



Impact of methotrexate on humoral and cellular immune responses to SARS-CoV-2 mRNA vaccine in patients with rheumatoid arthritis

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Abstract

The aim of this study was to longitudinally evaluate the undetermined impact of methotrexate (MTX) on the cumulative immunogenicity elicited by three doses of SARS-CoV-2 mRNA vaccination in patients with rheumatoid arthritis (RA). We prospectively evaluated vaccine-induced immune responses following the first dose, 1 and 6 months after the second dose, and 1 month after the third dose of BNT162b2 or mRNA-1273 in 144 SARS-CoV-2 naïve participants (70 patients with RA, 29 disease controls without immunosuppressive conditions, and 45 healthy controls). Humoral immune responses were assessed by quantifying anti-spike IgG antibody titers and the capacity of circulating antibodies to neutralize the ancestral SARS-CoV-2 strain and the Alpha, Delta, and Omicron variants. Vaccine-induced T-cell responses were measured using an interferon-gamma release assay. At 1 and 6 months after the second dose, anti-spike titers were highest in healthy controls, followed by disease controls and patients with RA. Multivariate analyses revealed that MTX treatment was significantly associated with a decrease in anti-spike titers, neutralizing activity, and SARS-CoV-2-specific interferon-gamma levels. Furthermore, MTX dose per body weight was negatively correlated with these two indices of humoral immune response. The third vaccine dose boosted anti-spike titers, especially in patients receiving MTX, while sera from these patients neutralized the Omicron variant far less robustly than those from healthy controls. In conclusion, MTX attenuated immunogenicity following two doses of SARS-CoV-2 mRNA vaccine in patients with RA, particularly resulting in dose-dependent suppression of the humoral immune response. Furthermore, MTX deteriorated the neutralizing activity against the Omicron variant, even after the third immunization.

Keywords SARS-CoV-2 · mRNA vaccine · Methotrexate · Rheumatoid arthritis · Immunogenicity

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Introduction

The coronavirus disease 2019 (COVID-19) pandemic, caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), remains a serious global health crisis. Particularly, individuals with immune-mediated inflammatory diseases (IMIDs), including rheumatoid arthritis (RA), are reported to be at increased risk of COVID-19-related hospitalization and mortality compared to the general population [1, 2].

Although the two novel mRNA-based SARS-CoV-2 vaccines have demonstrated robust immunogenicity and high protective efficacy in the general population, immunocompromised patients were excluded from clinical trials [3–6]. Accumulating evidence suggests that humoral immune responses to SARS-CoV-2 mRNA vaccines are attenuated when patients with IMIDs receive immunosuppressants or immunomodulatory drugs, including anti-CD20 B-cell-depleting agents (e.g., rituximab), glucocorticoids, mycophenolate mofetil, methotrexate (MTX), cytotoxic T lymphocyte-associated antigen 4-immunoglobulin (CTLA4-Ig), abatacept, and Janus kinase (JAK) inhibitors [7–12]. Although most reports focused only on anti-spike antibody titers and/or seroconversion rates, the quantity of antibodies required to prevent symptomatic infection remains unclear [13]. Instead, the neutralizing activity of circulating antibodies against the virus is highly predictive of immune protection against infection [14]. Furthermore, it is crucial to evaluate immune cross-reactivity with variants of concern (VOCs), as SARS-CoV-2 continues to mutate rapidly [15]. In addition, T-cell-mediated immunity is considered important to prevent clinical deterioration following SARS-CoV-2 infection [16].

Even with the development of various therapeutic agents, including biological disease-modifying anti-rheumatic drugs (bDMARDs), MTX remains the anchor drug for the treatment of RA, and is considered first-line therapy, based on global guidelines [17–19]. Each guideline has different recommendations for initial MTX doses and recommends that the dose should be adjusted while monitoring therapeutic efficacy and adverse events; thus, maintenance doses vary considerably among individuals [18–20]. Mycophenolate mofetil and prednisone have been reported to attenuate humoral immune responses to SARS-CoV-2 mRNA vaccines in a dose-dependent manner; however, the effect of MTX dose on the immunogenicity of these vaccines has not yet been evaluated [21, 22]. Furthermore, the effect of MTX on long-term immunogenicity following two doses of vaccine remains unclear, as does the humoral immune response to the third booster dose, including neutralization of VOCs.

The aim of this study was to evaluate the impact of MTX and its dose on humoral and cellular immune

responses to the first, second, and third doses of SARS-CoV-2 mRNA vaccines in patients with RA.

Methods

Study design

This prospective, longitudinal cohort study was conducted at two medical facilities (Kyoto University Hospital and Sugita Genpaku Memorial Obama Municipal Hospital) in Japan. Adults aged ≥ 18 years who were scheduled to receive a SARS-CoV-2 mRNA vaccine, and fell into one of the following three categories, were recruited between 1st February and 30th April 2021: Patients with a rheumatologist-confirmed diagnosis of RA; patients with hypertension, chronic heart disease, or sleep apnea syndrome who were not receiving systemic immunosuppressors (disease controls); and healthcare workers without underlying diseases (healthy controls). Medication type was used to classify patients with RA into four subgroups: Patients receiving MTX and bDMARDs ('MTX with bDMARDs'); patients receiving MTX without bDMARDs ('MTX without bDMARDs'); patients receiving bDMARDs without MTX ('bDMARDs without MTX'); and patients receiving neither MTX nor bDMARDs ('no MTX, no bDMARDs'). All participants received two or three doses of either BNT162b2 (Pfizer-BioNTech) or mRNA-1273 (Moderna). Per national guidelines, second doses were administered 3–4 weeks after the first dose and third doses were administered ≥ 6 months after the second dose.

The following were excluded from the analysis: Individuals with a history of COVID-19 or positive SARS-CoV-2 serology at baseline; patients receiving either CTLA4-Ig or JAK inhibitors, as these agents attenuate SARS-CoV-2 mRNA vaccine immunogenicity [7, 11, 12]. Furthermore, individuals with confirmed breakthrough infections and individuals with seroconversion of IgG antibodies against the SARS-CoV-2 nucleocapsid protein (anti-NCP) following vaccination were excluded from subsequent analyses because anti-NCP IgG is induced by COVID-19 infection rather than SARS-CoV-2 mRNA vaccination [23].

This study was approved by the Medical Ethics Committee of Kyoto University (reference number R2713) and Obama Municipal Hospital (reference number 2–14) and was conducted in accordance with the principles of the Declaration of Helsinki. All participants provided written informed consent before enrollment.

Procedure

We collected clinical data regarding demographics, underlying diseases, ongoing treatments, the dates and types of

administered vaccines, medication schedule modifications at the time of each vaccination, and breakthrough infections.

Blood samples were collected at five time points: ≤ 2 months before the first dose (T1) (median [interquartile range (IQR)]: 24 [6–43] days); approximately 2–3 weeks after the first dose (T2) (median [IQR]: 17 [14–18] days); approximately 3–4 weeks after the second dose (T3) (median [IQR]: 24 [20–29] days); and approximately 6 months after the second dose (T4) (median [IQR]: 181 [173–188] days). Additional blood sampling was performed 3–4 weeks after the third dose (T5) (median [IQR]: 24 [21–29] days) for healthy controls and patients with RA in the ‘MTX without bDMARDs’ or ‘no MTX, no bDMARDs’ groups.

