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CLINICAL RESEARCH

Hybrid Immunity Overcomes Defective Immune Response to COVID-19 Vaccination in Kidney Transplant Recipients

Nicolas Gemander[1], Delphine Kemlin[1,2], Stéphanie Depickère[3], Natasha S. Kelkar[4], Pieter Pannus[1], Shilpee Sharma[1], Alexandra Waegemans[1], Véronique Olislagers[1], Daphnée Georges[1,5], Emilie Dhondt[6], Margarida Braga[6], Leo Heyndrickx[6], Johan Michiels[6], Anaïs Thiriard[1], Anne Lemy[7], Marylène Vandevenne[5], Maria E. Goossens[3], André Matagne[5], Isabelle Desombere[8], Kevin K. Ariën[6,9], Margaret E. Ackerman[4,10], Alain Le Moine[1,2] and Arnaud Marchant[1]

[1]Institute for Medical Immunology and Université Libre de Bruxelles Centre for Research in Immunology, Université Libre de Bruxelles, Gosselies, Belgium; [2]Department of Nephrology, Dialysis and Transplantation, Erasme Hospital, Université Libre de Bruxelles, Brussels, Belgium; [3]Clinical Trial Unit, Scientific Direction Infectious Diseases in Humans, Sciensano, Brussels, Belgium; [4]Department of Microbiology and Immunology, Geisel School of Medicine at Dartmouth, Dartmouth College, Hanover, New Hampshire, USA; [5]Laboratory of Enzymology and Protein Folding, Centre for Protein Engineering, InBioS, University of Liège, Liège, Belgium; [6]Virology Unit, Department of Biomedical Sciences, Institute of Tropical Medicine, Antwerp, Belgium; [7]Department of Nephrology, Marie Curie Hospital, Charleroi, Belgium; [8]Laboratory of Immune Response, Scientific Direction Infectious Diseases in Humans, Sciensano, Brussels, Belgium; [9]Department of Biomedical Sciences, University of Antwerp, Antwerp, Belgium; and [10]Thayer School of Engineering, Dartmouth College, Hanover, New Hampshire, USA

Introduction: Comorbidities and immunosuppressive therapies are associated with reduced immune responses to primary COVID-19 mRNA vaccination in kidney transplant recipients (KTRs). In healthy individuals, prior SARS-COV-2 infection is associated with increased vaccine responses, a phenotype called hybrid immunity. In this study, we explored the potential influence of immune suppression on hybrid immunity in KTRs.

Methods: Eighty-two KTRs, including 59 SARS-CoV-2-naïve (naïve KTRs [N-KTRs]) and 23 SARS-CoV-2-experienced (experienced KTRs [E-KTRs]) patients, were prospectively studied and compared to 106 healthy controls (HCs), including 40 SARS-CoV-2-naïve (N-HCs) and 66 SARS-CoV-2-experienced (E-HCs) subjects. Polyfunctional antibody and T cell responses were measured following 2 doses of BNT162b2 mRNA vaccine. Associations between vaccine responses and clinical characteristics were studied by univariate and multivariate analyses.

Results: In naïve KTRs, vaccine responses were markedly lower than in HCs and were correlated with older age, more recent transplantation, kidney retransplantation after graft failure, arterial hypertension, and treatment with mycophenolate mofetil (MMF). In contrast, vaccine responses of E-KTRs were similar to those of HCs and were associated with time between transplantation and vaccination, but not with the other risk factors associated with low vaccine responses in naïve KTRs.

Conclusion: In conclusion, hybrid immunity overcomes immune suppression and provides potent humoral and cellular immunity to SARS-CoV-2 in KTRs.

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Compared to the general population, KTRs were at much higher risk of coronavirus disease 2019 (COVID-19) related severe disease and death during the first waves of the pandemic.[1,2] This higher risk has been attributed to immunosuppressive therapies required to prevent allograft rejection, a higher prevalence of comorbidities, and increased healthcare-associated exposure to the virus.[3,4] KTRs also developed lower humoral and cellular immune responses to primary COVID-19 vaccination as compared to healthy individuals and have been at higher risk of breakthrough infections.[5-12] Reduced vaccine responses were associated with recent induction therapy, cumulative

Correspondence: Nicolas Gemander, Institute for Medical Immunology and Université Libre de Bruxelles Centre for Research in Immunology (U-CRI), Université Libre de Bruxelles, Erasme campus. 808, route de Lennik. 1070 Anderlecht. Brussels, Belgium. E-mail: nicolas.gemander@ulb.be

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immunosuppressive treatments particularly with MMF or belatacept, and specific comorbidities such as lower kidney function.[8,13-16] Multiple doses of vaccines have been proposed as a mean to induce higher levels of immunity among KTRs.[17-21] However, about 50%, 40%, and 20% of patients remained low responders even following 3, 4, and 5 vaccinations, respectively.[19,20,22-24] Therefore, optimal vaccination strategies to protect this vulnerable population in the context of a pandemic remain uncertain and require further investigations.

In healthy adults, previous SARS-CoV-2 infection markedly modifies immunity induced by COVID-19 vaccination. This hybrid immunity is characterized by higher levels of neutralizing antibodies (nAbs) and antibody Fc-dependent effector functions as well as enhanced effector CD4 T cell responses as compared to COVID-19 vaccination alone.[25-30] In contrast, recent studies suggest a reduction of CD8 T cell responses to vaccination in subjects with previous SARS-CoV-2 infection.[31] The basis for this differential regulation of vaccine-induced immune response components after SARS-CoV-2 infection remains incompletely understood.

We and others have previously reported that KTRs preinfected with SARS-CoV-2 infection have higher binding and nAb responses to mRNA vaccination than patients who were SARS-CoV-2-naïve before vaccination.[32-35] This observation suggests that the factors reducing vaccine responses in naïve KTRs may have a limited impact on the response of previously infected patients. In this study, we provide a detailed characterization of hybrid immunity in KTRs. Beyond nAbs, we explored key components of humoral immunity to COVID-19, including SARS-CoV-2 spike (S) protein-specific antibody isotypes and subclasses, as well as IgG binding to Fcγ receptors and Fc-dependent effector functions, including antibody-dependent complement deposition (ADCD) and antibody-dependent cellular phagocytosis (ADCP).[36-39] In addition, we assessed S protein-specific T cell responses that are a key component of COVID-19 vaccine-induced immunity in KTRs.[9] We then explored how clinical factors underlying immune suppression influence vaccine-induced immunity in KTRs who were either naïve or infected with SARS-CoV-2 before vaccination.

METHODS

Study Design and Participant Characteristics

The study was designed to evaluate associations between clinical characteristics and immune responses to BioNTech/Pfizer BNT162b2 mRNA (Comirnaty) vaccination in COVID-19 naïve (N) and experienced (E) KTRs and HCs. KTRs were recruited from the department of nephrology, dialysis, and transplantation of the Hôpital Erasme, Belgium; and HCs were healthcare workers recruited from 2 Belgian nursing homes. Patients transplanted with multiple organs or with active invasive cancer were excluded from the study. All participants were adults of at least 18 years of age and provided written informed consent. Participants were enrolled before COVID-19 vaccination and then received 2 doses of the BNT162b2 vaccine (30 μg) 21 days apart, according to the Belgian national vaccination program. Patients recruited in this study were also included in our previously published report.[32]

The ethics committee of the Hôpital Erasme, Brussels, Belgium (references P2020/284 and A2021/131) and the Belgian Federal Agency for Medicines and Health Products (FAMHP, EudraCT 2021-000-412-28) approved the monocentric prospective phase IV investigator-initiated study of the immunogenicity of the BNT161b2 vaccine (Pfizer-BioNTech) in KTRs. HCs were included from a prospective cohort study named PICOV-VAC.[40,41] This latter study was approved by the Ethics Committee of the Hôpital Erasme, Brussels, Belgium (reference B4062020000134), the Federal Agency for Medicines and Health Products (2021-000401-24) and is registered on ClinicalTrials.gov (NCT04527614).

