CLINICAL SCIENCE

SARS-CoV-2 vaccination in rituximab-treated patients: B cells promote humoral immune responses in the presence of T-cell-mediated immunity

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ABSTRACT

Objectives Evidence suggests that B cell-depleting therapy with rituximab (RTX) affects humoral immune response after vaccination. It remains unclear whether RTX-treated patients can develop a humoral and T-cell-mediated immune response against SARS-CoV-2 after immunisation.

Methods Patients under RTX treatment (n=74) were vaccinated twice with either mRNA-1273 or BNT162b2. Antibodies were quantified using the Elecsys Anti-SARS-CoV-2 S immunoassay against the receptor-binding domain (RBD) of the spike protein and neutralisation tests. SARS-CoV-2-specific T-cell responses were quantified by IFN-γ enzyme-linked immunosorbent spot assays. Prepandemic healthy individuals (n=5), as well as healthy individuals (n=10) vaccinated with BNT162b2, served as controls.

Results All healthy controls developed antibodies against the SARS-CoV-2 RBD of the spike protein, but only 39% of the patients under RTX treatment seroconverted. Antibodies against SARS-CoV-2 RBD significantly correlated with neutralising antibodies (τ=0.74, p<0.001). Patients without detectable $CD19^+$ peripheral B cells (n=36) did not develop specific antibodies, except for one patient. Circulating B cells correlated with the levels of antibodies (τ=0.4, p<0.001). However, even patients with a low number of B cells (<1%) mounted detectable SARS-CoV-2-specific antibody responses. SARS-CoV-2-specific T cells were detected in 58% of the patients, independent of a humoral immune response.

Conclusions The data suggest that vaccination can induce SARS-CoV-2-specific antibodies in RTX-treated patients, once peripheral B cells at least partially repopulate. Moreover, SARS-CoV-2-specific T cells that evolved in more than half of the vaccinated patients may exert protective effects independent of humoral immune responses.

Key messages

What is already known about this subject?
► B cell-depleting therapy with rituximab (RTX) can lead to severe or prolonged disease courses after SARS-CoV-2 infection.
► B cell-depleting therapy with RTX affects humoral immune responses after vaccination. It is still unclear whether patients without measurable peripheral B cells after RTX treatment can develop antibodies against SARS-CoV-2 after vaccination and whether T-cell-mediated immune response is affected.

What does this study add?
► This study describes that patients who received RTX treatment and have no measurable peripheral B cells do not develop antibodies after SARS-CoV-2 vaccination. Patients with repopulated B cells can mount antibody responses after COVID-19 vaccination.
► T-cell-mediated immune response after COVID-19 vaccination was detected in the majority of patients after RTX treatment irrespective of the presence or absence of B cells and a humoral immune response.

How might this impact on clinical practice or future developments?
► RTX treatment should not preclude COVID-19 vaccination, since a robust T-cell response can be mounted even in the absence of circulating B cells.
► Delaying RTX treatment may be justified in patients with stable disease until peripheral B cells repopulate to allow development of a humoral response to vaccination.

INTRODUCTION

SARS-CoV-2 causes COVID-19 often resulting in a severe acute respiratory distress syndrome. Different vaccines have been developed as a critical factor to manage this global public health emergency. A major concern is the immunogenicity of vaccination during immunomodulatory therapies.[1–8] Among the immunosuppressive therapies, rituximab (RTX), a monoclonal antibody targeting CD20, represents an important treatment for various inflammatory diseases.[9] An increased risk of more severe disease courses and persistent viraemia have been reported in RTX-treated patients on SARS-CoV-2 infection.[10–13] RTX treatment in particular might

affect the COVID-19 disease course and the immunogenicity of SARS-CoV-2 vaccination, as reported previously.[1–7 14] Studying a small cohort of RTX-treated patients, we have recently provided some initial evidence that T-cell-mediated immune response is maintained even in the absence of a humoral anti-SARS-CoV-2 response. However, it remains unclear whether, or to which extent, repopulation of peripheral B cells is needed for antibody development in RTX-treated patients.[15]

To determine if or for how long it might be useful to withhold COVID-19 vaccination in RTX-treated patients, we assessed the cellular and humoral immune response and related it to numbers of peripheral B cells.

