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Supplemental Information

A self-amplifying mRNA SARS-CoV-2 vaccine candidate induces safe and robust protective immunity in preclinical models

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SUPPLEMENTAL FIGURES AND LEGENDS

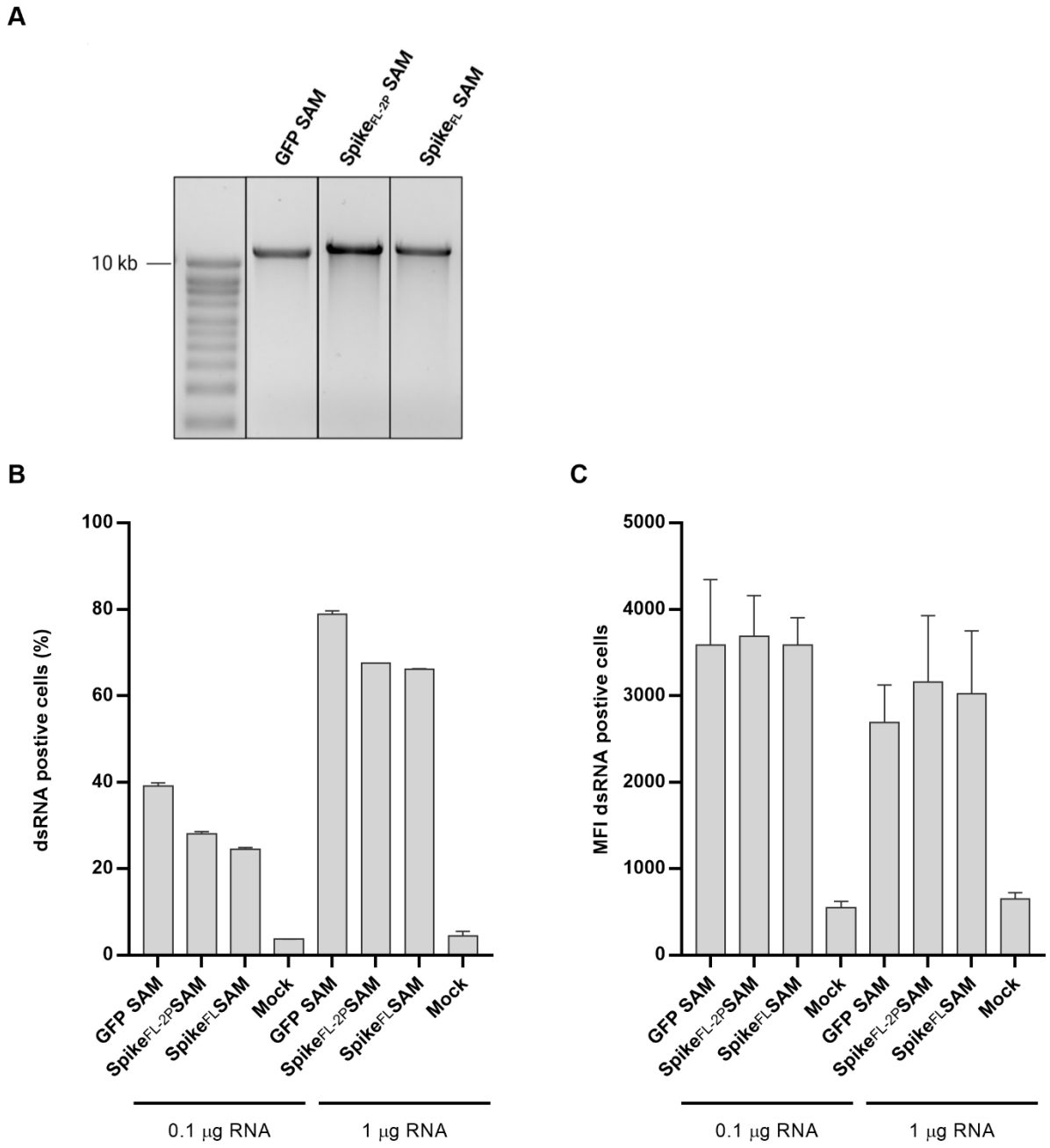

Figure S1. SARS-CoV-2 SAM *in vitro* characterization. (**A**) Quality of GFP and SARS-CoV-2 SAM RNA as measured by denaturing RNA agarose gel electrophoresis. (**B, C**) *In vitro* potency of SARS-CoV-2 SAM electroporated RNAs in BHK cells, as measured by (**B**) the percentage of double-stranded RNA (dsRNA) positive cells and (**C**) mean fluorescence intensity (MFI) by flow cytometry using an anti-dsRNA antibody. Mock, mock transfected cells; GFP, green fluorescent protein; $Spike_{FL-2P}$, prefusion stabilized spike sequence; $Spike_{FL}$, wild type full length spike sequence.

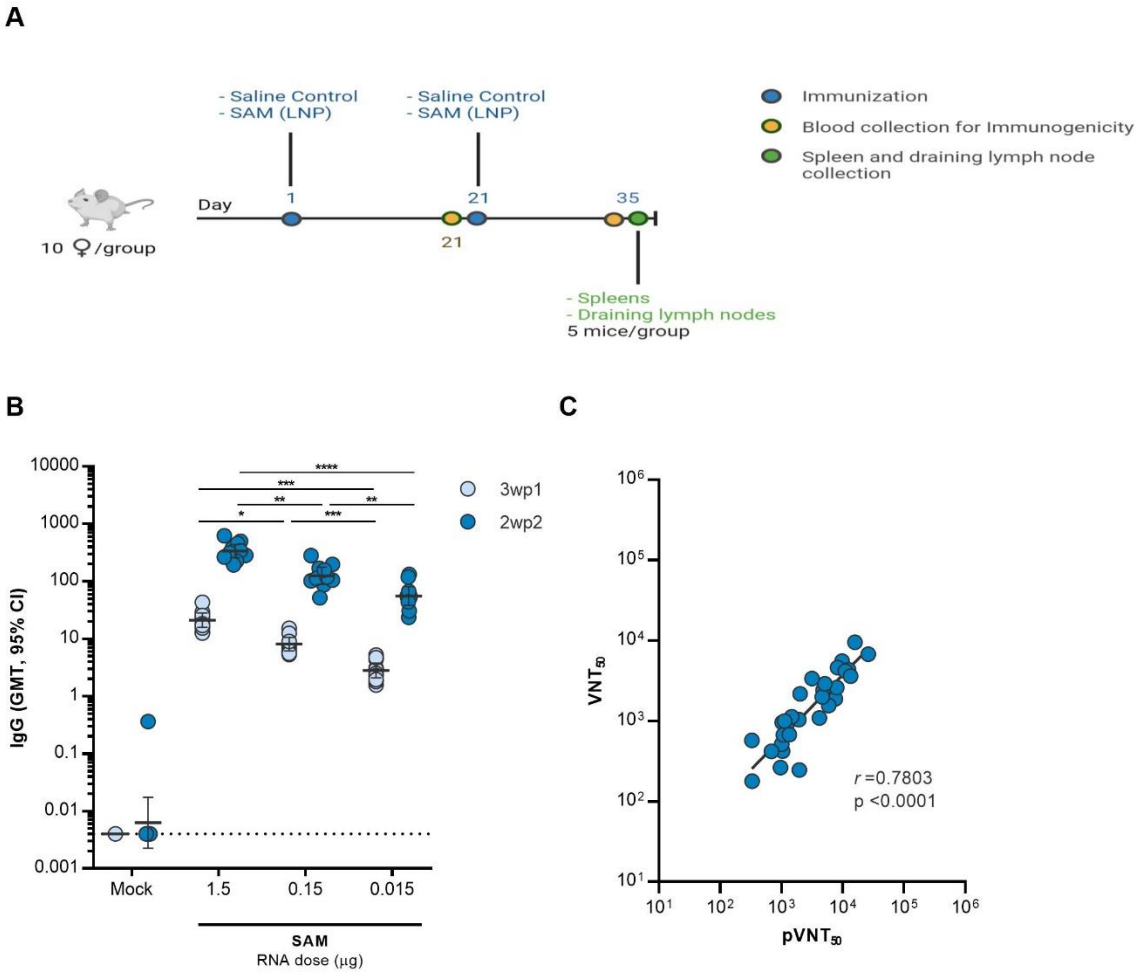

Figure S2. Mouse immunogenicity study design and humoral responses elicited by SARS-CoV-2 SAM (LNP) vaccine. (A) Study design. Female BALB/c mice (10 per group) were immunized intramuscularly (i.m.) with SARS-CoV-2 SAM (LNP) vaccine at a dose of 1.5 μg, 0.15 μg or 0.015 μg, or of saline as mock control. Sera were collected at Days 21 and 35 from all mice to measure binding and neutralizing antibody titers. At Day 35 spleens and draining inguinal lymph nodes were collected from 5 mice/group to characterize Spike-specific B cell and $CD4^+$ and $CD8^+$ T cell responses. **(B)** SARS-CoV-2 Spike-specific IgG binding antibody titers in sera collected 3 weeks after the first (3wp1) and 2 weeks after the second administration (2wp2) from mice immunized with SAM vaccine or saline control, as determined by a Luminex-based assay. Dots represent individual mice. The dotted line indicates the limit of detection. Geometric means (GMT) of each group ± 95% confidence interval (CI) are shown. Bars indicate significant differences between groups with *p* values marked by asterisks as *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$. Responses in sera collected 2wp2 from SAM vaccinated mice were significantly higher than those collected from the mock group. (**C**) Pearson correlation of VSV-SARS-CoV-2 pVNT50 (shown in Figure 2A) and live SARS-CoV-2 VNT_{50} from 30 mice (10 mice/dose group) immunized with SAM and collected at 2wp2.