Previous SARS-CoV-2 infection status was established according to the following criteria. Participants with a previous laboratory-confirmed SARS-CoV-2 infection were considered previously infected irrespective of prevaccination serology. All other participants with a baseline antireceptor binding domain (anti-RBD) IgG level <5 binding antibody units (BAU)/ml were considered infection naïve, and those with a level >20 BAU/ml were considered previously infected. Participants with a level >5 and <20 BAU/ml were further tested with a multiplexed Luminex assay, as previously described,[42] detecting IgG specific for 4 antigens, including SARS-CoV-2 RBD, spike subunit 1 (S1), spike subunit 2 (S2) and nucleocapsid. Participants with detectable IgG to ≥ 3 out of 4 antigens were considered previously infected.

The first vaccine dose was administered to HCs between January 21 and January 28, 2021, and to KTRs between March 2 and March 18, 2021. The second vaccine dose was administered to HCs between February 11 and February 18, 2021, and to KTRs between March 23 and April 8, 2021. Blood was collected to assess humoral and cellular immunity to SARS-CoV-2 just before the first vaccine dose (baseline, or day 0) and 4 weeks after the second dose (day 49).

Binding Antibodies

Levels of total IgG specific for the Wuhan SARS-CoV-2 RBD were measured using an enzyme-linked immunosorbent assay (Wantai SARS-CoV-2 IgG ELISA

[Quantitative]; CE-marked; WS-1396; Beijing Wantai Biological Pharmacy Enterprise Co., Ltd, China), as previously described.[41,43] Levels lower than the limit of detection of 5 BAU/ml were attributed the value 2.5 BAU/ml. Detailed methods are provided in the Supplementary Methods.

Antibody Avidity

Avidity of Wuhan SARS-CoV-2 RBD-specific IgG was measured with biolayer interferometry, as previously described.[44] Biolayer interferometry measurements were performed using the Fortebio HTX Octet instrument and Fortebio AR2G biosensors. Of note, IgG avidity was only calculated in KTRs and HCs who had detectable levels of RBD-binding IgG. Detailed methods are provided in the Supplementary Methods.

nAbs

Titers of antibodies neutralizing Wuhan SARS-CoV-2 were measured using a live virus neutralization assay, as previously described.[45] The Reed-Muench method was used to calculate the nAb titer that reduced the number of infected wells by 50% or 90%. Titers lower than the limit of detection of 50 IU/ml for 50% neutralization titer were attributed the value of 25 IU/ml. Detailed methods are provided in Supplementary Methods.

IgG Subclasses and Fc-Dependent Functions

Levels of SARS-CoV-2-specific antibody isotypes, subclasses, FcγR-binding profiles and ADCD were measured using a 96-well-based customized multiplexed immunoassay, as previously described.[42,46,47] Antigens used for multiplex assays included Wuhan SARS-CoV-2 S1 protein (Sanyou Biopharmaceuticals #PNA002), SARS-CoV-2 S2 protein (Sinobiological #40590-V08H1), SARS-CoV-2 RBD (Proteogenix #PX-COV-P046) and SARS-CoV-2 N protein (Sanyou Biopharmaceuticals #PNA006). Data were acquired with a BioPlex-200 (Bio-Rad, CA) and measured as median fluorescence intensity. Of note, systems serology analysis was done in all N-KTRs, E-KTRs, N-HCs and in a subset of 40 E-HCs. In addition, IgA, IgG2, and IgG4 were measured only in a subset of the cohort, including 14 N-KTRs, 22 E-KTRs, 10 N-HCs, and 18 E-HCs. ADCP was assessed using the human monocyte cell line THP-1 (ATCC #TIB-202), as previously described.[48] Bead phagocytosis was measured by flow cytometry with an LSR Fortessa Flow Cytometer (BD), and analysis was performed using FlowJo V10.8.1. A phagocytosis score was calculated as follows: Percentage of cells that phagocytosed beads × median fluorescence intensity of bead positive cells/10,000. Detailed methods are provided in Supplementary Methods.

Cellular Immune Responses

SARS-CoV-2 S1 and S2 of Wuhan strain-specific T cell frequencies were measured in peripheral blood mononuclear cells by flow cytometry following intracellular cytokine staining (BD Fastimmune, BD-Beckton Dickinson and Company-Biosciences, San Jose, CA), as previously described,[49,50] and analysis was performed using FlowJo V10.8.1. Percentages of CD4 and CD8 T cells expressing CD154 (only in CD4), interferon-γ, and IL-2 were measured. Gating strategies are shown in Supplementary Figure S1. The lower limit of quantitation was set at 0.0001% (after background subtraction). Detailed methods are provided in Supplementary Methods.

Statistical Analyses

Demographic characteristics of KTRs and HCs are presented as median (first quartile Q1 − third quartile Q3) for continuous variables and *n* (%) for categorical variables. The comparison of categorical variables was done using the $\chi 2$ test, or Fisher's exact test when appropriate. Single comparisons between other metrics were done using the 2-tailed Mann-Whitney U test. Simple comparisons between groups of RBD-binding IgG, IgG avidity, nAb, subclasses and isotypes, FcγR-binding, ADCD, ADCP, CD4, and CD8 T cells responses were performed using the 2-tailed Mann-Whitney U test and multiple comparisons were done using the analysis of variance Kruskal-Wallis test with Dunn's correction. Spearman correlation analysis was used for single continuous variate correlation analyses. Associations between immune variables (RBD IgG, IgG avidity, wild type 50% neutralization titer, S1 CD154+CD4, S1 IFNγ+CD4, and S1 IL2+CD4) and continuous clinical characteristics (age, body mass index, time between transplantation and vaccination, absolute lymphocyte count, plasmatic creatinine, estimated glomerular filtration rate, time between infection and vaccination) and categorical variables (sex, kidney retransplantation after graft failure described as transplantation rank with the number of transplantations, induction treatment, number of chronic immunosuppressive treatments, corticosteroids, MMF, azathioprine, tacrolimus, cyclosporine A, everolimus, donor-specific antibodies, arterial hypertension, diabetes, cardiovascular disease, chronic respiratory disease, chronic kidney insufficiency, noninvasive skin cancer or cancer remission, oxygen dependance, and intensive care unit hospitalization during SARS-CoV-2 infection) were explored by univariate and multivariate linear regressions. All variables with univariate $P < 0.1$ were included in the multivariate model and the best models after

multicollinearity assumption are shown in Supplementary Methods. RBD IgG avidity, levels of RBD-binding IgG, wild type 50% neutralization titer, S1 IgG1, S2 IgG1, RBD IgG1, S1 IgG3, S2 IgG3, RBD IgG3, S1 FcγRIIa, S2 FcγRIIa, RBD FcγRIIa, S1 FcγRIIIa, S2 FcγRIIIa, RBD FcγRIIIa, S1 ADCD, S2 ADCD, RBD ADCD, RBD ADCP, S1 CD154+CD4, S1 IFNγ+CD4, S1 IL2+CD4, S1 IFNγ+CD8, S1 IL2+CD8, S2 CD154+CD4, S2 IFNγ+CD4, S2 IL2+CD4, S2 IFNγ+CD8, and S2 IL2+CD8 responses after mRNA vaccination were selected for principal component analysis (PCA). Levels of IgG2, IgG4, and IgA were not selected for PCA because of missing data. PCA was applied to reduce the immunological features to a minimal set of features to the entire cohort on the one hand, and in N-KTRs and E-KTRs separately on the other hand. R packages stats[51] and ggplot2[52] were used to perform and visualize PCA in scaled and centered data. A 2-sided *P*-value less than 0.05 was considered statistically significant. Statistical analyses were done using GraphPad Prism 9.5.0 (GraphPad Software, San Diego, CA), R version 4.2.0 and Rstudio version 1.3.1073 with R version 4.2.1.[51]