METHODS
Patients
Patients under RTX treatment at our outpatient clinic were enrolled. All patients were vaccinated twice with an mRNA vaccine (either BioNTech/Pfizer BNT162b2 or Moderna mRNA-1273). Serum samples obtained after second vaccination were stored at the Biobank of the Medical University of Vienna, a centralised facility for the preparation and storage of biomaterial with certified quality management (International Organization for Standardization (ISO) 9001:2015).[16] Peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation and stored in liquid nitrogen until further use. Antibodies against the receptor-binding domain (RBD) were determined after the second vaccination.

Samples from healthy blood donors without exposure to SARS-CoV-2 were collected before the SARS-CoV-2 pandemic (June–November 2019) and served as prepandemic healthy controls. Sex-matched and age-matched individuals who were vaccinated twice with BNT162b2 served as healthy vaccination controls. Ethical approval for this study was granted by the ethics committee of the Medical University of Vienna, Austria (1291/2021; 559/2005; 1073/2021). Patients and/or the public were not involved in the design, conduct, reporting or dissemination plans of this research.

Quantification of CD19+ peripheral B cells
Immunological phenotyping was performed by flow cytometry (FACSCanto II, San Jose, California, USA) using the whole blood first stain and then lyse and wash method (Becton Dickinson). Lymphocyte subsets were characterised with a combination of the following monoclonal antibodies (all provided by Becton Dickinson): fluorescein isothiocyanate (FITC)-labelled anti-CD3, phycoerythrin (PE)-labelled anti-CD16+56+, peridinin-chlorophyll-protein (PerCP)-cy5.5-labelled anti-CD4, PE-Cy7-labelled anti-CD19, allophycocyanin (APC)-Cy7-labelled anti-CD8, V450-labelled anti-human leukocyte antigen (HLA)-DR, V500-labelled anti-CD45 and APC-labelled anti-CD14. Between 20 000 and 500 000 events were acquired to recover a significant B cell population of at least 50 cells. Results were expressed as proportion of CD19+ B cells among total lymphocytes.

Humoral immune responses
Anti-SARS-CoV-2 antibody testing
The Elecsys Anti-SARS-CoV-2 S immunoassay was used for the quantitative determination of antibodies to the RBD of the viral spike (S) protein.[17 18] The quantitation range is between 0.4 and 2500.0 U/mL. Tests were performed on a Cobas e801 analyser (Roche Diagnostics, Rotkreuz, Switzerland) at the department of laboratory medicine, Medical University of Vienna (certified acc. to ISO 9001:2015 and accredited acc. to ISO 15189:2012).

SARS-CoV-2 neutralisation test (NT)
The NT was performed as described previously.[19] Twofold serial dilutions of heat-inactivated serum samples were incubated with 50–100 tissue culture infectious dose 50% (TCID50) SARS-CoV-2 for 1 hour at 37°C before the mixture was added to Vero E6 (ATCC CRL-1586) cell monolayers. Incubation was continued for 3 days. NT titres were expressed as the reciprocal of the serum dilution required for protection against virus-induced cytopathic effects. NT titres ≥10 were considered positive.

T-cell responses
Peptides
For T-cell stimulation, PepMix SARS-CoV-2 peptide pools were purchased from JPT (Berlin, Germany). The pools cover the entire sequences of the SARS-CoV-2 S protein and comprise 15-mer peptides overlapping by 11 amino acids (aa). The S peptides are split into two subpools S1 (aa 1–643) and S2 (aa 633–1273). Peptides were dissolved in dimethyl sulfoxide and diluted in AIM-V medium for use in enzyme-linked immunosorbent spot (ELISpot) assays.

