPα-Fc-CD40L* and *TIGIT-Fc-LIGHT* mRNA encode functional fusion proteins
313   *in vivo*, which retain the pharmacodynamic and anti-tumor properties previously described for the
314   recombinant protein versions of these constructs.

315

316   **Discussion**

317   This study aimed to determine whether lipid encapsulated mRNA could be utilized to deliver complex Fc
318   fusion proteins that require self-association into hexamers through a mixture of covalent and non-
319   covalent interactions, via intravenous infusions. *SIRPα-Fc-CD40L* and *TIGIT-Fc-LIGHT* mRNA/LNP
320   formulations encoded functional fusion protein both *in vitro* and *in vivo*. The kinetics of both *SIRPα-Fc-
321   CD40L* and *TIGIT-Fc-LIGHT* detected concentrations through mRNA/LNP delivery was higher than that
322   achieved with a bolus delivery of recombinant fusion protein and was maintained across at least 96
323   hours. The progressive increase in expression of CD40L and LIGHT (contained within each fusion
324   protein), closely mirrors the native expression of these and other TNF ligands in the context of a
325   pathogen-induced immune response. In contrast, many TNF receptor agonist therapeutics that are
326   administered by intravenous infusion achieve maximal serum concentrations within a period of minutes

327 and then decay thereafter. Whether these distinct pharmacokinetic profiles contribute to distinct
328 differentiation states of the target cells for each TNF ligand is unknown at this time.

329 The primary site of *in vivo* protein production using intravenous administered mRNA/LNP is often shown
330 to be the liver, and consistently here, the highest concentrations of both SIRPα-Fc-CD40L and TIGIT-Fc-
331 LIGHT were detected in the serum and liver. Lower – but significant – amounts of both mRNA-encoded
332 proteins were detected in the spleen, lymph node, and within the tumor. The current study did not
333 differentiate between protein that was synthesized in a tissue versus proteins accumulated in that tissue
334 via diffusion or active transport from other compartments, however previous work has confirmed the
335 integration of mRNA within these tissues in similar studies ((6) and unpublished data). Regardless of the
336 mechanism, systemic exposure of both SIRPα-Fc-CD40L and TIGIT-Fc-LIGHT was increased when
337 administered as lipid encapsulated mRNA formulations.

338 The serum concentration of *SIRPα-Fc-CD40L* was high enough to enable the purification and SEC analysis
339 of the *in vivo* expressed protein, and remarkably, the prominent species was shown to exist as a
340 hexamer. When fusion proteins like SIRPα-Fc-CD40L or TIGIT-Fc-LIGHT are produced as recombinant
341 proteins from CHO cells, the final therapeutic exists primarily as a hexamer (10,11,22). The observation
342 here that intravenous infusion of mRNA/LNP results in hexameric TNF-ligand containing fusion proteins
343 *in vivo*, suggests that the necessary elements for oligomerization are present at the site of *in vivo*
344 translation. In future studies, it would be interesting to extend these observations to other routes of
345 administration, such as intramuscular and subcutaneous (23,24).

346 Previously published studies demonstrated anti-tumor activity for SIRPα-Fc-CD40L and TIGIT-Fc-LIGHT
347 fusion proteins in a range of preclinical models. Those data provided the pre-clinical basis to advance
348 SIRPα-Fc-CD40L (SL-172154) into clinical trials for patients with platinum resistant ovarian cancer, and
349 also patients with TP53 mutant AML and HR-MDS (NCT05483933 and NCT05275439, respectively),
350 where initial anti-tumor activity has been observed (10,11,25,26). Despite these data, a potential
351 limitation of this current study is that anti-tumor activity was only demonstrated in a single pre-clinical
352 mouse tumor model. Using the established CT26 tumor model, mRNA/LNP formulated *SIRPα-Fc-CD40L*
353 and *TIGIT-Fc-LIGHT* compared favorably to the recombinant protein counterparts. Treatment of mice
354 with large established tumors with SIRPα-Fc-CD40L or TIGIT-Fc-LIGHT recombinant protein extended
355 survival in comparison to groups treated with CD47 or TIGIT blocking antibodies. Survival was further
356 improved when the same fusion protein constructs were delivered as mRNA/LNPs, which is likely due to
357 increased exposure within the tumor, and because of prolonged pharmacodynamic effects compared to
358 the recombinant proteins. Delivery of *SIRPα-Fc-CD40L* as mRNA/LNP was associated with increased
359 tumor infiltration of antigen-specific CD8+ T cells, activated APCs, as well as high serum and tumor IL-2
360 levels that were distinct from other groups and persisted over the entire 96-hour time course. Whereas
361 the increase in IL-2 was consistent with *de novo* production, increases in many other serum cytokines
362 occurred within a few hours of treatment, and are likely due to the release of those cytokines and
363 chemokines from pre-formed granules in circulating immune cells, as has also been shown in human
364 cancer patients treated with SIRPα-Fc-CD40L(25,26). In other studies, elevation in chemokines like CCL3
365 and CCL20 were associated with infiltration of immunosuppressive myeloid and Th17 cells in the tumor
366 microenvironment(27). The current study was not focused on the assessment of these cell populations,
367 and future studies could aim to investigate this further.

