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Higher Proinflammatory Cytokines Are Associated With Increased Antibody Titer After a Third Dose of SARS-CoV-2 Vaccine in Solid Organ Transplant Recipients

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Background. Solid organ transplant recipients (SOTRs) are at increased risk for severe COVID-19 and exhibit lower antibody responses to SARS-CoV-2 vaccines. This study aimed to determine if prevaccination cytokine levels are associated with antibody response to SARS-CoV-2 vaccination. **Methods.** A cross-sectional study was performed among 58 SOTRs before and after two-dose mRNA vaccine series, 35 additional SOTRs before and after a third vaccine dose, and comparison to 16 healthy controls (HCs). Antispike antibody was assessed using the IgG Euroimmun ELISA. Electrochemiluminescence detection-based multiplexed sandwich immunoassays (Meso Scale Diagnostics) were used to quantify plasma cytokine and chemokine concentrations (n = 20 analytes) and compare concentrations between SOTRs and HCs, stratified by ultimate antibody response to the vaccine using Wilcoxon-rank-sum test with false discovery rates computed to correct for multiple comparisons. **Results.** In the study population, 100% of HCs, 59% of SOTRs after 2 doses and 63% of SOTRs after 3 doses had a detectable antibody response. Multiple baseline cytokines were elevated in SOTRs versus HCs. There was no significant difference in baseline cytokine levels between SOTRs with high versus low-titer antibodies after 2 doses of vaccine. However, as compared with poor antibody responders, SOTRs who went on to develop a high-titer antibody response to a third dose of vaccine had significantly higher prethird dose levels of several innate immune cytokines including IL-17, IL-2Ra, IL-6, IP-10, MIP-1α, and TNF-α (false discovery rates < 0.05). **Conclusions.** A specific inflammatory profile may be associated with developing higher antibodies in response to a third dose of SARS-CoV-2 vaccine in SOTRs.

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INTRODUCTION

Solid organ transplant recipients (SOTRs) are less likely than immunocompetent populations to develop a positive antibody response to the mRNA-based SARS-CoV-2 vaccines.[1-4] Additionally, SOTRs are more likely to die from COVID-19[5,6] and are at increased risk of breakthrough infections after vaccination.[7,8] Due to these factors, a third dose is now recommended for immunocompromised individuals.[9] These

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A.H.K., W.A.W., and A.A.R.T. conceived of the study and design. O.A. and Y.E. processed the samples and prepared them for the assays. A.H.K., K.H.W., S.E.B., S.S., and O.A. performed the assays and collected the cytokine and chemokine data. X.Z. and A.H.K. performed the analysis. A.H.K. wrote the original article. J.M.G.-W., S.L.K., J.R.B., A.L.C., J.N.B., C.M.D., D.L.S., W.A.W., and A.A.R.T. supervised the studies, provided material support, and contributed to the interpretation of results. All authors aided in editing the article.

Supplemental Visual Abstract; http://links.lww.com/TP/C346.

recommendations were based on several studies demonstrating that at least a portion of SOTRs who do not mount a positive immune response to 2 doses do respond to a third.[10-12] A large percentage (40%–50%) of SOTRs who do not respond to 2 doses of vaccine do not mount a humoral response even to a third dose of vaccine, and those who do have antibody titers markedly lower than healthy controls (HCs).[12,13] Therefore, understanding which SOTRs may respond to a third dose and which are unlikely to do so is critical to developing strategies to protect this vulnerable group.

The mechanisms underlying blunted immune response to mRNA vaccination in SOTRs are unknown. Some clinical factors associated with an increased likelihood of a positive vaccine response in SOTRs include younger age, receipt of the mRNA-1273 (Moderna) vaccine, and absence of antimetabolite maintenance immunosuppression.[14] Given the association between maintenance immunosuppression regimen and antibody response, we hypothesized that a certain inflammatory profile may represent resting immunoreactive potential and be associated with improved vaccine response, as was seen with influenza vaccination.[15]