To assess humoral immune responses to vaccination, IgG antibodies against the S1 domain of the SARS-CoV-2 spike protein (anti-S1) were quantitatively measured using enzyme-linked immunosorbent assay (ELISA) (Anti-SARS-CoV-2 QuantiVac ELISA [IgG]; EUROIMMUN, Lübeck, Germany) at each time point. We simultaneously measured anti-NCP via ELISA (Anti-SARS-CoV-2 NCP ELISA; EUROIMMUN) in a semi-quantitative manner.

Surrogate virus neutralization testing (sVNT) for SARS-CoV-2 was performed using two commercially available assay kits to evaluate the functional capacity of antibodies to neutralize the virus in vitro. Briefly, we first measured the neutralizing activities of sera collected at T3 and T4, respectively, against the ancestral SARS-CoV-2 strain using a EUROIMMUN sVNT assay kit (SARS-CoV-2 NeutralISA; EUROIMMUN). We defined a positive neutralizing response as $\geq 35\%$ inhibition, according to the manufacturer’s instructions. Next, we measured the neutralizing activities of sera collected at T5 against the ancestral SARS-CoV-2 strain, and the Alpha (B.1.1.7), Delta (B.1.617.2), and Omicron (B.1.1.529) variants, and compared these with those of sera collected at T3. The assay was conducted using a GenScript sVNT kit (cPass™ SARS-CoV-2 Neutralization Antibody Detection Kit; GenScript, Piscataway, NJ, USA), which defines a positive neutralizing response as $\geq 30\%$ inhibition.

We analyzed vaccine-induced SARS-CoV-2 spike-specific T-cell responses at T4 in patients with RA via IFN- γ release assays (IGRAs) (Interferon-gamma ELISA; EUROIMMUN). IFN- γ concentrations > 200 mIU/mL were defined as positive, according to the manufacturer’s instructions. A detailed description of the laboratory analysis is provided in Online Resource 1.

Statistical analyses

Categorical variables were presented as percentages and compared using Pearson’s Chi-square or Fisher’s exact tests. Continuous variables were presented as medians

(IQR) and compared using non-parametric Mann–Whitney U and Kruskal–Wallis tests, unless otherwise stated. Corrections for multiple comparisons were performed using Bonferroni corrections for categorical variables and the Steel–Dwass test for continuous variables, whenever we observed significant differences among ≥ 3 experimental groups. Correlations between two continuous variables were assessed using Spearman’s rank correlation coefficients (null hypothesis: $\rho = 0$). Multivariate linear regression analyses were performed to assess correlations between ≥ 3 variables, by calculating standardized partial regression coefficients (null hypothesis: $\beta = 0$). All tests were two-sided. $P < 0.05$ was considered statistically significant. All statistical analyses were conducted using JMP Pro 16.2.0 (SAS Institute Inc., Cary, NC, USA). Figures were generated using GraphPad Prism version 9.4.1 (GraphPad Software, San Diego, CA, USA).

Results

Participant characteristics

In total, 162 individuals were recruited to the study. The final analysis included 70 patients with RA, 29 disease controls, and 45 healthy controls (Fig. 1), after excluding 14 patients who received CTLA4-Ig ($n = 10$), or JAK inhibitors ($n = 4$), and four anti-NCP positive individuals at baseline (one disease control and three healthy controls) (Online Resource 2). Patients with RA were divided into four subgroups: ‘MTX with bDMARDs’ ($n = 20$); ‘MTX without bDMARDs’ ($n = 23$); ‘bDMARDs without MTX’ ($n = 13$); and ‘no MTX, no bDMARDs’ ($n = 14$) (Fig. 1). bDMARDs included tumor necrosis factor (TNF) and interleukin-6 (IL-6) inhibitors. We excluded two patients with RA, who had breakthrough infections, and four individuals (one patient with RA, two disease controls, and one healthy control) with anti-NCP seroconversion after vaccination (Online Resource 2) from subsequent analyses (Fig. 1).

The baseline characteristics of the study participants and details of administered vaccines are summarized in Table 1. Healthy controls were significantly younger than patients with RA and disease controls (median age: 41, 69, and 74 years, respectively; $P < 0.001$). Age and sex distributions and disease activity (represented as Disease Activity Score-28 with erythrocyte sedimentation rate [DAS28-ESR]) were similar among the four subgroups of patients with RA. Patients treated with bDMARDs had a longer duration of RA than the others. No patients reported pausing or discontinuing immunosuppressants around the time of vaccination.