RESULTS

Cohorts and Participant Characteristics

Eighty-six KTRs were enrolled in the study, including 63 N-KTRs and 23 E-KTRs. Four N-KTRs were excluded because a SARS-CoV-2 infection was diagnosed between the 2 doses of COVID-19 vaccine. No SARS-CoV-2 infection was diagnosed in E-KTRs between enrolment and day 49 postvaccination. Demographic and clinical characteristics were similar between N-KTRs and E-KTRs except for age, everolimus treatment, and estimated glomerular filtration rate (Table 1). A total of 106 healthcare workers enrolled in the parallel PICOV-VAC trial were included in our analysis as HCs, with 40 N-HCs and 66 E-HCs. Detailed characteristics of N-KTRs, E-KTRs, N-HCs, and E-HCs are summarized in Supplementary Table S1. HCs were younger than KTRs. As previously described for patients with chronic kidney disease,[53] most KTRs were male. All KTRs were taking immunosuppressive treatment and had a higher proportion of comorbidities as compared to HCs. SARS-CoV-2 infections among experienced participants were more commonly symptomatic among E-KTRs, who required more oxygen than E-HCs.

E-KTRs Have Higher Antibody Responses to COVID-19 mRNA Vaccination Than N-KTRs

At baseline, SARS-CoV-2 specific antibodies were not detected in naïve KTRs and HCs, whereas most previously infected participants had detectable antibodies (Figure 1). Intriguingly, E-KTRs had significantly higher levels of RBD IgG avidity and higher titers of nAb (Figure 1) as well as higher levels of RBD and S1 specific IgG3 (Figure 2a) than E-HCs. Following vaccination, N-KTRs had markedly lower levels of binding and nAbs as compared to N-HCs (Figure 1 and Figure 2b), as previously described.[5,6] In contrast, high antibody responses to vaccination were detected in both E-HCs and E-KTRs. On day 49, E-KTRs had markedly higher levels of SARS-CoV-2 specific IgG, IgG avidity and nAb (Figure 1) as well as IgG subclasses and IgA (Figure 2b) than N-KTRs. Antibody levels in vaccinated E-KTRs reached similar levels as those detected in E-HCs, and the higher levels of SARS-CoV-2 specific IgG3 detected at baseline in E-KTRs than in E-HCs were also detected after vaccination (Figure 2b).

E-KTRs Develop Higher IgG Fc-Dependent Effector Responses to mRNA Vaccination Than N-KTRs

To understand how prior SARS-CoV-2 infection influences vaccine-induced antibody Fc-dependent effector functions among KTRs, we measured the binding capacity of IgG to human Fc-receptors. At baseline, levels of spike-specific antibody binding to FcγRIIa and FcγRIIIa levels were similar in E-KTRs and E-HCs, except for S2-specific antibody binding to FcγRIIa that were significantly higher in E-KTRs. Consistent with binding IgG responses to vaccination, spike-specific antibody binding to Fcγ receptors were higher in E-KTRs than in N-KTRs and were comparable to those detected in E-HCs (Figure 3). To determine whether these FcγR-binding profiles translated to increased antibody effector functions, we measured ADCD and ADCP activities. At baseline and postvaccination, E-KTRs had similar levels of ADCD and ADCP as E-HCs, thus supporting a role for Fc-dependent effector functions in hybrid immunity acquired by KTRs (Figure 4).

E-KTRs Have Higher CD4 T Cell and Similar CD8 T Cell Responses to mRNA Vaccination Compared to N-KTRs

To assess the impact of previous SARS-CoV-2 infection on cellular immunity of KTRs, we explored T cell responses to the spike protein by flow cytometry. Prevaccination, E-KTRS had higher frequencies of CD4 T cell expressing CD154, IFNγ, and IL2 in response to both S1 and S2 than E-HCs (Figure 5a). Postvaccination, E-KTRs had significantly higher frequencies of CD4 T cells expressing CD154, IFNγ, and IL2 in response to both S1 and S2 than N-KTRs (Figure 5b). Frequencies of S1- and S2-specific CD4 T cells expressing CD154 were higher in E-KTRs than in

Table 1. Comparison of baseline characteristics between SARS-CoV-2-naïve and SARS-CoV-2-experienced KTRs

N (%) or Median (Q1–Q3)	N-KTRs *N* = 59	E-KTRs *N* = 23	*P* -value[i]
Age, yr	63 (54–70)	51 (45–63)	0.011
Female sex	27 (45.8)	6 (26.1)	0.167
Body mass index, KG/M^2	25.5 (21.4–29.5)	26.0 (22.6–27.6)	0.942
Time between KT and RNA vaccination, yr	9.7 (3.5–15.7)	8.1 (2.9–12.2)	0.227
Transplantation rank >1	7 (11.9)	4 (17.4)	0.493
Immunosuppression			
Induction (BX/ATG/MUROMONAB-CD3)	35/17/5 (61.4/29.8/8.8)[a]	14/8/2 (60.9/34.8/8.7)[b]	0.553
CS	45 (76.3)	19 (82.6)	0.744
MMF	30 (50.8)	10 (43.5)	0.723
AZA	15 (25.4)	7 (30.4)	0.855
TAC	39 (66.1)	14 (60.9)	0.851
CyA	8 (13.6)	0	0.098
EVE	14 (23.7)	12 (52.2)	0.026
Triple is chronic therapy	33 (55.9)	16 (69.6)	0.379
DSA prior vaccination	4 (6.9)[c]	1 (5)[d]	1
Comorbidities			
Arterial hypertension	50 (84.7)	18 (78.3)	0.522
Diabetes	22 (37.3)	10 (43.5)	0.792
Cardiovascular disease	16 (27.1)	8 (34.8)	0.678
Chronic respiratory disease	5 (8.5)	0	0.315
Chronic kidney insufficiency (EGFR <30 ml/min per 1.73 m^2)	8 (13.6)	1 (4.4)	0.231
Cancer	16 (27.1)	3 (13)	0.287
Biological data			
Absolute lymphocyte count,/mm^3	1390 (770–2108)[e]	1340 (1015–1970)[f]	0.493
EGFR, ml/min per 1.73 M^2	48 (36–61)	62 (43–73)	0.051
Plasmatic creatinine, mg/dl	1.40 (1.08–1.72)	1.17 (1.04–1.43)	0.213
SARS-CoV-2 infection			
Time between SARS-CoV-2 infection and RNA vaccination, D	NA	149 (126–335.5)[g]	NA
Asymptomatic	NA	6 (26.1)	NA
Need for supplemental oxygen	NA	3 (13.6)[h]	NA
Intensive care requirement	NA	1 (4.5)[h]	NA

ATG, antithymocyte globulin; AZA, azathioprine; BX, basiliximab; CS, corticosteroids; CyA, cyclosporine A; DSA, donor-specific antibodies; eGFR, estimated glomerular filtration rate; EVE, everolimus; IS, immunosuppressive; KT, kidney transplantation; KTR, kidney transplant recipient; MMF, mycophenolate mofetil; NA, not applicable or not available; RNA, ribonucleic acid; TAC, tacrolimus.
Continuous variables are expressed as median (Q1–Q3) and categorical variables as frequency (%).
[a]57 values.
[b]One immune KTR received BX and ATG.
[c]58 values.
[d]20 values.
[e]44 values.
[f]21 values.
[g]17 values.
[h]22 values.
[i]Qualitative variables were compared using a Fisher's Exact or chi-square test and quantitative variables were compared using a Mann-Whitney U test.

E-HCs. In contrast, CD8 T cell responses to S1 and S2 were similar in SARS-CoV-2-experienced groups at baseline and postvaccination across study groups, except for S1-specific CD8 T cells expressing IFNγ that were significantly higher in E-HCs than in N-HCs.

