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RESEARCH ARTICLE

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Hybrid immunity by two COVID-19 mRNA vaccinations and one breakthrough infection provides a robust and balanced cellular immune response as basic immunity against severe acute respiratory syndrome coronavirus 2

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Abstract

This longitudinal prospective controlled multicenter study aimed to monitor immunity generated by three exposures caused by breakthrough infections (BTI) after COVID-19-vaccination considering pre-existing cell-mediated immunity to common-corona-viruses (CoV) which may impact cellular reactivity against SARS-CoV-2. Anti-SARS-CoV-2-spike-IgG antibodies (anti-S-IgG) and cellular reactivity against Spike-(S)- and nucleocapsid-(N)-proteins were determined in fully-vaccinated (F) individuals who either experienced BTI (F+BTI) or had booster vaccination (F+Booster) compared to partially vaccinated (P+BTI) and unvaccinated (U) from 1 to 24 weeks post PCR-confirmed infection. High avidity anti-S-IgG were found in F+BTI compared to U, the latter exhibiting increased long-lasting pro-inflammatory cytokines to S-stimulation. CoV was associated with higher cellular

reactivity in U, whereas no association was seen in F. The study illustrates the induction of significant S-specific cellular responses in F+BTI building-up basic immunity by three exposures. Only U seem to benefit from pre-existing CoV immunity but demonstrated inflammatory immune responses compared to F+BTI who immunologically benefit from enhanced humoral and cellular immunity after BTI. This study demonstrates that individuals with hybrid immunity from COVID-19-vaccination and BTI acquire a stable humoral and cellular immune response that is maintained for at least 6 months. Our findings corroborate recommendations by health authorities to build on basic immunity by three S-protein exposures.

KEYWORDS
breakthrough infection, common corona viruses, COVID-19, hybrid immunity, mRNA vaccination, SARS-CoV-2

1 | INTRODUCTION

The majority of countries worldwide recommend basic immunity against COVID-19 by at least three severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) exposures: two exposures generated by COVID-19 vaccinations (primary COVID-19 vaccination) and a third exposure by either one booster vaccination or breakthrough infection (BTI). It has been speculated that pre-existing cell-mediated immunity to common corona viruses (CoV) may impact the cellular reactivity against SARS-CoV-2. The SARS-CoV-2 genome has a high degree of homology to CoV, such as OC43, HKU1, NL63, and 229E, with about 65% homologies.[1] The four structural proteins spike (S), envelope (E), membrane (M), and nucleocapsid (N) are expressed together with 16 nonstructural proteins and 9 accessory proteins from a positive-sense, single-stranded RNA.[2–4] The S-protein mediates viral cell entry by binding to the host angiotensin-converting enzyme 2 (ACE2) receptor and is substrate of ongoing immune escape mutations changing the transmissibility and antigenicity of the virus and resulting in variants of concern (VOC),[5,6] such as Omicron subvariants.[7]

Waning titers of neutralizing antibodies against S-protein (anti-S-IgG) have been reported within 24 weeks postimmunization associated with an increased risk of BTI.[8] In contrast to humoral immunity, cellular immune responses induced by vaccine-encoded S-protein are proposed to be robust and long-standing.[9] Recommendations by health authorities for basic immunity by three exposures are mainly based on antibody studies showing efficient neutralizing anti-S-IgG titers against VOCs.[10,11] Human challenge studies suggested that CoV may prime the cellular immune response and contribute to basic immunity against SARS-CoV-2.[12] This prospective longitudinal multicenter cohort study aimed to monitor basic humoral and cellular immunity generated against spike-SARS-CoV-2 by vaccination and/or BTI exposure taking into account baseline immunity by cross-reactivity to CoV. The study aimed to provide evidence of basic immunity against SARS-CoV-2 by three S-protein exposures as a reference for future vaccination recommendations.

2 | RESULTS

2.1 | Basic immunity demonstrated by high concentrations and avidity of anti-S-IgG antibodies

All individuals included in this study experienced mild to moderate symptoms of infection. None of them reported severe symptoms. Arterial hypertension is the comorbidity with the highest number of patients per group. No significant differences were established considering any of the comorbidity parameters described in Table 1. Significantly, higher anti-S-IgG concentrations were found in F+BTI compared to U (Figure 1A). In all groups, anti-S-IgG declined between w3–7 and w17–24, however, a much steeper decrease was seen in both, U and P++BTI, compared to F+BTI (Figure 1A, Supporting Information S1: Table 1). A group of individuals who had received a booster dose as a third S-protein exposure instead of experiencing BTI were included in this study (F+Booster) for comparison. As observed in the other groups, concentrations of anti-S-IgG were significantly reduced between w4 and w24 (Figure 1A, Supporting Information S1: Table 2).

Avidity maturation was assessed by binding strength of anti-S-IgG antibodies and revealed that at all time points, more individuals who were fully vaccinated demonstrated higher avidity (RAI > 60%) than U and P+BTI ($p < 0.0001$). The majority of individuals in the U and P+BTI groups showed low avidity anti-S-IgG (RAI < 40%) within the first 7 weeks postinfection and an increase from low to moderate and high avidity afterwards (Figure 1B, Supporting Information S1: Table 1). A positive correlation between anti-S-IgG and RAI was already established at w3-7 post BTI ($r = 0.621$, $p = 0.024$) in F+BTI. In contrast, a positive correlation between anti-S-IgG and RAI was found w17-24 postinfection in P ($r = 0.736$, $p = 0.010$) and U ($r = 0.750$, $p = 0.001$). Contrary to what is observed in individuals with BTI, F+Booster showed a significant reduction in avidity from

TABLE 1 Baseline characteristics of study participants.