T-cell IFN-γ ELISpot assay
For ex vivo ELISpot assays, PBMCs were thawed. A total of $1–2 \times 10^5$ cells per well were incubated with SARS-CoV-2 peptides (2 μg/mL; duplicates), AIM-V medium (negative control; 3–4 wells) or phytohemagglutinin (PHA) (L4144, Sigma; 0,5 μg/mL; positive control) in 96-well plates coated with 1.5 μg anti-IFN-γ (1-D1K, Mabtech) for 24 hours. After washing, spots were developed with 0.1 μg biotin-conjugated anti-IFN-γ (7-B6-1, Mabtech), streptavidin-coupled alkaline phosphatase (Mabtech, 1:1000) and 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (Sigma). Spots were counted using a Bio-Sys Bioreader 5000 Pro-S/BR177 and Bioreader software generation 10. Data were calculated as spot-forming cells (SFCs) per 10^6 PBMCs after subtraction of the spots from the negative control (mean spot number from three to four unstimulated wells).

Statistical analysis
According to the distribution, continuous variables are presented as mean with SD or median with IQR. Unpaired groups were compared depending on the distribution by either t-test and one-way analysis of variance or using non-parametrical tests such as the Kruskal-Wallis test. Categorical variables were analysed using Fisher's exact test. Associations between continuous variables were assessed via Kendall rank correlation coefficient (τ). Univariate and a multivariable logistic regression analysis was implemented to assess association of relevant variables with seroconversion. The investigators selected the variables included based on the expected relevance (age, concomitant medication and the number of peripheral B cells). GraphPad Prism (V.9.1.0) was used for the graphical presentation of the data. 'R' V.4.0.3 was used for the entire statistical analysis. The following packages were used: 'ggplot2', 'ggbeeswarm' and 'sjPlot' for creating plots and 'tableone' to create baseline tables.

RESULTS
Patient characteristics and sample collection
Seventy-four patients (mean age 61.7±13.3 years, 77% women) with immune-mediated inflammatory diseases (IMID) under B
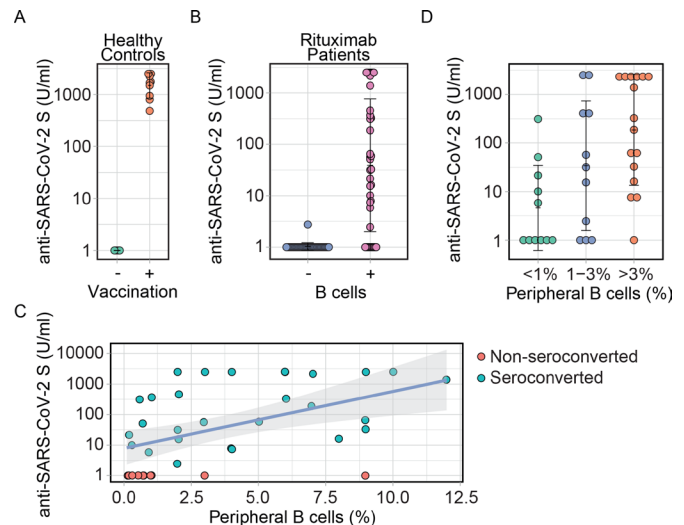
Table 1	Patient characteristics at baseline
n	74
Age (mean (SD))	61.7 (13.3)
Gender: woman (%)	57 (77.0)
Diagnosis, n (%)	
IgG4-related disease	2 (2.7)
Connective tissue diseases	22 (29.7)
Rheumatoid arthritis	33 (44.6)
Vasculitis	17 (23.0)
Peripheral B cells, absolute (median (IQR))	2.00 (0.00, 32.50)
Detectable B cells, n (%)	38 (51.4)
IgG (median (IQR)) (mg/dL)	820 (646, 1052)
IgM (median (IQR)) (mg/dL)	47 (26, 69)
Months between RTX and vaccination (mean (SD))	6.9 (6.0)
Days between vaccine and laboratory assessment (mean (SD))	21.9 (16.6)
Concomitant medication, n (%)	
Any csDMARD	42 (56.8)
Methotrexate	24 (32.4)
Mycophenolate mofetil	8 (10.8)
Hydroxychloroquine	7 (9.5)
Azathioprine	5 (6.8)
Leflunomide	4 (5.4)
Sulfasalazine	1 (1.4)
Immunoglobulin therapy	3 (4.1)
Prednisone	22 (29.7)
Vaccine, n (%)	
mRNA-1273	13 (17.6)
BNT162b2	61 (82.4)

