Supplementary Appendix

This appendix has been provided by the authors to give readers additional information about their work.

Supplement to: Hall VG, Ferreira VH, Ku T, et al. Randomized trial of a third dose of mRNA-1273 vaccine in transplant recipients. N Engl J Med. DOI: 10.1056/NEJMc2111462

SUPPLEMENTARY APPENDIX

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ADDITIONAL CONTRIBUTORS

The following study team members contributed to the trial implementation and analysis: Natalia Pinzon, Ilona Bahinskaya, Anila Yousuf, Laura Byers, Sonika Humar

METHODS

Patient population and Study Design

This study was conducted at the University Health Network organ transplant program and was a double-blind, randomized, controlled trial that compared a third dose of the mRNA-1273 (Moderna) vaccine versus placebo in solid organ transplant (SOT) recipients. Our primary hypothesis was that a third dose booster would result in increased vaccine immunogenicity compared to placebo. The institutional research ethics board approved the study. A notice of approval to conduct the study was also obtained from Health Canada due to off-label use of a third dose of mRNA-1273 vaccine. The study was registered on Clinicaltrials.gov (Identifier: NCT04885907) prior to enrolment of the first patient. All patients provided written informed consent.

We enrolled adult patients (aged ≥18 years) who had received an organ transplant (kidney, liver, heart, lung and pancreas, or combined organs) and had a functioning allograft. Patients were eligible if they had already received both doses of the mRNA-1273 vaccine at the 0,1-month interval. The intervention, third dose of mRNA-1273 (Moderna) vaccine versus placebo, took place 2 months post second dose of mRNA-1273 vaccine. Patients were enrolled without *a-priori* knowledge of their antibody response after the second dose. In the initial COVID-19 vaccine rollout at our transplant center, since there were limited data on vaccine safety, patients who were >6 months post-transplant were initially prioritized. Some of the patients in the current trial were enrolled in an observational study (n=84) that analyzed antibody and T-cell responses after the first and second doses of vaccine.[1]

Exclusion criteria for the current trial were as follows: 1) within 1-month post-transplant, 2) had a febrile illness within 1-week prior, 3) previous microbiologically confirmed COVID-19 infection, 4) active cytomegalovirus (CMV) infection, 5) received intravenous immunoglobulin in the 4 weeks prior, 6) received rituximab in the last 6 months, 7) had treatment for acute rejection in the 30 days prior, and 8) an allergic reaction to the previous mRNA-1273 vaccination.

Following consent, participants were randomized to receive either a third dose of mRNA-1273 vaccine versus normal saline placebo intramuscularly in a 1:1 ratio. Both study injections were 0.5ml. The contents of both mRNA-1273 and placebo vaccine for injection were concealed with opaque tape to ensure blinding. Randomization was performed using a computer-generated schedule in blocks of 4. Randomization was generated by someone not related to other aspects of study implementation. Allocation was concealed by ensuring the individual preparing syringes was not involved in other aspects of study implementation. The patients, and study team members who obtained consent, administered vaccine, and assessed adverse events were blinded to vaccine assignment. The laboratory team was also blinded to vaccine assignment. Patients were enrolled and vaccinated between May 25, 2021 to June 3, 2021. Patients were followed for outcomes till July 5, 2021.

In all participants, pre-third dose blood work was obtained 6±2 weeks after the second dose (mean 37±14 days placebo; 37±15 days mRNA-1273). The study vaccine was administered to participants' left or right deltoid muscle by a blinded study team member. A subgroup of patients also had bloodwork pre-first dose, and pre-second dose (n=84). All patients were negative for anti-RBD pre-first dose (data not shown).

Outcomes: Antibody responses

The primary outcome was the measurement of anti-spike receptor binding domain antibodies (anti-RBD) using the Roche Elecsys anti-SARS-CoV-2 S enzyme immunoassay) as described in Hall et al (Am J Transplant in press).[1] Sera were collected 4±1 weeks after the third dose of vaccine. Testing was performed as per manufacturer's instructions in a certified biochemistry laboratory. This assay is a double-antigen sandwich electrochemiluminescence immunoassay that quantitatively detects antibodies to RBD of the spike protein. It has a lower limit of detection (LOD) of 0.4 U/mL although positive detection is defined as ≥ 0.8 U/mL. During the trial, a surrogate virus neutralization assay became available in Canada and was added as a secondary endpoint before any post-3rd dose sera was collected. Neutralizing antibodies were assessed via the SARS-CoV-2 Surrogate Virus Neutralization Test (SVNT) assay (GenScript), according to the manufacturer's specifications. The assay was originally described by Tan et al. in Nature Biotechnology[2] and has been used in several peer-reviewed studies to assess neutralizing antibodies.[3-5] This assay has received emergency use authorization from the FDA. Briefly, serum is incubated with horseradish peroxidase (HRP)-conjugated spike-RBD and transferred to ACE2 coated wells. Neutralizing antibodies present in serum will inhibit RBD-ACE2 interactions. The assay provides the percent neutralization, with <30% classified as negative; 30-100% represents a range of low-to-high neutralization ability. The negative and positive percent agreement with conventional plaque reduction neutralization test $(PRNT)_{50}$ and $PRNT_{90}$ assays is approximately 100%. The manufacturer reported sensitivity and specificity for the assay is 93.80% and 99.4%, respectively. Although a previous diagnosis of COVID-19 was an exclusion criteria, in order to further confirm that measured immune responses were not due to natural infection, post-3rd dose sera were tested for anti-nucleocapsid protein antibody. This would not be expected to be positive in response to vaccine. Testing was done using a commercially available chemiluminescent microparticle immunoassay (Abbott Laboratories, USA) as per manufacturer's instructions. As recommended by the manufacturer, an index measurement of ≥1.4 was considered positive.