MATERIALS AND METHODS

Study Participants

The SOTR participants were enrolled in a national prospective, observational cohort: COVID-19 antibody testing of recipients of solid organ transplants and patients with chronic diseases, which was approved by the Johns Hopkins IRB (00248540), as previously described.[1,11] All SOTR participants were recruited virtually and provided detailed transplant history as well as oral informed consent (waiver of written consent granted). All vaccines were also administered independently in the community. For participants in the 2-dose cohort, blood samples were obtained 0–4 wks before and 2 wks after vaccine doses. The 3-dose cohort consisted of participants who did not have a substantial antibody response after the 2-dose series. For this cohort, blood samples were obtained (0–22 d or median 1 [IQR 0–5] d) before dose 3 (median of 83 d after dose 2) and 2 wks after dose 3.

HC participants were enrolled under Johns Hopkins IRB00027183, and all received 2 doses of BNT162b2 (Pfizer). All HCs provided written informed consent and contributed limited demographic data (eg, sex, race, decade of age).

Blood was collected in acid citrate dextrose or heparin tubes, and plasma was isolated by centrifugation and stored at –80°C until cytokines were measured.

SARS-CoV-2 Antibody Detection

Plasma specimens were analyzed using the Euroimmun Anti-SARS-CoV-2 ELISA (Mountain Lakes, NJ), which measures anti-SARS-CoV-2 IgG specific to spike (S1 subunit). All ELISA kits were purchased from the manufacturer, and assays were performed according to the manufacturers' instructions. The Euroimmun results are reported as an arbitrary unit ratio (AU), which is the optical density of the sample divided by calibrator provided.[16,17] Plasma IgG in World Health Organization binding antibody units (BAU) was measured using the chemiluminescent Meso Scale Diagnostics (MSD, Rockville, MD) V-PLEX COVID-19 Respiratory Panel 3 Kit according to the manufacture's protocol at a dilution of 1:5000. BAU were calculated by multiplying AU by the manufacturer's verified conversion factor.

Pseudoneutralization/ACE2 Inhibition Measurement

Plasma from study participants was thawed and ACE2 blocking/pseudoneutralization was measured using the MSD ACE2 V-PLEX SARS-CoV-2 kit according to the manufacturer's instructions at a dilution of 1:100.

Cytokine Measurement

Twenty cytokines and chemokines were measured in plasma from HCs and SOTRs before receiving the first dose of a 2-dose SARS-CoV-2 vaccine series, and immediately before a third dose of vaccine among SOTRs. The choice of cytokines and chemokines was based on previous studies on SARS-CoV-2 infection and studies of cytokines in SOTRs before receiving influenza vaccines.[15,18,19]

Plasma was thawed and cytokines (interferon [IFN]-α2a, IFN-γ, IFN-λ, interleukin [IL]-10, IL-15, IL-16, IL-17A, IL-18, IL-1RA, IL-21, IL-22, IL-2Ra, IL-6, IL-7, IL-8, interferon-γ-inducible protein 10 [IP-10], monocyte chemoattractant protein-1 [MCP-1], macrophage inflammatory protein 1α [MIP-1α], tumor necrosis factor [TNF]-α, vascular endothelial growth factor [VEGF]) were measured using a custom multiplex kit from MSD according to the manufacture's protocol, and data were acquired on a MESO QuickPlex SQ 120.

Statistical Analysis

Each sample was tested twice, and the mean value of 2 runs was used in analysis. The actual read of the values were kept for samples below the lower limit of detection. Below fitted curve values were set to 0. The SOTR dose 2 and dose 3 cohorts were subdivided based on antibody response after the second dose (for the dose 2 cohort) or after the third dose (for the dose 3 cohort) into low-titer (<3.5 AU) and high-titer (≥3.5 AU) based on the classification of low-titer versus high-titer convalescent plasma set by the FDA.[20] Cytokines and chemokines prevaccine were then compared among HCs, low-titer SOTRs, and high-titer SOTRs to determine if 1 or more cytokines or chemokines were associated with development of a high-titer response to the 2-dose or 3-dose series. Wilcoxon-Rank-Sum test was used to compare median cytokine values between groups. Multiple comparisons were adjusted using Benjamini-Hochberg procedure with a false discovery rate (FDR) cutoff of 0.05. Sensitivity analyses were also performed to assess differences between type of vaccine received (mRNA-1273 versus BNT162b), use of antimetabolite immunosuppression, and seroconversion before third dose as described later.