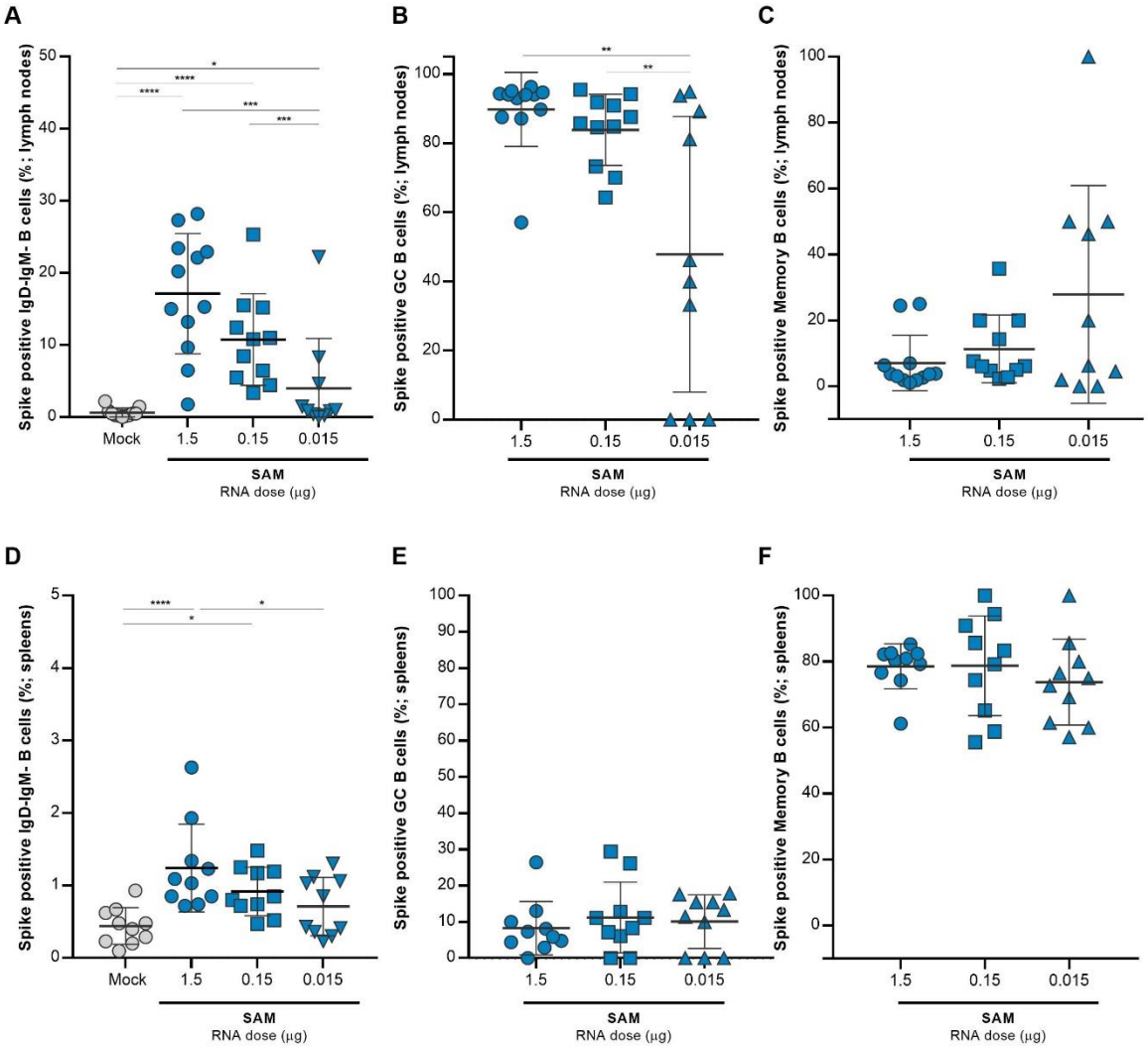

Figure S3. Spike-specific germinal center (GC) and memory B cell responses in inguinal draining lymph nodes and spleens of mice immunized with SARS-CoV-2 SAM (LNP) vaccine. Inguinal draining lymph nodes (**A-C**) and spleens (**D-F**) of immunized mice were harvested 2wp2 and analyzed for frequency of SARS-CoV-2 Spike-specific class switched B cells (defined as live $CD3^-CD19^+IgM^-IgD^-Spike^-AF488^+$) (**A, D**); frequency of Spike-specific GC B cells (defined as live $CD3^-CD19^+IgD^-IgM^-Spike\text{-}AF488^+CD95^+GL7^+$ cells) (**B, E**), and frequency of Spike-specific memory B cells (defined as live $CD3^-CD19^+IgD^-IgM^-Spike\text{-}AF488^+CD95^-CD38^+$) cells (**C, F**). Data are combined from two independent experiments at 2wp2 (5 mice/group/experiment). Means ± standard deviation (SD) for each group are shown, and each data point represents an individual mouse. Bars indicate significant differences between groups with *p* values marked by asterisks as *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$.

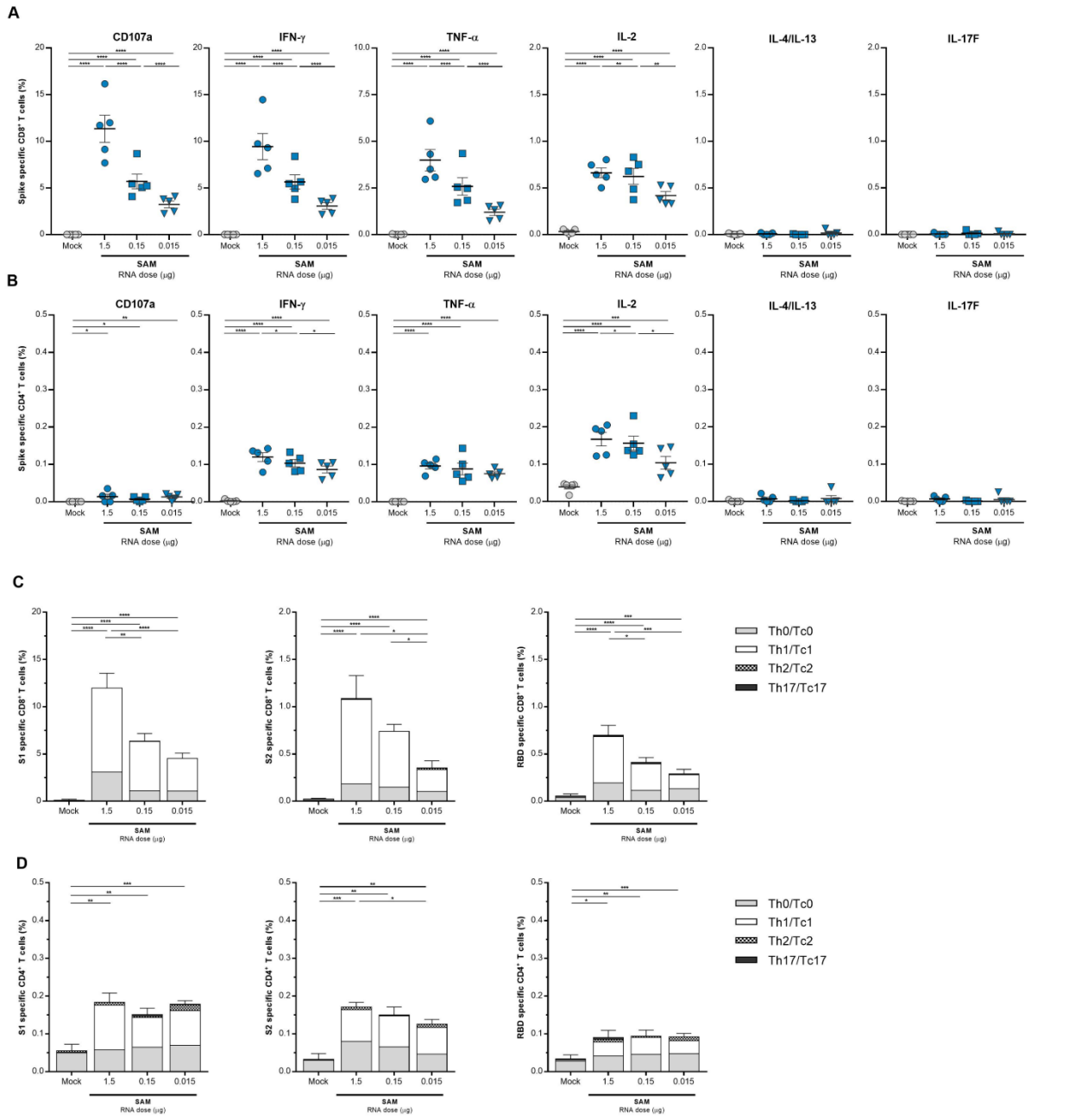

Figure S4. Spike-specific T cell responses induced by SARS-CoV-2 SAM (LNP) vaccine in mice. $Spike_{FL\text{-}2P}$-specific $CD8^+CD44^+$ (**A**) and $CD4^+CD44^+$ (**B**) T cell cytokines from splenocytes of individual immunized mice, as measured by flow cytometry at 2wp2. (**C, D**) S1, S2 or RBD specific T cell responses measured by flow cytometry after *ex vivo* stimulation of splenocytes harvested from immunized mice at 2wp2. The stacked bars (**C, D**) indicate distribution of the Tc0/Tc1/Tc2/Tc17 cytotoxic cells within the total $CD8^+$ (**C**) and the Th0/Th1/Th2/Th17 T helper cells within the $CD4^+$ (**D**) Spike-specific T cells (Mean ± standard error of mean (SEM)). Bars indicate significant differences between groups with *p* values marked by asterisks as *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$.