368 One of the barriers to delivering biologics using lipid encapsulated mRNAs has been toxicity and
369 immunogenicity associated with the LNP component. Recent advances in LNP and mRNA optimization
370 have reduced these risks, and clinical studies currently underway in children with propionic acidemia
371 (NCT04159103) have demonstrated that mRNA-3957 was well tolerated for over a year of chronic
372 therapy (9). These findings are important as the use of lipid encapsulated nucleic acid technologies
373 continue to broaden into other disease areas that require chronic and sometimes lifelong treatment, for
374 example in various oncology indications or autoimmune disorders. The compelling expression of
375 complex biologics via mRNA/LNP formulations presented here, opens the door to exploring nucleic acid
376 delivery of other therapeutic scaffolds in broader populations of patients, previously thought to be
377 unreachable by an mRNA cargo. Recent studies have shown that lipid encapsulated mRNA can also be
378 used to facilitate *in vivo* production of functional monoclonal antibodies and T cell engagers, which do
379 not require oligomerization but instead require assembly of heavy and light chains independently
380 translated from distinct open reading frames(28-31). The speed, cost, and efficiency of mRNA/LNP
381 manufacturing, as well as improved expression, biodistribution, and pharmacodynamics, could result in
382 future approaches where biologics are developed and assessed in parallel with their RNA counterparts,
383 to provide optionality for the optimal targeting of particular receptors or ligands, based on desired PK
384 and PD characteristics that could be uniquely provided by the delivery of a biologic or an mRNA/LNP.
385 The studies herein demonstrate that such approaches may not be limited to individual cytokines,
386 antibodies, or enzymes, but could also apply to complex biologics such as SIRPα-Fc-CD40L and TIGIT-Fc-
387 LIGHT.