csDMARD, conventional synthetic disease-modifying antirheumatic drug; RTX, rituximab.


Figure 1 Humoral immune response to SARS-CoV-2 vaccination in rituximab (RTX)-treated patients. Antibodies to the receptor-binding domain (RBD) of the viral spike (S) protein were determined using an anti-SARS-CoV-2 immunoassay. (A.) Antibody levels were determined in prepandemic healthy controls (n=5) and in vaccinated healthy controls (n=10). (B.) Antibody levels were determined in RTX-treated patients (n=74) without (-) and with (+) detectable CD19$^+$ peripheral B cells. (C.) Scatter plot of antibody levels to the RBD of the S protein and the percentage of CD19$^+$ peripheral B cells with linear regression line including a 95% CI. (D.) Antibody levels grouped in patients according to the percentage of CD19$^+$ peripheral B cells. Mean±SD deviation is shown.

cell-depleting therapy with RTX received two vaccinations with either BNT162b2 (Pfizer/BioNTech) (n=61, 82%) or mRNA-1273 (Moderna) (n=13, 18%). Blood was collected at a mean of 21.9 days (range: 7–49 days) after the second vaccination to determine cellular and humoral immune response. None of the patients had a clinical history of, or developed a, SARS-CoV-2 infection during the observation period. Most patients with IMID had rheumatoid arthritis (45%), followed by connective tissue diseases (30%), vasculitides (23%) and IgG4-related disease (3%) (table 1). The mean time between the last RTX treatment and the first COVID-19 vaccination was 6.9 (±6.0) months. Forty-three percent of the patients received RTX monotherapy, while 57% received comedication with conventional synthetic (cs) disease-modifying antirheumatic drugs (DMARDs) such as methotrexate (MTX) (n=24), mycophenolate mofetil (MMF) (n=8), hydroxychloroquine (n=7) and leflunomide (n=4); 30% of the patients received a therapy with low-dose prednisone (mean: 5.5±3.6 mg). Fity-one percent of the patients had detectable B cells.

Humoral immune responses to COVID-19 vaccination

Antibodies against the SARS-CoV-2 RBD of the S protein were analysed after the second dose of BNT162b2 or mRNA-1273 vaccine. Healthy individuals who received two vaccinations with BNT162b2 (n=10) and unvaccinated prepandemic healthy individuals (n=5) served as controls. None of the prepandemic healthy controls but all healthy vaccinated controls had detectable antibodies (figure 1A).

In 29 of the 74 RTX-treated patients (39%), seroconversion was seen (online supplemental table 1). Among patients in whom peripheral B cells were not detectable (n=36), anti-RBD antibodies were also not detectable, with one exception (figure 1B). In patients with detectable peripheral B cells (n=38), seroconversion rate was 74%; levels of peripheral B cells correlated significantly with antibody levels (τ=0.4, p<0.001) (figure 1C). Comparison of antibody levels in patients with different proportions of peripheral B cells revealed that 45% of patients with more than 0% but less than 1% peripheral B cells (n=11) were able to mount an antibody response, suggesting that the mere presence of peripheral B cells allows seroconversion irrespective of the B cell count (figure 1D).

