Supplementary Material

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Supplementary methods:

Study design and patient recruitment

This is a prospective multicenter observational cohort study of antiviral immune responses against SARS-CoV-2 variants following the third SARS-CoV-2 mRNA vaccine dose in adult KTRs. Full exclusion criteria are listed below. Subjects were enrolled consecutively. Enrolled KTRs had received two doses of mRNA-1273 or BNT162b2 SARS-CoV-2 mRNA vaccines and were planned to receive a third dose of the same vaccine as per national guidelines**.** Participants had a baseline visit prior to the third vaccine dose and a follow-up visit one month following the third vaccine dose, where blood and urine samples were collected. Samples from fifteen pre-pandemic kidney transplant control patients and five pre-pandemic healthy controls were used for comparison.

Exclusion criteria:

- Age<18 years
- Unstable allograft function (>20% variation in last two eGFR values measured at least one week apart)
- Cellular or antibody-mediated rejection during the previous six months
- Multi-organ transplantation
- Pregnant or lactating females
- Allergy to any component of mRNA-1273 or mRNA-BNT162b2 vaccines
- Investigational drug use within 30 days of enrollment
- Receipt of non-live vaccine with 2 weeks or live viral vaccine within 4 weeks of SARS-CoV-2 vaccination

- Acute or chronic illness at the time of vaccination which in the opinion of the investigator will alter immune response (e.g. human immunodeficiency virus infection, primary immunodeficiency disease, disseminated or untreated malignancy, or systemic infection).

Study approval:

The study was approved by the institutional review board at Mass General Brigham (IRB 2021P000043). All subjects signed written informed consent forms prior to enrollment in the study. The study was conducted in accordance with the Declaration of Helsinki and the Declaration of Istanbul. Data are reported in compliance with the STROBE statement reporting guidelines.

Outcomes

The primary outcome was the development of antiviral humoral responses following the third vaccine dose as assessed by 1) measurement of antibodies directed against the spike protein and receptor-binding domain (RBD) of WT and Alpha (B.1.1.7), Beta (B.1.351), Gamma (P.1), Delta (B.1.617.2) and Omicron (B.1.1.529) variants of SARS-CoV-2 by a Luminex-based multiplex assay and by enzyme-linked immunosorbent assay (ELISA); and 2) Antibodies neutralization capacity against WT, Delta and Omicron variants of SARS-CoV-2 using a surrogate virus neutralization test.[S1] Secondary objectives included 1) the occurrence of any severe or grade 4 related adverse events; 2) development of breakthrough SARS-CoV-2 infection; and 3) monitoring for rejection using serum creatinine, urine protein-to-creatinine ratio, donor-derived cell-free DNA and donor-specific antibodies (DSAs).

Sample collection and processing

Blood and urine samples were collected from KTRs prior to and one month after the third vaccine dose. Blood and urine samples were sent to the clinical lab (for serum creatinine and urine protein-to-creatinine ratio quantification), our research laboratories (for anti-HLA antibody, anti-viral antibody immune assays) and CareDx, Inc. (for donor-derived cell-free DNA levels).

Serum and plasma were obtained from peripheral blood by centrifuging for 15 minutes at 2,500 RPM at room temperature then stored in cryogenic tubes at -80°C.

Antibody quantification by Luminex-based multiplex assay:

Total antibodies (IgM, IgA and IgG) directed against multiple WT SARS-CoV-2 antigens (spike protein trimer, S1 region, receptor-binding domain [RBD] region and nucleocapsid [NC]) and the spike protein from multiple SARS-CoV-2 variants (alpha [B.1.1.7], Beta [B.1.351], Gamma [P.1] and Delta [B.1.617.2]) were measured pre- and post-vaccination using the Coronavirus Ig Total Human 15-Plex ProcartaPlex™ Panel (Invitrogen™, catalog no. PPX-15-MXFVM2D). The 15-Plex panel consists of the previously described 11-PLEX panel[S17,18] with the addition of four SARS-CoV-2 variants listed above. Briefly, capture beads were added to each well and then controls and samples (in a 1:1,000 dilution) were added. The plate was incubated for two hours at room temperature, then washed and detection antibody was added to each well. After a 30-minute incubation at room temperature, the plate was read using MAGPIX system (Luminex, Austin, TX) and the data was analyzed using xPONENT software (Luminex, Austin, TX). As indicated by the manufacturer, a positive result was defined as a sample to low-control MFI ratio above 1.3. An indeterminate result was defined as an MFI ratio between 1.0 and 1.3, while a MFI ratio <1.0 was