	Unvaccinated (U)	Partially vaccinated (P+BTI)	Fully vaccinated (F+BTI)	Fully vaccinated (F+Booster)
n	18	11	18	37
Age mean (years)	37.8	44.5	42.6	48.1
(Minimum–maximum)	(20–68)	(21–75)	(21–63)	(22–66)
Male (*n*; %)/female (f; %)	4 (22.2)/14 (77.8)	5 (45.5)/6 (54.5)	5 (27.8)/13 (72.2)	4 (10.8)/33 (89.2)
Time last vaccination (weeks)	N/A	5.3	16.3	14.0
Mean (minimum–maximum)		(2.0–7.8)	(10.0–22.3)	(4.0–24.0)
Variant of concern	Alpha: 4 (22.2)	Alpha: 6 (54.5)	Alpha: 3 (16.7)	N/A
(VOC) *n* (%)	Delta: 6 (33.3)	Delta: 5 (45.5)	Delta: 14 (77.8)	
	ND: 8 (44.4)		ND: 1 (5.6)	
Symptoms				N/A
Severity Scale				
Asymptomatic	0 (0.0%)	2 (18.1%)	1 (5.6%)	
Mild	2 (11.1%)	5 (45.5%)	5 (27.8%)	
Moderate	15 (83.3%)	4 (36.4)	12 (66.7%)	
Severe	0 (0.0%)	0 (0.0%)	0 (0.0%)	
No information	1 (5.6%)			
Comorbidities				N/A
Arterial hypertension	3 (16.7%)	4 (36.4%)	3 (16.7%)	
Asthma	2 (11.1%)		2 (11.1%)	
Allergic rhinitis	2 (11.1%)	1 (9.1%)	2 (11.1%)	
Dust mite allergy	1 (5.6%)		1 (5.6%)	
Endometriosis	1 (5.6%)		1 (5.6%)	
Animal hair allergy	1 (5.6%)	1 (9.1%)	1 (5.6%)	
Hashimoto thyroiditis	1 (5.6%)	1 (9.1%)	1 (5.6%)	
Tachycardia		1 (9.1%)		
Third kidney		3 (27.3%)		
None	7 (38.9%)		7 (38.9%)	

Note: Individuals of the unvaccinated group (U), partially vaccinated group who had breakthrough infection (P+BTI) and fully vaccinated who had either BTI (F+BTI) or booster vaccination (F+Booster) were consecutively recruited in the third (Alpha) and fourth (Delta) SARS-CoV-2 waves in Germany[13] at the University Hospital Würzburg, Germany, and the Institute for Microbiology and Hygiene, Regensburg, Germany, April to November 2021. Individuals with immunosuppressive or immunomodulatory therapy, malignant disease, autoimmunity or monogenetic, or acquired immunodeficiency were excluded. F+Booster reported no comorbidities and no BTI.

Abbreviations: N/A, not appropriate; ND, not determined.

w4 to w24 after booster vaccination. Five individuals showed RAI below 60% at w24 (Figure 1B, Supporting Information S1: Table 2).

2.2 | The IFNγ cellular response to S- and N-protein is stable in fully vaccinated individuals

The IFNγ cellular response to S-protein was positive in most samples in all groups already at w1 after infection and stayed stable over time in most cases, particularly in F+BTI (Figure 2A). The N-specific cellular response tends to lower levels at later time points in P+BTI and U, whereas it stayed positive in F+BTI (Figure 2B, Supporting Information S1: Tables 3–5). Cellular immune responses to S-protein positively correlated with the response to N-protein at w1 post BTI ($r = 0.890$, $p = 0.001$) and later (w2: $r = 0.664$, $p = 0.003$; w3–7: $r = 0.691$ $p = 0.003$; w17–24: $r = 0.635$, $p = 0.008$) in F+BTI. In contrast in group P+BTI, correlations of cellular immune responses to S- and N-protein