Outcomes: T-Cell mediated immunity assessment

As a pre-specified secondary outcome, SARS-CoV-2 specific CD4+ and CD8+ T-cell responses were assessed pre and post intervention in patients who consented for additional blood. Methods are described in Hall et al.[1] and have previously been validated using healthy controls post-vaccine and post-recovery from COVID-19 infection samples. Briefly, peripheral blood mononuclear cells (PBMCs) were isolated from whole blood and cryopreserved for batch testing. For testing, a total of 10^6 PBMCs were rested for two hours and incubated with overlapping peptides encompassing the full SARS-CoV-2 spike protein. Peptides consisted mainly of 15-mer sequences with 11 amino acid overlaps (PepTivator®, Miltenyi Biotec)(final concentration of 5 µg/mL per peptide). Cells were incubated overnight with peptides, a CD28/CD49d co-stimulatory antibody cocktail (BD Biosciences) and a

protein transport inhibitor to prevent cytokine release (ThermoFisher Scientific). Intracellular cytokine staining (ICS) was used to measure the frequency of spike-specific T-cells. IFN-γ and IL-2 were used as the markers for this study, as has been also reported with other mRNA-1273 vaccine studies [6,7]. The positive control was PMA/ionomycin and the negative (media) control was cells treated with media alone. Following overnight incubation at 37ºC, PBMCs were stained with a viability dye (Zombie Aqua, Biolegend), Fc blocked (BD Biosciences) and incubated with a surface marker antibodies (CD3, CD4, CD8). Cells were then fixed, permeabilized and incubated with antibodies for intracellular markers (IFN-γ, and IL-2). Flow cytometry was performed on an LSR II BGRV (BD Biosciences) instrument. A representative gating strategy and a patient with a positive CD4+ and a negative CD8+ response is shown in Figure S4a-c. As a robust, validated, yet conservative measure of vaccine-induced T-cell responses, we specifically measured frequencies of CD4+ and CD8+ T-cells that expressed two cytokines (IFN-γ and IL-2 positive; after subtraction of concurrent untreated comparator). Polyfunctional T-cells are commonly used to assess vaccine-induced immunogenicity [8-10]. A minimum number of 100,000 live, CD3+ T-cells were required for samples to be included in the flow analysis.

Outcomes: Safety and adverse events

All patients were followed closely for the duration of the study. Safety assessments included monitoring through a participant-directed vaccine diary for local and systemic adverse events each day for the 7 days after injection (Figure S3a-d). Adverse events were categorized by the Food and Drug Administration toxicity grading scale for volunteers in vaccine trials as follows; grade 1 (no interference in daily activities), grade 2 (some interference in daily activities), grade 3 (participants unable to perform daily activities) and grade 4 (potentially life threatening) [11]. In addition, study team members contacted all participants every 2 weeks by phone call and chart review for episodes of acute organ rejection, hospitalization, other adverse events, or COVID-19 infection for at least 4 weeks post-intervention.

Primary endpoint and sample size calculation

The primary endpoint was based on anti-RBD response following the third vaccine dose. A positive threshold of anti-RBD titer of ≥100 U/ml was chosen *a priori* (as per clinicaltrials.gov registration) after careful consideration. Anecdotally we had noted several transplant patients developing severe disease post-vaccine despite low levels of detectable anti-RBD antibody. In a dose-finding challenge study in rhesus macaques (McMahan et al, Nature 2021), the threshold anti-RBD ELISA titer of approximately 100 U/ml was required for protection against SARS-CoV-2 infection.[12] This was corroborated by a comprehensive analysis by Khoury et al. (Nature 2021)[13] that looked at correlates of protection in people using data from seven current vaccines and from convalescent cohorts. They estimated a 50% protective neutralization level equates to approximately an anti-RBD titer of 54 U/ml with a 95% CI 30-96 U/ml.[13] This validates our primary endpoint as the upper bound of the estimated 95% confidence interval that correlates with 50% protective neutralization. In addition, Marinelli et al in their prospective cohort study found a median convalescent anti-RBD antibody titre of 64.3 U/ml (interquartile range (IQR) 5.7 – 185) in solid organ transplant patients infected with COVID-19.[14]

Our primary hypothesis was that a third dose booster would result in increased vaccine immunogenicity compared to placebo. Our sample size calculation was based on the hypothesis that at least one-third (33%) of participants in the treatment arm (3-doses) will meet the primary end-point vs. 10% in the placebo arm (2-doses). Based on this endpoint estimate, for two independent study groups to achieve a power of 80% and alpha level 0.05; at 1:1 randomization, a sample size of 98 patients total (49 per arm) was required for analyses using an uncorrected chi-squared test. The function power.prop.test in the R statistical software was used to calculate this sample size. To account for a potential inability to obtain follow-up blood in all patients, a sample size of 120 patients was selected as the enrolment target.