Comparative univariate analysis of seroconverted and non-seroconverted patients revealed a statistical significance for time since last RTX administration and first vaccination (p=0.001), but not for comedication, type of vaccine or diagnosis (online supplemental table 1). Accordingly, time since the last RTX treatment was significantly correlated with B cell levels (τ=0.43, p<0.001) and antibody levels (τ=0.37, p<0.001) (online supplemental figure 1A,B). Multivariable logistic regression analysis showed that the percentage of peripheral B cells contributed significantly to seroconversion (OR 2.4, 95% CI 1.63 to 4.15) when adjusted for age, csDMARDs and prednisone (figure 2). The calculated McFadden's R^2 of 0.41 indicates a good model fit. Time since the last RTX treatment did not show an additional effect on seroconversion nor did it improve model fit (likelihood-ratio test, p=0.777) (online supplemental figure 1C).

Forty-two patients (57%) received comedication with csDMARDs (table 1). Among them, 24 (32%) were treated with MTX and eight (11%) with MMF; 22 (30%) received
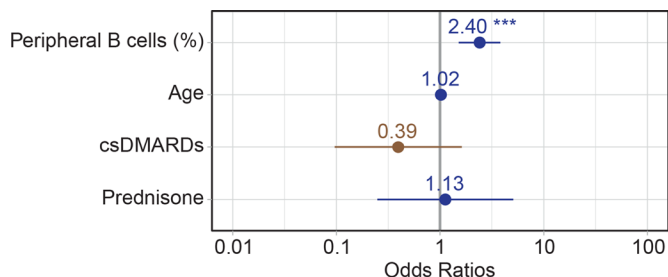
Figure 2 ORs of logistic regression assessing seroconversion in vaccinated rituximab-treated patients. csDMARDs, conventional synthetic disease-modifying antirheumatic drugs.

prednisone. No difference was observed in the levels of antibodies against the SARS-CoV-2 RBD of the S protein in the presence or absence of comedication with csDMARDs (online supplemental figure 1D). Although the OR might suggest a negative impact of comedication with csDMARDs on seroconversion, it does not reach statistical significance nor does the omission of csDMARDs alter the model fit (likelihood-ratio test, p=0.184) (figure 2). Of note, 58.3% of the MTX-treated and 75% of the MMF-treated patients did not seroconvert.

Neutralising activity against SARS-CoV-2
Neutralising antibodies against SARS-CoV-2 were measured in 36 RTX-treated patients after the second vaccination. Patients who did not have detectable antibodies against the SARS-CoV-2 RBD as determined in the immunoassay also did not have neutralising antibodies (online supplemental figure 2A). Accordingly, RBD-specific antibody levels (U/mL) significantly correlated with neutralising activity (τ=0.74, p<0.001) (online supplemental figure 2B). Except for one patient, who also developed anti-RBD antibodies, no neutralising antibodies could be detected in patients without peripheral B cells (online supplemental figure 2C). Neutralising antibody levels correlated significantly with levels of peripheral B cells (τ=0.54, p<0.001) (online supplemental figure 2D). Overall, these data suggest that seroconversion reflects functionally protective antibody responses.