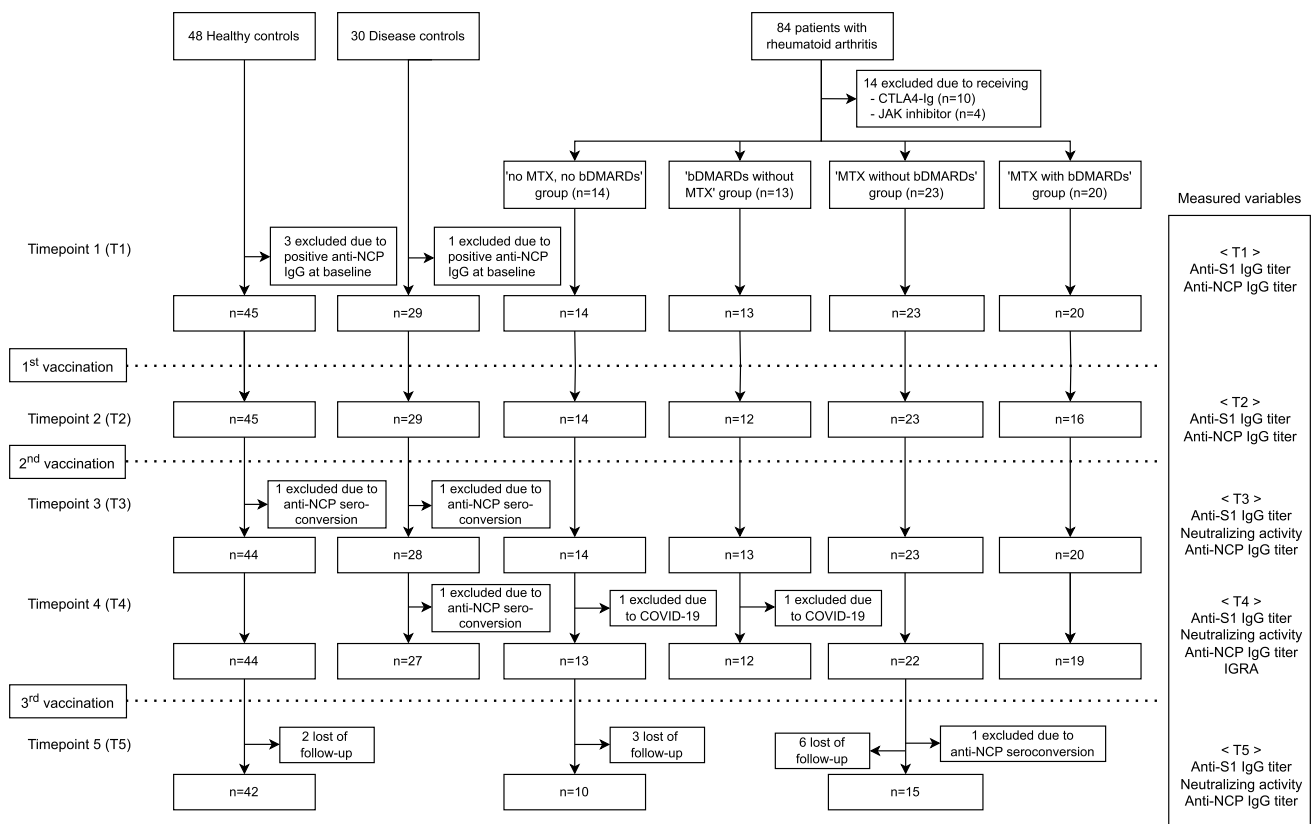


Fig. 1 Study flow diagram. Patients with rheumatoid arthritis were classified into four subgroups—patients receiving neither MTX nor bDMARDs ('no MTX, no bDMARDs'); patients receiving bDMARDs without MTX ('bDMARDs without MTX'); patients receiving MTX without bDMARDs ('MTX without bDMARDs');

and patients receiving MTX and bDMARDs ('MTX with bDMARDs'). bDMARDs, biological disease-modifying anti-rheumatic drugs; CTLA4-Ig, cytotoxic T lymphocyte-associated antigen 4-immunoglobulin; IGRA, interferon-gamma release assay; JAK, Janus kinase; MTX, methotrexate; NCP, nucleocapsid protein

Humoral immune responses to two doses of vaccine were attenuated in patients with RA

First, we assessed the humoral immune responses to two doses of vaccine across all study participants. Our longitudinal evaluation showed that anti-S1 titers significantly increased at T2 and T3 across all groups, but significantly decreased from T3 to T4 (Fig. 2A). Anti-S1 titers at T2, T3, and T4 were highest among healthy controls, followed by disease controls and patients with RA (Fig. 2B). Neutralizing activity against the ancestral SARS-CoV-2 strain also decreased significantly from T3 to T4 across all groups (Fig. 2C). All healthy and disease controls had positive neutralizing responses at T3. However, the proportion of patients with RA with positive neutralizing responses (83%) was significantly decreased compared to the control groups ($P=0.009$ and $P=0.049$, respectively). At T4, the proportion of individuals with positive neutralizing responses was highest among healthy controls and lowest among patients with RA (Fig. 2D).

MTX, but not bDMARDs, attenuated humoral immune responses to two doses of vaccine

Next, we analyzed patients with RA and the relative effects of MTX and bDMARDs on humoral immune responses to the first and second vaccine doses. Anti-S1 titers increased significantly at T2 and T3 across all four groups, except at T2 for the 'MTX with bDMARDs' group (Fig. 3A). The anti-S1 titers of the 'MTX without bDMARDs' group, at both T2 and T3, were significantly lower than those of either the 'no MTX, no bDMARDs' or 'bDMARDs without MTX' groups. There was no significant difference between the latter two groups (Fig. 3B). The anti-S1 titers and proportions of positive neutralizing responses at T3 were significantly lower for the MTX-using group than for the non-MTX-using group (Online Resource 3A, B). Taken together, these results indicate that MTX, but not bDMARDs, attenuated humoral immune responses to the first and second vaccine doses.

Table 1 Baseline characteristics of study participants

		Healthy controls (n = 45)	Disease controls (n = 29)	Rheumatoid arthritis (n = 70)			
				no MTX, no bDMARDs (n = 14)	bDMARDs without MTX (n = 13)	MTX without bDMARDs (n = 23)	MTX with bDMARDs (n = 20)
Age, years		41 (35–46)	74 (69–77)	73 (67–82)	67 (64–73)	71 (62–78)	66 (59–72)
Sex (female)		24 (53.3)	16 (55.2)	11 (78.6)	11 (84.6)	19 (82.6)	18 (90.0)
Underlying disease							
Bronchial asthma		N/A	5 (17.2)	1 (7.1)	3 (23.1)	1 (4.4)	2 (10.0)
Sleep apnea syndrome		N/A	5 (17.2)	1 (7.1)	0 (0.0)	0 (0.0)	0 (0.0)
Hypertension		N/A	20 (69.0)	6 (42.9)	5 (38.5)	5 (21.7)	5 (25.0)
Chronic heart disease		N/A	11 (37.9)	3 (21.4)	1 (7.7)	3 (13.0)	0 (0.0)
Chronic kidney disease		N/A	1 (3.5)	1 (7.1)	2 (15.4)	1 (4.4)	0 (0.0)
Diabetes mellitus		N/A	4 (13.8)	3 (21.4)	2 (15.4)	3 (13.0)	0 (0.0)
Malignant tumor		N/A	1 (3.5)	1 (7.1)	1 (7.7)	0 (0.0)	0 (0.0)
Disease parameters							
Disease duration, years		N/A	N/A	8.0 (2.5–14.0)	15.0 (6.0–21.0)	7.0 (5.0–11.0)	14.0 (9.3–21.0)
RF-positive		N/A	N/A	8 (61.5)	9 (69.2)	15 (65.2)	17 (85.0)
ACPA-positive		N/A	N/A	6 (46.2)	10 (76.9)	14 (60.9)	16 (80.0)
DAS28-ESR		N/A	N/A	2.9 (2.3–3.5)	2.5 (1.5–2.9)	2.5 (2.1–3.6)	2.5 (1.8–2.9)
Ongoing therapy							
MTX		N/A	N/A	0 (0.0)	0 (0.0)	23 (100.0)	20 (100.0)
TNF inhibitor ^a		N/A	N/A	N/A	5 (38.5)	N/A	12 (60.0)
IL-6 inhibitor ^b		N/A	N/A	N/A	8 (61.5)	N/A	8 (40.0)
Tacrolimus		N/A	N/A	5 (35.7)	0 (0.0)	0 (0.0)	1 (5.0)
Salazosulphapyridine		N/A	N/A	3 (21.4)	1 (7.7)	1 (4.4)	0 (0.0)
Iguratimod		N/A	N/A	3 (21.4)	2 (15.4)	2 (8.7)	2 (10.0)
Corticosteroids		N/A	N/A	6 (42.9)	5 (38.5)	1 (4.4)	4 (20.0)
Others ^c		N/A	N/A	1 (7.1)	2 (15.4)	0 (0.0)	0 (0.0)
Vaccine type							
1st/2nd	BNT162b2	45 (100.0)	29 (100.0)	14 (100.0)	12 (92.3)	23 (100.0)	17 (85.0)
	mRNA-1273	0 (0.0)	0 (0.0)	0 (0.0)	1 (7.7)	0 (0.0)	3 (15.0)
3rd	BNT162b2	42 (100.0)	N/A	9 (90.0)	N/A	10 (62.5)	N/A
	mRNA-1273	0 (0.0)	N/A	1 (10.0)	N/A	6 (37.5)	N/A