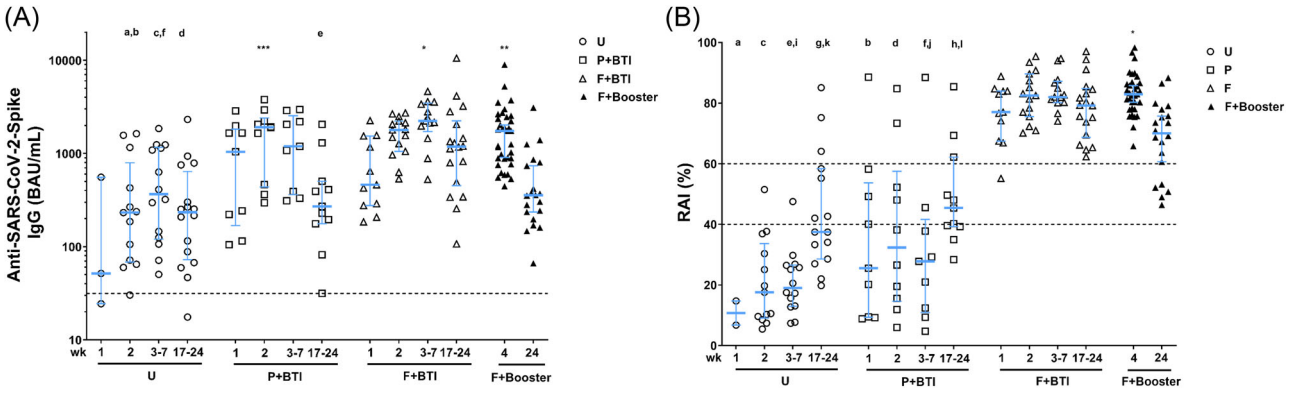

FIGURE 1 Anti-SARS-CoV-2-Spike-IgG (anti-S-IgG) concentrations (A) and relative avidity index (RAI) (B). (A) Concentrations of anti-S-IgG (BAU/mL) were determined by ELISA in serum samples from unvaccinated (U), partially vaccinated (P+BTI), and fully vaccinated (F+BTI) individuals at Week 1 (w1), w2, w3–7, and w17–24 after PCR-confirmed SARS-CoV-2 infection. Lines indicate the median ± interquartile range (whiskers). Dashed horizontal line indicate the lower limit of cut-off of the ELISA-assay (31.5 BAU/mL). Independent variables were determined by Kruskal–Wallis-test and Bonferroni-corrected. a indicates comparison at w2 groups U versus P+BTI ($p = 0.002$), b: at w2 U versus F+BTI ($p = 0.001$), c: at w3-7 U versus F+BTI ($p = 0.0002$), f: at w3–7 U versus F+Booster (w4) ($p = 0.004$), d: at w17–24 U versus F+BTI ($p = 0.0002$) and e: at w17–24 P+BTI versus F+BTI ($p = 0.045$). Comparison of dependent samples at different time points was performed by Friedman test group P+BTI (w2 vs. w17–24, $^{***}p < 0.0001$) and group F+BTI (w3-7 vs. w17–24, $^{*}p < 0.01$). Group F+Booster (w4 vs. w24, $^{**}p < 0.001$). (B) The relative avidity index (RAI) was determined at w1, w2, w3–7, and w17–24 after PCR-confirmed SARS-CoV-2 infection. Dashed lines indicate the border for low avidity (RAI < 40%), moderate avidity (40% ≤ RAI < 60%), and high avidity (RAI ≥ 60%). Significant values were determined by Kruskal–Wallis-test and corrected by Bonferroni. a: w1 (U vs. F+BTI, $p = 0.028$), b: w1 (P+BTI vs. F+BTI, $p = 0.016$), c: w2 (U vs. F+BTI, $p < 0.0001$), d: w2 (P+BTI vs. F+BTI, $p = 0.005$), e: w3–7 (U vs. F+BTI, $p < 0.0001$), i: w3–7/w4 (U vs. F+Booster, $p = 0.0003$), f: w3–7 (P+BTI vs. F+BTI, $p = 0.05$), j: w3–7/w4 (P vs. F+Booster, $p = 0.001$) g: w17–24 (U vs. F+BTI, <0.0001), h: w17–24 (P+BTI vs. F+BTI, $p = 0.03$), k: w17–24/w24 (U vs. F+Booster, $p = 0.001$), l: w17–24/w24 (P+BTI vs. F+Booster, $p = 0.020$). Comparison of dependent samples at different time points was performed by Friedman test group F+Booster (w4 vs. w24, $^{*}p < 0.01$).

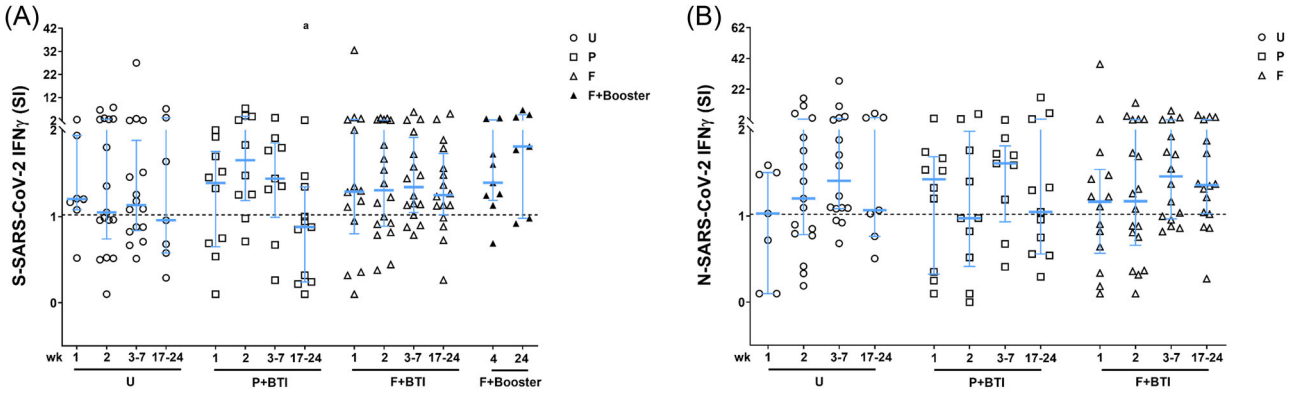

FIGURE 2 Interferon-gamma (IFNγ) production after stimulation of PBMCs with SARS-CoV-2-specific spike (S) and nucleocapsid (N) protein. IFNγ production upon SARS-CoV-2 spike (A) and nucleocapsid protein (B) stimulation of PBMCs from unvaccinated (U), partially vaccinated (P), and fully vaccinated (F) individuals at Week (wk) 1, Weeks 2, Weeks 3–7, and Weeks 17–24 after PCR-confirmed SARS-CoV-2 infection was determined by ELISpot. Spot forming units (SFU) normalized to 10^6 PBMCs were determined and used to calculate the stimulation index (SI) as: SFU with antigen (spike, nucleocapsid or positive control)/SFU of negative control. Medium was used as negative and staphylococcal enterotoxin B (20 ng/mL) (SEB; Sigma) as positive control. Positive T cell reactivity was considered for SI values above 1.04 as determined by pilot experiments with defined negative prepandemic samples. Significant values were determined by Kruskal–Wallis-test and corrected by Bonferroni. a: w17–24/24 (P+BTI vs. F+Booster, $p = 0.037$). Lines indicate the median ± interquartile range (whiskers).

were present only at w1 ($r = 0.794$, $p = 0.006$) and at w17–24 ($r = 0.0767$, $p = 0.016$) post BTI. In U, positive correlations were found at w3–7 ($r = 0.524$, $p = 0.037$) and at w17–24 ($r = 0.964$, $p < 0.001$). In U und F+BTI, age did neither correlate with antibody nor with cellular responses (Figure 5A–D). Group P demonstrated a negative correlation between age and cellular immune responses against S-protein (Figure 5E,F) or against N-protein ($r = -0.772$, $p = 0.009$). The cellular response to S-protein was significantly higher in F+Booster compared to P+BTI at w24 (Figure 2A).