Integrated Analysis of Immune Response to mRNA Vaccination in Naïve and SARS-CoV-2 Experienced KTRs and HCs

To visualize and characterize differences in immune response features across individuals and groups, dimensionality reduction was performed using PCA.[54] PCA resulted in 2 components with eigenvalues >1 that described relationships between immunological parameters following mRNA vaccination. The 2 major components, PC1 and PC2, accounted for 50.8% of the total variance (Figure 6a). N-KTRs, N-HCs, and E-HCs formed distinct clusters whereas responses among E-KTRs were more diffuse, encompassing N-HCs and E-HCs. Within KTRs and HCs, PC1 and PC2 were distinct between SARS-CoV-2-naïve and experienced subjects (Figure 6b). As shown in Figure 6c, humoral immune response features dominated the main principal component, PC1, where T cell response features as well as IgG3 responses, contributed most to PC2. Together, these analyses indicate that N-KTRs and E-KTRs have unique vaccine response profiles across many different immune effectors. The potential contribution of clinical

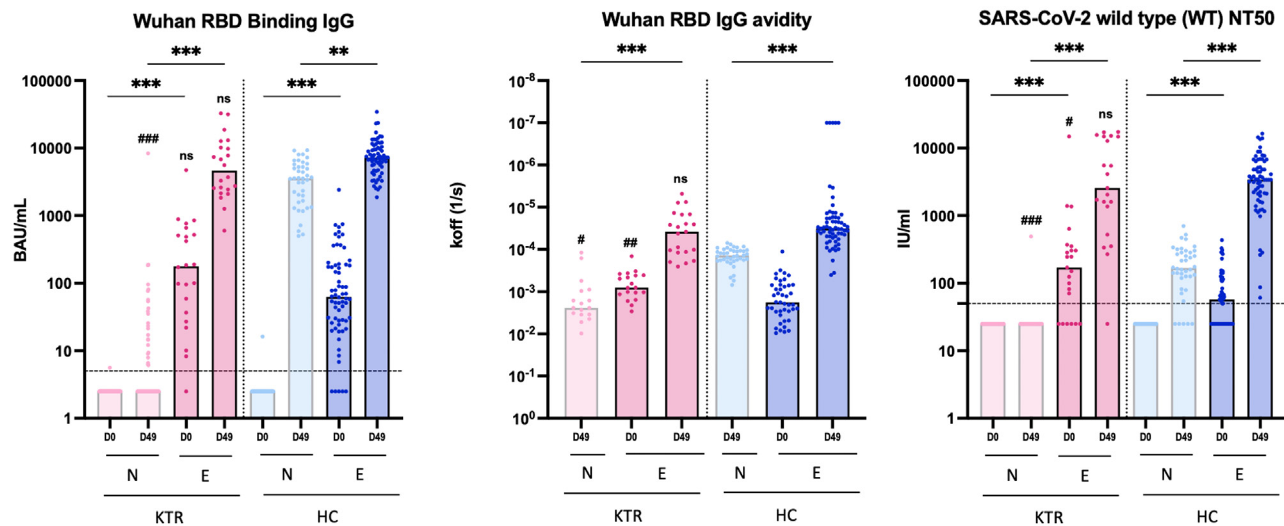

Figure 1. Binding IgG, RBD IgG avidity, and neutralizing antibody responses to SARS-CoV-2 mRNA vaccination in naïve and experienced KTRs. Serum levels of SARS-CoV-2 RBD specific binding IgG (BAU: binding antibody units), RBD specific IgG avidity (koff: dissociation rate constant) and titers of neutralizing antibodies (NT50: 50% neutralization titer) were measured before vaccination (D0) and 1 month after 2 doses of mRNA vaccine (D49) in naïve KTRs (N-KTRs, light pink), experienced KTRs (E-KTRs, dark pink), naïve HCs (N-HCs, light blue) and experienced HCs (E-HCs, dark blue). Bars indicate median values. Horizontal grid lines indicate a technical negative signal (blank). Groups were compared using the analysis of variance Kruskal-Wallis test with Dunn's correction. For within HC or KTR comparisons, ns; $^{a}P < 0.05$; $^{b}P < 0.01$; $^{c}P < 0.001$. For comparisons between HC and KTR, ns; $^{d}P < 0.05$; $^{e}P < 0.01$; $^{f}P < 0.001$. HC, healthy control; KTR, kidney transplant recipient; ns, not significant; RBD, receptor binding domain.

factors to these different profiles was assessed by multivariate analyses. As shown in Table 1, N-KTRs were older, included fewer patients taking everolimus and had poorer kidney function than E-KTRs. Multivariate linear regression showed that previous SARS-CoV-2 infection was the only significant variable determining the differences of immune response features between N-KTRs and E-KTRs (Supplementary Table S2).

Immune Response to mRNA Vaccination in N-KTRs and E-KTRs Do Not Correlate With the Same Clinical Characteristics

The contrast between the low responses to mRNA vaccination in N-KTRs and the potent hybrid immunity acquired in E-KTRs suggests a different role for immunosuppression-related factors in the 2 study groups. Univariate and multivariate linear regressions were used to explore correlations between demographic and clinical factors and the immune response features that differed most between N-KTRs and E-KTRs.

In N-KTRs, levels of RBD binding IgG were negatively correlated with age, arterial hypertension, kidney retransplantation after graft failure and MMF treatment; and were positively correlated with azathioprine treatment in univariate analyses (Table 2 and Supplementary Table S3). Significant correlations were also observed in multivariate analyses, except for azathioprine treatment (Table 2 and Supplementary Table S3). RBD IgG avidity and titers of nAb were not included in these analyses because of their very low values in N-KTRs. Several clinical parameters were correlated with S1-specific CD4 T cell responses in univariate analyses (Supplementary Tables S4, S5, and S6). In multivariate analyses, only absolute lymphocyte counts were negatively correlated with frequencies of SI-specific IFNγ+ CD4 T cells (Supplementary Table S5). Together, these results indicate that clinical factors associated with immune suppression negatively correlate with humoral immune responses in N-KTRs, with limited correlations with cellular immune responses.

In E-KTRs, levels of RBD binding IgG were positively correlated with time between transplantation and vaccination, and nAb titers were positively correlated with absolute lymphocyte count in univariate analyses but no significant correlation was observed in multivariate analyses (Table 2, Supplementary Tables S7 and S8). RBD IgG avidity was positively correlated with time between transplantation and vaccination, and with muromonab-CD3 treatment in univariate analysis; and only time between transplantation and vaccination was significantly correlated in multivariate analyses (Supplementary Table S9). Univariate analyses indicated significant correlations between demographic and clinical factors and S1-specific CD4 T cell responses (Supplementary Tables S10, S11, and S12). In