Statistical analysis

The safety analysis was performed in all patients who received the study vaccine regardless of whether they returned for follow-up serum (intention-to-treat population) with the exception of one patient in the placebo group who withdrew from the study. Demographics and safety analysis were summarized using descriptive statistics. The immunogenicity analysis was performed in those who received the third vaccine dose and returned for follow-up serum (per-protocol population). The outcomes were designed to evaluate vaccine immunogenicity by assessment of pre- and post-3rd dose blood. The primary endpoint was a post-intervention anti-RBD titer of $\geq$ 100 U/ml as a threshold of response/no-response. The hypothesis of no difference in outcomes between placebo and mRNA-1273 groups was assessed in the primary analysis with a Yates-corrected chi-square test; an unadjusted relative risk (RR) was calculated and a 95% confidence interval (CI) for the unadjusted RR was calculated using the normal approximation to the distribution of the log-relative risk. For the primary outcome, an adjusted marginal relative risk (RR) was also computed, along with a 95% CI. To obtain the adjusted RR estimate, a logistic regression model was used with baseline log(anti-RBD) titer as a covariate; the RR was obtained through a model-based approach.[15] This fitted a logistic regression model and used the estimated parameters to predict the probability of a response. First the responses were predicted using the baseline log(anti-RBD) titer as a covariate and assuming everyone was in the vaccinated group. Then the responses were predicted using the baseline log(anti-RBD) titer as a covariate assuming everyone was in the non-vaccinated group. The ratio of the averages of the two sets of probabilities was used as the estimate of the marginal adjusted RR. The 95% BCa CI for this RR was calculated based on 4000 bootstrap replications of the calculation of this model-based RR. Confidence intervals for the fold-change from pre-to post, and the absolute values of anti-RBD titers were computed using the nonparametric bootstrap with 4000 replicates and the BCa method.

For secondary outcomes, confidence internals for the differences in median, percent virus neutralization, and SARS-CoV-2-specific T-cells were computed using the nonparametric bootstrap with 4000 replicates and the BCa method. Confidence intervals for the RR for secondary binary outcomes were calculated using the normal-based approximation. For the purposes of quantitative statistical analysis for each of the assays, where required, values below threshold (e.g. LOD) were coded as threshold/2. Statistical significance for the primary outcome was defined as a p value < 0.05. Because no provision for correcting for multiplicity when conducting tests for secondary outcomes was pre-specified, all secondary results were reported as point estimates and 95% CIs. The widths of the CIs have not been adjusted for multiplicity, so the intervals should not be used to infer definitive treatment effects for secondary outcomes. All statistical analysis was done using R version 4.03 (R

Core Team (2020). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL https://www.R-project.org/) and Prism GraphPad version 9.1.1.

No interim analysis was planned or performed.