2.3 | Higher IFNγ production in response to CoV-specific S-protein in unvaccinated individuals

As cellular cross-reactivity to CoV may bias the SARS-CoV-2-specific cellular response particularly during the earliest time point after SARS-CoV-2 infection, cell-mediated immunity against S-protein derived from CoV types was assessed (Figure 3A). Four individuals from group F+BTI, two from P+BTI and one from U showed negative cellular reactivity against CoV (Figure 3B). Comparing cellular responses against SARS-CoV-2-specific S-protein in fully vaccinated individuals between individuals with and without cellular responses in the CoV panel, no difference could be found at any time point regarding humoral or cellular responses, including reactivity against SARS-CoV-2-derived S-protein and N-proteins and cytokine release (data not shown). Individuals with positive response to CoV showed no significant difference regarding cellular reactivity against SARS-CoV-2-S-proteins between the groups (Figures 3B and S1, Supporting Information S1: Table 7). The IFNγ production upon stimulation with M-peptide pools did not vary among the three study groups (Figure S1). However, in P+BTI, the trend toward higher SFU against CoV correlated with younger age ($r = -0.933$, $p = 0.0001$), with the SARS-CoV-2-S- and N-protein-specific SI ($r = 0.857$, $p = 0.007$ and $r = 0.714$, $p = 0.047$, respectively) and IFNγ release ($r = 0.810$; $p = 0.015$) at w1. In U, SFU after stimulation with CoV antigens at w1 resulted in a positive correlation with SI induced by SARS-CoV-2-S-protein ($r = 0.587$, $p = 0.045$). SI induced by CoV positively correlated with SI induced by SARS-CoV-2-N-protein at w2 ($r = 0.700$, $p = 0.016$). In U, a significant correlation of SFU caused by CoV antigens was seen with TNF-α production induced by SARS-CoV-2-S-protein at w1 ($r = 0.941$, $p = 0.005$). No correlations of CoV-induced SFU with any immunogenicity parameters were present in group F+BTI.

2.4 | Unvaccinated individuals demonstrate a pro-inflammatory cytokine profile after SARS-CoV-2 infection

The concentrations of IL-2, IL-4, IL-5, IL-6, IL-9, IL-10, IL-13, IL-17A, IL-17F, IL-22, IFNγ, and TNF-α were determined in cell-culture supernatants from U, P+BTI, and F+BTI following stimulation with SARS-CoV-2-specific S-protein. Significantly, higher concentrations of IL-2, IL-6, IL-17A, IFNγ and TNF-α were found in group U compared to P+BTI or F+BTI, respectively (Figure 4, Supporting Information S1: Table 8). Low levels of IL-4, IL-5, IL-9, IL-10, IL-13, IL-17F, and IL-22 were found in all individuals after stimulation with S-protein at all observation time points (Supporting Information S1: Table 8).

Correlations with age, time since last vaccination and immunological parameters were determined at each time point (Figure 5).

IL-6 is known as an important cytokine for B cell differentiation and antibody maturation. Therefore, the association of IL-6 concentrations with humoral and cellular immunogenicity markers was longitudinally analyzed. In group F+BTI, early responses with higher IL-6 concentrations at w1 were significantly associated with immunological parameters at later time points, for example anti-S-IgG concentrations at w2 ($r = 0.636$, $p = 0.035$), w3–7 ($r = 0.767$, $p = 0.026$) and w17–24 ($r = 0.584$, $p = 0.028$), RAI at w3–7 ($r = 0.783$, $p = 0.013$) and SFU at w1 (Figure 5E), and at w3–7 ($r = 0.560$, $p = 0.047$). IL-6 concentrations at later time points were not significantly associated with antibody or S-specific

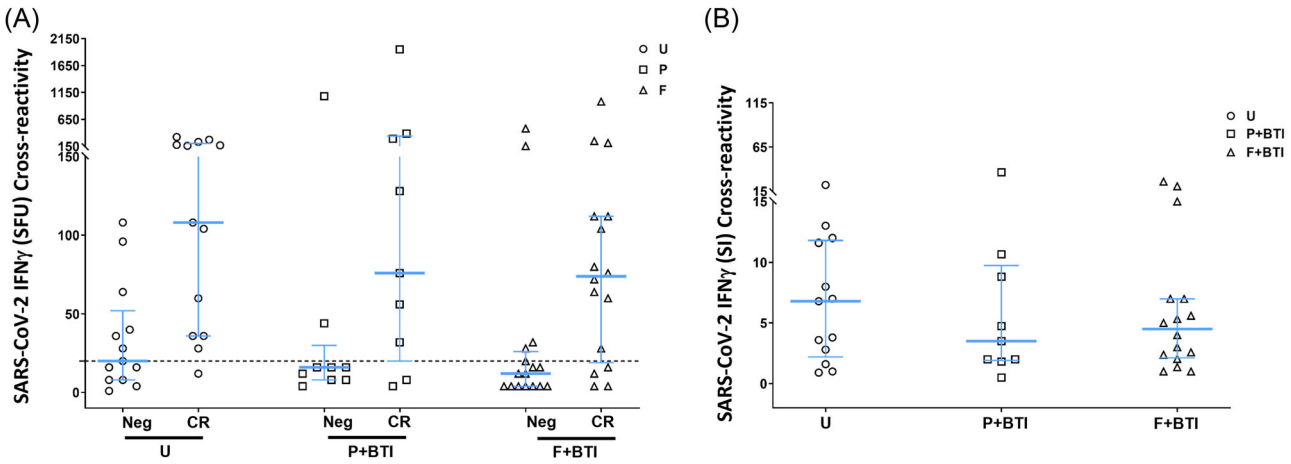

FIGURE 3 Interferon-gamma (IFNγ) response to spike peptides from common corona viruses (CoV). The IFNγ production was determined by ELISpot as indicated in material and methods. PBMCs from unvaccinated (U), partially (P+BTI), and fully vaccinated (F+BTI) individuals at Week 1 were stimulated with a panel-set of peptides with high homology with endemic coronavirus (cross-reactivity, CR). (A) Spot forming units (SFU)/1×10^6 cells for negative control (no stimuli) and CR are shown. Positive cross-reactivity was considered when more than 20 SFU/10^6 cells were found in the cross-reactivity panel (dashed line). (B) Stimulation index (SI) was calculated SFU with antigen (cross-reactivity spike peptides from CoV)/SFU of negative control. Lines indicate median ± interquartile range (whiskers). CR, cross-reactive panel; Neg, negative control, no antigen stimulation.