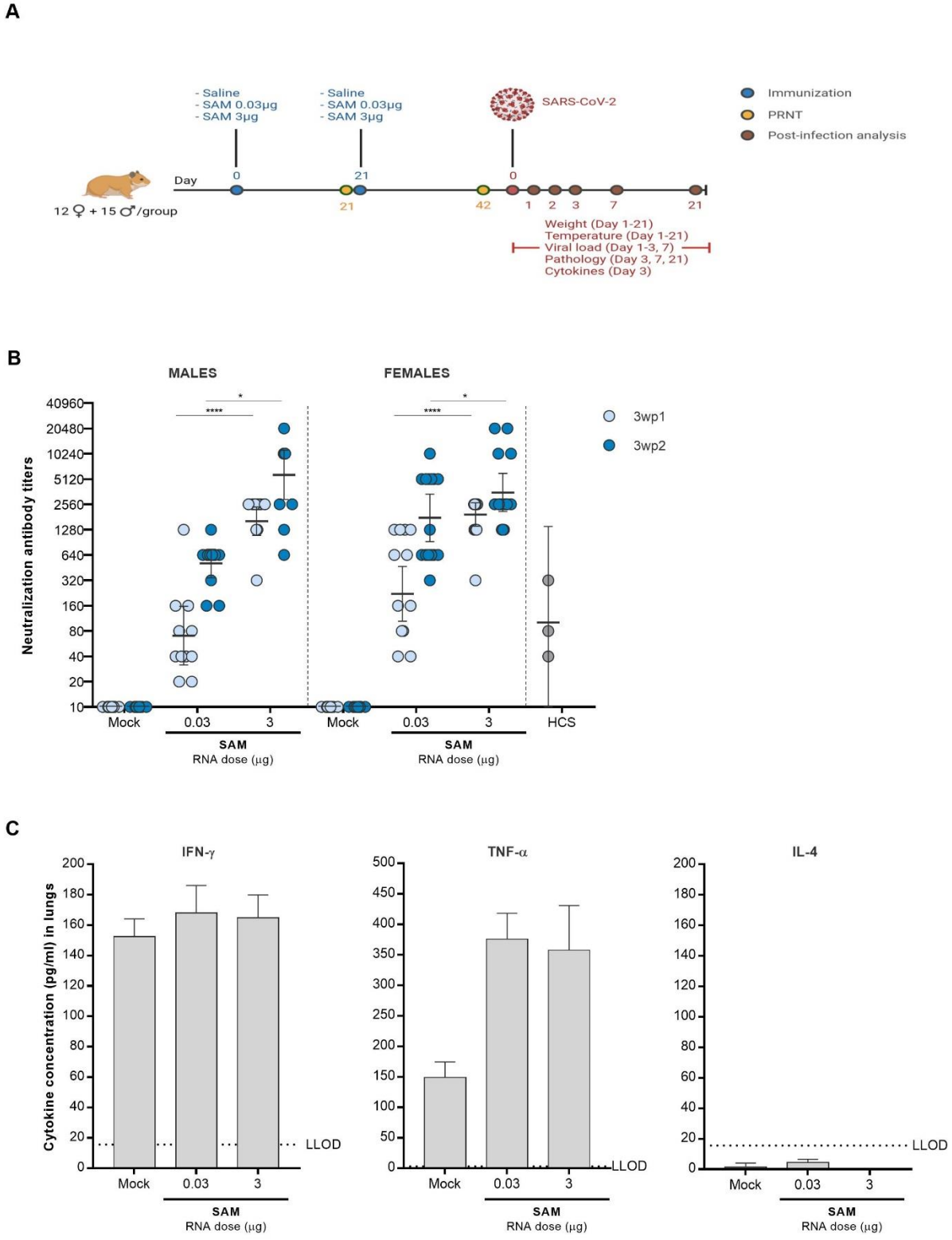

Figure S5. Immunogenicity and efficacy of SARS-CoV-2 SAM (LNP) vaccine candidate in hamsters. (A) Study design. Male and female Golden Syrian hamsters were injected i.m. with 0.03µg or 3 µg of SARS-CoV-2 SAM (LNP) vaccine or with saline (mock) at Days 0 and 21. Sera were collected at Day 21 (3wp1) and 42 (3wp2) to measure neutralizing antibody titers. At Day 42, animals were challenged intranasally with SARS-CoV-2 (isolate USA-

WA1/2020) (10^4 PFU) under BSL-3 containment level. At 1, 2, 3, 7, and 21 dpi hamsters (n=8 per group) were euthanized for tissue collection. **(B)** Serum SARS-CoV-2 neutralizing antibody titers as measured by plaque reduction neutralization assay (PRNT). Neutralizing antibody titers were compared to a panel of COVID-19 human convalescent sera (HCS; n=3) samples. Bars denote GMT±95% CI. **(C)** Analysis of cytokines in the supernatants from dissociated lung cells collected at 3 dpi and cultured without stimulation. Bars denote group means ± SD. Horizontal dotted line denotes lower limit of detection (LLOD).

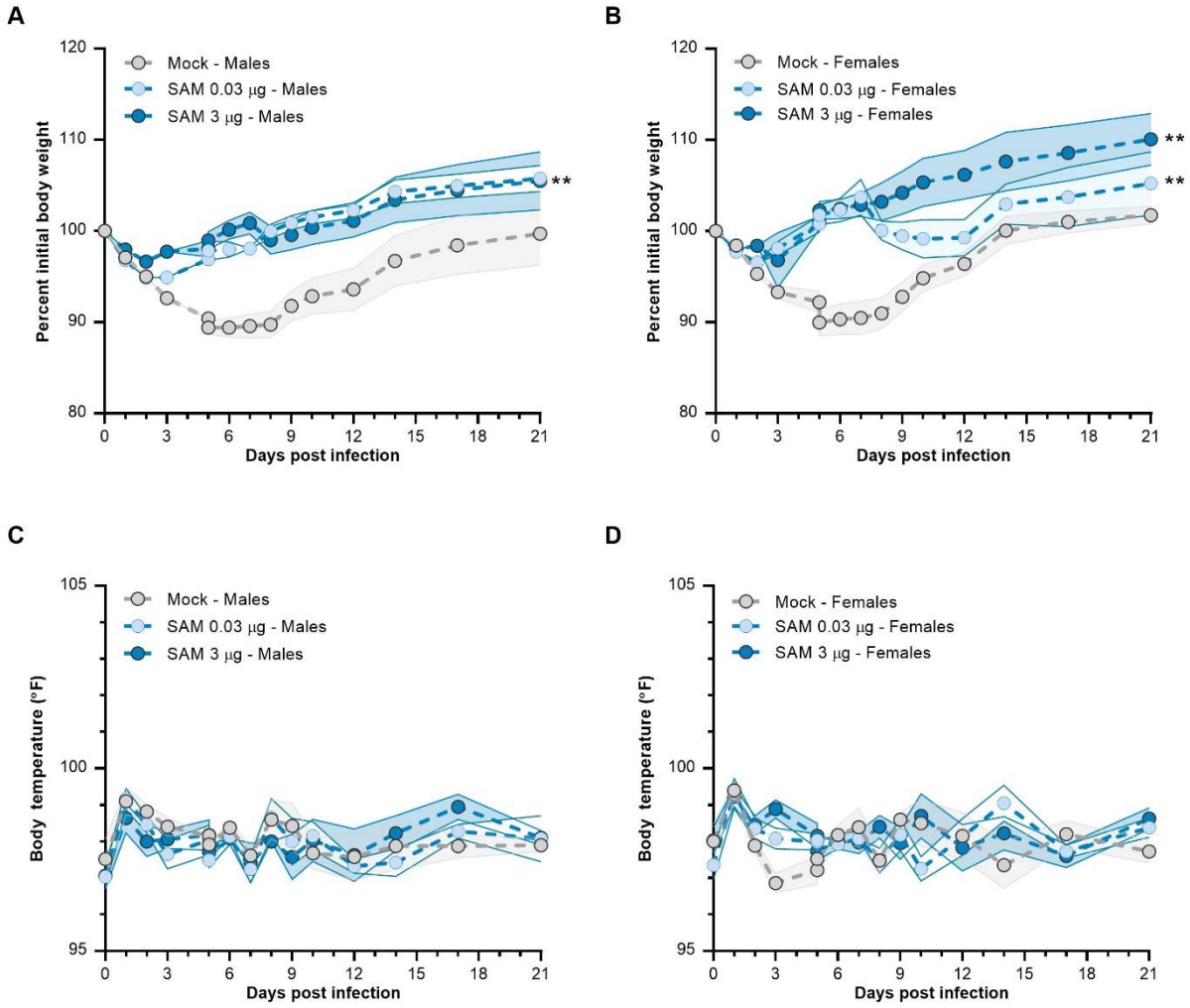

Figure S6. Weight and body temperature changes following SARS-CoV-2 challenge of vaccinated hamsters. (**A-B**) Percent weight change in male (**A**) and female (**B**) animals over 21 days post infection. Mean weights are highlighted by colored symbols. Shaded area represents SEM. **, $p < 0.01$, as compared to the mock vaccinated animals. (**C-D)** Body temperature measured daily in male (**C**) and female (**D**) animals over 21 days post infection. Mean temperature are highlighted by color symbols. Shaded area represents SEM.