RESULTS

Figure S1. Study flow chart

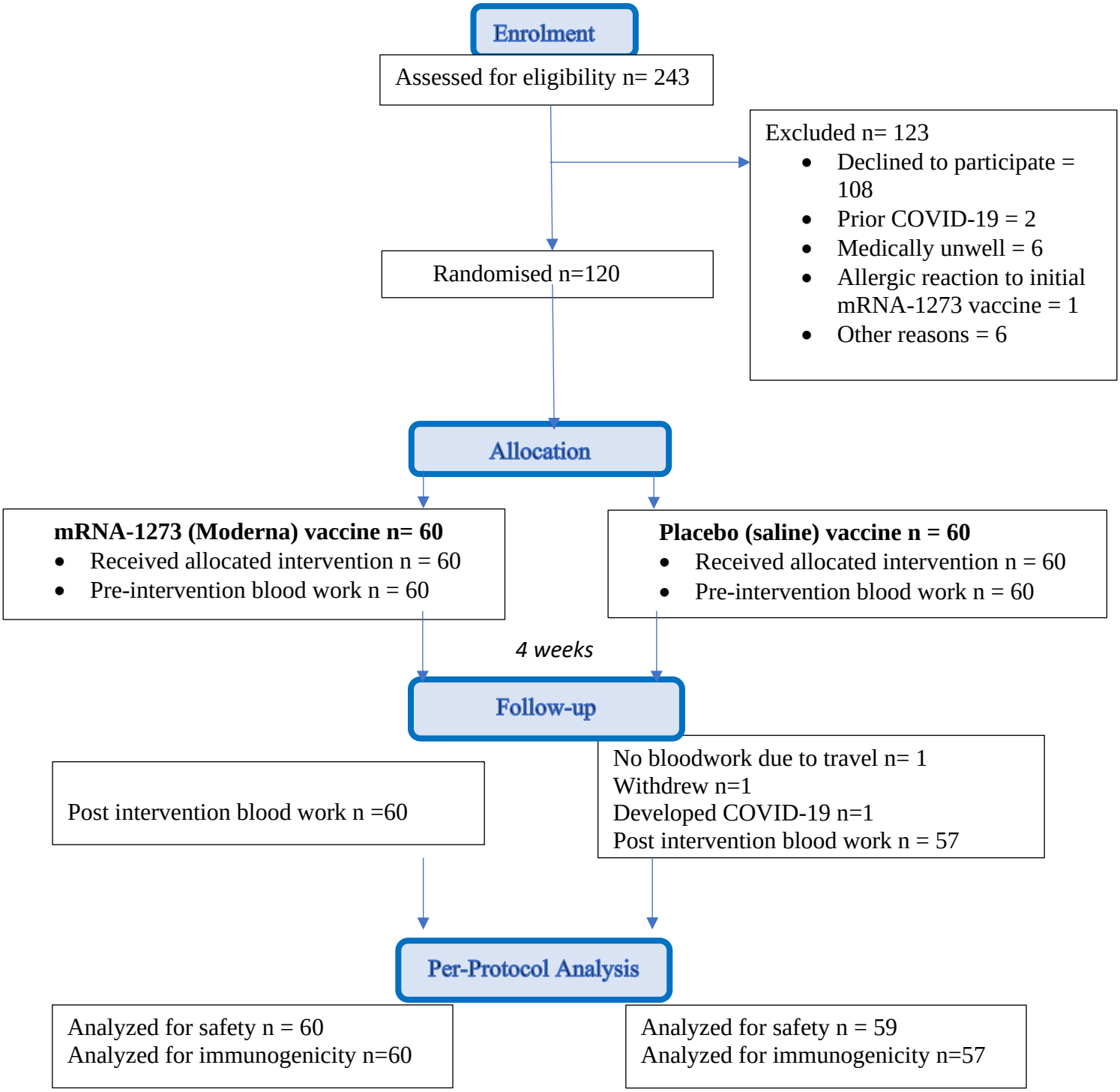


Table S1. Patient characteristics at enrolment, mRNA-1273 vaccine vs placebo groups

Characteristic	mRNA-1273 (n=60)	Placebo (n=60)
Age (years), median (IQR)	66.9 (64.0 – 71.8)	65.9 (62.9 – 70.3)
Male sex, n (%)	37 (61.7%)	42 (70.0%)
Time from transplantation to intervention (years), median (IQR)	3.57 (1.99 – 6.75)	2.20 (1.44 – 5.55)
Rejection within the preceding 3 months n (%)	1 (1.7%)	1 (1.7%)
Anti-thymocyte globulin in the preceding 6 months	0	0
Type of transplant (%)		
Thoracic	21 (35.0%)	26 (43.3%)
Lung	11	18
Heart	10	8
Abdominal	39 (65.0%)	34 (56.7%)
Kidney	20	9
Pancreas and Kidney-Pancreas	15	9
Liver	4	16
Immunosuppression		
Prednisone (%)	50 (83.3%)	42 (70.0%)
Prednisone daily dose, mg; median (IQR)	5 (5–5)	5 (5-7.5)
Calcineurin inhibitor (%)	59 (98.3%)	59 (98.3%)
Tacrolimus	47 (78.3%)	46 (76.7%)
Tacrolimus trough level, ng/mL (IQR)	7.6 (5.9 – 9.8)	6.7 (5.3 – 8.6)
Cyclosporine	12 (20.0%)	13 (21.7%)
Mycophenolate mofetil/ mycophenolate sodium (%)	44 (73.3%)	46 (76.7%)
Mycophenolate daily dose; mg, median (IQR)	1080 (720-1440)	720 (585 – 1440)
Azathioprine (%)	8 (13.3%)	4 (6.7%)
Sirolimus (%)	6 (10.0%)	5 (8.3%)
Lymphocyte count at time of intervention (10^3 cells/μL), median (IQR)	1.15 (0.90 – 1.60)	1.3 (0.825 – 1.70)
Pre-third dose anti-RBD titre (U/ml), median (IQR)	0.37 (0.2 – 27.64)	0.44 (0.2 – 18.19)
Anti-RBD ≥ 100 U/ml pre-3rd dose	7 (11.7%)	5 (8.8%)*

Positive surrogate virus neutralization assay pre-3rd dose (threshold ≥30%)	22 (36.7%)	18 (31.6%)*

*denominator represents per-protocol population (n=57)

Table S2: Immunogenicity outcomes comparing third dose of mRNA-1273 vs. placebo

Outcome	**mRNA-1273 (Moderna) n=60**	**Placebo N=57**	**Comparison and 95% confidence interval***
Anti-RBD ≥ 100 U/ml post-3rd dose	33 (55.0%)	10 (17.5%)	RR 3.1 (1.7-5.8) unadjusted; RR 2.9; (2.0-4.7) adjusted for baseline anti-RBD
Absolute anti-RBD titer (U/ml) post 3rd dose; mean (SD), median [IQR]	3145 (7517) 313.8 [0.2-2191]	86 (231) 1.19 [0.2-63.4]	Ratio of means: 36.5 (12.9-94)
Anti-nucleocapsid antibody post-third dose positive; n (%)	0 (0%)	1(1.8%)**	
Positive surrogate virus neutralization assay post-3rd dose (threshold ≥30%)	36 (60.0%)	14 (24.6%)	RR 2.4 (1.5-4.0)

*Widths of confidence intervals have not been adjusted for multiplicity and cannot be used to infer treatment effects for secondary outcomes.
**Borderline positive anti-nucleocapsid antibody level (1.56 U/ml); Patient had positive anti-RBD titer (>100 U/ml) both pre-and post-3rd dose.