defined as a negative result. The thresholds indicated by the manufacturer are based on results from 39 polymerase chain reaction confirmed SARS-CoV-2 infected and 160 SARS-CoV-2 non-infected individuals as per the product manual. The assay results have been shown to be highly correlated with results from ELISA and neutralization assays, and to have a specificity and sensitivity for detecting the four WT SARS-CoV-2 antigens ranging from 98.2-100% and 83.4-93.7% respectively.[S18] In our study, pre-pandemic healthy controls and pre-pandemic kidney transplant recipients were used as negative controls (Figure 2B), and immunocompetent individuals with prior SARS-CoV-2 vaccination and/or infection were used as positive controls.

Antibody quantification by ELISA:

IgG antibodies directed against the RBD of WT, Delta (B.1.617.2) and Omicron (B.1.1.529) variants of SARS-CoV-2 were measured using an enzyme-linked immunosorbent assay (ELISA). Briefly, 96-well flat-bottom plates were coated with 100 µL of RBD protein for the respective variants (SinoBiological, catalog no. 40592-V08H, 40592-V08H90, and 40592-V08H121) at a concentration of 1 µg/mL. The plates were incubated overnight, washed with washing buffer then blocking solution (Thermo Scientific, catalog no. 37520) was added for 1 hour at room temperature (RT). The plates were washed then 100 µL of serum (diluted 1:3,000 in sample diluent solution, R&D Systems, catalog no. DY007) were added to each well and the plates were incubated for 2 hours at RT. The plates were then washed, 100 µL of anti-human IgG secondary antibody (BioLegend, catalog no. 410902, diluted 1:10,000) was added, and the plate was incubated for 1 hour at RT. 3,3',5,5'-Tetramethylbenzidine (TMB, ThermoFisher, catalog no. N301) was added and the plate was incubated for 15 minutes at RT. Finally, a stop solution (R&D Systems, catalog no. DY007) was added, and the plate was read at 450nm using SpectraMax iD3 microplate reader

(Molecular Devices, San Jose, CA). In the absence of a commercially available monoclonal human anti-Omicron RBD IgG standard, antibody levels are reported as OD450.

Pre-pandemic healthy controls and pre-pandemic kidney transplant recipients were used as negative controls (Figure 2B). Immunocompetent individuals with prior SARS-CoV-2 vaccination and/or infection were used as positive controls. The intra-assay coefficients of variation were 2.5%, 4.2% and 4.2% for the WT, Delta and Omicron anti-RBD antibodies respectively supporting the reliability of the assay. The inter-assay coefficients of variation were 9.0%, 10.4% and 9.7% for the WT, Delta and Omicron anti-RBD antibodies respectively, supporting the reproducibility of the assay. All the samples tested for this study were run at the same time. Results for anti-WT RBD antibody OD450 values measured by ELISA and anti-WT RBD antibody MFIs measured by Luminex-based multiple were highly correlated (Spearman's ρ=0.862, p=3.0 x10^{-31}). Furthermore, anti-RBD antibody OD450 values measured by ELISA for each variant and its respective neutralization percentage by surrogate virus neutralization test were moderately-to-highly correlated as well (Spearman's ρ=0.872 and p=8.6 x10^{-33} for WT, Spearman's ρ=0.800 and p=6.4 x10^{-24} for the Delta variant, and Spearman's ρ=0.503 and p=7.1 x10^{-8} for the Omicron variant).