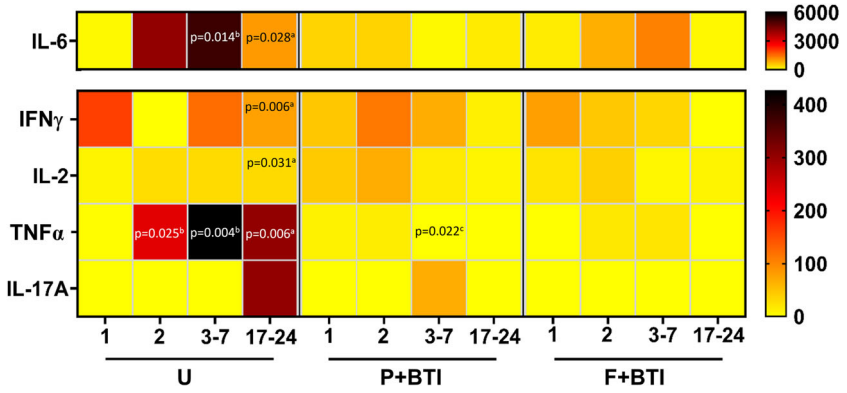

FIGURE 4 Cytokine profile after SARS-CoV-2 infection. The concentrations of IL-6, IFNγ, IL-2, TNF-α, and IL-17A (pg/mL) in plasma samples were determined from unvaccinated (U), partially vaccinated (P), and full-vaccinated (F) individuals at Week (wk) 1, Week 2, Weeks 3–7, and Weeks 17–24 after PCR-confirmed SARS-CoV-2 infection after stimulation with S-protein in a cytokine release assay as indicated in material and methods. Significant values were determined by Kruskal–Wallis-test and corrected by Bonferroni. Comparison at specific time points: a: U versus F, b: U versus P, c: P. versus F.

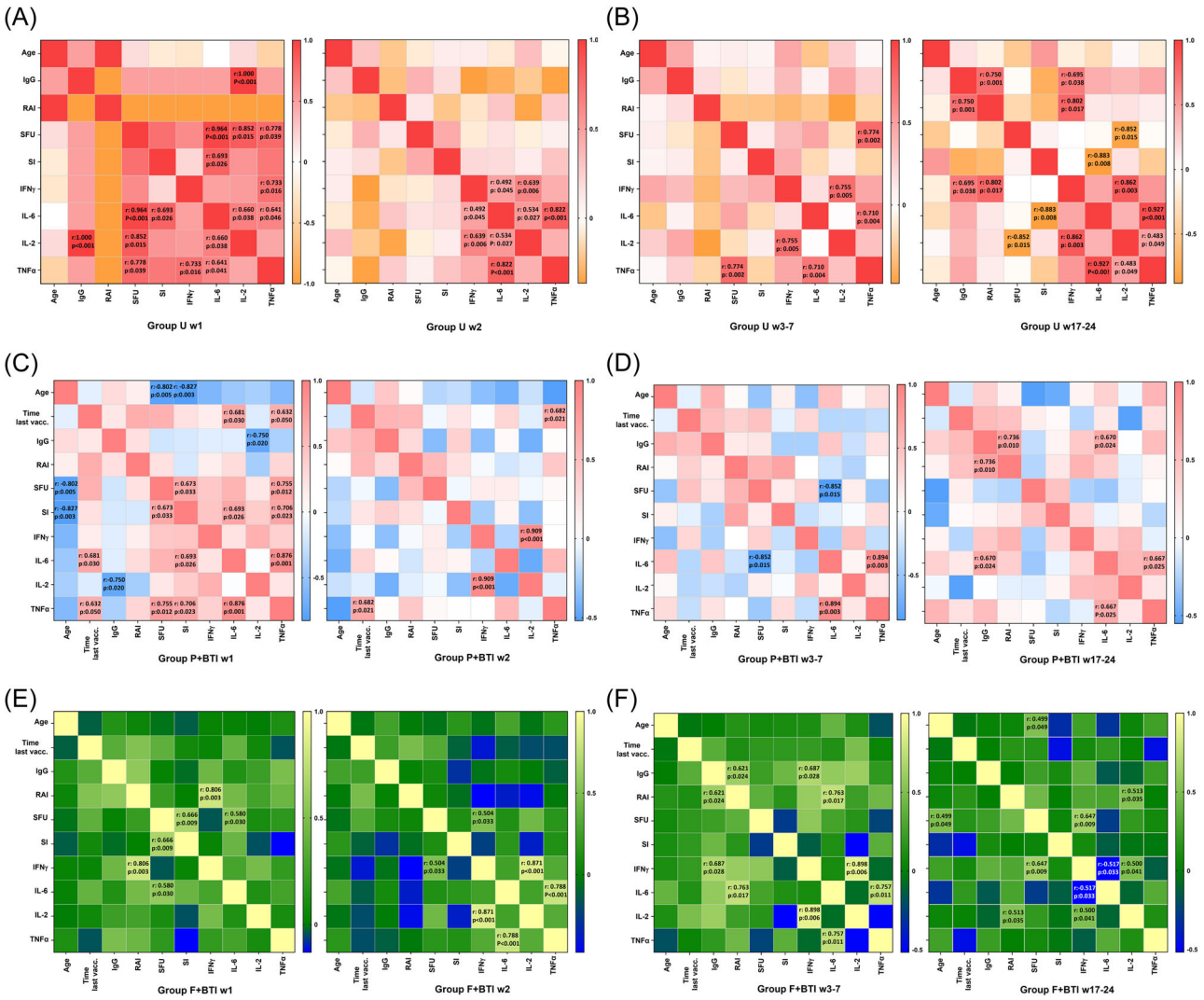

FIGURE 5 Correlations of age, time since last vaccination and immunological parameters at each time point. Immunological parameters (anti-SARS-CoV-2-spike IgG concentrations, IgG; relative avidity index, RAI; IFNγ-sproducing spot forming units/10^6 cells, SFU; stimulation index, SI, cytokine concentrations in release assays, interferon-gamma (IFNγ), Interleukin-6 (IL-6), Interleukin-2 (IL-2), and tumor-necrosis-factor-alpha (TNF-α) were correlated to age (years) and time since last vaccination (weeks) using Spearman's rank correlation coefficient (*r*) in each group, unvaccinated individuals (group U) (A and B) partially vaccinated individuals (group P+BTI) (C and D), and fully vaccinated individuals (group F+BTI) (E and F), and at Week 1 (w1), w2, w3–7, and w17–24. A $p < 0.05$ indicates significance.

cellular responses, except at w3–7 when an association was found with higher RAI (Figure 5F). In U, higher IL-6 concentrations at w1 were significantly associated with higher SFU at w1 (Figure 5A) and later at w3–7 ($r = 0.933$, $p = 0.001$).