Data are presented as n (%) or median (IQR)

ACPA, anti-citrullinated protein antibody; DAS28-ESR, Disease Activity Score-28 with erythrocyte sedimentation rate; IL-6, interleukin-6; MTX, methotrexate; N/A, not applicable; RF, rheumatoid factor; TNF, tumor necrosis factor

^aGolimumab (n = 6), infliximab (n = 4), etanercept (n = 4), adalimumab (n = 2), and certolizumab (n = 1)

^bTocilizumab (n = 13) and sarilumab (n = 3)

^cCyclosporine (n = 1), bucillamine (n = 1), and mizoribine (n = 1)

Most patients with RA exhibited cellular, but not humoral, immune responses at 6 months after two vaccine doses

Longitudinal evaluation showed that anti-S1 titers and neutralizing activity against the ancestral SARS-CoV-2 strain significantly decreased at T4 compared to T3 across all four groups (Fig. 3A, C). At T4, there was no significant difference in anti-S1 titers or the proportions of positive neutralizing responses among the groups (Fig. 3B, D). Next, we

performed IGRAs to evaluate the vaccine-elicited cellular immune response at T4 in patients with RA. We found that 74% (35/47) of samples were IGRA-positive. The concentrations of IFN- γ secreted by SARS-CoV-2 spike-specific T-cells in the ‘MTX without bDMARDs’ group were significantly lower than those in the ‘bDMARDs without MTX’ group ($P = 0.042$) (Fig. 3E). There was a significant difference in IFN- γ levels between the MTX-using and non-MTX-using groups ($P = 0.006$) (Online Resource 3C); however, the proportions of positive IGRA results did not reach the

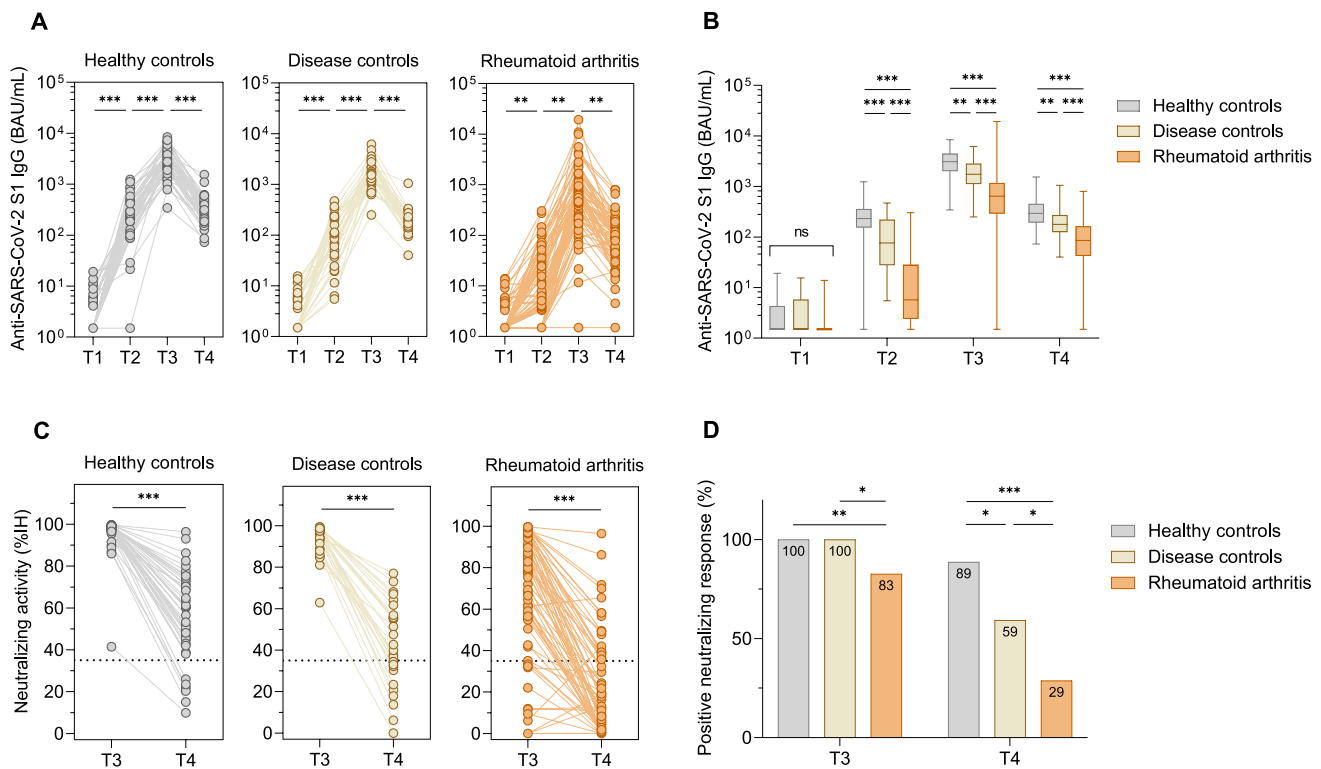


Fig. 2 Humoral immune responses to the first and second doses of SARS-CoV-2 mRNA vaccine in healthy controls, disease controls, and patients with rheumatoid arthritis. **A** Anti-SARS-CoV-2 S1 IgG antibody titers at each time point (T1–T4). **B** Boxplots (median [interquartile range]) of anti-SARS-CoV-2 S1 IgG antibody titers at each time point (T1–T4). **C** Neutralizing activity against the ancestral SARS-CoV-2 strain after the second dose (T3 and T4). Dotted line denotes the cut-off value of 35% IH for scoring a positive neutralizing response. **D** Proportion of positive neutralizing responses after the second dose (T3 and T4). The numbers above the bar graph represent the number of participants in each group. Statistical analyses were performed using Kruskal–Wallis tests with subsequent Steel–Dwass tests for multiple comparisons (A, B), Mann–Whitney U (C) or Fisher’s exact tests with subsequent Bonferroni corrections for multiple comparisons (D). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. ns, not significant

threshold for significance (Fig. 3F and Online Resource 3D). There was no significant correlation between neutralizing activity and IFN- γ levels at T4 (Fig. 3G). Furthermore, 49% (23/47) of samples were negative for neutralizing responses and were IGRA-positive, while only 6% (3/47) were positive for neutralizing responses and were IGRA-negative. We examined the correlation between immunosuppressant use and vaccine immunogenicity at T4 in patients with RA (Fig. 3H). Interestingly, all cases of poor humoral and cellular immune responses were observed in patients receiving MTX.