Figure S2: Fold-change in anti-RBD titer for placebo and mRNA-1273 groups. For calculation of fold-change, values below the threshold were assigned a value of 0.2 U/mL. The mean fold change was 75 times as high in the treatment arm vs. the placebo arm (95% CI: 21-220).* Horizontal lines in box represent median fold change.
*Widths of confidence intervals have not been adjusted for multiplicity and cannot be used to infer treatment effects for secondary outcomes.

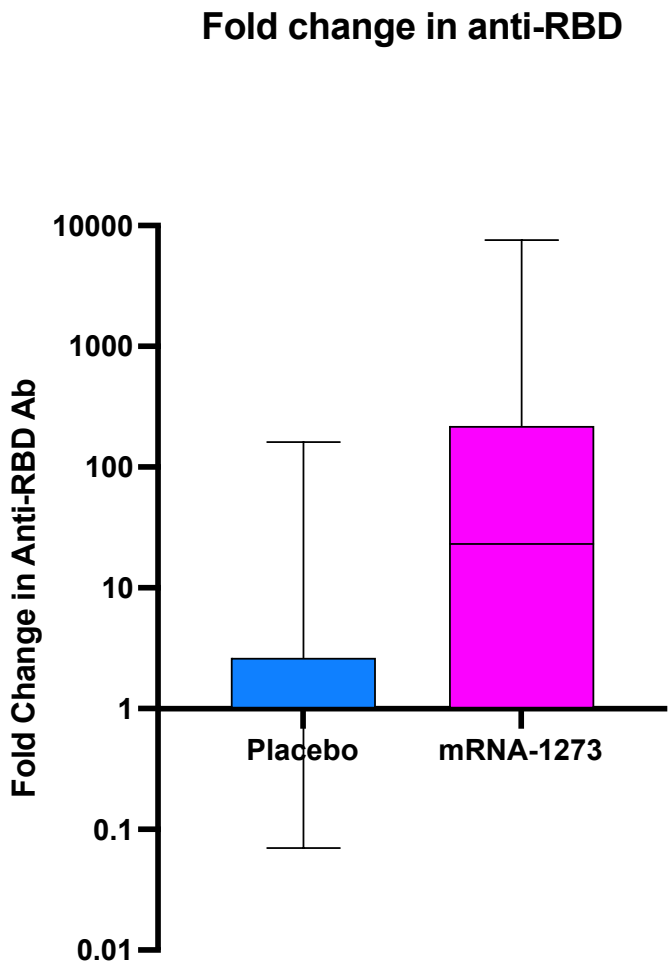
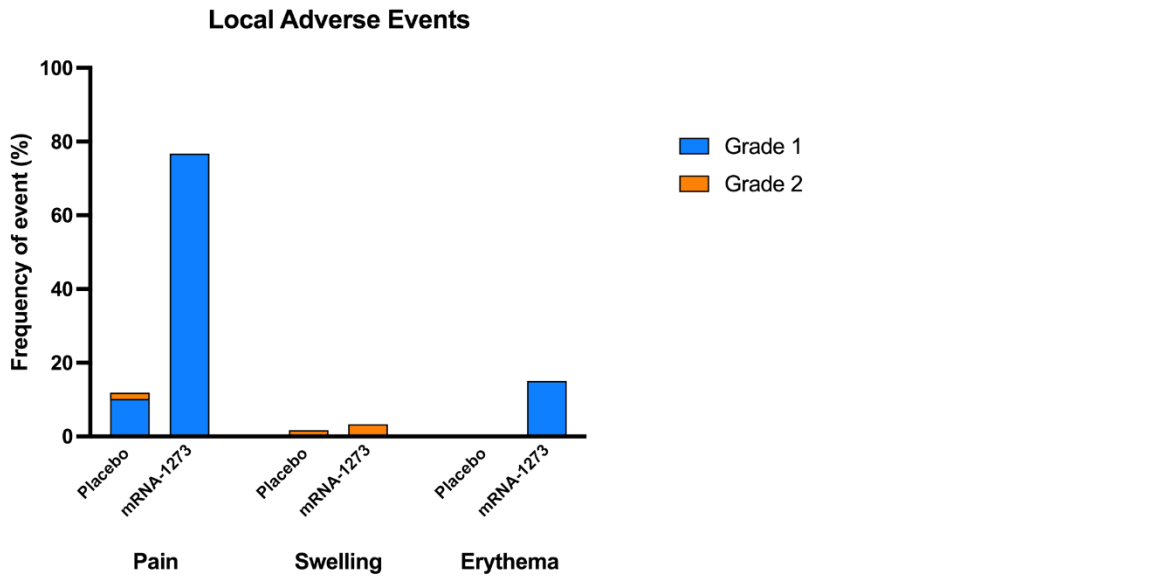


Figure S3a-d. Local and systemic adverse events in mRNA-1273 vaccine and placebo groups. In addition, in the 4 weeks post-intervention, patients sought outpatient medical attention for diverticulitis (n=1; placebo), blurry vision (n=1; placebo). Hospitalization occurred in three patients in the placebo arm, one each for COVID-19 infection, fall, gastrointestinal bleed. No cases of clinically treated or biopsy proven acute rejection occurred in either group.