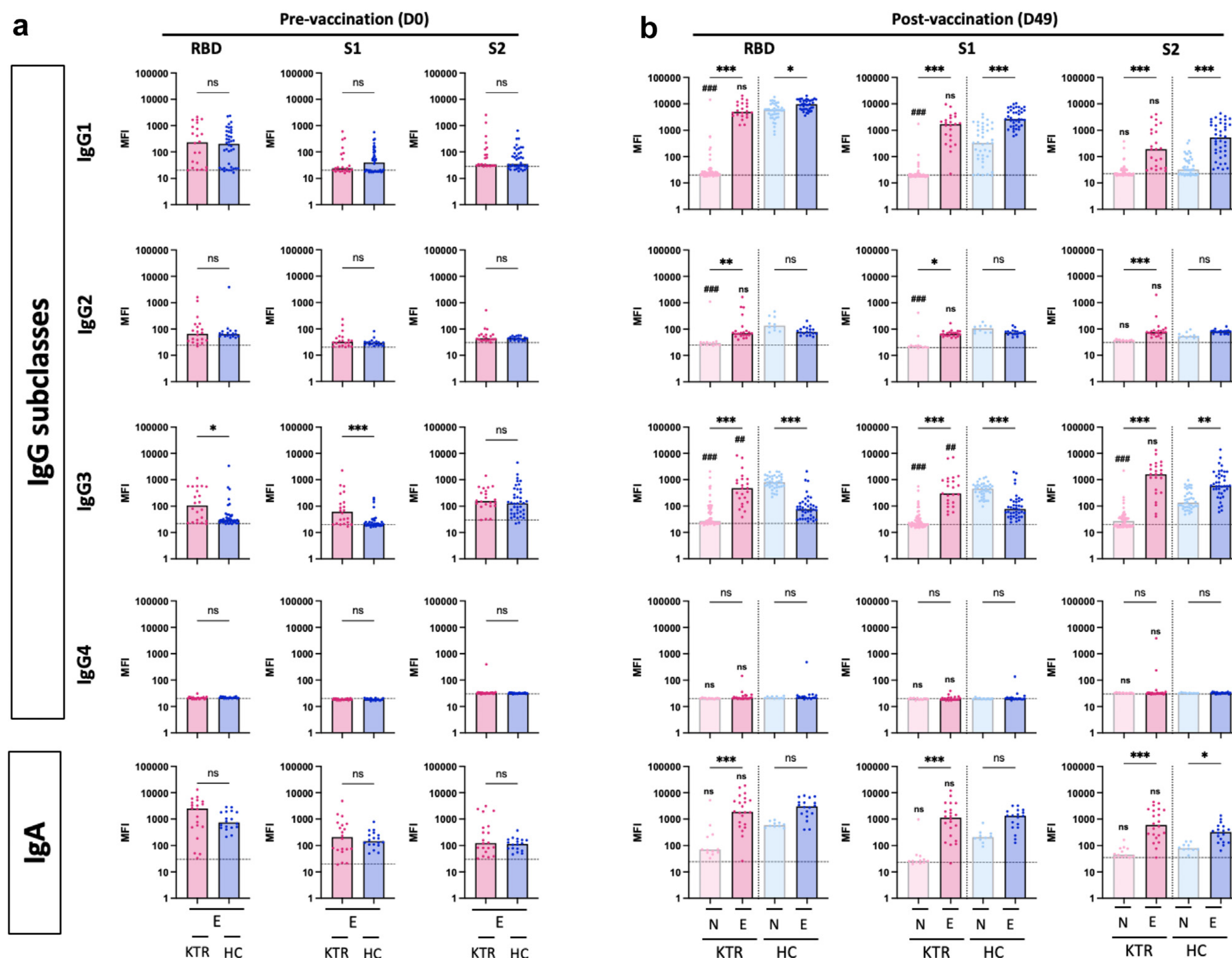

Figure 2. IgG Subclasses and IgA responses to SARS-CoV-2 mRNA vaccination in naïve and experienced KTRs. Serum levels of SARS-CoV-2 RBD specific, spike S1 subunit specific, and spike S2 subunit specific IgG1, IgG2, IgG3, IgG4 and IgA were measured before vaccination (D0, panel a) and 1 month after vaccination (D49, panel b) in naïve KTRs (N-KTRs, light pink), experienced KTRs (E-KTRs, dark pink), naïve HCs (N-HCs, light blue) and experienced HCs (E-HC, dark blue). Bars indicate median values. Horizontal grid lines indicate a technical negative signal (blank). Groups were compared using the analysis of variance Kruskal-Wallis test with Dunn's correction. For within HC or KTR comparisons, $^{a}P < 0.05$; $^{b}P < 0.01$; $^{c}P < 0.001$. For comparisons between HC and KTR, ns; $^{d}P < 0.05$; $^{e}P < 0.01$; $^{f}P < 0.001$. HC, healthy control; KTR, kidney transplant recipient; MFI, median fluorescent intensity; ns, not significant; RBD, receptor binding domain.

multivariate analyses, frequencies of S1-specific IFNγ+ CD4 T cells and IL2+ CD4 T cells were negatively correlated with time between SARS-CoV-2 infection and vaccination (Supplementary Tables S11 and S12). To further explore the role of demographic and clinical factors in immune response features in N-KTRs and E-KTRs, we performed PCA and analyzed their correlations with dominant PCs (Supplementary Figures S2 and S3). In line with multivariate regression analyses, in N-KTRs, PC1 was correlated with time between transplantation and vaccination, kidney retransplantation after graft failure, arterial hypertension, and azathioprine treatment (Supplementary Table S13). In E-KTRs, PC1 was correlated with time between transplantation and vaccination and PC2 was correlated with time between infection and vaccination (Supplementary Table S14). Together, these data indicate that, except for time between transplantation and vaccination, factors associated with low vaccine responses in N-KTRs were not correlated with vaccine responses in E-KTRs.

DISCUSSION

The defective immune response of KTRs to mRNA vaccination remains a concern for the protection of this vulnerable population against emerging infectious diseases. The acquisition of hybrid immunity to SARS-CoV-2 provides proof-of-principle that KTRs can develop high immune responses to viruses despite their state of immune suppression. This study provides a comprehensive analysis of hybrid immunity in KTRs as compared to HCs and provides evidence that vaccine responses in E-KTRs are not correlated with most factors associated with decreased responses in N-KTRs.

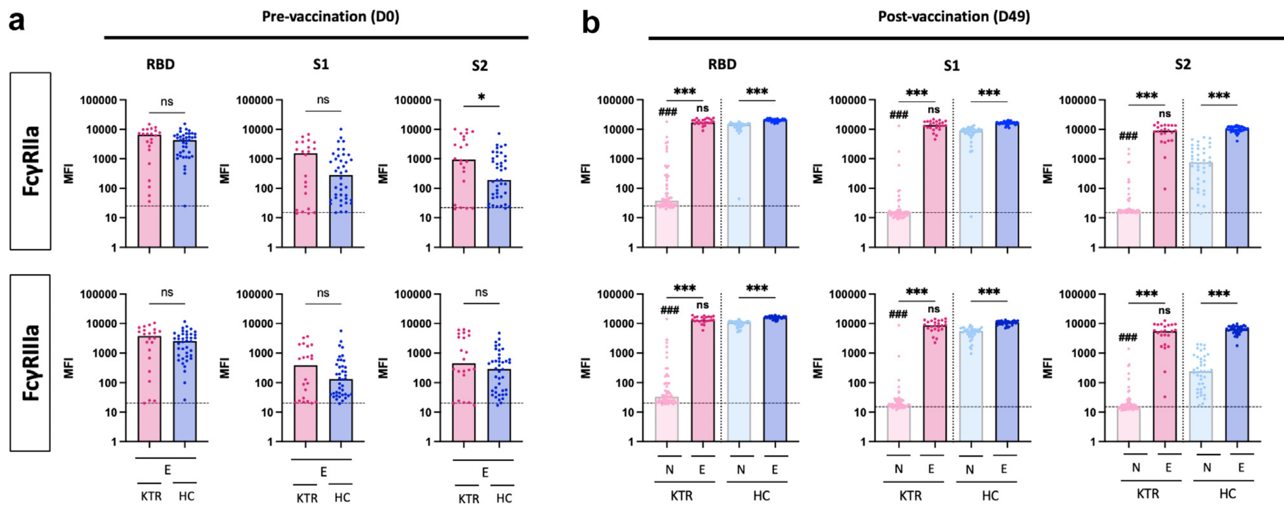

Figure 3. Fcγ receptors binding antibody responses to SARS-CoV-2 mRNA vaccination in naïve and experienced KTRs. Serum levels of SARS-CoV-2 RBD specific, spike S1 subunit specific, and spike S2 subunit specific antibodies binding the Fcγ receptors, FcγRIIa and FcγRIIIa were measured before vaccination (D0, panel a) and 1 month after vaccination (D49, panel b) in naïve KTRs (N-KTRs, light pink), experienced KTRs (E-KTRs, dark pink), naïve HCs (N-HCs, light blue) and experienced HCs (E-HC, dark blue). Bars indicate median values. Horizontal grid lines indicate a technical negative signal (blank). Groups were compared using the analysis of variance Kruskal-Wallis test with Dunn's correction. For within HC or KTR comparisons, ns; $^{a}P < 0.05$; $^{b}P < 0.01$; $^{c}P < 0.001$. For comparisons between HC and KTR, ns; $^{d}P < 0.05$; $^{e}P < 0.01$; $^{f}P < 0.001$. HC, healthy control; KTR, kidney transplant recipient; MFI, median fluorescent intensity; ns, not significant; RBD, receptor binding domain