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RESULTS

Cohort Characteristics

A total of 93 SOTRs participants were included in the study along with 16 HCs. The SOTR cohort (50% <60 y of age) was older than the HCs (100% <60 y of age) with more females (54% versus 31%) (**Table 1**). The SOTR cohort was subdivided into those who received a 2-dose vaccine regimen (SOTR dose 2) and those who received a third dose (SOTR dose 3). Both SOTR groups were similar

TABLE 1.
Demographic and clinical characteristics of the cohort

	SOTR dose 2 (N = 58), n (%)	SOTR dose 3 (N = 35), n (%)	P[a]	HC (N = 16), n (%)
Age				
20–39	15 (26)	3 (9)	0.108	7 (44)
40–59	15 (26)	13 (37)		9 (56)
60–79	28 (48)	19 (54)		0 (0)
Sex				
Female	31 (53)	19 (54)	1.000	5 (31)
Male	27 (47)	16 (46)		11 (69)
Race				
White	53 (91)	33 (94)	0.417	11 (69)
Asian	3 (5)	0 (0)		3 (19)
African American	1 (2)	0 (0)		2 (13)
Multiple race	1 (2)	1 (3)		0 (0)
Other	0 (0)	1 (3)		0 (0)
Type of vaccine received				
First 2 doses				
BNT162b2	33 (57)	17 (49)	0.665	–
mRNA-1273	25 (43)	17 (49)		–
Missing	0 (0)	1 (3)		–
Third dose				
BNT162b2	–	8 (23)	–	–
mRNA-1273	–	14 (40)		–
Ad26.COV2.S	–	13 (37)		–
Graft transplanted				
Kidney	37 (67)	19 (54)	0.815	–
Heart	5 (9)	3 (9)		–
Liver	12 (21)	9 (26)		–
Lung	2 (3)	1 (3)		–
Pancreas	0 (0)	1 (3)		–
Multi[b]	2 (0)	1 (3)		–
Antirejection medication[c]				
Prednisone	38 (66)	17 (49)	0.130	–
Calcineurin Inhibitors	52 (90)	28 (80)	0.226	–
mTOR inhibitors	8 (14)	3 (9)	0.526	–
Antimetabolites	40 (69)	23 (66)	0.820	–
Treated for rejection in the past 6 mo				
Not treated	52 (90)	32 (91)	1.000	–
Was treated	3 (5)	1 (3)		–
Missing	3 (5)	2 (6)		–
Postvaccine SARS-CoV-2 IgG				
Negative	24 (41)	13 (37)	0.827	0 (0)
Positive	34 (59)	22 (63)[d]		16 (100)
Low titer	32 (55)	19 (54)	1.000	0 (0)
High titer	26 (45)	16 (46)		16 (100)

One participant was included in both SOTR dose 2 cohort and SOTR dose 3 cohort.
[a]*P*-value comparing SOTR dose 2 and SOTR dose 3 cohort were computed using 2-tailed Fisher's exact test.
[b]In the SOTR dose 2 cohort, 1 participant received both kidney and heart transplants, and 1 participant received both kidney and liver transplants; in the SOTR dose 3 cohort, 1 participant received both kidney and pancreas transplants, and 1 participant received both lung and other specified transplant.
[c]Antirejection medication use was not mutually exclusive.
[d]Six participants in the dose 3 cohort were seropositive before receiving a third dose.
HC, healthy control; IgG, immunoglobulin G; mRNA, mRNA; mTOR, mechanistic target of rapamycin; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; SOTR, solid organ transplant recipient.

with regard to age, sex, race, type of transplanted organ, baseline use of immunosuppressants, and type of vaccine received. Fifty-nine percent of the SOTR dose 2 cohort seroconverted after the 2-dose vaccine series. Among the SOTR dose 3 cohort, 6 (17%) had already seroconverted after 2 doses and after 3 doses a total of 22 (63%) were then seropositive (**Table 1**).