MTX-mediated attenuation of vaccine-induced humoral immune responses was dose-dependent

Additional analyses were performed to assess the effects of MTX use or dose on vaccine immunogenicity. Univariate analysis demonstrated negative correlations between MTX dose per body weight and anti-S1 titers at T3 ($P < 0.001$), neutralizing activity at T3 ($P < 0.001$), and IFN- γ levels at T4 ($P = 0.026$) (Fig. 4). Multivariate analyses with

adjustment for potential confounders, such as age, sex, vaccine type, and oral corticosteroid (OCS) use and dose, all of which are associated with immunogenicity following SARS-CoV-2 mRNA vaccination [7, 10, 24–27], were conducted using two different linear regression models. In model 1, which included the presence or absence of MTX and OCS use as categorical variables in the covariates, MTX use was negatively correlated with anti-S1 titer at T3, neutralizing activity at T3, and released IFN- γ levels at T4. In model 2, which included doses per body weight of MTX and OCS as continuous variables in the covariates, MTX dose was negatively correlated with anti-S1 titer and neutralizing activity at T3 and T4 (Table 2).

adjustment for potential confounders, such as age, sex, vaccine type, and oral corticosteroid (OCS) use and dose, all of which are associated with immunogenicity following SARS-CoV-2 mRNA vaccination [7, 10, 24–27], were conducted using two different linear regression models. In model 1, which included the presence or absence of MTX and OCS use as categorical variables in the covariates, MTX use was negatively correlated with anti-S1 titer at T3, neutralizing activity at T3, and released IFN- γ levels at T4. In model 2, which included doses per body weight of MTX and OCS as continuous variables in the covariates, MTX dose was negatively correlated with anti-S1 titer and neutralizing activity at T3 and T4 (Table 2).

MTX attenuated neutralizing activity against the Omicron variant, even after the third immunization

We evaluated the effect of MTX on humoral immune responses, including neutralization of VOCs, following the third vaccine dose. To this end, we analyzed sera collected at

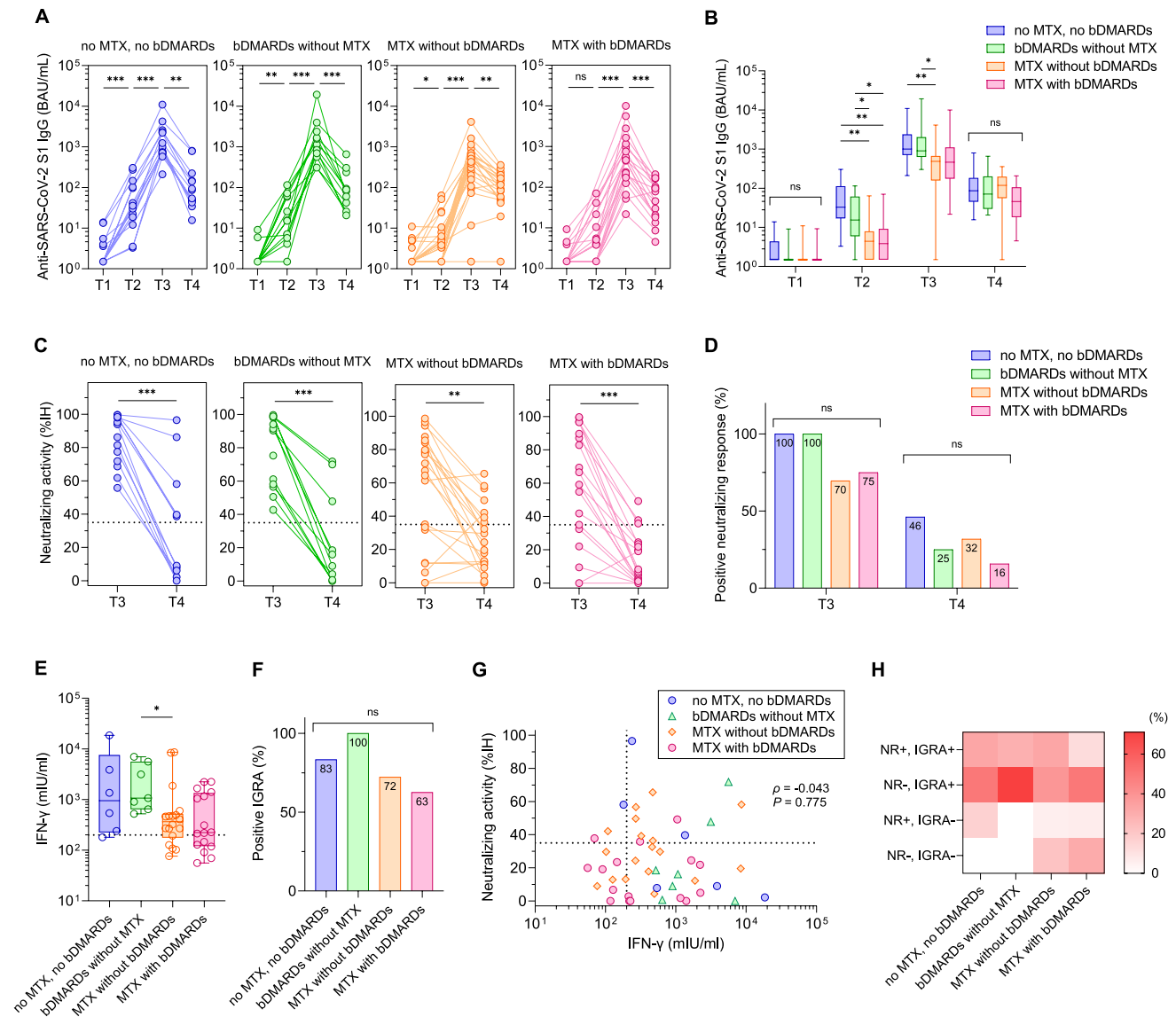


Fig. 3 Humoral and cellular immune responses to the first and second doses of SARS-CoV-2 mRNA vaccine in patients with rheumatoid arthritis grouped by MTX and bDMARD use. **A** Anti-SARS-CoV-2 S1 IgG antibody titers at each time point (T1–T4). **B** Boxplots (median [interquartile range]) of anti-SARS-CoV-2 S1 IgG antibody titers at each time point (T1–T4). **C** Neutralizing activity against the ancestral SARS-CoV-2 strain after the second dose (T3 and T4). The dotted line denotes the cut-off value of 35% IH for positive neutralizing responses. **D** Proportion of positive neutralizing responses after the second dose (T3 and T4). **E** Boxplots (median [interquartile range]) of IFN- γ levels secreted by SARS-CoV-2 spike-specific T-cells at T4. **F** Positivity rate of IGRA at T4. **G** Correlation between

neutralizing activity against the ancestral SARS-CoV-2 strain and IFN- γ levels secreted by SARS-CoV-2 spike-specific T-cells at T4. **H** Heatmap of the correlation between immunosuppressant use and vaccine-elicited immunogenicity. Horizontal and vertical dotted lines denote the cut-off values of 35% IH and 200 mIU/mL for positivity, respectively. Spearman rank correlation coefficient (ρ) and p -values (P) are indicated. Statistical analyses were performed using Kruskal–Wallis and Steel–Dwass tests (A, B, E), Mann–Whitney U (C), and Fisher’s exact tests with Bonferroni corrections (D, F). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. bDMARDs, biological disease-modifying anti-rheumatic drugs; IFN- γ , interferon-gamma; IGRA, IFN- γ release assay; MTX, methotrexate; ns, not significant