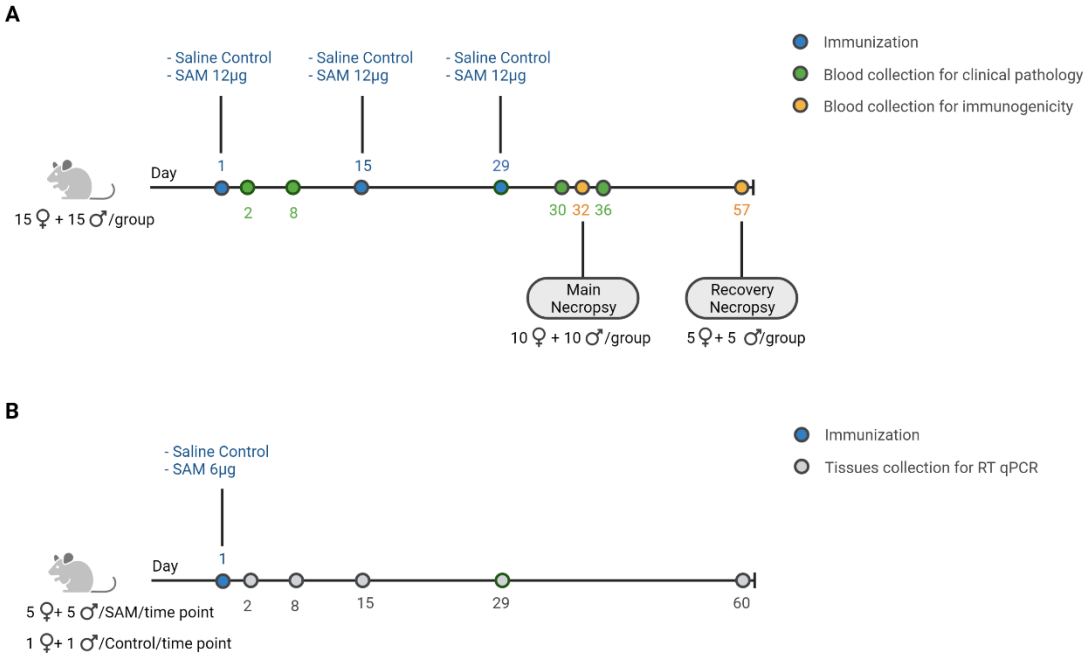

Figure S7. Repeated-dose toxicology and biodistribution experimental design. **(A)** Repeated dose study. Male and female Sprague-Dawley rats were injected i.m. on Day 1, 15, and 29 with either SARS-CoV-2 SAM (LNP) or saline. Ten animals/sex/group constituted the main study group, necropsied at Day 32, and 5 animals/sex/group were in the recovery group, necropsied at Day 57. **(B)** Biodistribution study. Male and female Sprague-Dawley rats were injected once with either SARS-CoV-2 SAM (LNP) or saline. The treated group was divided in 5 subgroups of 10 animals (n=5/sex) and the control group was divided in 5 subgroups of 2 animals (n=1/sex). Each subgroup corresponded to the 5 time points of necropsy where organs were collected for RT-qPCR analysis.

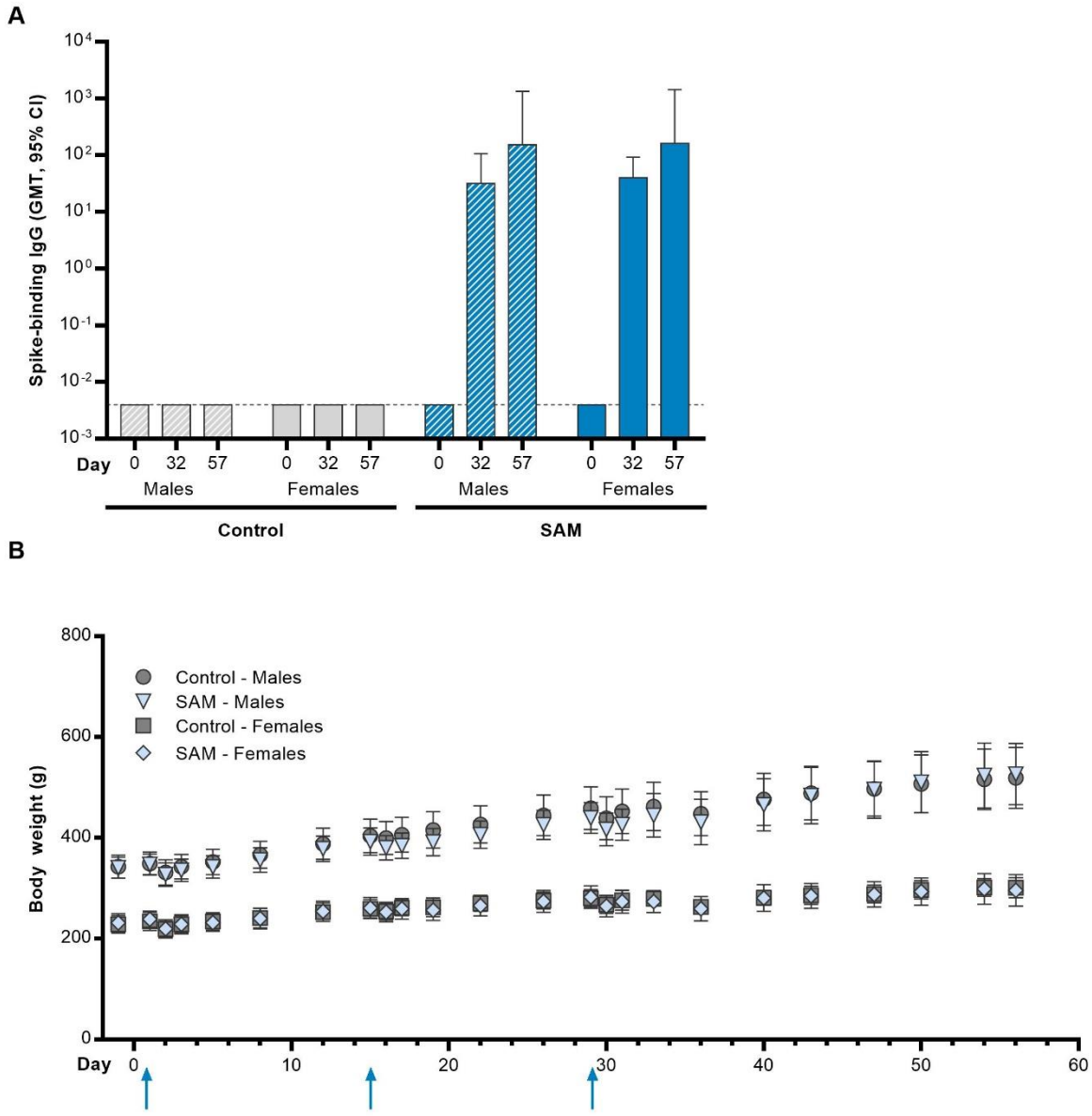

Figure S8. Rat immune responses and body weight changes after SARS-CoV-2 SAM (LNP) vaccination. (A) Spike-specific antibody (IgG) titers were measured in the sera of rats prior to immunization (Day 0), 3 days (Day 32), and 28 days (Day 57) following the third immunization. The graph shows GMT±95% CI. (**B**) The body weights were measured at multiple time points during the 57 days of the study. The graph shows mean body weights with SD. Arrows indicate the vaccine administration.

Table S1. Repeated-dose study. Effect of SARS-CoV-2 SAM (LNP) injection on selected clinical pathology parameters in male Sprague-Dawley rats.

	Day 2		Day 8		Day 30		Day 36		Day 57	
	Control	SAM	Control	SAM	Control	SAM	Control	SAM	Control	SAM
White blood cells [× 10^9/L]	9.724 (1.782)	10.461 (2.277)	9.602 (0.904)	10.828 (1.674)	10.210 (1.682)	**14.334 (3.402) ****	8.818 (0.944)	11.124 (2.030)	7.792 (1.933)	8.626 (1.854)
Neutrophils [× 10^9/L]	1.154 (0.330)	**7.691 (1.747) *****	1.454 (0.293)	1.848 (0.825)	1.732 (0.329)	**10.223 (4.893) *****	1.558 (0.430)	1.978 (0.527)	1.662 (0.597)	1.808 (0.845)
Lymphocytes [× 10^9/L]	8.165 (1.501)	**2.443 (0.661) *****	7.744 (0.768)	8.465 (1.485)	8.373 (1.337)	**3.590 (2.639) ****	6.770 (0.915)	8.590 (1.982)	5.720 (1.754)	6.358 (1.380)
Monocytes [× 10^9/L]	0.209 (0.099)	0.161 (0.070)	0.178 (0.048)	0.268 (0.138)	0.217 (0.075)	0.236 (0.104)	0.250 (0.037)	0.264 (0.123)	0.192 (0.086)	0.256 (0.127)
Eosinophils [× 10^9/L]	0.072 (0.030)	0.068 (0.023)	0.104 (0.051)	0.110 (0.018)	0.114 (0.052)	0.161 (0.110)	0.142 (0.060)	0.162 (0.059)	0.142 (0.056)	0.108 (0.042)
Basophils [× 10^9/L]	0.054 (0.013)	**0.031 (0.012) ****	0.062 (0.013)	0.055 (0.006)	0.062 (0.014)	0.048 (0.022)	0.032 (0.004)	**0.048 (0.015) ***	0.034 (0.023)	0.038 (0.004)
Red blood cells [× 10^{12}/L]	8.173 (0.618)	8.525 (0.331)	8.614 (0.280)	**7.573 (0.486) ****	8.929 (0.398)	8.475 (0.885)	8.338 (0.859)	8.120 (0.859)	8.534 (0.516)	9.056 (0.436)
Hemoglobin [g/L]	158.8 (7.2)	163.6 (6.4)	168.6 (2.9)	**143.3 (12.6) ****	161.8 (4.1)	152.8 (16.3)	154.2 (12.0)	148.0 (10.7)	154.4 (14.9)	164.8 (8.9)
Hematocrit [L/L]	0.464 (0.023)	0.479 (0.020)	0.486 (0.009)	**0.428 (0.038) ***	0.476 (0.014)	0.442 (0.051)	0.438 (0.036)	0.428 (0.030)	0.456 (0.044)	0.494 (0.027)
Fibrinogen [g/L]	2.79 (0.73)	**6.89 (0.50) *****	3.21 (0.24)	3.27 (0.55)	3.45 (0.69)	**4.47 (1.42) ***	3.04 (0.33)	**3.79 (0.60) ***	3.24 (0.36)	3.16 (0.38)
AST [U/L]	133.2 (22.9)	137.9 (30.6)	116.4 (12.5)	**158.8 (24.7) ****	173.1 (50.7)	165.7 (20.7)	114.6 (26.6)	115.2 (10.1)	114.6 (24.0)	129.6 (23.4)
ALT [U/L]	44.7 (6.2)	45.1 (3.0)	57.4 (11.5)	56.6 (6.5)	55.0 (14.9)	57.7 (5.8)	48.0 (9.2)	44.8 (5.4)	47.2 (8.0)	43.0 (6.4)
ALP [U/L]	250.9 (75.8)	243.8 (52.5)	239.8 (17.0)	284.6 (40.3)	165.4 (40.1)	174.4 (32.1)	151.8 (11.3)	168.6 (30.8)	130.4 (19.5)	135.8 (14.0)
Albumin [g/L]	45.2 (2.49)	**40.65 (1.05) *****	46.36 (1.62)	**43.90 (0.64) ***	45.37 (2.23)	**39.15 (1.69) *****	45.06 (1.29)	**39.82 (2.08) ****	45.76 (3.19)	44.76 (1.24)
Globulin [g/L]	17.7 (1.40)	**23.15 (1.82) *****	19.66 (1.65)	19.20 (1.97)	21.83 (1.06)	**25.42 (1.87) *****	20.96 (1.45)	19.40 (2.51)	23.88 (3.42)	21.16 (2.37)
Albumin/ Globulin ratio	2.653 (0.232)	**1.765 (0.146) *****	2.370 (0.204)	2.304 (0.223)	1.992 (0.157)	**1.548 (0.131) *****	2.154 (0.106)	2.084 (0.339)	2.022 (0.201)	2.134 (0.222)