Prevaccine Cytokines in the 2-dose Cohort (HC Versus SOTR Dose 2)

Overall, the median levels of baseline cytokines and chemokines in the HCs were lower than both SOTR subgroups. After correcting for multiple comparisons IFN-α2a, IFN-γ, IFN-λ1, IL-16, IL-18, IL-1RA, IL-21, IL-22, IL-2Ra, IL-6, IL-7, IL-8, IP-10, MCP-1, MIP-1α, TNF-α, and VEGF were all significantly lower in the HCs compared with both the low-titer SOTR group and the high-titer SOTR group (FDR < 0.05) (**Figure 1**). IL-15 was also significantly lower in HCs compared with the low-titer group but not the high-titer group. When comparing the low-titer SOTR group to the high-titer SOTR group several cytokines and chemokines were higher in the high-titer group including IFN-γ, IL-17, IL-18, IL-6, IL-8, and TNF-α, but none of these differences reached statistical significance after correcting for multiple comparisons (**Figure 1**). When subdividing the SOTR dose 2 cohort into those who had any positive antibody response after receiving 2 doses of an mRNA vaccine (positive) and those with an undetectable response (negative), the same trends were observed; however, no statistically significant differences were detected after correcting for multiple comparisons (**Figure S1, SDC,** http://links.lww.com/TP/C345).

Prethird Dose Cytokines in the SOTR Dose 3 Cohort

Cytokines and chemokines were also measured in the SOTR dose 3 cohort before receiving a third dose