T3 and T5 from healthy controls and patients with RA in the ‘no MTX, no bDMARDs’ and ‘MTX without bDMARDs’ groups. Anti-S1 titers at T5 were significantly higher than those at T3 across all three groups (Fig. 5A). Notably, the significant difference in anti-S1 titers among these groups at T3 was ameliorated at T5 (Fig. 5B). A robust positive

correlation was confirmed between anti-S1 titers and neutralizing activity against the ancestral SARS-CoV-2 strain, and the Alpha and Delta variants, at T3 ($P < 0.001$ for each comparison). However, even antibodies with high anti-S1 titers only partially neutralized the Omicron variant at T3 (Online Resource 4A). In contrast, we observed a positive

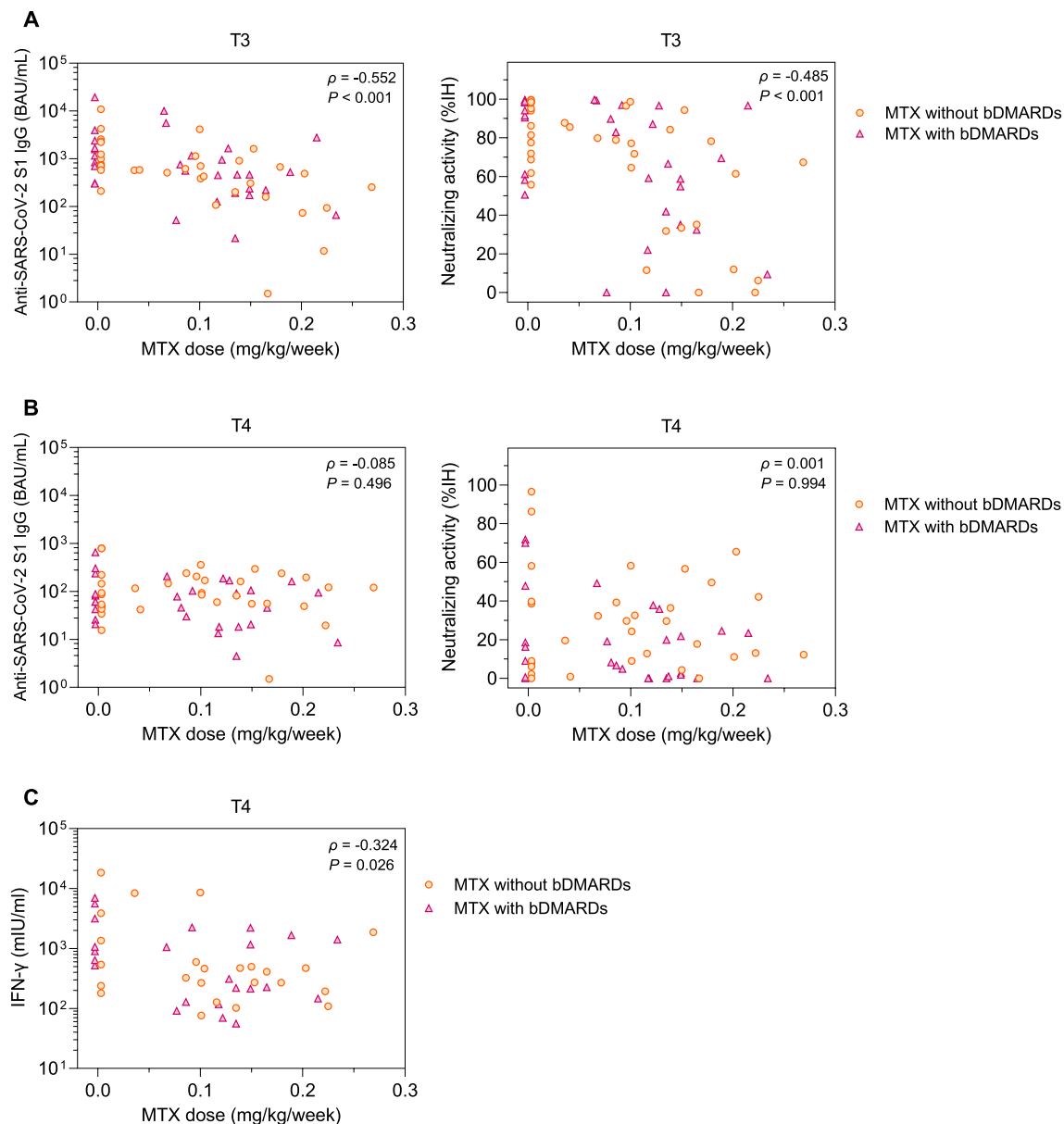


Fig. 4 Correlation between MTX dose and humoral or cellular immune response following the second dose of SARS-CoV-2 mRNA vaccine. **A** Correlation between MTX dose per body weight and anti-SARS-CoV-2 S1 IgG antibody titers at T3 (left) or neutralizing activity against the ancestral SARS-CoV-2 strain at T3 (right). **B** Correlation between MTX dose per body weight and anti-SARS-CoV-2 S1 IgG antibody titers at T4 (left) or neutralizing activity against the ancestral SARS-CoV-2 strain at T4 (right). **C** Correlation between MTX dose per body weight and IFN- γ levels secreted by SARS-

CoV-2 spike-specific T cells at T4. Orange dots: patients receiving MTX without bDMARDs ('MTX without bDMARDs') or those receiving neither MTX nor bDMARDs ('no bDMARDs, no MTX'). Red triangles: patients receiving MTX and bDMARDs ('MTX with bDMARDs') or those receiving bDMARDs without MTX ('bDMARDs without MTX'). Spearman rank correlation coefficients (ρ) and p -values (P) are denoted in each scatter plot. bDMARDs, biological disease-modifying anti-rheumatic drugs; IFN- γ , interferon-gamma; MTX, methotrexate

correlation between anti-S1 titers and neutralizing activity against the Omicron variant at T5 ($P < 0.001$) (Online Resource 4B). The third vaccine dose improved the neutralizing activity of antibodies at T5 against all tested VOCs and the ancestral SARS-CoV-2 strain (Online Resource 5). However, there was a significant gap across all groups'

neutralizing activity against the Omicron variant relative to the other two variants and the ancestral SARS-CoV-2 strain, even at T5 ($P < 0.001$ for each comparison) (Fig. 5C). Sera collected from the 'MTX without bDMARDs' group at T3 less effectively neutralized the ancestral SARS-CoV-2 strain (80%), as well as the Alpha (67%) and Delta (73%) variants,

Table 2 Multivariate linear regression analyses of factors associated with immune response after two doses of SARS-CoV-2 mRNA vaccine