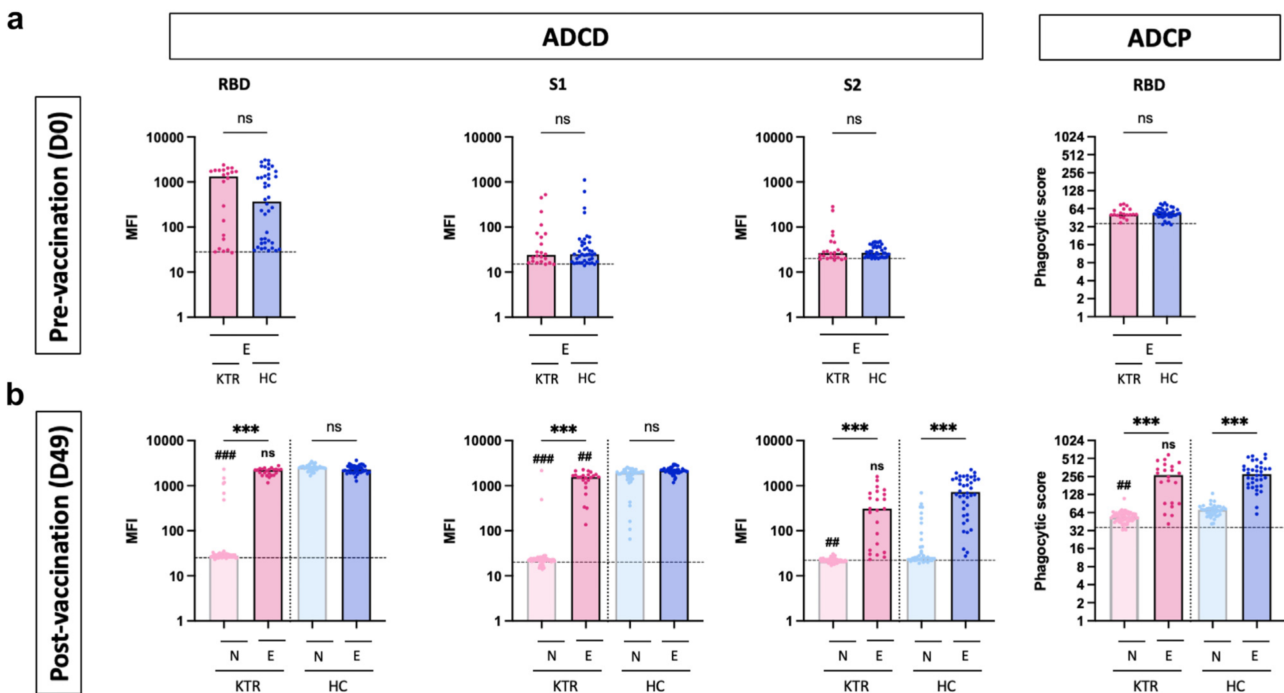

Figure 4. IgG-dependent complement deposition and phagocytosis responses to SARS-CoV-2 mRNA vaccination in naïve and experienced KTRs. Serum levels of SARS-CoV-2 RBD specific, spike S1 subunit specific, and spike S2 subunit specific IgG promoting complement deposition (ADCD) and cellular phagocytosis (ADCP) were measured before vaccination (D0, panel a) and 1 month after vaccination (D49, panel b) in naïve KTRs (N-KTRs, light pink), experienced KTRs (E-KTRs, dark pink) naïve HCs (N-HCs, light blue) and experienced HCs (E-HCs, dark blue). Levels of ADCD are expressed as MFI. Levels of ADCP are expressed as phagocytic score (see methods). Bars indicate median values. Horizontal grid lines indicate a technical negative signal (blank). Groups were compared using the analysis of variance Kruskal-Wallis test with Dunn's correction. For within HC or KTR comparisons, ns; $^{a}P <0.05$; $^{b}P < 0.01$; $^{c}P < 0.001$. For comparisons between HC and KTR, ns; $^{d}P < 0.05$; $^{e}P < 0.01$; $^{f}P < 0.001$. HC, healthy control; KTR, kidney transplant recipient; MFI, median fluorescent intensity; ns, not significant; RBD, receptor binding domain.