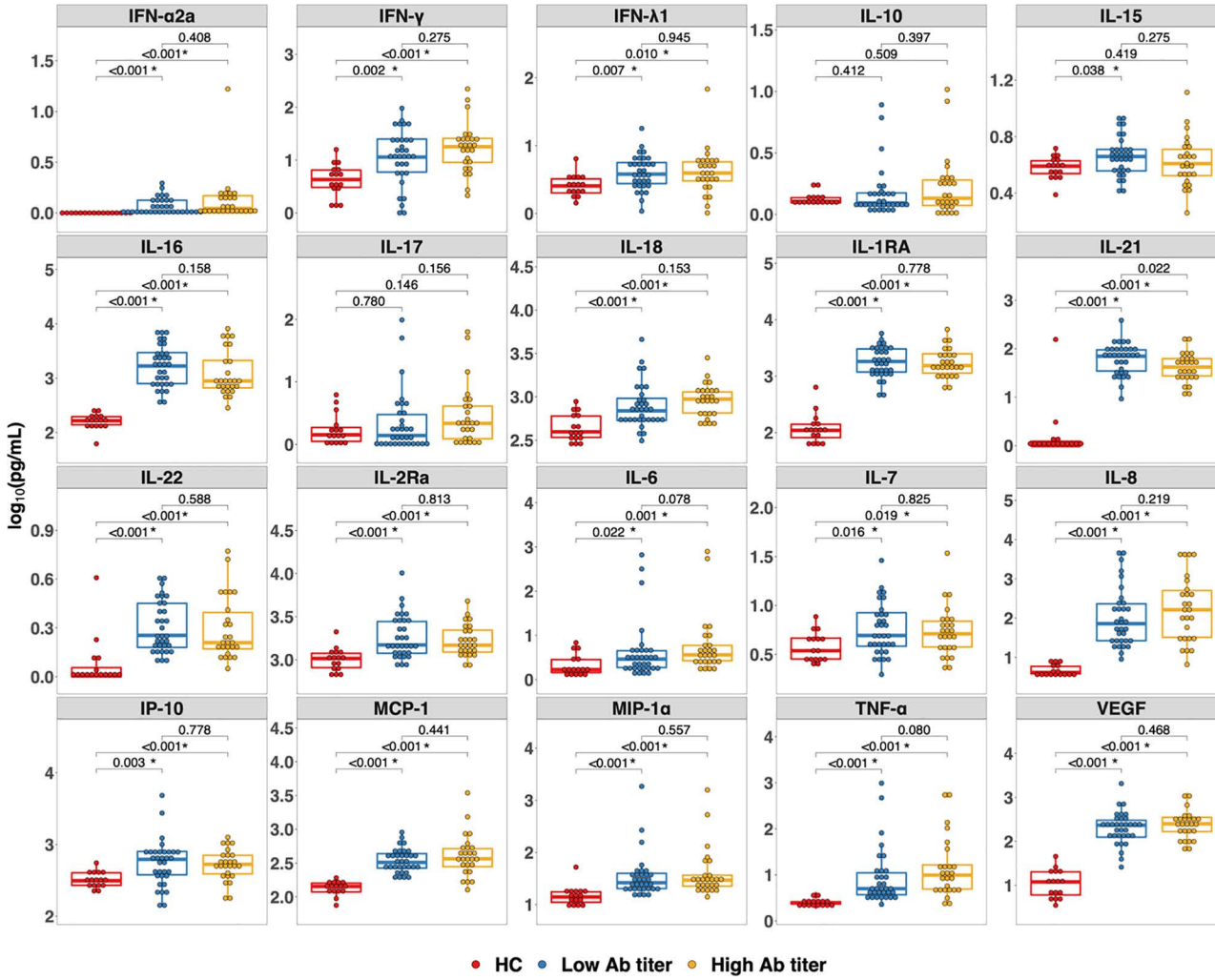

FIGURE 1. Cytokines in SOTRs and healthy controls before first dose of a 2-dose mRNA-based vaccine series. Each cytokine or chemokine measured is indicated in the gray header above each panel. Differences among the healthy controls (red, n = 16), SOTRs who developed low-titer responses (blue, n = 32), and SOTRs who developed high-titer responses (yellow, n = 26) after vaccination were determined by a 2-tailed Wilcoxon-Rank-Sum test. Multiple comparisons were controlled using the Benjamini-Hochberg procedure with false discovery rate of 0.05. Significant *P* after adjusting for multiple comparisons are marked with *. The boxplots represent the interquartile range. The median is represented by a horizontal line in the box. The lower and upper whiskers represent 1.5× the interquartile range beyond the quartiles. Each dot represents an individual sample. Low-titer (ratio < 3.5) and high-titer (ratio ≥ 3.5) were based on the classification of low-titer vs high-titer convalescent plasma set by the FDA.[20] Ab, antibody; HC, healthy control; IFN, interferon; IL, interleukin; IP-10, Interferon-γ-inducible protein 10; MCP, monocyte chemoattractant protein; MIP, macrophage inflammatory protein; SOTR, solid organ transplant recipient; TNF, tumor necrosis factor; VEGF, vascular endothelial growth factor.

of SARS-CoV-2 vaccine. This cohort was also subdivided based on low-titer or high-titer antibody response after the third dose. When comparing these groups with the HCs, there were again differences between the HCs and the SOTR cohorts. Specifically, IL-15, IL-16, IL-18, IL-1RA, IL-21, IL-22, IL-2Ra, TNF-α, VEGF were significantly lower in HCs compared with the low-titer group and IFN-α2a, IFN-λ1, IL-16, IL-17, IL-18, IL-1RA, IL-21, IL-22, IL-2Ra, IL-6, IL-8, IP-10, MIP-1α, TNF-α, VEGF were significantly lower in HCs compared with the high-titer group (FDR < 0.05) (**Figure 2**). When comparing the low-titer to high-titer SOTR groups, IL-17, IL-2Ra, IL-6, IP-10, MIP-1α, and TNF-α were all significantly higher in the high-titer group even after adjusting for multiple comparisons (FDR < 0.05) (**Figure 2**). When comparing cytokines and chemokines in SOTRs with no detectable antibody response after a third dose to those with any positive response, these same cytokines and chemokines levels were higher in the positive subgroup, but the differences did not reach statistical significance after adjustment for multiple comparisons (**Figure S2, SDC,** http://links.lww.com/TP/C345).