TNF-α, an important factor of innate immune responses, at w1 was associated with higher SFU at w2 ($r = 0.584$, $p = 0.028$) in F+BTI. A similar effect was seen in U, TNF-α concentrations at w1 correlated with SFU at w1 (Figure 5A), and at w3–7 ($r = 0.708$, $p = 0.049$). In P+BTI, TNF-α correlated with SFU at w1 (Figure 5C). TNF-α responses at w2 were associated with higher SFU at w3–7 ($r = 0.636$, $p = 0.048$).

IL-2, a factor for expansion of activated T cells, showed correlation to SFU at w1 in U (Figure 5A).

In U, at w17-24, high IFNγ production was associated with higher anti-S-IgG and RAI (Figure 5B), whereas IFNγ concentrations did not show correlations in the other two groups P+BTI and F+BTI. In F+BTI, the RAI significantly correlated with the IFNγ response to S-protein at w1 (Figure 5E), whereas this correlation was not seen in U or P+BTI.

A correlation of cytokine concentrations with time after mRNA vaccination was only seen for group P but not for the other groups U and F. At w1, IL-6 and TNF-α responses were positively associated with longer time since single mRNA vaccination in P+BTI (Figure 5C). At w2, TNF-α responses were still associated with time after single mRNA vaccination.

3 | DISCUSSION

The emergence of new SARS-CoV-2 immune-escape VOCs shows that prevention of severe disease should not exclusively be based on the neutralizing activity of anti-S-IgG, but depends also on T-cell immunity to probably more conserved regions of the S-protein.[13,14] Therefore, the generation of immunity may be dependent on the development of a robust and stable cellular response from both B- and T-cells. This study corroborates that healthy individuals who had received two mRNA vaccinations and had experienced BTI may have immunological benefits from a robust B-cell response by the generation of S-specific high avidity antibodies compared to those who either had a single vaccine dose or were not vaccinated as recently published.[15] Accumulation of somatic mutations in S-specific memory B-cells[16] generating antibodies with high binding capacity against S-protein antigens may be accelerated by complete primary vaccination in combination with BTI rather than by one exposure to S-protein during primary infection. In a previous study, we have demonstrated that individuals who had at least three exposures not only increased the concentration of S-specific IgG antibodies, but also improved avidity and neutralization capacity.[11] Similar effects were also observed in a cohort of patients undergoing hemodialysis who had further exposures by either vaccine-encoded S-protein or by natural infection with Omicron variant.[10] The avidity maturation was significant better in those individuals who had hybrid immunity compared to those who had exclusively three COVID-19-vaccinations at w24. This may be explained by the broader selection process of high-affinity B-cell clones by vaccine-encoded S-specific mRNA and wildtype S-protein.

The cell-mediated immune response to S-protein provides the second base of immunity against severe courses of COVID-19 and has drawn increasing attention in the waves with immune-escape VOCs in which antibodies in general failed to provide protection against infection and transmission. Our data indicate a robust and stable cellular response to S- and N-proteins in fully vaccinated individuals already at w1 after BTI. Unvaccinated individuals or those who had received only one vaccine dose reached N-specific cell-mediated immunity after 3–7 weeks. Only in F+BTI, SFU derived by S-protein stimulation correlated with SFU derived by N-protein, but not in P+BTI and U. This contradicts the idea of suppressed N-specific cellular responses in individuals who had exclusively vaccine-derived S-protein exposures.[17]

Interestingly, in both U and P+BTI groups the S-specific cellular response tended to drop at w17–24, which was not observed in F+BTI and in F+Booster in accordance with previous publications.[18] In SARS-CoV-2-infected individuals, the frequency of CD4+ and CD8+ memory T-cells was depleted up to 50% between 6 and 8 months postinfection.[19] Thus, our results support the idea that repeated exposures to S-protein either by vaccine-encoded S-protein or by BTI may pulse the cellular response which is not affected by the COVID-19 severity, comorbidities, age, or sex of the individuals.

Human challenge studies and pediatric cohorts have suggested that cross-reactivity toward CoV may promote specific T-cell reactivity against SARS-CoV-2 resulting in protection or less severe diseases than found in individuals without CoV contacts.[20,21] Cross-reactive T-cell epitopes have been identified in the N-protein, in the S2-region and in nonstructural proteins.[22–24] Several studies have reported the presence of cross-reactive T-cells in SARS-CoV-2-unexposed individuals[25–27] that recognize many epitopes localized outside the S-protein-region.[28] Although in this study, cross-reactivity was limited to the S-protein, individuals who showed cross-reactivity had significantly higher S-specific immune responses than those with no cross-reactive cellular response. This effect was seen in all groups with a trend towards higher SFU counts in U than found in P+BTI and F+BTI. T-cell clonal selection of immunodominant epitopes derived from vaccine-encoded S-protein may narrow the diversity of S-reactive T-cells which may partly explain the trend toward lower SFU counts against S-protein in P+BTI and F+BTI.

Comparing the four individuals from group F+BTI who were negative for cross-reactivity to those who were positive, no difference in humoral and cellular immune responses toward SARS-CoV-2-S- or N-protein were found. This indicates that the effect of cross-reactivity to CoV may be overestimated particularly in fully vaccinated individuals.