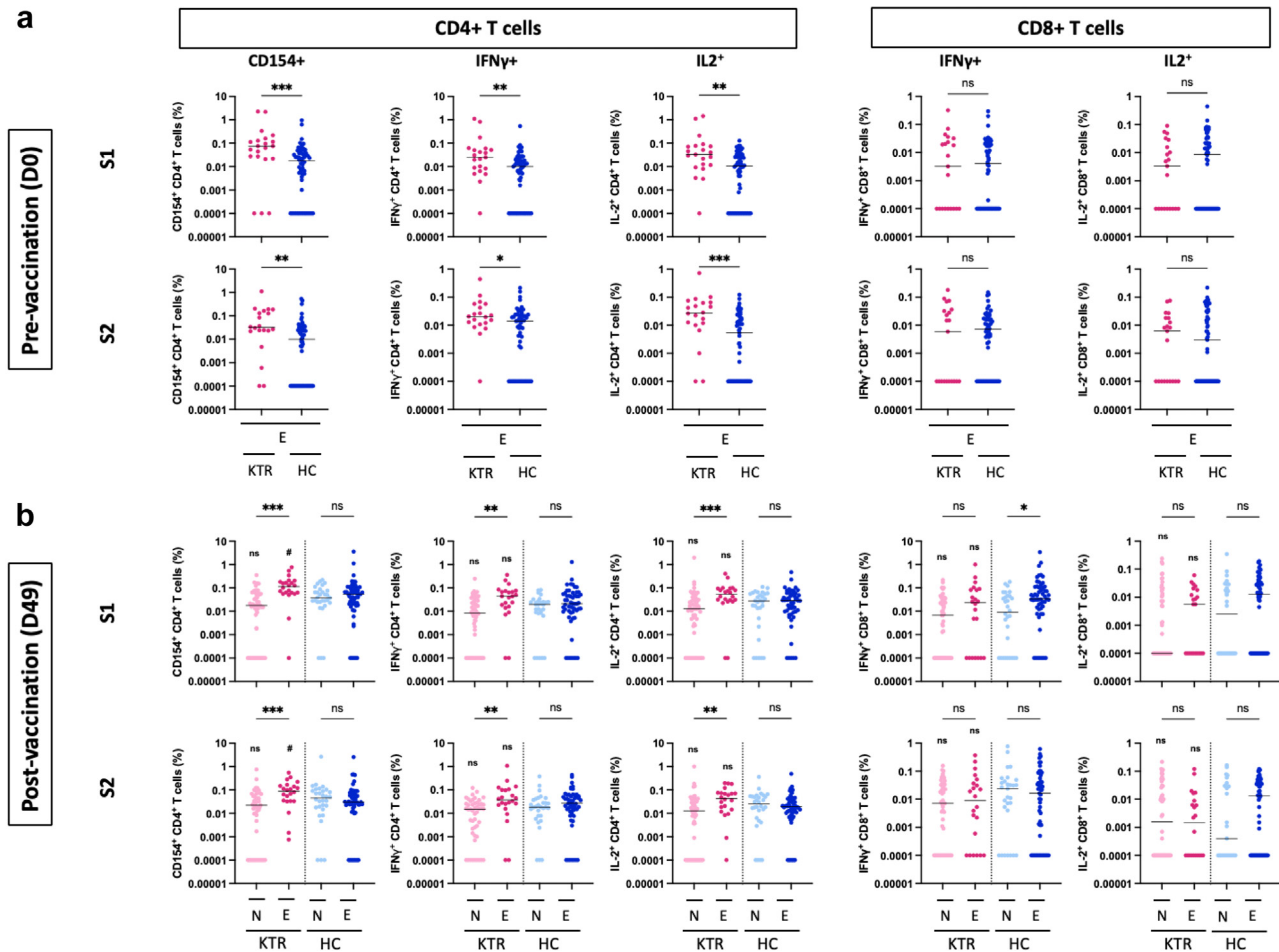

Figure 5. CD4 and CD8 T cell responses to SARS-CoV-2 mRNA vaccination in naïve and experienced KTRs. Percentage of SARS-CoV-1 spike S1 subunit and spike S2 subunit specific CD4 T cells expressing CD154, IFNγ and IL2, and of CD8 T cells expressing IFNγ and IL-2 were measured in peripheral blood before vaccination (D0, panel a) and 1 month after vaccination (D49, panel b) in naïve KTRs (N-KTRs, light pink), experienced KTRs (E-KTRs, dark pink), naïve HCs (N-HCs, light blue) and experienced HCs (E-HC, dark blue). Bars indicate median values. Groups were compared using the analysis of variance Kruskal-Wallis test with Dunn's correction. HC, healthy control; KTR, kidney transplant recipient; ns, not significant.
For within HC or KTR comparisons, ns; $^{a}P < 0.05$; $^{b}P < 0.01$; $^{c}P < 0.001$.
For comparisons between HC and KTR, ns; $^{d}P < 0.05$; $^{e}P < 0.01$; $^{f}P < 0.001$.

Although the mechanisms underlying hybrid immunity remain incompletely understood, priming of B and T cell responses by natural infection probably plays a central role. Before vaccination, polyfunctional antibody and T cell responses to SARS-CoV-2 spike protein were detected in E-KTRs. Whereas the levels of RBD binding antibodies were similar in E-KTRS and E-HCs before vaccination, E-KTRs had higher RBD IgG avidity and higher titers of nAbs. This observation suggests higher germinal center reactions following SARS-CoV-2 infection in E-KTRs, a possibility supported by the higher frequency of CD4 T cells expressing CD154, with potential B cell help capacity, in E-KTRs than in E-HCs. At baseline, E-KTRs also had higher frequencies of CD4 T cells expressing IFNγ and IL2 than E-HCs. In contrast, frequencies of spike specific CD8 T cells were similar in the 2 groups. The higher levels of immune effectors in E-KTRs as compared to E-HCs following SARS-CoV-2 infection could be related to several factors. Symptomatic COVID-19 was associated with more intense immune responses.[55] This factor is unlikely to play a dominant role because most E-KTRs had experienced mild COVID-19 and because RBD binding antibody levels were similar in the 2 groups. Previous studies have shown defective antibody and T cell responses to SARS-CoV-2 in KTRs than in controls during the early phase of the infection.[56,57] In contrast, KTRs and controls were shown to have similar levels of SARS-CoV-2 specific antibodies and T cells during the convalescent phase,[58] in line with our observations. The delayed acquisition of antibody and T cell responses in KTRs as compared to controls may be related to the duration of SARS-CoV-2 antigen exposure that may have further stimulated B cells and CD4 T cells. In support of this hypothesis,

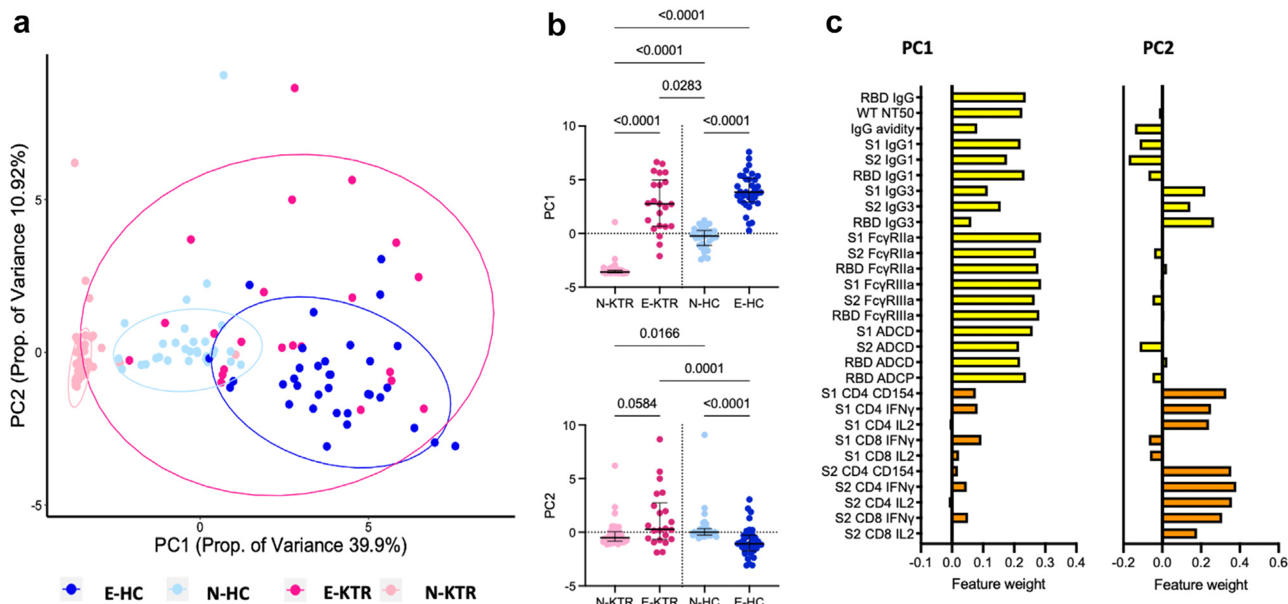

Figure 6. Principal component analysis of immune responses to SARS-CoV-2 mRNA vaccination in naïve and experienced KTRs. (a) Scatter plot of PCA including all immune response parameters except IgG2, IgG4 and IgA measured 1 month after vaccination (D49) in naïve KTRs (N-KTRs, light pink), experienced KTRs (E-KTRs, dark pink), naïve HCs (N-HCs, light blue) and experienced HCs (E-HC, dark blue). (b) Comparison of PC1 and PC2 values between groups by analysis of variance Kruskal-Wallis test with Dunn's correction with $P < 0.1$. (c) Relative weighting of individual immune response parameters in PC1 and PC2. HC, healthy control; KTR, kidney transplant recipient, PC, principal component; PCA, principal component analysis.

prolonged viral excretion was observed in KTRs following COVID-19.[59] Prolonged antigen stimulation may have also played a role in the higher levels of the IgG3 observed in E-KTRs at baseline, because this subclass is typically produced following recent antigen exposure. Alternatively, the high levels of antibodies and T cells detected in convalescent KTRs may also involve a better survival of patients with highest immune responses in the early phase of the infection. This possibility is not supported by longitudinal studies showing delayed acquisition of antibodies in KTRs compared to controls.