To provide another measure of the immune response between the low-titer and high-titer group, BAU and pseudoneutralization were measured in the dose 3 cohort using a quantitative ELISA and ACE2-spike inhibition assay, respectively. Those who developed high-titer responses as measured by the Euroimmun assay had significantly higher BAU and pseudoneutralization compared with the low-titer group (**Figure S3, SDC,** http://links.lww.com/TP/C345). The median BAU values (IQR) were 15 (1–49) for the low-titer group and 644 (250–1648) for the high-titer group.

Subgroup Analysis in the SOTR Dose 3 Cohort

In an effort to understand whether other factors influenced these findings, subgroups were explored. To determine if type of vaccine received impacted cytokine levels

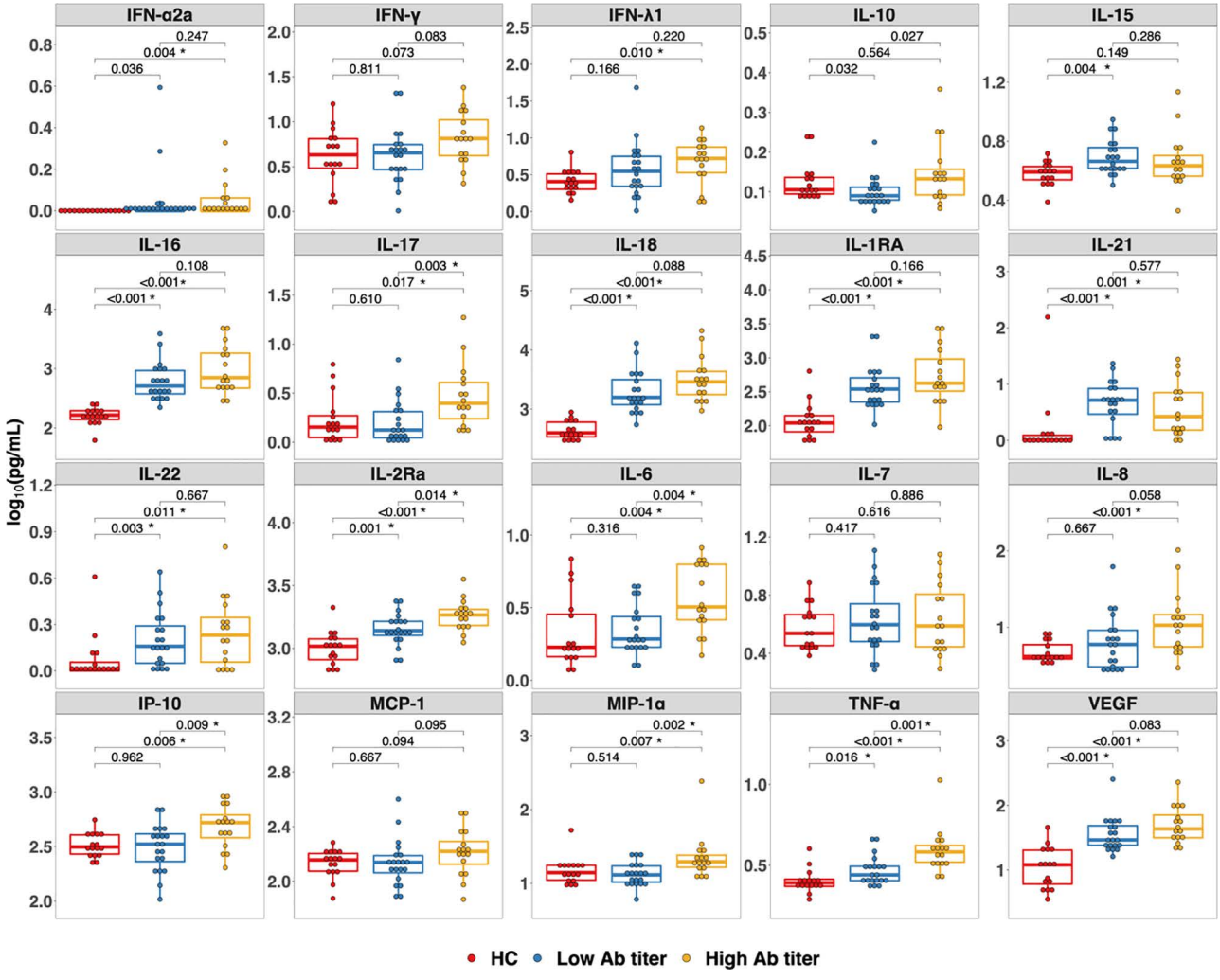

FIGURE 2. Cytokines in SOTRs before a third dose of SARS-CoV-2 vaccine and healthy controls before first dose of a 2-dose mRNA-based vaccine series. Each cytokine or chemokine measured is indicated in the gray header above each panel. Differences among the healthy controls (red, n = 16), SOTRs who developed low-titer responses (blue, n = 19), and SOTRs who developed high-titer responses (yellow, n = 16) after a third dose of SARS-CoV-2 vaccine were determined by a 2-tailed Wilcoxon-Rank-Sum test. Multiple comparisons were controlled using the Benjamini-Hochberg procedure with false discovery rate of 0.05. Significant *P* after adjusting for multiple comparisons are marked with *. Low-titer (ratio < 3.5) and high-titer (ratio ≥ 3.5) were based on the classification of low-titer vs high-titer convalescent plasma set by the FDA.[20] Ab, antibody; HC, healthy control; IFN, interferon; IL, interleukin; IP-10, Interferon-γ-inducible protein 10; MCP, monocyte chemoattractant protein; MIP, macrophage inflammatory protein; SOTR, solid organ transplant recipient; TNF, tumor necrosis factor; VEGF, vascular endothelial growth factor.

before a third dose, cytokines from those SOTRs who received BNT162b were compared with those who received mRNA-1273. No cytokines or chemokines were significantly different between these 2 groups (**Figure S4, SDC,** http://links.lww.com/TP/C345). The use of antimetabolite immunosuppression has been associated with decreased response to the SARS-CoV-2 vaccines[14] and could influence cytokine levels. Therefore, cytokines were compared between the subgroup that was on antimetabolite immunosuppression and the subgroup that was not; there were no significant differences (**Figure S5, SDC,** http://links.lww.com/TP/C345). As described, 6 patients from the dose 3 cohort already had seroconverted before a third dose. Cytokines from these patients