All values are means (SD). Statistically significant differences are shown in bold. AST, aspartate aminotransferase; ALT, alanine aminotransferase; ALP, alkaline phosphatase. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$, compared with control.

Table S2. Repeated-dose study. Effect of SARS-CoV-2 SAM (LNP) injection on selected clinical pathology parameters in female Sprague-Dawley rats.

	Day 2		**Day 8**		**Day 30**		**Day 36**		**Day 57**	
	Control	SAM	Control	SAM	Control	SAM	Control	SAM	Control	SAM
White blood cells [× 10^9/L]	8.882 (2.375)	9.026 (2.360)	8.903 (2.415)	9.446 (3.882)	8.922 (2.514)	10.177 (2.155)	8.338 (2.533)	10.186 (4.993)	6.150 (3.129)	6.534 (2.294)
Neutrophils [× 10^9/L]	0.800 (0.413)	**5.451 (0.953) *****	0.913 (0.148)	1.330 (0.919)	0.795 (0.390)	**6.336 (1.975) *****	1.215 (0.342)	1.226 (0.760)	1.006 (0.517)	0.698 (0.209)
Lymphocytes [× 10^9/L]	7.689 (2.106)	**3.253 (1.762) *****	7.585 (2.228)	7.674 (2.994)	7.719 (2.456)	**3.463 (1.397) *****	6.716 (2.096)	8.472 (4.699)	4.824 (3.116)	5.540 (2.035)
Monocytes [× 10^9/L]	0.184 (0.059)	**0.123 (0.069) ***	0.213 (0.116)	0.184 (0.083)	0.186 (0.068)	0.159 (0.118)	0.183 (0.062)	0.206 (0.135)	0.150 (0.051)	0.140 (0.053)
Eosinophils [× 10^9/L]	0.104 (0.036)	0.119 (0.042)	0.093 (0.081)	0.136 (0.050)	0.100 (0.037)	0.119 (0.052)	0.115 (0.066)	0.132 (0.064)	0.090 (0.068)	0.080 (0.032)
Basophils [× 10^9/L]	0.037 (0.014)	0.027 (0.012)	0.040 (0.008)	0.044 (0.023)	0.048 (0.022)	0.047 (0.042)	0.040 (0.022)	0.042 (0.026)	0.028 (0.019)	0.026 (0.011)
Red blood cells [× 10^{12}/L]	7.769 (0.546)	7.772 (0.589)	7.960 (0.449)	7.652 (0.320)	8.101 (0.393)	7.974 (0.872)	7.783 (0.706	7.860 (0.299)	7.458 (1.378)	8.244 (0.536)
Hemoglobin [g/L]	149.3 (11.1)	150.8 (11.3)	154.3 (10.2)	147.0 (7.5)	154.6 (7.5)	153.5 (15.6)	151.8 (11.8)	151.8 (6.5)	145.2 (26.9)	160.4 (5.4)
Hematocrit [L/L]	0.426 (0.029)	0.431 (0.033)	0.440 (0.027)	0.422 (0.011)	0.444 (0.023)	0.444 (0.048)	0.428 (0.034)	0.426 (0.017)	0.420 (0.080)	0.470 (0.014)
Fibrinogen [g/L]	2.59 (0.13)	**6.40 (0.54) *****	2.69 (0.22)	2.32 (1.32)	2.31 (0.33)	**4.59 (0.64) *****	2.45 (0.17)	2.45 (0.47)	2.14 (0.39)	2.27 (0.06)
AST [U/L]	123.8 (25.5)	**162.0 (52.5) ***	126.8 (23.5)	161.0 (38.7)	126.1 (26.4)	**297.3 (407.0) ***	100.8 (17.7)	109.4 (27.7)	140.2 (28.5)	118.0 (33.5)
ALT [U/L]	37.6 (7.3)	74.0 (17.4)	44.0 (4.7)	65.6 (37.0)	52.1 (10.1)	114.0 (144.1)	48.4 (7.8)	43.8 (10.8)	52.6 (14.3)	51.4 (23.9)
ALP [U/L]	129.9 (23.0)	117.8 (18.8)	129.3 (25.3)	127.4 (24.2)	81.3 (17.3)	90.9 (20.1)	67.4 (13.0)	75.8 (16.7)	54.8 (18.1)	56.4 (10.0)
Albumin [g/L]	51.97 (2.28)	**47.44 (4.46) ****	53.53 (1.21)	**48.18 (2.49) ****	53.74 (2.42)	**48.49 (5.23) ****	56.02 (1.99)	**47.88 (3.28) ****	56.22 (2.99)	55.58 (2.52)
Globulin [g/L]	16.11 (2.30)	**20.12 (1.14) *****	15.95 (0.85)	17.46 (1.21)	19.98 (1.80)	21.55 (2.44)	17.60 (2.58)	17.90 (2.19)	17.18 (2.72)	19.22 (2.27)
Albumin/ Globulin ratio	3.271 (0.362)	**2.364 (0.259) *****	2.363 (0.223)	**2.766 (0.210) ****	2.703 (0.189)	**2.259 (0.185) ****	3.236 (0.454)	**2.694 (0.217) ***	3.334 (0.482)	2.926 (0.385)

All values are means (SD). Statistically significant differences are shown in bold. AST, aspartate aminotransferase; ALT, alanine aminotransferase; ALP, alkaline phosphatase. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$, compared with control.

Table S3. Repeated-dose study. Mean absolute, relative-to-body and relative-to-brain weights of selected lymph nodes and spleens in control and SARS-CoV-2 SAM (LNP)-treated rats.

	Main (Day 32)				Recovery (Day 57)			
	Males (n=10)		Females (n=10)		Males (n=5)		Females (n=5)	
	Control	SAM	Control	SAM	Control	SAM	Control	SAM
Iliac lymph node								
Absolute [g]	0.03209 (0.01109)	0.04974 (0.02782)	0.02726 (0.01022)	**0.03966 (0.01497)***	0.03794 (0.01650)	0.03684 (0.00780)	0.03172 (0.00972)	0.02138 (0.01097)
Relative to body weight [%]	0.00751 (0.00273)	0.01230 (0.00659)	0.01052 (0.00414)	**0.01545 (0.00598)***	0.00782 (0.00394)	0.00725 (0.00082)	0.01094 (0.00272)	0.00784 (0.00445)
Relative to brain weight [%]	1.49067 (0.49993)	2.36712 (1.31305)	1.37129 (0.52016)	**1.98901 (0.74210)***	1.69281 (0.74787)	1.66112 (0.29990)	1.55610 (0.47545)	1.04615 (0.56875)
Popliteal lymph node								
Absolute [g]	0.04953 (0.03412)	0.04962 (0.01440)	0.02469 (0.00668)	**0.03614 (0.01255)***	0.02572 (0.00652)	0.02172 (0.00839)	0.01660 (0.00675)	0.02066 (0.00696)
Relative to body weight [%]	0.01146 (0.00750)	0.01240 (0.00365)	0.00953 (0.00262)	**0.01393 (0.00438)***	0.00522 (0.00131)	0.00426 (0.00152)	0.00580 (0.00249)	0.00750 (0.00289)
Relative to brain weight [%]	2.33749 (1.67971)	2.36011 (0.67201)	1.23894 (0.34208)	**1.81346 (0.59482)***	1.14765 (0.29757)	0.97799 (0.36840)	0.81281 (0.32381)	1.00206 (0.34223)
Inguinal lymph node								
Absolute [g]	0.05122 (0.02257)	0.04930 (0.01350)	0.03509 (0.02477)	0.05173 (0.03941)	0.02370 (0.01440)	0.03278 (0.01681)	0.02046 (0.00446)	0.02398 (0.00501)
Relative to body weight [%]	0.01190 (0.00500)	0.01224 (0.00309)	0.01322 (0.00873)	0.02002 (0.01497)	0.00463 (0.00246)	0.00639 (0.00288)	0.00713 (0.00150)	0.00866 (0.00220)
Relative to brain weight [%]	2.38723 (1.04494)	2.35144 (0.66499)	1.74379 (1.19519)	2.59969 (1.94192)	1.04118 (0.60265)	1.46450 (0.70299)	1.00289 (0.21710)	1.16150 (0.24581)
Spleen								
Absolute [g]	0.78034 (0.09648)	**0.95305 (0.17387)***	0.54410 (0.04282)	**0.66476 (0.13521)***	0.76534 (0.12726)	0.78698 (0.14753)	0.48404 (0.12677)	0.56048 (0.11678)
Relative to body weight [%]	0.18373 (0.01439)	**0.23739 (0.04064)****	0.20930 (0.02012)	**0.25764 (0.04400)****	0.15549 (0.02262)	0.15594 (0.02502)	0.16807 (0.03831)	0.19938 (0.03611)
Relative to brain weight [%]	36.52873 (4.03627)	**45.36674 (8.08238)***	27.29450 (1.86765)	**33.39376 (6.32573)***	34.09256 (5.03680)	35.65214 (6.65324)	23.82166 (6.73888)	27.09681 (5.47487)

All values are means (SD). Statistically significant differences are shown in bold. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$, compared with control.