Antibody neutralizing capacity:

We evaluated the neutralizing function of antibodies using a surrogate virus neutralization test (GenScript cPass kit, catalog no. L00847-A), which has been shown to be highly correlated with conventional live virus neutralization test[S1,8,19] and pseudovirus neutralization assays.[S8,9] Patient sera (diluted 1:10) were incubated at 37°C for 30 minutes with horseradish peroxidase-conjugated recombinant SARS-CoV-2 WT RBD, Delta variant RBD (HRP-RBD, GenScript catalog no.

Z03614-20) or Omicron variant RBD (SinoBiological catalog no. 40592-V49H7-B combined with ThermoFisher catalog no. 434423) in a 1:1 ratio. The Delta variant RBD peptide had L452R and T478K mutations. The Omicron variant RBD peptide had G339D, S371L, S373P, S375F, K417N, N440K, G446S, S477N, T478K, E484A, Q493R, G496S, Q498R, N501Y, Y505H, T547K mutations.

The mix of sera and HRP-RBD was added to each well of a capture plate pre-coated with human angiotensin converting enzyme 2 protein (ACE2) then incubated at 37°C for 15 minutes. Neutralizing antibodies form complexes with HRP-RBD that remain in the supernatant are removed with washing, while non-neutralizing antibodies-HRP-RBD complexes and unbound HRP-RBD bind to ACE2 and are captured on the plate. After washing, TMB solution was added, and the plate was incubated in the dark for 15 minutes at room temperature. Finally, a stop solution was added, and the plate was read at 450nm using SpectraMax iD3 microplate reader (Molecular Devices, San Jose, CA).The equation for percentage inhibition is $(1-[OD_{sample} \div OD_{negative\ control}]) \times 100\%$. As indicated by the manufacturer, inhibition of ≥30% was considered a positive result for neutralization.[S1]

The negative control included in the kit (data not shown), pre-pandemic healthy controls and pre-pandemic kidney transplant recipients were used as negative controls (Figure 2D). The positive control included in the kit in addition to immunocompetent individuals with prior SARS-CoV-2 vaccination and/or infection were used as positive controls with neutralization results of 96-98% against the WT, Delta and Omicron variants.

Donor-derived cell-free DNA assay:

Circulating ddcfDNA levels were measured as per published AlloSure® protocol (CareDx, Inc., Brisbane, CA).[S20,21] Briefly, duplicate samples of venous blood were collected using Streck cell-free DNA BCT® tubes then shipped to CareDx, Inc. laboratories (Brisbane, CA), where plasma was separated by centrifugation then cell-free DNA was extracted using the circulating nucleic acid kit (Qiagen, cat no. 55114) following manufacturer's instructions. Plasma ddcfDNA levels were measured using a next-generation sequencing assay utilizing 266 single nucleotide polymorphisms, which allows quantification of ddcfDNA without requiring genotyping of the donor or recipient. Results are reported as percentage of total circulating cell-free DNA. Percentages above 0.5-1.0% are associated with an increased risk of allograft rejection.[S21,22]

Anti-human leukocyte antigen antibody assays

Screening for anti-human leukocyte antigen (HLA) antibodies was performed post-vaccination using mixed class I & II kit (One Lambda, catalog no. LSM12). Briefly, patient sera and negative control sera (One Lambda, catalog no. LS-NC) were added to HLA-coated beads and incubated for 30 minutes at room temperature. After washing, PE-conjugated goat anti-human IgG secondary antibody (One Lambda, catalog no. LS-AB2) was added then incubated for 30 minutes. The results were read using LABScan3D™ (One Lambda, Los Angeles, CA) and analyzed using HLA Fusion™ software (One Lambda, Los Angeles, CA). A sample to negative control serum mean fluorescent intensity (MFI) ratio >3.5 was considered positive per our histocompatibility lab's standards. Patients with a positive anti-HLA antibody screen after the third vaccine then had anti-HLA antibody class I and II testing using single-antigen beads (LABScreen™ Single Antigen Class I – Combi, catalog no. LS1A04 and Class II – Group 1, catalog no. LS2A01, One Lambda)

with an identical protocol to determine which anti-HLA antibodies were present to determine if they were donor-specific. Baseline (pre-third vaccine) sera were then tested to determine if DSAs had been present prior to vaccination or were *de novo* (defined as new DSAs with MFI >1,000).