T-cell-mediated immune responses to COVID-19 vaccination
To investigate whether the patients mounted a SARS-CoV-2-specific T-cell response, we analysed PBMCs from 45 patients after the second COVID-19 vaccination. All healthy vaccinated controls had detectable SARS-CoV-2-specific T-cell responses, and the prepandemic controls had low or no background responses (figure 3A,B). Interestingly, 26 out of 45 patients (58%) had detectable cellular responses to the S peptide pools (S1/S2). Among them, 12/26 (46%) did have detectable RBD-specific antibodies as compared with 14/26 (54%) who did not seroconvert after the second vaccination. Nineteen of the 45 patients (42%) did not have a T-cell-mediated immune response to COVID-19 vaccination. Among them, 6/19 (32%) had antibodies after vaccination in the absence of a detectable T-cell response. Thirteen out of nineteen (68%) were negative in the RBD immunoassay. Thus, overall, 13 out of 45 patients (29%) developed neither a T-cell response nor antibodies against SARS-CoV-2 (online supplemental table 3). Comparative analysis of seroconverted and non-seroconverted patients without a T-cell response revealed a statistical significance for time since last RTX administration and first vaccination (p=0.006) and for peripheral B cells (p=0.003), but not for age, comedication, type of vaccine or diagnosis (online supplemental table 4). No
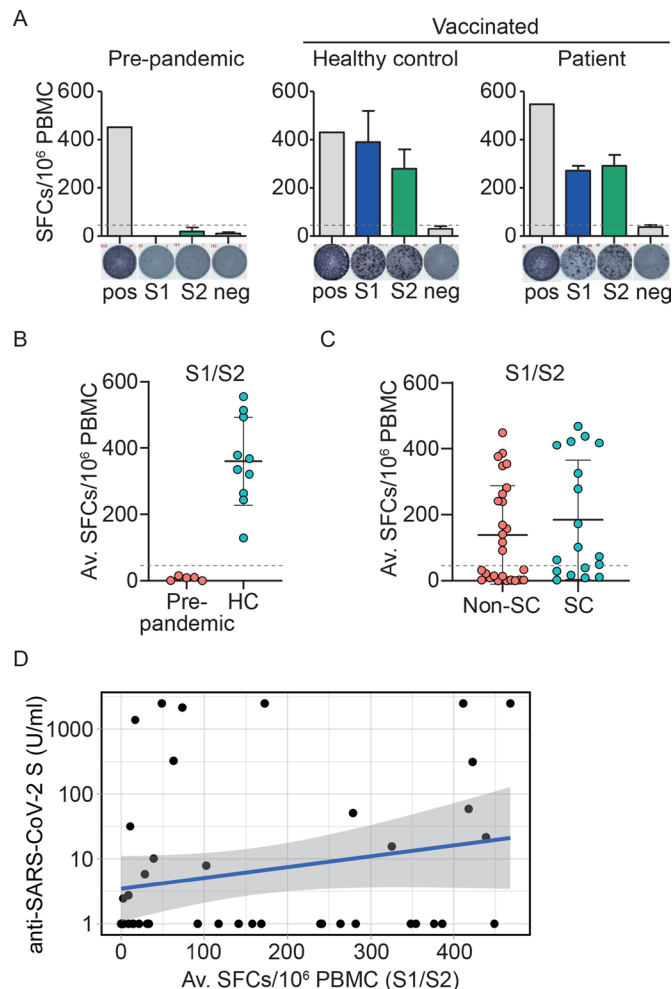
Figure 3 SARS-CoV-2-specific T-cell responses in rituximab (RTX)-treated patients. (A.) Representative ex vivo interferon (IFN)-γ enzyme-linked immunosorbent spot (ELISpot) results from peripheral blood mononuclear cells (PBMCs) stimulated with spike subunit S1 and S2 peptide pools shown for one prepandemic control, one representative vaccinated healthy control and one representative RTX-treated patient. Y-axis indicates the number of spot-forming cells (SFCs) per 10^6 PBMCs. (B.) Average of SFCs/10^6 PBMCs from S1 and S2 peptide pools is shown for each subject in prepandemic (n=5) or vaccinated (n=10) healthy controls (HC). (C.) Composite ELISpot results from 45 patients divided into seroconverted (SC) and non-SC RTX-treated patients. Data show average of SFCs/10^6 PBMCs from S1 and S2 peptide pools. Dotted lines represent the cut-off as defined by the mean SFC count+three times the SD from the prepandemic controls. (D.) Scatter plot of antibodies to the receptor-binding domain of the spike protein and average of SFCs/10^6 PBMCs from S1 and S2 peptide pools with linear regression line including a 95% CI. Mean±SD deviation is shown. Av., average; neg, negative; pos, positive.

significant difference between patients with and without T-cell response was found with respect to seroconversion (p=0.371). SFC responses tended to be higher in seroconverted than non-seroconverted RTX-treated patients, but these differences did not reach statistical significance (figure 3C). In line with these data, no significant correlation between the SFC responses to S peptide pools and antibody levels against the RBD of the S protein was observed (figure 3D).