SUPPLEMENTAL METHODS

SARS-CoV-2 SAM synthesis

In vitro RNA transcription and purification was performed as previously described[6]. Briefly, DNA plasmids encoding the RNA SAM vaccines sequence were linearized by restriction digestion with BspQI at the precise 3′ end of the SAM sequences and purified by phenol-chloroform extraction. Linearized DNA templates were in vitro transcribed using T7 RNA polymerase (NEB) and subsequently capped using a vaccinia capping enzyme (NEB). Denaturing agarose gel electrophoresis was performed to evaluate the integrity of the RNA as previously described.[6]

LNP formulation preparation

LNP encapsulating SAM were prepared through flash precipitation by mixing an ethanolic solution of lipids with an aqueous solution of RNA as described previously.[45] Lipid excipient components (proprietary ionizable lipid, 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC), cholesterol and 1,2-dimyristoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000] (DMPE-PEG2000)) were dissolved in ethanol at mole ratio of 40:10:48:2[6]. SAM was in a citrate buffer solution at pH 6.0. The lipid mixture was rapidly mixed with SAM in a microfluidic mixing chip at a flow rate of 1:2, respectively, using a NanoAssemblr Benchtop System (Precision Nanosystems, Vancouver BC, Canada). The formed nanoparticles were held briefly at room temperature (RT) and then transferred to a Tris-buffered solution. Concentration of SAM (LNP) was adjusted to target concentrations by dilution. Analytical characterization included size and polydispersity determination (Zetasizer DLS, Malvern Instruments), appearance, pH, osmolality, RNA concentration and entrapment by fluorometric assay using Ribogreen RNA dye, endotoxin content by LAL assay, and lipid identity and composition by HPLC. All LNP prepared had a hydrodynamic radius of less than 120 nm, and mRNA entrapment of greater than 80% as determined by Ribogreen assay (as described in [6,46]). Final materials were aseptically transferred into glass vials, stoppered, capped and frozen at -80 ± 10°C.

IN VITRO CHARACTERIZATION OF SAM CONSTRUCTS

RNA potency assay and intracellular protein expression

To determine the efficiency of SAM to launch the self-amplification cycle, approximately 1×10^6 Baby Hamster Kidney (BHK) or C2C12 mouse myoblast cells were electroporated (one pulse, 120 V, 25 milliseconds (ms)) with 0.1 µg or 1µg of SAM respectively, together with mouse thymus carrier RNA (Takara) up to 4.2 µg total RNA. Cells were seeded in six-well plates in DMEM medium containing 1% fetal bovine serum (FBS) and Penicillin–Streptomycin (P/S) and incubated at 37°C and 5% CO_2 for 18 hours. Cells were collected, fixed, permeabilized with Cytofix/Cytoperm (BD Biosciences) and then stained with allophycocyanin (APC) (Zenon Allophycocyanin Labeling Kit, Invitrogen) conjugated anti-dsRNA antibody (J2 monoclonal IgG2a antibody, Bioclass) or with an anti-S2 antibody (1A9 mouse monoclonal IgG1, GeneTex) followed by a goat anti-mouse IgG Alexa 488 secondary antibody (Invitrogen). Cells were acquired on a Fortessa X20 SORP flow cytometer (BD Biosciences) and analyzed by FlowJo v10 software (FlowJo, BD Biosciences).

Immunoblot analysis

Approximately 1×10^6 BHK cells were electroporated (one pulse, 120 V, 25 ms) with 1.0 µg of SAM together with mouse thymus carrier RNA (Takara) up to 4.2 µg total RNA. Cells were seeded in six-well plates in DMEM containing 1% FBS and P/S. At 18 hours after electroporation, cells were lysed under nonreducing conditions and whole-cell lysates subjected to SDS–polyacrylamide gel electrophoresis and blotted to a nitrocellulose membrane using a semi-dry transfer system (ThermoFisher). A recombinant Spike protein (Acro Biosystems) was loaded as control. SARS-CoV-2 Spike and actin proteins were detected using the mouse anti-S2 antibody (1A9 mouse monoclonal IgG1, GeneTex) and rabbit anti-actin antibody (Millipore) at a dilution of 1:1,000, followed by IRDye 800CW–conjugated donkey anti-mouse IgG secondary and IRDye 680RD Goat anti-Rabbit antibody (LI-COR) at a 1:15,000 dilution. Protein bands were visualized on the Odyssey fluorescent imager (LI-COR).

Flow cytometry analysis of surface-bound Spike protein and its binding to hACE2 receptor.

Approximately 1×10^6 C2C12 mouse myoblast cells were prewashed in Opti-MEM low serum media (Gibco), electroporated (one pulse, 120 V, 25 ms) with SAM RNA (1 µg) and mouse thymus carrier RNA up to 4.2 µg total RNA (Takara). Cells were seeded in six-well plates with 1% FBS and P/S containing DMEM growth media and incubated at 37°C and 5% CO_2 for 18 hours. At 4 hours post transfection, Brefeldin A (BFA, BD Biosciences) was added to cells as needed following the manufacturer's recommendation. Cells were collected, washed, and stained with an anti-Spike antibody (1A9 mouse monoclonal IgG1, GeneTex) followed by a goat anti-mouse IgG Alexa 488 secondary antibody (Invitrogen). Cells were fixed with 1.5% paraformaldehyde in phosphate buffered saline (PBS) (Thermo Fisher), acquired on a Fortessa X20 SORP flow cytometer (BD Biosciences) and analyzed by FlowJo v10 software (BD Biosciences).

To assess the binding of hACE2 to Spike protein on the surface of SAM transfected cells, hACE2 recombinant protein was diluted 1:150 (0.31 mg/ml stock; Acro Biosystems) in PBS containing 2.5% FBS. Cells were spun down and incubated with the diluted hACE2 protein on ice for 30 minutes. Samples were washed and stained with a goat anti-hACE2 polyclonal antibody (R&D systems) followed by a rabbit anti-goat IgG Alexa 488 (Invitrogen). Cells were fixed with 1.5% paraformaldehyde in PBS, acquired on a Fortessa X20 SORP flow cytometer (BD Biosciences) and analyzed by FlowJo v10 software (BD Biosciences).

Human COVID-19 convalescent-phase serum (HCS) panel

Human samples were obtained with informed consent. All recruitment, sample collection, and experimental procedures using human samples have been approved by relevant institutional review boards and by GSK human sample management board. Human SARS-CoV-2 infection/COVID-19 convalescent sera (n=22) were drawn from donors 23-61 years of age, mostly from females, after PCR-confirmed diagnosis and at least 28 days after the participants were asymptomatic. Sera were obtained from CHU Tivoli, Belgium.

Ethical statement

All nonclinical studies were conducted in accordance with the GSK Policy on the Care, Welfare and Treatment of Laboratory Animals and were reviewed by the Institutional Animal Care and Use Committee by the ethical review process at the institution where the work was performed. All studies followed ARRIVE Guidelines as applicable and were conducted in compliance with provisions of the USDA Animal Welfare Act, the Public Health Service Policy on Humane Care and Use of Laboratory Animals and the U.S. Interagency Research Animal Committee Principles for the Utilization and Care of Research Animals.

MOUSE IMMUNOGENICITY STUDY

SARS-CoV-2 Spike-specific IgG binding antibody titers

The titers of Spike-specific IgG binding antibodies were measured using a Luminex-based assay (Luminex Corporation). The antigen used in this assay was a Spike ectodomain protein, encoding amino acid residues 1-1208 of Wuhan-Hu-1 strain (GenBank: MN908947) with proline substitutions at residues 986 and 987, a "GSAS" substitution at the furin cleavage site (residues 682–685), a C-terminal Foldon trimerization motif, a TEV protease cleavage site, a double StrepTag, and an 8x HisTag. The antigen sequence was synthesized, cloned into an in-house mammalian expression vector, similar in design to the one reported by Wrapp and co-authors, and produced and purified as previously reported.[16] Luminex microspheres were covalently coupled with the Spike protein using N-hydroxysulfosuccinimide and N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide (EDC) according to the manufacturer's instructions. The microspheres were mixed with serial dilutions of mouse serum, incubated for 1 hour at room temperature (RT), washed with PBS, and suspended for 1 hour at RT in r-Phycoerythrin-conjugated anti-mouse IgG (Fcγ-specific, Jackson Immunoresearch) at a 1:50 dilution. The beads were then washed and resuspended in PBS. Fluorescence intensity was measured using a FlexMap 3D instrument (Luminex Corporation). A standard composed of a mix of 3 monoclonal antibodies to the Spike protein (anti-S2 1A9 mouse monoclonal IgG1 (GeneTex); anti-S2, mouse monoclonal IgG1 (MP Biomedical); anti-S1, human monoclonal IgG1 (Absolute Antibody)), diluted into naïve mouse serum, was assigned a value of 100 AU (Assay Units) and a standard curve of concentrations was plotted. Sample titers were interpolated on a 4-parameter logistic fit of the standard curve. Data points for calculation of sample titers were selected from within the range of 10% to 70% of the lower and upper asymptote of the standard

curve. The final sample titer was the average of the back calculated titers for dilutions falling within the acceptable range of the standard curve.