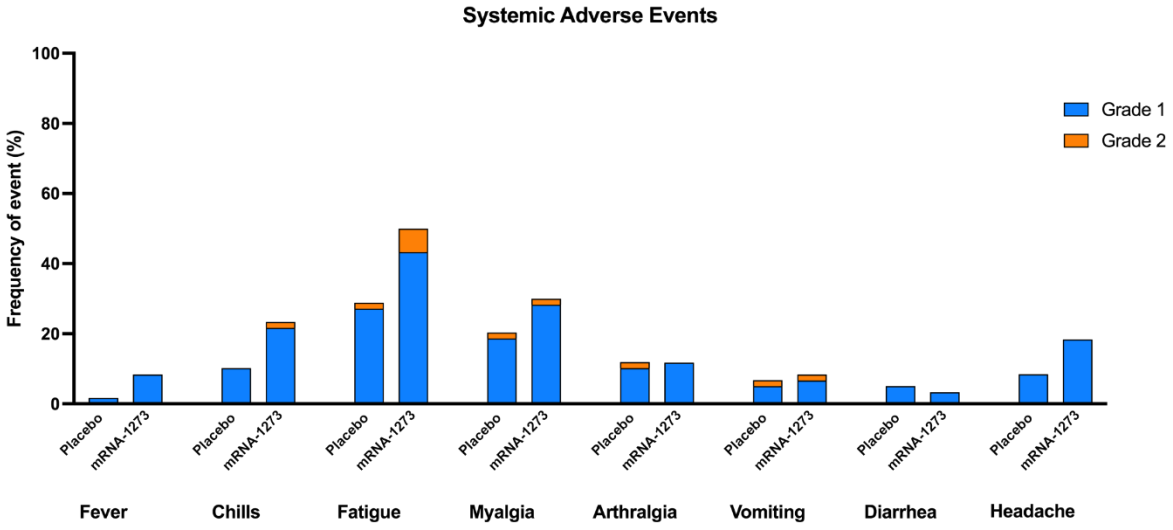


Table S3. Local and Systemic Adverse Events from Third dose mRNA-1273 or Placebo*

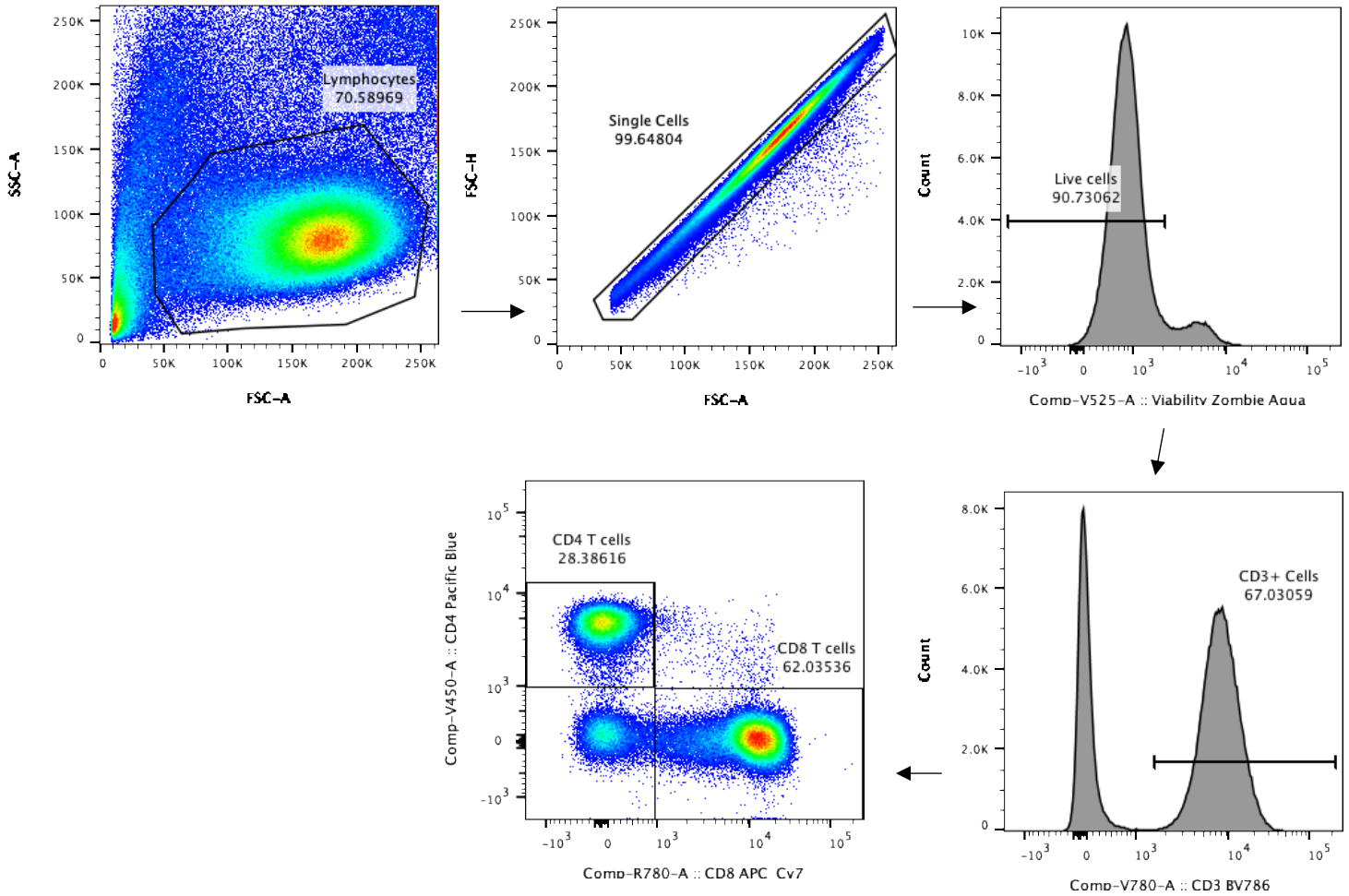
	mRNA-1273, n = 60		**Placebo, n = 59**	
	Grade 1, n (%)	**Grade 2, n (%)**	**Grade 1, n (%)**	**Grade 2, n (%)**
Local adverse event				
Pain	46 (76.7)	0	6 (10.2)	1 (1.7)
Erythema	0	2 (3.3)	0	1 (1.7)
Swelling	9 (15.0)	0	0	0
Systemic adverse event				
Fever	5 (8.3)	0	1 (1.7)	0
Chills	13 (21.7)	1 (1.7)	6 (10.2)	0
Fatigue	26 (43.3)	4 (6.7)	16 (27.1)	1 (1.7)
Myalgia	17 (28.3)	1 (1.7)	11 (18.6)	1 (1.7)
Arthralgia	7 (11.7)	0	6 (10.2)	1 (1.7)
Headache	11 (18.3)	0	5 (8.5)	1 (1.7)
Nausea or vomiting	4 (6.7)	1 (1.7)	3 (5.1)	0
Diarrhea	2 (3.3)	0	3 (5.1)	0
Rash	0	0	0	1 (1.7)