were compared with those who had not seroconverted before a third dose, but no significant differences were observed (**Figure S6, SDC,** http://links.lww.com/TP/C345). Finally, given that the third dose cohort was composed of SOTRs who had minimal response to 2 doses, we compared the cytokines in this group before a third dose to the cytokines in the 2-dose cohort before any vaccination. IFN-γ, IL-16, IL-1RA, IL-21, IL-8, IP-10, MCP-1, MIP-1α, TNF-α, and VEGF were all significantly lower in the dose 3 cohort while IL-18 was higher in the dose 3 cohort (**Figure S7, SDC,** http://links.lww.com/TP/C345).

DISCUSSION

Despite widespread recognition of poor humoral immune responses to SARS-CoV-2 vaccination in SOTRs, mechanisms underlying these suboptimal responses and prediction of response to additional vaccine doses remain obscure. Data in this series suggest that a specific pattern of immune activation, as measured by plasma cytokines and chemokines, may distinguish those SOTRs who are likely to develop higher antibody titers to a third dose of SARS-CoV-2 vaccine. The majority of cytokines identified as significantly different are innate immune cytokines, consistent with the role of the innate immune system in initiating and modulating the adaptive, including the antibody, response to pathogens. IL-6, TNF-α, MIP-1α, and IP-10 are all produced by innate immune cells such as macrophages and natural killer cells in response to pathogen recognition.[21-23] IL-17 and IL-2Ra can be produced by innate immune cells, but are also associated with ongoing T-cell activation.[24,25] Several of these cytokines, particularly TNF-α and IL-6, have previously been shown to be important in developing immune response against viral vaccines.[15,26,27] Thus, these data possibly reflect a role for the innate immune system in the response to SARS-CoV-2 vaccines in SOTRs. Given that these cytokines were elevated in the high-titer group, this may signify a state of less immunosuppression and greater ability to respond to additional vaccine antigen exposure. This is further supported by the fact that the dose 2 cohort had mostly higher cytokine and chemokine levels than the dose 3 cohort which consisted of participants with low or no response to the initial 2-dose series. However, despite this trend in SOTRs, HCs had overall very distinct cytokine and chemokine profiles. This likely reflects major alterations in baseline immune state following organ transplantation and subsequent use of immunosuppressive medications. Furthermore, this suggests that strategies to enhance the response of SOTRs to SARS-CoV-2 vaccines should be evaluated independently of strategies used for other populations.