388

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References

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393   1.   Kiaie SH, Majidi Zolbanin N, Ahmadi A, Bagherifar R, Valizadeh H, Kashanchi F, *et al.* Recent
394   advances in mRNA-LNP therapeutics: immunological and pharmacological aspects. J
395   Nanobiotechnology **2022**;20:276
396   2.   Zong Y, Lin Y, Wei T, Cheng Q. Lipid Nanoparticle (LNP) Enables mRNA Delivery for Cancer
397   Therapy. Adv Mater **2023**:e2303261
398   3.   August A, Attarwala HZ, Himansu S, Kalidindi S, Lu S, Pajon R, *et al.* A phase 1 trial of lipid-
399   encapsulated mRNA encoding a monoclonal antibody with neutralizing activity against
400   Chikungunya virus. Nat Med **2021**;27:2224-33
401   4.   Jiang L, Park JS, Yin L, Laureano R, Jacquinet E, Yang J, *et al.* Dual mRNA therapy restores
402   metabolic function in long-term studies in mice with propionic acidemia. Nat Commun
403   **2020**;11:5339
404   5.   de Picciotto S, DeVita N, Hsiao CJ, Honan C, Tse SW, Nguyen M, *et al.* Selective activation and
405   expansion of regulatory T cells using lipid encapsulated mRNA encoding a long-acting IL-2
406   mutein. Nat Commun **2022**;13:3866
407   6.   Hewitt SL, Bai A, Bailey D, Ichikawa K, Zielinski J, Karp R, *et al.* Durable anticancer immunity from
408   intratumoral administration of IL-23, IL-36gamma, and OX40L mRNAs. Sci Transl Med **2019**;11
409   7.   Khattak AC, M.; Meniawy, T.; Ansstas, G.; Medina, T.; Taylor, M.H.; Kim, K.B.; McKean, M.; Long,
410   G.V.; Sullivan, R.J.; Faries, M.; Tran, T.; Cowey, C.; Pecora, A.; Segar, J.; Atkinson, V.; Gibney, G.T.;
411   Luke, K.; Thomas, S.; Buchbinder, E.; Hou, P.; Zhu, L.; Zaks, T.; Brown, M.; Aanur, P.; Meehan,
412   R.S.; Weber, J.S. A personalized cancer vaccine, mRNA-4157, combined with pembrolizumab
413   versus pembrolizumab in patients with resected high-risk melanoma; Efficacy and safety results
414   from the randomized, open-label Phase 2 mRNA-4157-P201/Keynote-942 trial. American
415   Association of Cancer Research **2023**
416   8.   Khattak AW, J.S.; Menjawy, T.; Taylor, M.H.; Ansstas, G.; Kim, K.B.; McKean, M.; Long, G.V.;
417   Sullivan, R.J.; Farier, M.B.; Tran, T.; Cowey, C.L.; Medina, T.M.; Segar, J.M.; Atkinson, V.; Gibney,
418   G.T.; Luke, J.J.; Buchbinder, E.I.; Meehan, R.S.; Carlino, M.S. Distant metastasis-free survival
419   results from the randomized, phase 2 mRNA-4157-P201/KEYNOTE-942 trial. American Society of
420   Clinical Oncology **2023**
421   9.   Attarwala H, Lumley M, Liang M, Ivaturi V, Senn J. Translational
422   Pharmacokinetic/Pharmacodynamic Model for mRNA-3927, an Investigational Therapeutic for
423   the Treatment of Propionic Acidemia. Nucleic Acid Ther **2023**;33:141-7
424   10.   de Silva S, Fromm G, Shuptrine CW, Johannes K, Patel A, Yoo KJ, *et al.* CD40 Enhances Type I
425   Interferon Responses Downstream of CD47 Blockade, Bridging Innate and Adaptive Immunity.
426   Cancer Immunol Res **2020**;8:230-45
427   11.   Yoo KJ, Johannes K, Gonzalez LE, Patel A, Shuptrine CW, Opheim Z, *et al.* LIGHT (TNFSF14)
428   Costimulation Enhances Myeloid Cell Activation and Antitumor Immunity in the Setting of PD-
429   1/PD-L1 and TIGIT Checkpoint Blockade. J Immunol **2022**;209:510-25
430   12.   Lakhani NJS, D.; Richardson, D.L.; Dockery, L.E., Van Le, L.; Call, J.; Rangwala, F.; Scholl, A.; Wang,
431   G.; Ma, B.; Metenou, S.; Pandite, L; Hamilton, E. Phase 1 dose escalation study of SL-172154
432   (SIRPα-Fc-CD40L) in platinum-resistant ovarian cancer. American Society of Clinical Oncology
433   **2023**
434   13.   Shuptrine CW, Perez VM, Selitsky SR, Schreiber TH, Fromm G. Shining a LIGHT on myeloid cell
435   targeted immunotherapy. Eur J Cancer **2023**;187:147-60
436   14.   Kucka K, Wajant H. Receptor Oligomerization and Its Relevance for Signaling by Receptors of the
437   Tumor Necrosis Factor Receptor Superfamily. Front Cell Dev Biol **2020**;8:615141

438   15.   Fromm GdS, S.; Schreiber, T.H. Reconciling Intrinsic Properties of Activating TNF Receptors by
439   Native Ligands Versus Synthetic Agonists. Frontiers in Immunology **2023**

440   16.   Croft M, Benedict CA, Ware CF. Clinical targeting of the TNF and TNFR superfamilies. Nat Rev
441   Drug Discov **2013**;12:147-68

442   17.   Nelson J, Sorensen EW, Mintri S, Rabideau AE, Zheng W, Besin G*, et al.* Impact of mRNA
443   chemistry and manufacturing process on innate immune activation. Sci Adv **2020**;6:eaaz6893

444   18.   Sabnis S, Kumarasinghe ES, Salerno T, Mihai C, Ketova T, Senn JJ*, et al.* A Novel Amino Lipid
445   Series for mRNA Delivery: Improved Endosomal Escape and Sustained Pharmacology and Safety
446   in Non-human Primates. Mol Ther **2018**;26:1509-19