VSV-SARS-CoV-2 Spike pseudovirus neutralization assay

Neutralizing antibodies from HCS panel and immunized mice sera were assessed using a cell-based pseudovirus neutralization assay. A recombinant replication-deficient Vesicular stomatitis virus (VSV) vector that encodes a luciferase protein in place of VSV-G was pseudotyped with a Spike glycoprotein that lacked the last 19 amino acids of the cytoplasmic tail, derived from SARS-CoV-2 Wuhan, Alpha (B.1.1.7; HV 69-70 del, Y144 del, N501Y, A570D, D614G, P681H, T716I, S982A, D1118H mutations) or Beta (B.1.351; L18F, D80A, D215G, del242-244, R246I, K417N, N501Y, E484K, D614G, A701V mutations) variants or Delta (B.1.617.2; T19R, G142D, del156-157, R158G, L452R, T478K, D614G, P681R, D950N).[20]

Vero E6 cells expressing the hACE-2 receptor were seeded in 96-well white plates at 20,000 cells/well to reach a cell confluence of 80%. Serum samples or controls were diluted in duplicate in DMEM containing 10% FBS and P/S (growth media) at a starting dilution of 1/25, 1/250 or 1/2500 (for mouse samples), or 1/10, 1/100 or 1/1000 (for human samples), followed by a serial dilution (2-fold dilutions, 6 times). Pseudovirus was added to diluted serum samples and pre-incubated for 1 hour at 37°C with 5% CO_2. VSV-SARS-CoV-2 Spike pseudovirus diluted in cell growth media was used as the reference for 100% infectivity. The mixtures were then added to the pre-seeded Vero E6 cell layers and plates were incubated for 18-20 hours at 37°C with 5% CO_2. Media were removed, ONE-Glo EX Luciferase Assay Substrate (Promega) was added to cells, and incubated for 3 minutes at RT with shaking. Luminescence was measured using a microplate reader (SpectraMax i3x) and SoftMax Pro v6.5.1 (Molecular Devices). Luminescence results for each dilution were used to generate a titration curve using a 4-parameter logistic regression (4PL). Titers were established by determining the reciprocal of the serum dilution leading to a 50% reduction in signal compared to virus-only wells (pseudovirus 50% neutralization titers, $pVNT_{50}$). This was established using the 50% flanking points of the 4PL titration curve for each sample. The final titer was calculated as the average of replicates.

Live virus SARS-CoV-2 neutralization assay

A neutralization assay using a previously described strain of SARS-CoV-2 (isolate USA-WA1/2020), that had been rescued by reverse genetics and engineered by the insertion of a mNeonGreen (mNG) gene into open reading frame 7 of the viral genome, was used to measure neutralizing antibodies in the sera collected from mice 2 weeks after the second immunization.[19] Briefly, Vero CCL-81 cells (1.2×10^4) in DMEM containing 2% FBS and P/S were seeded in each well of a 96-well plate, and the cells were incubated overnight at 37°C and 5% CO_2. On the following day, serum samples were two-fold serially diluted in 2% FBS and P/S DMEM and incubated with mNG SARS-CoV-2 at 37°C for 1 hour. The virus-serum mixture was transferred to the Vero CCL-81 cell plate at a final multiplicity of infection (MOI) of 0.5. For each serum, the starting dilution was 1/20 with 9 two-fold dilutions to the final dilution of 1/5,120. After incubation for 16 hours at 37°C, 25 µl of Hoechst 33342 solution (400-fold diluted in Hank's Balanced Salt Solution, Gibco) was added to each well to stain the cell nucleus. The plate was sealed, incubated at 37°C for 20 minutes, and quantified for mNG fluorescence on CytationTM 7 (BioTek). The raw images were acquired using 4×objective and processed using the default setting. The total cells (indicated by nucleus staining) and mNG-positive cells were quantified for each well. Infection rates were determined by dividing the mNG-positive cell number by the total cell number. Relative infection rates were obtained by dividing the infection rates of serum-treated wells with the infection rate of the non-serum treated controls well. The curves of the relative infection rates versus the serum dilutions (log10 values) were plotted using Prism 8 (GraphPad). A nonlinear regression method was used to determine the dilution fold that neutralized 50% of mNG fluorescence (VNT50). Each serum was tested in duplicates. All mNG SARS-CoV-2 reporter neutralization assay was performed at the BSL-3 facility at University of Texas Medical Branch.

SARS-CoV-2 Spike-specific B cell responses in mice

Characterization of Spike-specific B cells from spleens and draining inguinal lymph nodes collected from immunized mice (2 independent experiments, 5 mice/group/experiment) at 2 weeks post the second immunization (2wp2) was performed using multi-parametric flow cytometry. Single cell suspensions prepared from spleen, or pairs of inguinal lymph nodes from each mouse, were stained with near-IR live/dead cell stain (Invitrogen) for 20 minutes

at RT, washed and incubated with mouse Fc block (BD Biosciences) in PBS plus 1% FBS (HyClone, Thermo Scientific) for 10 minutes at 4˚C. Approximately 1-2 $\times 10^6$ splenocytes or lymph node cells were stained for 30 minutes at 4˚C with the following commercial monoclonal antibodies: anti-CD3 (BUV737), anti-CD19 (BV786), anti-IgD (BV421), anti-IgM (BUV395), anti-GL7 (AF647), anti-CD95 (BV711), anti-CD138 (BB700), anti-CD38 (BV650), anti-CD80 (PECF594), anti-CD73 (PE-Cy7), and anti-CD273 (PE). All monoclonal antibodies were from BD Biosciences, except anti-CD73 PE-Cy7 which was from Biolegend. To identify Spike-specific B cells, samples were stained with 1 µg per 1-2 × 10^6 cells of SARS-CoV-2 Spike protein labelled with Alexa Fluor 488 (BD Biosciences) included in the antibody mix. Stained cells were analyzed on a FACSymphony A5 SORP cell analyzer (BD Biosciences). Data from two independent experiments were combined. Spike-specific B cells were identified within class-switched B cell population (identified as $CD3^-CD19^+IgM^-IgD^-$ B cells), which was further subdivided into memory B cells ($CD95^-CD38^+$), and germinal center B cells ($GL7^+CD95^+$). CD80, PD-L2 and CD73 markers were also used to provide further characterization of the Spike-specific B cells elicited by the SAM (LNP) vaccine.

Analysis of SARS-CoV-2 Spike-specific T cell responses in mice

Splenocytes were collected from mice at 2wp2 and SARS-CoV-2 Spike-specific T cell responses were assessed by intracellular cytokine staining and multi-parametric flow cytometry. Briefly, single cell suspensions of 1-2 $\times 10^6$ live splenocytes were plated in 96-well U-bottom plates (Corning) and incubated for 2 hours at 37ºC with 1 µg/ml of $Spike_{FL\text{-}2P}$ specific (Genscript), S1, S2 or RBD specific peptide pools (JPT Peptides) containing 15mer peptides overlapping by 11 amino acids or with cell culture medium (no peptide, for background subtraction) as control in complete RPMI and in the presence of anti-CD28 co-activation and anti-CD107a-PE degranulation markers (BD Biosciences). Golgi transport inhibitor BFA (BD Biosciences) at 1 µg/ml was added after 2 hours and the splenocytes incubated for an additional 12 hours at 37ºC. Cells were then stained with 1:1000 diluted near-IR LIVE/DEAD stain (Invitrogen) for 20 minutes at RT. Cells were subsequently washed, fixed and permeabilized with Cytofix/Cytoper (BD Biosciences) for 20 minutes at 4ºC, blocked with 1:15 diluted mouse Fc Block (anti-CD16/CD32) in 1X Perm/Wash Buffer (BD Biosciences) for 10 minutes at RT, and stained with antibody mix T cell surface markers (anti-CD3-BV711, anti-CD4-BUV395, anti-CD8-BUV805), activation marker (anti-CD44-PECF594), and intracellular cytokines (anti-Interferon (IFN)-γ-BV786, anti-Interleukin (IL)-2-APCR700, anti-Tumor Necrosis Factor (TNF)-α-BV650 (BD Biosciences), anti-IL-17F-A647 (Biolegend), anti-IL-4-FITC, anti-IL-13-FITC (eBiosciences)) in 1X Perm/Wash with Brilliant Stain Buffer (BD Biosciences) for 30 minutes at RT. All incubation steps after cell viability staining were conducted in the dark. Finally, the cells were washed and suspended in PBS plus 1% BSA (Gibco, Thermo Scientific), then acquired on BD FACSymphony A5 SORP cell analyzer and data were analyzed using FlowJo v10 software (BD Biosciences).