While this study demonstrated similar trends in innate cytokines in the high-titer group within the 2-dose cohort, these differences did not reach statistical significance. This could be explained by the relatively small sample size but could also be due to greater heterogeneity of immune state within the 2-dose cohort. An important consideration in interpreting these data is that the 3-dose cohort comprised SOTRs who had a poor response to 2 doses, and therefore, were uniformly composed of those with weak or no response. As a result, it may be easier to distinguish differences in response among this more immunological homogenous group with more uniformly low baseline SARS-CoV-2 response.

Although these results support that cytokine profiles may identify the SOTRs who are likely to benefit from a third dose of SARS-CoV-2 vaccine, this study does have several limitations. This was a single cohort, single-center, observational study. The samples were not collected at precisely the same time relative to vaccination, different transplanted organ recipients were allowed to participate, and sample size was relatively small. This heterogeneity limits the general applicability of the results. Although a broad cytokine and chemokine panel was used, this analysis was limited to a subset of these immune signaling molecules and there may be other unmeasured cytokines and chemokines that contribute to vaccine response. While some significant differences were detected, the sample sizes were relatively small, and a larger study might allow identification of additional inflammatory markers associated with robust vaccine responses. Furthermore, comprehensive medical histories or clinical laboratory data were not available for these participants. Therefore, it was not possible to determine whether prior episodes of rejection, infections, immunosuppressant drug levels, or absolute lymphocyte count (ALC) may influence the findings. Moreover, the majority of the SOTRs in this observational study were kidney and/or liver transplant recipients reflecting the demographics of organ transplantation in the United States. Therefore, it is unclear whether these findings would hold for lung or heart transplant recipients who were underrepresented in this study. The HCs were younger than the SOTR group, and therefore some of the observed differences in resting cytokine levels may be related to age rather than SOTR status. Additionally, cellular responses to the vaccine were not measured in this study, though they are thought to be reduced compared with healthy controls.[3] We also are unable to comment on whether these antibody responses are truly reflective of protection from COVID-19. While the ACE2 inhibition assay reported here has excellent correlation with live-virus neutralization, it is not a perfect correlation, therefore it is not clear if these cytokine signatures are associated with true neutralization and/or protection from infection.[28-30]

While it is impractical to measure twenty cytokines on an individual before vaccination, several of the cytokines associated with a more robust third dose response (such as IL-6 and TNF-α) are available for clinical use. Furthermore, in a more controlled study these cytokines could be correlated with laboratory tests that are routinely available (such as ALC and C-reactive protein)

which could be used to guide recommendations about additional vaccine doses or other strategies to increase protection from COVID-19.

Despite these limitations, these novel findings highlight that specific cytokine profiles may be associated with greater antibody responses in some SOTRs. Should this prove to be true in a larger study, this could be used to target strategies for enhancing vaccine responses in specific SOTRs. These results indicate that there might be identifiable and measurable characteristics in SOTRs that could be used to stratify this population into those who would most benefit from additional doses of currently available SARS-CoV-2 vaccines and those who may require alternative protection strategies (ie, novel vaccine platforms, immunosuppression reduction before vaccination, or passive immunoprophylaxis with antibody infusions). Additional investigations with a larger cohort will be required to determine if the data presented here can be extrapolated to the greater transplant population.

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