However, from cellular responses, it seems that the P+BTI group showed a different behavior than individuals of group U and F+BTI. Particularly, the age correlation seen between older age and higher SFU against S- and N-proteins was not found in U and F+BTI as well as the significant association between younger age and higher SFU to

CoV. Cross-reactivity has been discussed as a cause of less severe COVID-19 disease in children and teenagers.[28–30]

Unvaccinated individuals showed a strong IL-6 response as well as TNF-α production after S-protein stimulation compared to P+BTI and F+BTI. One hallmark of severe infection is the overproduction of IL-6 and TNF-α[31,32] during cytokine storm that is associated with poor control of virus spread and may produce systemic symptoms.[32,33] In our study, significant elevated levels of pro-inflammatory cytokines, such as IFNγ, TNF-α, IL-6, and IL-17A, were found at early stage in unvaccinated individuals after SARS-CoV-2 infection, which was not observed in vaccinated individuals who experienced BTI. These findings suggest that the memory response in vaccinated individuals during BTI may result in a modulated cellular immune response against S-protein and less inflammatory activity of S-specific immune cells. Although, long-COVID symptoms were not studied in this analysis, in other studies inflammatory cytokine responses were associated to long- and post-COVID syndromes.[34,35] Interestingly, higher IL-2 concentrations at Weeks 17–24 were associated with lower S-specific IFNγ SFU, which may indicate an insufficient IFNγ production despite IL-2 production probably suggests immune dysbalance in group U. Others have shown that persons who had received booster vaccination increased secretion of IFNγ in both CD4+ and CD8+ T-cells and had a better prognosis and disease control in the case of BTI.[35] Individuals of group P+BTI showed higher IL-6 and TNF-α concentrations with longer time after one mRNA vaccination and an inverse correlation with IL-6 and IFNγ SFU to S-protein stimulation at w3–7 and a direct correlation with higher anti-spike-IgG in the long-term at w17–24. These findings indicate that the adaptive immune response seems to require sufficient time to develop and mature cellular and humoral responses.[36] Group F+BTI showed a significant association between high IL-6 production and high anti-S-IgG concentrations and avidities as well as high SFU at earliest time points which strongly indicates the significant cellular recall response in fully vaccinated individuals.

To our knowledge, this is the first study showing the longitudinal course of S-specific cellular responses over 6 months observation period in fully vaccinated individuals after BTI compared to unvaccinated controls and individuals who had exposure to a single dose of mRNA vaccine. Effectiveness in preventing reinfection is an important point to be mentioned and of relevance for clinical practice. From our previous publication,[15] we calculated a vaccine effectiveness of 94.2% ($n = 3$ BTI in 52 partially vaccinated individuals) and 100% ($n = 0$ BTI in fully vaccinated 101 individuals) in the vaccinated groups P+BTI and F+BTI, respectively, until w17–24 compared to 91.3% ($n = 4$ of 46 unvaccinated individuals) in group U who experienced a further SARS-CoV-2 infection in the observation period ($p < 0.001$, comparison between group F+BTI and U). In this study, two of the U group experienced BTI in the observation period between w5–7 and w14–24 (effectiveness 78.9%), none in the P+BTI and F+BTI group. In this study, exclusively Delta and Alpha VOC infections were studied, thus translation to other immune escape VOCs, such as Omicron, is limited.

Further, due to the study design, previous SARS-CoV-2 exposures before study inclusion could not be assessed and thus, may impair the analysis and interpretation of the results. Correlations of immunogenicity parameters may not be interpreted as causal inferences and thus, should be taken with caution and may be more useful for deduction of hypotheses than for confirmative issues.

However, the study illustrates the induction of a high anti-S-IgG antibody binding capacity and of a strong and stable S-specific cellular immune response with a modulated pro-inflammatory cytokine production in individuals who had completed primary vaccination against COVID-19 and experienced BTI with immune escape VOCs. In contrast, unvaccinated individuals who experienced their first SARS-CoV-2 infection showed a delayed humoral and cellular immune response to S-protein and a long-lasting pro-inflammatory cytokine profile by S-specific immune cells. Those individuals who had high SFU to CoV had higher SFU to S-protein which corroborates the potential role of cellular recall responses against CoV which may promote S-specific cellular reactivity. However, in vaccinated individuals the role of previous CoV infection may be overestimated regarding impact on humoral and cellular responses. Our data strongly support the immunological benefit of stronger and more durable hybrid immunity. SARS-CoV-2 infection alone seems to be not sufficient to generate significant anti-S-IgG avidity, although the cellular response is similar to vaccinated individuals. The trend to the higher inflammatory immune response in unvaccinated individuals may be of disadvantage regarding inflammatory diseases in the postinfection course of COVID-19.[34,35] This study provides evidence for the constitution of basic humoral and cellular immunity after three S-protein exposures by two COVID-19 vaccinations and one BTI also on the cellular level which may corroborate recommendations by health authorities to build on basic immunity generated by three S-protein exposures.

4 | MATERIAL AND METHODS

4.1 | Study groups

Nonhospitalized adults (age ≥18 years) with a newly diagnosed (≤14 days) PCR-confirmed SARS-CoV-2 infection were consecutively enrolled following a previously established protocol by the CoVaKo consortium, a prospective multicenter controlled cohort study collecting samples and data from April 13, 2021 until November 25, 2021 in Bavaria, Germany, covering the third (prevalent VOC Alpha) and fourth (Delta) SARS-CoV-2 waves in Germany (Table 1).[15] Samples were allocated into three groups according to their COVID-19 vaccination status including wild-type-based SARS-CoV-2 mRNA vaccines (Comirnaty, BioNTech/Pfizer or Spikevax, Moderna): (1) full primary vaccinated individuals (group F+BTI, $n = 18$) with two vaccinations regardless of vaccine type with ≥14 days between second vaccination and SARS-CoV-2 detection, (2) partially vaccinated individuals (group P+BTI, $n = 11$) with either one vaccination or two vaccinations <14 days before SARS-CoV-2 detection, and (3)

unvaccinated individuals (group U, $n = 18$). Peripheral venous blood samples were obtained after 1 week (w1), 2 weeks (w2), 3–7 weeks (w3–7), and 17–24 weeks (w17–24) after PCR-confirmed SARS-CoV-2 infection collecting samples and data from April 13, 2021 until November 25, 2021 in Bavaria, Germany, covering the third (prevalent VOC Alpha) and fourth (Delta) SARS-CoV-2 waves in Germany (Table 1).[15] For comparison, a group of individuals with a booster dose (F+Booster, $n = 37$) without BTI were included in this study.[10]

The study was performed according to the principles of the declaration of Helsinki 1964 and its later amendments and approved by the local ethics committees (protocol number 100/21_z).