447   19.   Al Shoyaib A, Archie SR, Karamyan VT. Intraperitoneal Route of Drug Administration: Should it
448   Be Used in Experimental Animal Studies? Pharm Res **2019**;37:12

449   20.   Kunert R, Reinhart D. Advances in recombinant antibody manufacturing. Appl Microbiol
450   Biotechnol **2016**;100:3451-61

451   21.   Johnson MLS, L.L., Hong, D.; Schoffski, P.; Galvao, V.; Rangwala, F.; Hernandez, R.; Gonzalez, L.;
452   Ma, B.; Pandite, L.; Brana, I. Phase 1 dose escalation of an agonist redirected checkpoint (ARC)
453   fusion protein, SL-279252 (PD1-Fc-OX40L), in subjects with advanced solid tumors or
454   lymphomas (NCT03894618). Society for the Immunotherapy of Cancer **2022**

455   22.   Fromm G, de Silva S, Johannes K, Patel A, Hornblower JC, Schreiber TH. Agonist redirected
456   checkpoint, PD1-Fc-OX40L, for cancer immunotherapy. J Immunother Cancer **2018**;6:149

457   23.   Munter R, Christensen E, Andresen TL, Larsen JB. Studying how administration route and dose
458   regulates antibody generation against LNPs for mRNA delivery with single-particle resolution.
459   Mol Ther Methods Clin Dev **2023**;29:450-9

460   24.   Pardi N, Tuyishime S, Muramatsu H, Kariko K, Mui BL, Tam YK*, et al.* Expression kinetics of
461   nucleoside-modified mRNA delivered in lipid nanoparticles to mice by various routes. J Control
462   Release **2015**;217:345-51

463   25.   Lakhani N.J. SD, Richardson D.L., Dockery L.E., Van Lee L., Call J., Rangwala F., Scholl A., Wang G.,
464   Ma B., Metenou S., Pandite L., Hamilton E. Phase 1 dose escalation study of SL-172154 (SIRPα-
465   Fc-CD40L) in platinum-resistant ovarian cancer. American Association of Clinical Oncology
466   **2023**;Annual Meeting

467   26.   Daver N. SAS, Bixby D., ChaiHo W., Zeidner J.F., Maher K., Stevens D., Stahl M., Yee K., Curran
468   E.K., Ito S., Sochacki A., Sallman D., Hernandez R., Metenou S., Ma B., Kato K., Zeidan A.M.
469   Safety, Pharmacodynamic, and Anti-Tumor Activity of SL-172154 as Monotherapy and in
470   Combination with Azacitidine (AZA) in Relapsed/Refractory (R/R) Acute Myeloid Leukemia
471   (AML) and Higher-Risk Myelodysplastic Syndromes/Neoplasms (HR-MDS) Patients (pts).
472   American Society of Hematology **2023**;Annual Meeting

473   27.   Nagarsheth N, Wicha MS, Zou W. Chemokines in the cancer microenvironment and their
474   relevance in cancer immunotherapy. Nat Rev Immunol **2017**;17:559-72

475   28.   Mukherjee J, Ondeck CA, Tremblay JM, Archer J, Debatis M, Foss A*, et al.* Intramuscular delivery
476   of formulated RNA encoding six linked nanobodies is highly protective for exposures to three
477   Botulinum neurotoxin serotypes. Sci Rep **2022**;12:11664

478   29.   Hotz C, Wagenaar TR, Gieseke F, Bangari DS, Callahan M, Cao H*, et al.* Local delivery of mRNA-
479   encoded cytokines promotes antitumor immunity and tumor eradication across multiple
480   preclinical tumor models. Sci Transl Med **2021**;13:eabc7804

481   30.   Liu X, Barrett DM, Jiang S, Fang C, Kalos M, Grupp SA*, et al.* Improved anti-leukemia activities of
482   adoptively transferred T cells expressing bispecific T-cell engager in mice. Blood Cancer J
483   **2016**;6:e430

484   31.   Wu L, Wang W, Tian J, Qi C, Cai Z, Yan W*, et al.* Engineered mRNA-expressed bispecific antibody
485   prevent intestinal cancer via lipid nanoparticle delivery. Bioengineered **2021**;12:12383-93