Statistics

Continuous variables are presented as means (± standard deviation) or as medians (with interquartile ranges or full ranges) depending on normality of distribution. Categorical variables are presented as frequencies and percentages. For continuous variables, differences between paired samples were assessed using a paired t-test, a Wilcoxon matched-pairs signed rank test, a repeated measures ANOVA or Friedman test as appropriate. For comparisons between continuous variables between three or more groups, if testing reached statistical significance, then pairwise testing was performed to determine significant differences between groups, using Dunn's correction to adjust for multiple comparisons. For categorical variables, the differences in proportions were calculated using a Pearson's Chi squared test or Fisher's exact test as appropriate. Univariate logistic regression was used to evaluate associations between exposure variables and the outcome of neutralizing responses to WT, Delta and Omicron variants of SARS-CoV-2. Due to the low number of events in the Omicron group, multivariable logistic regression was not performed to prevent model overfitting. All tests used were two-sided and a two-sided α level of 0.05 was considered to be statistically significant. SPSS v24 (Chicago, IL) and GraphPad Prism v9.1.2 (San Diego, CA) were used for statistical analysis and creation of figures.

Supplementary tables

Table S1. Baseline characteristics of kidney transplant recipients.

Baseline characteristic	**n=51**
Age at enrollment (years), median (IQR)	63 (54-69)
Months between transplantation and 1st vaccine dose, median (range)	43 (4-443)
Female sex, n (%)	23 (43)
Previous kidney transplant, n (%)	
None	47 (92)
One	3 (6)
Two	1 (2)
Cause of ESKD, n (%)	
Glomerular disease	21 (41)
Polycystic kidney disease	9 (18)
Diabetic nephropathy	5 (10)
Other genetic kidney disease	5 (10)
Lithium toxicity	2 (4)
Other or unknown	9 (18)
Pre-transplant RRT, n (%)	
None	19 (37)
Hemodialysis	27 (53)
Peritoneal dialysis	5 (10)
Donor source, n (%)	
Living related	11 (22)
Living unrelated	22 (43)
Deceased	18 (35)
Cold ischemia time (hours), median (IQR)	1.3 (0.8-12.1)
KDPI (%), median (IQR)	57 (42-69)
HLA ABDR mismatches, median (IQR)	4 (3-5)
Class I PRA (%), median (range)	0 (0-69)
Class II PRA (%), median (range)	0 (0-97)
Pre-transplant DSA, n (%)	2 (4)

DSA at the time of vaccination, n (%)	6 (12)
Induction immunosuppression, n (%)	
Anti-thymocyte globulin	28 (55)
Basiliximab	16 (31)
Data not available	7 (14)
Maintenance immunosuppression, n (%)	
Calcineurin inhibitor	
Cyclosporine	1 (2)
Tacrolimus	26 (51)
Trough level in ng/mL, median (IQR)	5.7 (4.9-6.6)
mTOR inhibitor	
Everolimus	2 (4)
Sirolimus	2 (4)
Belatacept	22 (43)
Mycophenolate	35 (69)
Total daily dose in mg, median (IQR)	1,000 (875-1,000)
Azathioprine	5 (10)
Total daily dose in mg, median (range)	62.5 (50-100)
Prednisone	42 (82)
Total daily dose in mg, median (IQR)	5 (5-5)
History of allograft rejection, n (%)	11 (22)
Months between most recent rejection and 1st vaccine dose, median (IQR)	30 (9-51)
Serum creatinine (mg/dL), median (IQR)	1.25 (1.05-1.59)
Estimated GFR (ml/min/1.73 m^2), mean ± SD	56 ± 17
Urine protein to creatinine ratio (g/g), median (IQR)	0.12 (0.07-0.36)
Donor-derived cell free DNA (%), median (IQR)	0.15 (0.00-0.23)
Previous SARS-CoV-2 infection, n (%)	4 (8)
mRNA vaccine received, n (%)	
BNT162b2	48 (94)
mRNA-1273	3 (6)
Days between 1st and 2nd vaccine dose, median (IQR)	21 (21-21)
Days between 2nd and 3rd vaccine dose, median (IQR)	186 (181-193)