Among the 45 patients, 25 (56%) received comedication with any csDMARD (online supplemental table 2). Among them, 16

(36%) were treated with MTX and three (7%) with MMF; 12 (27%) patients received prednisone. Comparative univariate analysis revealed no difference for comedication, prednisone dose or age between patients with and without T-cell-mediated response to SARS-CoV-2 (online supplemental table 3). Of note, MMF was reported to influence the humoral and cellular immune response. In our data, exclusion of MMF-treated patients did not alter the analysis (data not shown).

Time-resolved humoral and T-cell-mediated immune responses to COVID-19 vaccination

To investigate the dynamics of humoral and T-cell-mediated immune responses, anti-RBD antibody levels and SARS-CoV-2-specific T-cell responses were analysed at two different time points. SARS-CoV-2-specific antibodies were determined on average 15 and 37 days after the second vaccination in a subgroup of 42 patients. As shown in online supplemental figure 3A, no difference in antibody levels against SARS-CoV-2 were found 5 weeks after the first laboratory testing. Likewise, no difference was observed for SARS-CoV-2-mediated T-cell responses on average 15 and 42 days after the second vaccination in a subgroup of nine patients (online supplemental figure 3B). These data suggest a robust humoral and T-cell-mediated immune response to COVID-19 vaccination over a period of 5 weeks after second vaccination.

DISCUSSION

B cells play a critical role in the development of humoral immune responses. In the presented study, we could show that B cell depletion in RTX-treated patients affects the humoral but does not necessarily abolish T-cell-mediated immune responses to COVID-19 vaccination. These data are in line with recent reports, suggesting that RTX might affect antibody responses to COVID-19 vaccination.[14 20–23] However, here, we could show that a humoral response can be mounted once peripheral B cells are present and that the numbers of peripheral B cells correlate with levels of antibodies against the RBD of the S protein. These findings suggest a qualitative and quantitative dependence of a successful humoral response to COVID-19 vaccination on peripheral B cells. Recent data indicate a role of csDMARDs on humoral immune responses.[20 21 24 25] Within our data, no clear effect on seroconversion was observed, which might be due to the small size of the patient cohort with csDMARDs. Larger cohorts are certainly needed to sufficiently address the impact of comedication on humoral immune responses. In line with recent reports, impaired humoral immune response was independent of the diagnosis.[26] Antibodies against the SARS-CoV-2 RBD significantly correlated with neutralising activity supporting a protective antibody response.

Our data also showed that a subset of RTX-treated patients could develop robust SARS-CoV-2-specific T-cell immunity in response to vaccination. T-cell-mediated immune response was observed in seroconverted and non-seroconverted patients suggesting that the absence of peripheral B cells is the primary mediator of an impaired humoral but not cellular immune response. More extensive trials will undoubtedly be needed to understand the exact role of T-cell immunity in protection against SARS-CoV-2 infection and if the current findings also pertain to other vaccines. Further analysis of additional intracellular cytokines would be beneficial for a more detailed characterisation of the T-cell-mediated immune response. Recent reports show an effect of MTX on the cellular immunity.[24] No clear statement can be made based on our data most likely due to the limited number of patients on csDMARDs.

The most recent EULAR recommendations for vaccination in adult patients with autoimmune inflammatory rheumatic diseases recommend that vaccines should ideally be administered before B cell-depleting biological therapy is started or, when patients are on such a treatment already, at least 6 months after the start but 4 weeks before the next course.[27] Our data suggest that a humoral immune response can be obtained once B cells have recovered, which may drive a new vaccination strategy in these individuals. However, since higher levels of peripheral B cells predict an enhanced humoral immune response, delaying RTX treatment in clinically stable patients or waiting for a robust number of peripheral B cells in treated patients with a low risk for COVID-19 may be justified. On the other hand, a T-cell-mediated immunity can be mounted even in the absence of peripheral B cells, indicating that RTX treatment may not have to preclude SARS-CoV-2 vaccination if B cell repopulation is delayed, as happens in some patients.

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