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Figure Legends

Figure 1. Proof of concept: Can mRNA/LNP formulations encode active multimeric biologics *in vivo*? (A) Complex, hexameric bifunctional therapies that simultaneously block an immune checkpoint (CP) or tumor associated antigen (TAA) and agonize TNF receptor/ligand (TNFR / TNFL) pathways have been successfully manufactured as fusion protein biologics and are under clinical investigation in multiple oncology trials. The mRNA/LNP generation of similar multimeric therapeutics could influence certain attributes of pharmacokinetics and pharmacodynamics, including the (B) *in vivo* production from multiple tissue compartments over an extended time-course as compared to the fusion protein exposure kinetics. (C) Fc-linked hexameric fusion proteins produced from mammalian production cell lines are secreted as monomers, that go through a stepwise process of disulfide mediated dimerization, followed by tetramer and then hexamer formation through non-covalent interactions between neighboring TNF-ligand domains. (D) The resulting hexameric fusion protein uniquely activates TNF-receptors which require trimerization for signaling. Figure generated using Biorender.

Figure 2. mRNA constructs encoded functional hexameric bifunctional therapeutic fusion proteins. (A) mRNA-encoded human SIRPα-Fc-CD40L or (B) TIGIT-Fc-LIGHT were transfected into HEK293T cells and after 48 hours, the resulting protein was affinity purified using FcXL resin. The purified protein migrated as expected in SDS-PAGE under non-reduced, BME reduced (R) and deglycosylated (DG) conditions, and was detected by three separate domain-specific antibodies. (C)(D) Per cell production of mRNA generated protein was quantitated using a dual MSD PK assay and two separate VHH reagents developed to be hexameric-fusion protein specific, for capture and detection. The mRNA generated supernatant concentrations were matched to that of fusion protein reference material (FP Ref.) and both the FP ref. and mRNA-generated protein (mRNA) were assessed head-to-head for (C) SIRPα-Fc-CD40L in (1) a dual MSD potency assay, (2) CD40 cell-based NFκB activity assay, and (3) an *in vitro* macrophage/tumor phagocytosis assay. (D) Human *TIGIT-Fc-LIGHT* was also encoded by mRNA and characterized by western blot and a dual PK MSD assay. The resulting mRNA-generated protein concentrations were normalized to a FP ref. and assessed head-to-head in (1) a dual MSD potency assay, (2) a U2OS/NFκB/NIK/LTβR+ cell-based reporter assay, and (3) by qPCR assessing the expression of CCL2 and CXCL8 in the LTβR+ human tumor cell line A375.

Figure 3. mRNA-encoded fusion proteins were generated *in vivo* as hexameric proteins and demonstrated extended serum and tissue exposure kinetics. (A) Mice were given a single dose through tail vein of 200 μg of the FP ref., 12.5 μg of the corresponding mRNA/LNP formulation, or vehicle. After 0.5, 1, 2, 6, 24, 48, 72, and 96 hours, (B) serum, and (C) liver, spleen, and iLNs were collected from each animal (n=3 per group, per time point), and PK was assessed using MSD assay formats that captured one end of the fusion protein and detected the other. (D) Serum was collected from mRNA/LNP treated mice, pooled, and purified using a single-step ProA capture and elute method. The resulting protein was analyzed using SEC alongside of an existing human SIRPα-Fc-CD40L FP ref. control. (E) Serum cytokines were assessed in non-tumor bearing mice over the entire time-course, including the kinetic increase in IL-2 over a 96-hour period. (F) Other cytokines were more transiently increased, usually within 1-2 hours of the treatment. Part A was generated using Biorender.

Figure 4. mRNA-encoded fusion proteins detected in the tumor and induced on-target PD activity, anti-tumor response, and extended survival. A CT26 tumor study was designed to assess the anti-tumor activity of mRNA/LNP formulated fusion proteins compared to that of the FP ref. material. Mice were inoculated on the hind flank with CT26 tumors and when the tumor volume reached ~90 mm^3, treatment began. FP ref. were given at doses of 200 μg through IP injection, mRNA/LNPs at 12.5 μg through IV injection, and benchmark antibodies at 100 μg through IP injection. Treatments were given on days 0, 3, 7, 10, 14, and 17. One cohort of treated animals was assessed for tumor growth and survival over a 30-day period, and another cohort of animals was euthanized on day 8 of the time-course, 24 hours after the day 7 dose, to assess (A) therapeutic protein concentrations within the tumor and