ALC at time of 3rd vaccine dose, cells/mm^3, median (IQR)	1,395 (930-1,983)
ACE inhibitor or ARB use, n (%)	11 (22)

ACE: Angiotensin converting enzyme. ALC: Absolute lymphocyte count. ARB: Angiotensin receptor blocker. CMV: Cytomegalovirus. EBV: Epstein-Barr virus. ESKD: end-stage kidney disease. GFR: glomerular filtration rate. HLA: human leukocyte antigen. KDPI: kidney donor profile index. mTOR: mammalian target of rapamycin. PRA: panel reactive antibodies. RRT: renal replacement therapy. SD: standard deviation.

Table S2. Characteristics associated with developing neutralizing antibodies responses against wild-type, Delta and Omicron variants of SARS-CoV-2 after third dose of mRNA vaccination in kidney transplant recipients.

Variable	OR (95% CI) for developing neutralizing response against		
	WT	**Delta**	**Omicron**
Age (per year)	0.93 (0.87-0.99)	0.93 (0.86-0.98)	1.04 (0.97-1.15)
Living vs deceased donor	0.68 (0.20-2.21)	0.86 (0.26-2.77)	0.50 (0.08-2.98)
Years since transplantation (per year)	1.02 (0.96-1.10)	1.03 (0.96-1.11)	1.08 (1.00-1.16)
eGFR (per 10 ml/min/1.73 m^2)	1.21 (0.87-1.72)	1.31 (0.94-1.88)	0.97 (0.59-1.61)
ALC (per 1,000 cells/mm^3)	1.22 (0.63-2.50)	1.30 (0.67-2.67)	1.19 (0.42-3.12)
Steroid-based vs steroid-free regimen	0.15 (0.01-0.93)	0.14 (0.01-0.85)	1.08 (0.15-22.20)
Belatacept vs non-belatacept-based regimen	0.32 (0.09-1.00)	0.26 (0.08-0.83)	0.23 (0.01-1.57)
Mycophenolate-based vs non-mycophenolate-based regimen	0.90 (0.26-3.01)	1.17 (0.34-3.88)	0.18 (0.02-1.05)

Table S3. Breakthrough SARS-CoV-2 infection after third dose of vaccination.

Study ID	Months after KT	Days after 3rd dose	Maintenance IS regimen	Neutralizing response			Treatment	Outcome
				WT	Delta	Omicron		
M10	25	99	Tacrolimus, MPA and prednisone	-	-	-	Bamlanivimab-etesevimab	Recovered
M36	90	78	Belatacept (monotherapy)	-	-	-	Bamlanivimab-etesevimab	Recovered
M37	44	89	Belatacept and prednisone	+	+	-	Casirivimab-imdevimab	Recovered

IS: immunosuppression. KT: kidney transplantation. MPA: mycophenolic acid. WT: Wild-type.

Table S4. Allograft status before and four weeks after third SARS-CoV-2 mRNA vaccine dose.

Biomarker	Before third dose	After dose	*p* value
Serum creatinine (mg/dL), median (IQR)[‡]	1.25 (1.05-1.59)	1.28 (1.05-1.49)	0.212[†]
UPCR (g/g), median (IQR)[§]	0.12 (0.07-0.36)	0.13 (0.08-0.37)	0.257[†]
ddcfDNA (%), median (IQR)[¥]	0.15 (0.00-0.23)	0.15 (0.00-0.26)	0.434[†]

ddcfDNA: donor-derived cell-free DNA. IQR: interquartile range. UPCR: urine protein-to-creatinine ratio. [‡]n=51. [§]n=46. [¥]n=45. [†]Statistic by Wilcoxon matched-pairs signed rank test.

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