	1 month after the 2nd dose (T3)				6 months after the 2nd dose (T4)					
	Anti-S1 IgG titer		Neutralizing activity		Anti-S1 IgG titer		Neutralizing activity		IFN- γ	
	β	P-value	β	P-value	β	P-value	β	P-value	β	P-value
Model 1										
Age	-0.16	0.191	-0.01	0.955	-0.06	0.633	-0.20	0.114	0.37	0.013*
Sex (female)	0.11	0.327	0.09	0.424	0.16	0.204	0.09	0.463	0.14	0.326
Vaccine (mRNA-1273)	0.19	0.119	0.24	0.037*	0.21	0.087	0.07	0.568	0.14	0.330
Oral corticosteroid use	-0.14	0.259	-0.17	0.153	-0.13	0.332	-0.24	0.080	-0.01	0.967
MTX use	-0.32	0.011*	-0.45	< 0.001***	-0.26	0.051	-0.20	0.138	-0.28	0.047*
Model 2										
Age	-0.29	0.025*	-0.10	0.384	-0.16	0.209	-0.30	0.025*	0.34	0.030*
Sex (female)	0.14	0.212	0.11	0.271	0.17	0.159	0.10	0.419	0.15	0.296
Vaccine (mRNA-1273)	0.10	0.370	0.17	0.118	0.17	0.168	0.04	0.763	0.11	0.430
Prednisone equivalent dose (per body weight)	-0.32	0.014*	-0.20	0.100	-0.31	0.021*	-0.38	0.007**	-0.00	0.976
MTX dose (per body weight)	-0.44	< 0.001***	-0.59	< 0.001***	-0.34	0.011*	-0.27	0.047*	-0.27	0.073

Valuables with $P < 0.05$ are indicated in bold

IFN- γ , interferon-gamma; MTX, methotrexate

* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

when compared to those from healthy controls (all 100%) (Fig. 5D). All groups acquired positive neutralizing activity against the ancestral SARS-CoV-2 strain and the Alpha and Delta variants at T5, whereas the proportion of positive neutralizing responses to the Omicron variant at T5 was lower in the 'MTX without bDMARDs' group than in healthy controls (33% vs. 88%, respectively, $P < 0.001$).

Discussion

This longitudinal cohort study revealed that MTX, but not bDMARDs, such as TNF and IL-6 inhibitors, attenuated immunogenicity following two doses of the SARS-CoV-2 mRNA vaccine in patients with RA, particularly resulting in a dose-dependent suppression of the humoral immune response. The third vaccine dose boosted anti-spike titers and improved neutralizing activity against the ancestral strain and variants, especially in patients receiving MTX, but only one-third of these patients had neutralizing activity against the Omicron variant.

Although several studies have evaluated the effects of MTX on humoral immune responses after two doses of SARS-CoV-2 mRNA vaccine in patients with IMIDs, the findings are inconsistent. Some studies observed decreased responses following MTX treatment [28–31], while others reported no impact on immunogenicity [7, 32–34]. These contradictory results may be attributed to background heterogeneity (e.g., in age, sex, and IMIDs) or inconsistent

methods of evaluating immune responses. In contrast, we focused solely on patients with RA, and performed robust multivariate analysis, adjusted for age and other potential confounders, to reliably detect drug-specific effects on vaccine immunogenicity.

B-cell response to vaccination is crucial for vaccine-specific antibody production [35, 36]. While MTX has been shown to hamper lymphocyte proliferation in patients with inflammatory arthritis [37], its effects on B-cell dynamics following vaccination are poorly understood. One study investigating pneumococcal conjugate vaccines noted that expansion of circulating plasmablasts and switched memory B cells following vaccination was absent in patients with RA who were treated with MTX [38]. Similarly, a recent study on the SARS-CoV-2 mRNA vaccine suggested that MTX treatment adversely affects B-cell recall responses following mRNA-1273 vaccination in patients with RA with previous SARS-CoV-2 infection [39]. The present study indicates that MTX may also exert an adverse, dose-dependent effect on B-cell dynamics following SARS-CoV-2 mRNA vaccination in COVID-19 naïve individuals.

Data on the impact of MTX on long-term immunogenicity following SARS-CoV-2 mRNA vaccination are scarce. We showed significant negative correlations between MTX dose and anti-S1 titers, as well as neutralizing activity against the ancestral SARS-CoV-2 strain, even 6 months after the second vaccine dose. A previous study showed that humoral immune responses to the SARS-CoV-2 mRNA vaccine wane over time, more prominently in men, older adults,