531 (B) average serum and tumor cytokines for treated animals. (C) Individual tumor growth curves are shown for
532 each treatment group. In addition, the average day in which all animals within a treatment group reached tumor
533 burden is quantitated and shown within each graph, and also depicted as a vertical dotted line. The number of
534 animals in each group and any animals that completely rejected the established tumor are also shown within the
535 graphs. For example, the vehicle treated group reached tumor burden at an average of 13.13 days, 8 animals were
536 in this group, and 0/8 rejected the tumor. (D) The Kaplan-Meir graph depicts group survival over the time-course
537 and the Mantel-Cox test was used to assess group significance. (E) A cohort of animals was euthanized on day 8,
538 24 hours after the third dose on day 7. Tumors were excised, dissociated, and the immune infiltrate was assessed
539 by flow cytometry. Truncated violin plots depict the median, first, and third quartiles. (F) Combination efficacy of
540 FP and mRNA therapeutics was assessed with anti-PDL1 (clone 10F.9G2), which was delivered at doses of 200 µg
541 alone or in the combinations shown on days 0, 3, 7, 10, and 14. Tumor growth inhibition on day 15 of the time
542 course is shown in comparison to the vehicle control group and significance between groups was calculated using
543 unpaired t-test. (G) A cohort of treated animals from the combination study was euthanized on day 8, 24 hours
544 after the third dose on day 7. Tumors were excised, dissociated, and the immune infiltrate was assessed by flow
545 cytometry. Cell populations were normalized to those of the anti-PDL1 monotherapy (set at a value of 1) group to
546 visualize the contribution of each combination.

547

548

Figure 1

A.

Evaluation of Complex Biologics Delivered as:

(1) Multimeric Fusion Protein

CP/TAA Blocker
(e.g. SIRPα, TIGIT)

Fc Linker

TNFL Agonist
(e.g. CD40L, LIGHT)
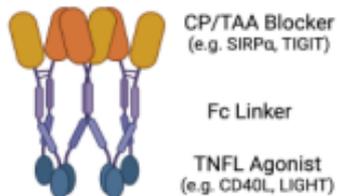
(2) mRNA/LNP Formulation
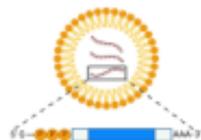
Can mRNA/LNPs generate functional oligomeric proteins *in vivo*?

B.

In Vivo Production of mRNA Encoded Protein

Fusion Protein

mRNA/LNP

Potential Drug Exposure Over Time
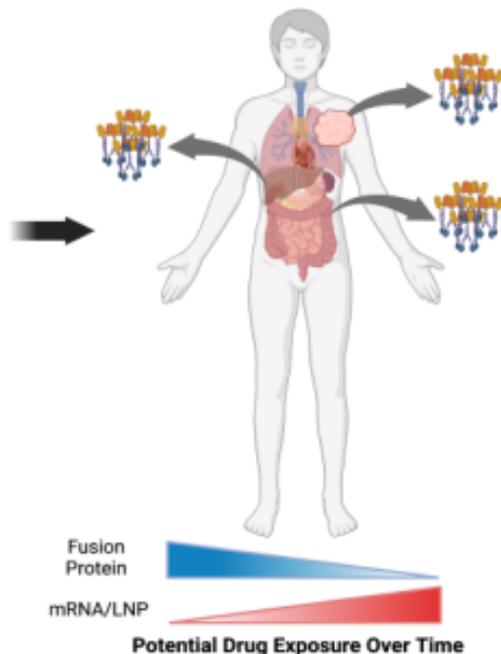
C.

1. Secretion of Monomer

2. Dimerization

Disulfide Bonds

3. Two Dimers Form Tetramer

TNF Ligand Domain Forms Homo-Trimer (non-covalent interactions)
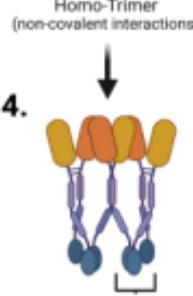
4. Tetramer and Free Dimer Form Hexamer

Third Dimer Completes the Second Trimer

D.

Combined Checkpoint Blockade / Immune Agonism

Immune Cell

Receptor

CP & TAA

Tumor Cell

Figure 2

A. mRNA Encoded SIRPα-Fc-CD40L

B. mRNA Encoded TIGIT-Fc-LIGHT

C.

D.

Figure 3

Figure 4