Table 2. Univariate and multivariate linear regressions related to humoral response after mRNA vaccination in N-KTRs and E-KTRs

	Univariate linear regression			Multivariate linear regression		
RBD binding IgG	Estimate (B)	95% CI	*P*-value	Estimate	Standard error	*P*-value
N-KTRS						
Age	−0.02	−0.03, 0.00	0.037	−0.014452	0.005833	0.016
Transplantation rank						
1	0.39	−0.17, 0.96	0.2			
2	−0.51	−1.1, 0.09	0.093	−0.641834	0.231526	0.008
Time between KT and RNA vaccination	0.01	−0.01, 0.03	0.3			
Arterial hypertension	−0.75	−1.2, −0.28	0.002	−0.611656	0.197785	0.003
MMF	−0.61	−0.94, −0.28	<0.001	−0.664619	0.141061	<0.001
AZA	0.75	0.38, 1.1	<0.001			
E−KTRS						
Age	0.01	−0.01, 0.02	0.2			
Transplantation rank						
1	−0.26	−0.78, 0.26	0.3			
2	0.01	−0.71, 0.72	>0.9			
Time between KT and RNA vaccination	0.03	0.00, 0.05	0.063	0.02502	0.01270	0.063
Arterial hypertension	0.30	−0.17, 0.77	0.2			
MMF	0.08	−0.34, 0.49	0.7			
AZA	0.34	−0.09, 0.78	0.12			

AZA, azathioprine; CI, confidence interval; E-KTRs, SARS-CoV-2-experienced kidney transplant recipients; KT, kidney transplantation MMF: mycophenolate mofetil; N-KTRs: SARS-CoV-2-naïve kidney transplant recipients; RBD, receptor binding domain.

Significant univariate ($P < 0.1$) and multivariate ($P < 0.05$) linear regressions with log(10) RBD binding IgG after vaccination in N-KTRs and E-KTRs, respectively. For the univariate analyses, all variables that were significant ($P < 0.1$) in N-KTRs and/or E-KTRs are reported for both groups. A multivariate analysis per group was then done, including only the variables ($P < 0.1$) significant in its own group.

Postvaccination, N-KTRs had lower humoral responses to the spike protein than N-HCs, as previously reported.[5-8,60-62] Levels of RBD binding IgG were negatively correlated with age, arterial hypertension, kidney retransplantation after graft failure, and MMF treatment, confirming previous reports.[8,13-15,34] Humoral and cellular responses to vaccination were markedly higher in E-KTRs than in N-KTRs. E-KTRs produced high levels of high avidity binding IgG and IgA and high titers of nAbs in response to mRNA vaccination. They also displayed high binding to Fcγ receptors, including FcγRIIa, a receptor promoting antibody-dependent phagocytosis by myeloid cells, and FcγRIIIa, a receptor promoting activation of natural killer cells by IgG. These high levels of FcγR binding IgG were associated with high levels of ADCP and ADCD. E-KTRs also had high frequencies of CD4 T cells expressing CD154, IFNγ and IL2. This higher level of vaccine-induced immunity in E-KTRs than in N-KTRs is likely to provide increased protection against breakthrough infections. Indeed, we recently reported that both antibody and T cell responses to vaccination correlated with the risk of breakthrough infection in N-KTRs.[9] In contrast to CD4 T cells, E-KTRs did not show higher frequencies of spike-specific CD8 T cells than N-KTRs. A dissociation between CD4 and CD8 T cell responses to mRNA vaccination was also observed in healthy adults who acquired hybrid immunity to SARS-CoV-2.[31] However, Gao *et al.*[31] reported lower CD8 T cell responses to vaccination in SARS-CoV-2-experienced than in naïve subjects, a phenomenon that was not observed in our study. The factors associated with decreased vaccine responses in N-KTRs did not correlate with either antibody or T cell responses to vaccination in E-KTRs. Time between transplantation and vaccination were correlated with RBD IgG avidity, but not with the other immune vaccine response features, suggesting the immune suppression induced at the time of transplantation has some impact on vaccine response in E-KTRs. A negative correlation was also observed between T cell responses and time between SARS-CoV-2 infection and vaccination, suggesting waning of cellular immune response priming.

An important strength of this study is that KTRs and HCs were recruited before the administration of the first dose of COVID-19 vaccine and were included in parallel studies with standardized protocols and procedures. A limitation of the study is its relatively small sample size, in relation to the monocentric recruitment of KTRs. However, differences in vaccine responses between groups and risk factors associated with low vaccine responses in N-KTRs that were previously reported in larger studies could be confirmed in our study. Further studies of hybrid immunity in other KTR populations are needed to validate our observations. Other limitations are the lack of follow-up with the study population and the focus of the analysis on the immune response to the vaccine strain. The study was focused on the response to primary immunization because it offered the best model to compare vaccine-induced immunity and their determinants independently of postvaccination exposure to SARS-CoV-2 variants.

In conclusion, this study shows that KTRs can acquire potent humoral and cellular immune responses to COVID-19 mRNA vaccination when they have been primed by natural infection and that these responses are not correlated with factors associated with low vaccine responses in SARS-CoV-2-naïve patients. Understanding the cellular and molecular bases of hybrid immunity in KTRs should help the development of optimized vaccination strategies against emerging pathogens for this vulnerable population.

DISCLOSURE

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DATA AVAILABILITY STATEMENT

The datasets used and analyzed during the current study are available from the corresponding author on reasonable request, following material transfer agreement.

AUTHOR CONTRIBUTIONS

NG, DK, ALM, and AM conceptualized the study. DK and PP wrote the clinical study protocol. DK obtained permission from the ethics committee and A.F.M.P.S. MEG, ALM, and AM secured the funding of the study. NG, DK and AL conducted the clinical study. ALM overviewed the clinical study. NG, DK, SS, AW, VO, DG, ED, MB, LH, JM, and MV conducted the laboratory analyses. PP, SS, AT, AMat, ID, KKA, MEA, and AM overviewed the laboratory analyses. NG, DK, SD, NK, DG, and LH contributed to data analysis and interpretation. NG, DK, SD, MEA, and AM interpreted the data. NG, PP, and AM drafted the manuscript. All co-authors reviewed, edited, and approved the manuscript.

SUPPLEMENTARY MATERIAL

Supplementary File (PDF)

Supplementary Methods.

Figure S1. Representative gating of CD3 T cells, CD4 T cells and CD8 T cells.

Figure S2. Principal component analysis of immune responses to SARS-CoV-2 mRNA vaccination in N-KTRs.

Figure S3. Principal component analysis of immune responses to SARS-CoV-2 mRNA vaccination in E-KTRs.

Table S1. Baseline characteristics of the study population.

Table S2. Multivariate linear regression analysis of factors associated with the immune response to mRNA vaccination in N-KTRs compared to E-KTRs.

Table S3. Univariate and multivariate linear regression analyses of spike RBD specific binding IgG responses to mRNA vaccination in N-KTRs.

Table S4. Univariate and multivariate linear regression analyses of spike S1 specific CD154+CD4 T cell responses to mRNA vaccination in N-KTRs.

Table S5. Univariate and multivariate linear regression analyses of spike S1 specific IFNγ+CD4 T cell responses to mRNA vaccination in N-KTRs.

Table S6. Univariate and multivariate linear regression analyses of spike S1 specific IL2+CD4 T cell responses to mRNA vaccination in N-KTRs.

Table S7. Univariate and multivariate linear regression analyses of spike RBD specific binding IgG responses to mRNA vaccination in E-KTRs.

Table S8. Univariate and multivariate linear regression analyses of SARS-CoV-2 neutralizing antibody responses to mRNA vaccination in E-KTRs.

Table S9. Univariate and multivariate linear regression analyses of spike RBD specific IgG avidity responses to mRNA vaccination in E-KTRs.

Table S10. Univariate and multivariate linear regression analyses of spike S1 specific CD154+CD4 T cell responses to mRNA vaccination in E-KTRs.

Table S11. Univariate and multivariate linear regression analyses of spike S1 specific IFNγ+CD4 T cell responses to mRNA vaccination in E-KTRs.

Table S12. Univariate and multivariate linear regression analyses of spike S1 specific IL2+CD4 T cell responses to mRNA vaccination in E-KTRs.

Table S13. Association between clinical parameters and principal components of immunological parameters in N-KTRs.

Table S14. Association between clinical parameters and principal components of immunological parameters in E-KTRs.

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