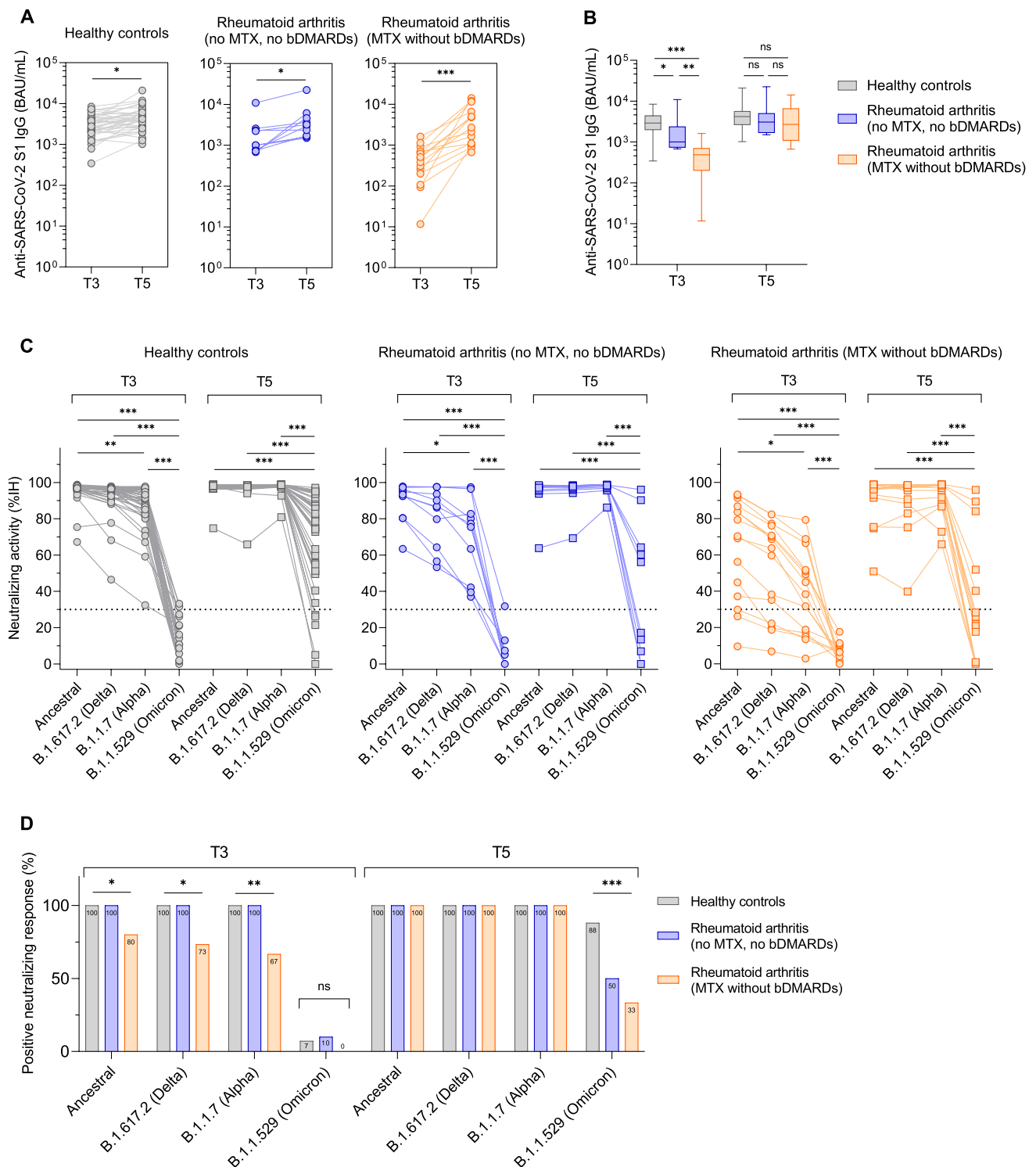


Fig. 5 Humoral immune responses to the third dose of SARS-CoV-2 mRNA vaccine in healthy controls and patients with rheumatoid arthritis. **A** Anti-SARS-CoV-2 S1 IgG antibody titers at T3 and T5. **B** Boxplots (median [interquartile range]) of anti-SARS-CoV-2 S1 IgG antibody titers at T3 and T5. **C** Neutralizing activity against the ancestral SARS-CoV-2 strain and variants of concern (VOCs) [B.1.617.2 (Delta) variant, B.1.1.7 (Alpha) variant, and B.1.1.529 (Omicron) variant] at T3 and T5. Solid lines connect samples from the same participant. Dotted line denotes the cut-off value of 30% IH

for scoring a positive neutralizing response. **D** Proportion of positive neutralizing responses against the ancestral SARS-CoV-2 strain and VOCs at T3 and T5. Statistical analyses were performed using the Mann–Whitney U (A), Kruskal–Wallis tests with Steel–Dwass corrections (B, C), or Fisher’s exact test with Bonferroni correction (D). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. bDMARDs, biological disease-modifying anti-rheumatic drugs; MTX, methotrexate; ns, not significant

and individuals with immunosuppressive conditions [40]. A recent study on patients with IMIDs showed that those treated with MTX had significantly reduced antibody titers and neutralizing activity at 3 and 6 months after a second dose of COVID-19 vaccine compared to those not treated with MTX [41]. The results of our study were consistent with these findings. Furthermore, we found that the long-term effects of MTX on humoral immune responses were dose-dependent.

In addition to the humoral immune response, we evaluated SARS-CoV-2-specific T-cell responses in patients with RA using IGRAs 6 months after the second vaccine dose. Most patients showed positive results, suggesting that, unlike humoral immunity, cellular immunity may be longer lasting. However, in multivariate analysis, we observed that MTX treatment was associated with a decrease in IFN- γ levels at 6 months after the second dose. Previous studies have reported that T-cell responses in patients with IMIDs taking MTX were significantly suppressed shortly after two doses of BNT162b2 [28, 33, 42]. A study using the same assay kit as used in this study reported a median IFN- γ value of 926 [IQR: 463–2272] mIU/mL at 180 days after the second dose of BNT162b2 in 271 SARS-CoV-2 naïve healthcare workers with a median age of 47 years [43]. This value aligns generally with the measurement data of the non-MTX group in our study (a median of 1068 [IQR: 530–4743] mIU/mL) and indicates considerable variability among individuals. Because IGRA was not evaluated in healthy and disease control groups, it is difficult to rigorously evaluate whether the observed variations in IFN- γ levels in patients with RA are related to the disease itself or treatment factors such as MTX. However, our findings suggest that MTX exerts a long-lasting, detrimental effect on cellular immune responses to the SARS-CoV-2 mRNA vaccine.

We showed that all patients receiving MTX exhibited marked increases in anti-S1 titers and positive neutralizing responses to the ancestral SARS-CoV-2 strain, and the Alpha and Delta variants, after the third vaccine dose, consistent with a previous study [42]. Furthermore, we found that, even after the third immunization, only half of the serum samples from patients receiving neither MTX nor bDMARDs, and one-third of serum samples from those receiving MTX but not bDMARDs, could neutralize the Omicron variant. To our knowledge, this is the first study to evaluate the neutralizing activity of circulating antibodies against the Omicron variant after the third vaccine dose. Several previous studies on healthy individuals, including older adults, reported that a third dose of SARS-CoV-2 mRNA vaccine achieves neutralizing activity against the Omicron variant to a similar degree as other VOCs, in most cases [44–46], which was confirmed in our study. However, we also found that patients with RA, especially those taking MTX, may have insufficient neutralizing activity against the Omicron variant, even after booster

immunization, indicating that these patients require continuous monitoring for breakthrough infections.

This study has several limitations that should be acknowledged. First, the sample size was limited, which may have resulted in an inability to detect smaller effect sizes discernible to more highly powered analysis. Second, the MTX doses administered to patients in this cohort were comparatively lower than those commonly administered in Western countries. Therefore, our findings should be validated in Western countries. Third, most patients with RA were positive for rheumatoid factor (RF), which may result in the false-positive detection of SARS-CoV-2 antibodies by ELISA [47]. However, previous studies have shown that, unlike IgM antibody titers, SARS-CoV-2 IgG and IgA antibody titers did not show false-positive results in RF-positive serum samples [48, 49]. Additionally, in this study, the potential effect of RF could be mitigated by diluting samples for more than 101 times as instructed in the protocol. Fourth, we did not evaluate T-cell responses to VOCs, because the stimulating antigen used in this assay was derived from the ancestral SARS-CoV-2 strain. Importantly, recent research on healthy volunteers showed that ancestral SARS-CoV-2-specific T-cells extracted from peripheral blood collected 6 months after two doses of BNT162b2 cross-recognized the Omicron variant [50]. The impact of MTX on VOC cross-recognition by SARS-CoV-2 mRNA vaccine-elicited T-cells requires further clarification.

In conclusion, our study provides evidence that MTX attenuates immunogenicity following two doses of SARS-CoV-2 mRNA vaccine in patients with RA, particularly resulting in a robust dose-dependent suppression of humoral immune responses. The third vaccine dose boosted humoral immunity in patients treated with MTX, while neutralizing activity against the Omicron variant remained insufficient. Further research is warranted to fully elucidate the effects of MTX and its dose on vaccine-elicited immunogenicity, including B- and T-cell dynamics.

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Author contributions MS, II, MN, MH, HIt, HU and TH contributed to the study design. MS, II, MT, KMurat, KMurak, Hik, IO, NH, KN, YH, MH, HIt and AM were involved in recruitment of study participants and sample collection. MS, IO, NH, KN and YH performed the assays. MS, II, IO, NH, AM, HU and TH are responsible for data analysis and interpretation. MS and II wrote the first draft of the manuscript. MT, KMurat, KMurak, Hik, IO, NH, KN, YH, MN, MH, HIt, HU, AM and TH were involved in critical proof reading of the manuscript. All

authors have approved the final version of the manuscript. II had full access to all of the data in this study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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Declarations

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Ethical approval This study was approved by the Medical Ethics Committee of Kyoto University (reference number R2713) and Obama Municipal Hospital (reference number 2–14) and was conducted in accordance with the principles of the Declaration of Helsinki.

Consent to participate All participants provided written informed consent before enrollment.

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