4.2 | Anti-S-IgG concentration and avidity

Serum anti-S-IgG concentrations and avidities were determined by ELISA and an adapted protocol using thiocyanate as chaotropic agent (Institute Virion/Serion).[11,37] Anti-S-IgG was considered positive for values ≥31.5 binding antibody units (BAU)/mL. Anti-S-IgG was expressed as relative avidity index (RAI).[38]

4.3 | Cellular reactivity against S-, N-protein and CoV

Peripheral blood mononuclear cells (PBMCs) were prepared by density gradient centrifugation (FicoLite-H; Linaris) and stored in liquid nitrogen according to laboratory standards. Interferon-gamma (IFNγ) release upon SARS-CoV-2-specific antigen stimulation with either 10 µg/mL of the wild-type SARS-Cov-2 spike ectodomain S1-S2 antigen (S) or 20 µg/mL N-protein (both purchased by Institute Serion/Virion) was determined after 18 h by Enzyme-linked-Immunospot-Assay (ELISpot).[10,11] Spot forming units (SFU) were quantified using the C.T.L immunospot reader (Bonn, Germany) using the ImmunoSpot 4.0.16 (Cellular technology; Shaker Heights) software and referred as SFU/1 × 10^6 cells as well as stimulation index (SI). The T-SPOT discovery SARS-CoV-2 kit (Oxford Immunotec) was used to check reactivity to S- (region S1), N-, and M-(membrane) SARS-CoV-2 and for cross-reactivity to CoV-specific antigens using derived peptides pools at the earliest time point at w1 according to the manufacturer's instructions. Peptides included in the cross-reactivity CoV panel may include the most frequent CoV, such as CoV-NL63, CoV-229E, CoV-HKU1, CoV-OC43 (personal communication) and were not included in S, N, or M panel to increase their specificity (Oxford Immunotec).[39] F+Booster were screened negative for pre-existing SARS-CoV-2 or CoV infection-derived immunity.

4.4 | Cytokine analysis

Cytokine production was determined following stimulation of whole blood samples in precoated tubes with S-protein in an IFNγ release assay (SARS-CoV-2 IGRA Stimulation tube set; Euroimmun). After overnight incubation, supernatants were collected by centrifugation and concentrations of Interleukin-(IL)-2, IL-4, IL-5, IL-6, IL-9, IL-10, IL-13, IL-17A, IL-17F, IL-22, IFNγ, and tumor-necrosis-factor-alpha (TNF-α) were measured using a bead-based assay according to the manufacturer's instructions (LEGENDplex™; Biolegend). Analysis was performed by FACSCanto II (BD Biosciences) using LEGENDplex Data Analysis Software (Biolegend).

4.5 | Statistics

Variables were tested for normal distribution using Kolmogorov–Smirnov-test. Nonparametric continuous independent variables were analyzed using Mann–Whitney *U* or Kruskal–Wallis test. Bonferroni correction was performed to consider bias by multiple testing. Dependent variables were analyzed by Wilcoxon-signed-rank test or Friedman test. Correlations were assessed with Spearman rank correlation coefficient. Statistical tests were performed using SPSS 29.0 statistics software (IBM). A $p < 0.05$ was considered significant.

AUTHOR CONTRIBUTIONS

Conceptualization and study design: Martina Prelog, Klaus Überla, Ralf Wagner, Johannes Liese, Philipp Steininger, and Ulrike Protzer. *Methodology and laboratory investigation*: Giovanni Almanzar, Lorena Richter, Nicola Hepp, Kimia Koosha, Tim Vogt, Astrid Stein, Lars Ziegler, Claudia Asam, Christof Geldmacher, Manuela Weps, Valeria Schwägerl, Claudia Asam, Isabell Wagenhäuser, and Julia Reusch. *Participant recruitment and study visits*: Valeria Schwägerl, Lars Ziegler, Andre Fuchs, Manuel Krone, and Claudia Asam. *Statistical analysis*: Martina Prelog and Giovanni Almanzar. *Discussion and manuscript writing*: Martina Prelog and Giovanni Almanzar.

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CONFLICT OF INTEREST STATEMENT

Martina Prelog received honoraria for presentations at scientific meetings from Abbvie, Chugai-Roche, GSK, Janssen, Lilly, Moderna, MSD, Novartis, Pfizer, Sanofi and SOBI, received honoraria for advisory boards from Abbvie, GSK, Moderna, Novartis, Janssen, Pfizer and BioNTech, received travel scholarships from Chugai-Roche, GSK,

Novartis and Pfizer, received financial support for conduction of investigator-initiated research from Baxter, Chugai-Roche, GSK, Moderna, MSD, Novartis, Pfizer and SOBI; Martina Prelog declares to have no conflict of interest regarding the manuscript. Ulrike Protzer received personal fees from Abbott, Abbvie, Arbutus, Gilead, GSK, J&J, MSD, Roche, Sanofi, Sobi, and Vaccitech. Ulrike Protzer is co-founder, share-holder, and board member of SCG Cell Therapy Inc. Manuel Krone receives honoraria from GSK and Pfizer outside the submitted work. The remaining authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request. Data will be made available on request to either Prof. Dr. Martina Prelog (martina.prelog@uni-wuerzburg.de) or Dr. Giovanni Almanzar (Almanzar_G@ukw.de).

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Additional supporting information can be found online in the Supporting Information section at the end of this article.

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