*No Grade 3 or 4 adverse events were reported in either group

Figure S4a-c. Representative Gating Strategy for T-cell Analysis.

(A) The sequential gating strategy for identifying CD4+ and CD8+ T-cells. Arrows indicate the sequence of hierarchical gates. Number in each plot refers to the frequency of the gated subset in the parental population. (B, C) Plots showing IFN-γ (y-axis) vs. IL-2 (x-axis) production, gated on CD4+ T-cells (B) or CD8+ T-cells (C). Plots are taken from the same study participant. Plots show T-cells stimulated with SARS-CoV-2 spike peptides, or media alone for the unstimulated control. Left two panels show post-second dose response, and right two panels show T-cell responses following administration of a third dose of mRNA-1273, demonstrating a good polyfunctional CD4+ T-cell response but minimal CD8+ T-cell response. There is no clear clinical correlate of disease protection defined for T-cell response magnitude.

A



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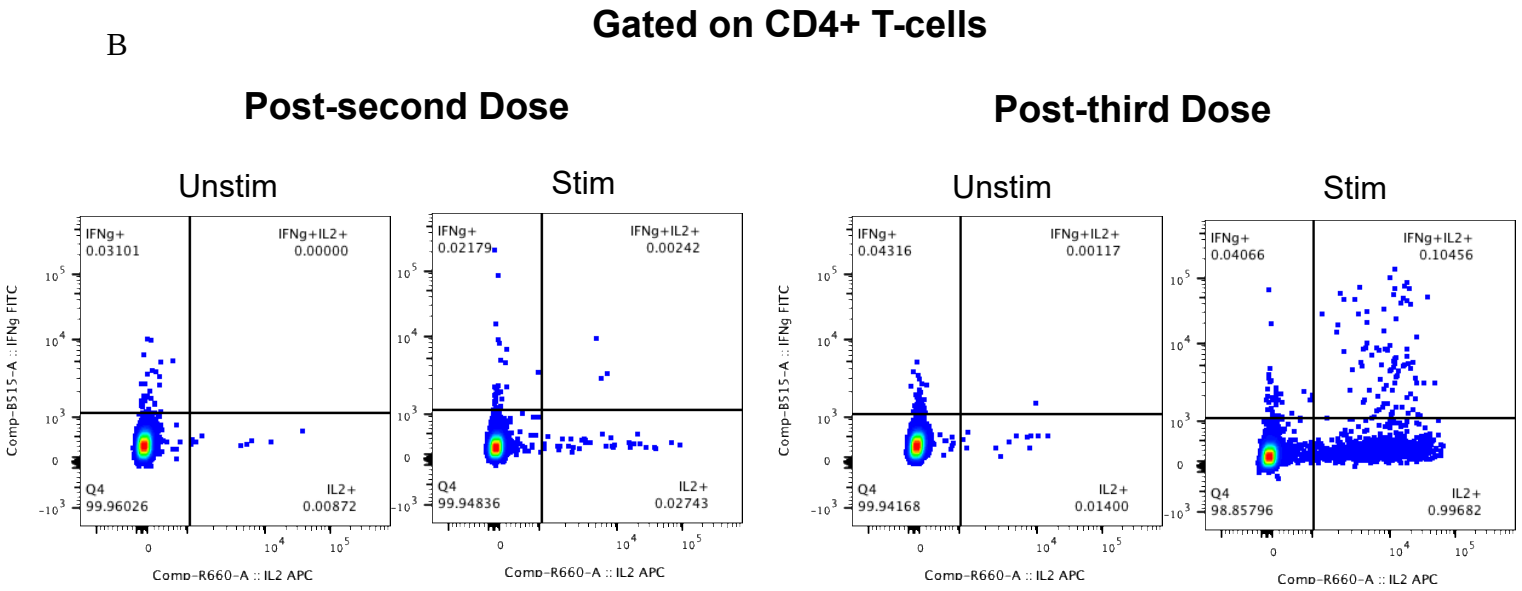
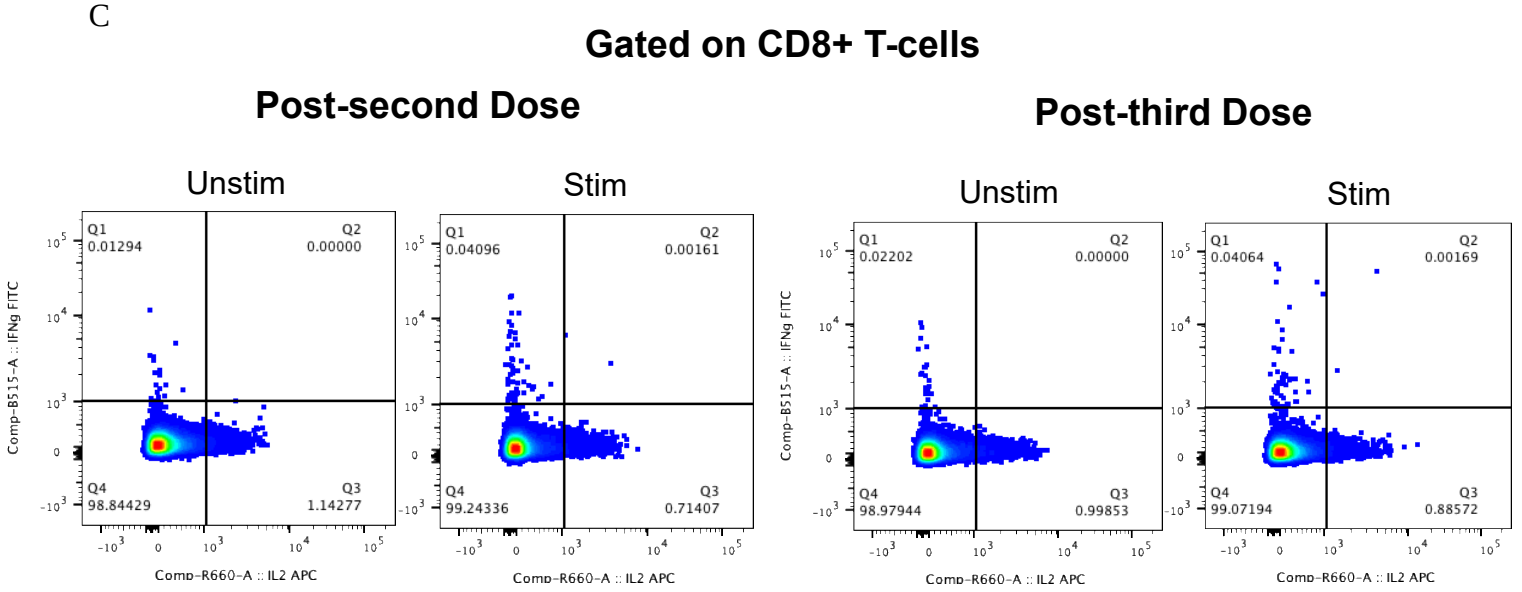

Gated on CD4+ T-cells
Post-second Dose
Post-third Dose
Unstim
Stim
Unstim
Stim
IFNg+
0.03101
IFNg+IL2+
0.00000
Q4
99.96026
IL2+
0.00872
IFNg+
0.02179
IFNg+IL2+
0.00242
Q4
99.94836
IL2+
0.02743
IFNg+
0.04316
IFNg+IL2+
0.00117
Q4
99.94168
IL2+
0.01400
IFNg+
0.04066
IFNg+IL2+
0.10456
Q4
98.85796
IL2+
0.99682
Comp-B515-A :: IFNg FITC
Comp-R660-A :: IL2 APC

C


Gated on CD8+ T-cells
Post-second Dose
Post-third Dose
Unstim
Stim
Unstim
Stim
Q1
0.01294
Q2
0.00000
Q4
98.84429
Q3
1.14277
Q1
0.04096
Q2
0.00161
Q4
99.24336
Q3
0.71407
Q1
0.02202
Q2
0.00000
Q4
98.97944
Q3
0.99853
Q1
0.04064
Q2
0.00169
Q4
99.07194
Q3
0.88572
Comp-B515-A :: IFNg FITC
Comp-R660-A :: IL2 APC

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