For analysis, cytokine responses (IFN-γ, TNF-α, IL-2, IL-4/IL-13, IL-17F,and CD107a) were measured on activated, $CD44^+$ T cell populations and subjected to Boolean analysis to yield 128 unique cytokine populations, including 64 unique populations for the $CD4^+$ compartment and 64 unique populations for the $CD8^+$ compartment. The IL-4 and IL-13 Th2 cytokines were assessed on the same FITC channel. The frequencies of each Boolean population were summed to yield the total cytokine response. The phenotype of T cell subsets was assessed as Th1 (defined as IFN-γ^+ IL-4^-IL-13^-; may or may not be IL-2^+, TNF-α^+, or $CD107a^+$), Th2 (defined as IL-4^+ and/or IL-13^+ and IFN-γ^-; may or may not be IL-2^+, TNF-α^+, or $CD107a^+$), Th17 (defined as IL-$17F^+$ and IFN-γ^-, IL-4^-IL-13^-; may or may not be IL-2^+, TNF-α^+, or $CD107a^+$), and Th0 (defined as IL-2^+ and/or TNF-α^+, and IFN-γ^-IL-$17F^+$IL-4^-IL-13^- ; may or may not be $CD107a^+$) for both $CD4^+$ (designated as Th0/Th1/Th2/Th17 T helper subsets) and $CD8^+$ (designated as Tc0/Tc1/Tc2/Tc17 T cytotoxic subsets) T cells. Additionally, polyfunctionality was assessed for selected vaccine groups using Pestle v2 and Simplified Presentation of Incredibly Complex Evaluations (SPICE v6.1) software (Joshua Nozzi & Mario Roederer; NIAID). The Spike-specific IL-17F and IL-4/IL-13 levels were negligible and thus excluded from polyfunctional SPICE analysis.

HAMSTER IMMUNOGENICITY AND EFFICACY STUDY

Plaque reduction neutralization test with hamster sera

Neutralizing antibodies in sera of vaccinated hamsters were determined by plaque reduction neutralization test as previously described.[47] Briefly, serum was heat-inactivated for 30 minutes at 56°C and diluted two-fold in BA-1 media starting at a 1:5 dilution on a 96-well plate. An equal volume of SARS-CoV-2 virus (isolate USA-WA1/2020; 100 PFU) was added to the serum dilutions and the sample-virus mixture was gently mixed. The plates were incubated

for 1 hour at 37°C. Following incubation, serum-virus mixtures were plated onto Vero plates. Antibody titers were recorded as the reciprocal of the highest dilution in which >90% of virus was neutralized. All hamsters were confirmed negative for the presence of antibodies against SARS-CoV-2 prior to vaccination.

Cytokine profiling in cultured hamster lung cells

Lungs were homogenized using syringe plunger and passed through a 70 µm filter to prepare single cell suspension. Erythrocytes were lysed by treating cells with Gey's RBC lysis buffer (0.15 M NH4Cl, 10 mM HCO3) for 2 minutes with constant shaking in between. After 2 minutes, complete media (RPMI-1640 with 10% FBS, 10% non-essential amino acids, 10% P/S and 10% L-Glutamine) was added to dilute the RBC lysis buffer. Cells were centrifuged at 500 x g for 10 minutes to pellet down cells and wash the pellet once with complete media. After washing, cells were suspended in 1mL complete media, homogenized and equal number of cells were added to each well in a U-bottom 96 well plate. Cells were cultured in 37°C CO_2 incubator without stimulation. After 24 hours the cultures were transferred into 96 well v-bottom plate, centrifuged at 500g for 7 minutes and supernatant was collected and transferred into 96 well flat bottom plates that were stored at -80°C until assay for cytokines. Supernatants were assayed for hamster IFN-γ, TNF-α, and IL-4 using competitive ELISA commercial kits (G Biosciences), following manufacturer's recommendations and provided standard curves to obtain cytokine concentrations.

Histopathology

Hamster tissues (trachea, lung, heart, liver, spleen, brain, kidney, adrenal glands, and thymus) were fixed in 10% neutral buffered formalin for at least 72 hours at RT prior to sectioning and slide preparation for standard Hematoxylin and Eosin (H&E)-stained sections. Additional sections of lung were stained with picrosirius red to evaluate the degree of pulmonary fibrosis. Tissue sections were evaluated by two board-certified veterinary pathologists and a consensus was agreed on all findings and interpretation. Key pathologic features were recorded as separate findings and graded on a 5-point scale (minimal, mild, moderate, marked, very marked/severe).

REPEATED-DOSE TOXICITY STUDY IN SPRAGUE-DAWLEY RATS

The welfare of the animals was maintained in accordance with the general principles outlined in the current Guidelines published by the Canadian Council on Animal Care and the Guide for the Care and Use of Laboratory Animals, a National Research Council Canada publication. The Institutional Animal Care and Use Committee (IACUC) at Citoxlab (now part of Charles River Laboratories, Inc) approved the study plans. The repeated dose toxicology and biodistribution studies were conducted in an Organization for Economic Cooperation and Development (OECD) member country and in accordance with the OECD Test Guidelines and the Principles of Good Laboratory Practice (GLP). The non-clinical development plan was defined in accordance with the World Health Organization (WHO) "Guidelines on Non-clinical Evaluation of Vaccines"[48] and the Food and Drug Administration (FDA) Guidance for Industry, "Considerations for Plasmid DNA Vaccines for Infectious Disease Indications".[49]

Clinical examinations

The animals were checked twice daily for mortality and signs of illness. Briefly, local skin reactions at the injection sites, such as erythema or edema, were recorded prior to dosing, and 6, 24, and 48 hours after each injection. The body weight of each animal was recorded once before group allocation, prior to first injection (Day −1), daily for 2 consecutive days following each injection and twice weekly until the next dose/study termination, including the day of necropsy. On each dosing occasion, rectal body temperature was measured for all surviving animals prior to vaccination and approximately 2, 4, 6, 12, 18, 24, and 48 hours post dosing. Ophthalmological examinations (funduscopy and biomicroscopy) were performed on all animals once during the pre-dosing period, after the last dosing occasion, and on recovery animals at the end of the recovery period.

Blood sampling, blood chemistry, hematology and serology

Hematology, coagulation and blood biochemistry investigations were performed at Days 2 (main animals), 8 (recovery animals), 30 (main animals), and 36 (recovery animals). Blood samples for the determination of anti-Spike IgG binding antibodies were collected from the jugular vein (non-terminal collection) of all animals before the

first vaccination (beginning of the treatment period) and from the abdominal aorta (under anesthesia with isoflurane) during necropsy (terminal collection) on Days 32 (main animals) and 57 (recovery animals). A Luminex-based antibody binding assay (Luminex Corporation) was used as described for mouse Spike-specific IgG binding antibody titers evaluation. A PE-conjugated anti-rat IgG (Fcγ-specific, Jackson Immunoresearch) at a 1:50 dilution was used for detection.

Necropsy, tissue processing and histopathological examination

At Days 32 and 57 (main and recovery animals, respectively), the rats were anesthetized by isoflurane and euthanized by exsanguination after blood collection. They were subjected to a detailed external (particularly the injection sites) and internal macroscopic examination for pathological changes. Adrenal glands, brain, draining lymph nodes (iliac, inguinal, popliteal), other lymph nodes (mandibular, mesenteric), epididymites, heart, kidneys, liver, lungs, ovaries, pituitary gland, prostate, spleen, testes, thymus, thyroids with parathyroid, and uterus were weighed and subsequently preserved in phosphate-buffered neutral 10% formaldehyde (except testes in Davidson's fixative) for microscopic evaluation. Other organs were preserved for examination, including aorta, cecum, colon, diaphragm, duodenum, esophagus, eyes and optic nerves (fixed in modified Davidson's fixative), femoral bone, Harderian glands, injection sites (muscle with skin and subcutis), ileum, jejunum (and Peyer's patches), lacrymal glands, larynx, nasal cavities, olfactory bulbs, rectum, salivary glands, oviducts, pancreas, sciatic nerve, seminal vesicles, triceps muscle, skin (with subcutis and mammary glands), spinal cord, sternum with bone marrow, stomach, tongue, trachea, ureters, urinary bladder, and vagina. Tissues intended for histological examination, according to a standard list of protocol designated tissues, were prepared by embedding in paraffin wax, sectioning and staining with hematoxylin and eosin. Briefly, representative tissue samples were fixed in 10% neutral buffered formalin, processed to slide and stained with H&E using routine histological methodology. Primary and peer-review pathology evaluation were both performed by board-certified pathologists. Any test article-related findings were graded on a standard, 5-point semiquantitative scale (minimal, mild, moderate, marked or severe).

Evaluation of tissue samples for biodistribution by RT-qPCR

After the determination of RNA concentration and verification of RNA integrity, samples were subjected to Reverse Transcription Quantitative Polymerase Chain Reaction (RT-qPCR). Extracted material was used as template for reverse transcription followed by PCR amplification with an RT-qPCR test carried out on a QuantStudio 5 Real-Time PCR System (Applied Biosystems). The primers and probes were designed to target a non-structural backbone region of the SAM construct. The sequence of the forward primer was 5′- GACGGACCGACAAGTCTCTA -3’; the sequence of the reverse primer was 5′- GGTGGTGTCAAAGCCTATCCA -3′, and the sequence of the internal Taqman probe was 5′- 6FAM - TCACCAAGC/ZEN/CAATAAGGGAGTT/IABkFQ-3'. An amount of 1 µg RNA in 5 µL was incubated with a 20 µL mix containing 5 µL TaqMan fast virus 1-step master mix (Thermofisher Scientific), 8.8 µL DNase/RNase-free water, 0.2 µM of each primer and 0.2 µM of hybridization probe. The amplification profile was 1 cycle of 5 minutes at 50°C (reverse transcription), one cycle of 20 seconds at 95°C (polymerase activation), and 40 cycles of 3 seconds at 95°C, followed by 30 seconds at 60°C (alternating denaturation and annealing/extension). Amplification was detected in real-time during the elongation step at 60°C by following the fluorescent signal generated by the degradation of the probe. The assay lower and upper limits of quantitation, limit of detection and inter- and intra-assay precision have been previously described.[40]