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ORIGINAL RESEARCH

Methotrexate treatment hampers induction of vaccine-specific CD4 T cell responses in patients with IMID

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ABSTRACT

Objectives Methotrexate (MTX) is one of the most commonly used medications to treat rheumatoid arthritis (RA). However, the effect of MTX treatment on cellular immune responses remains incompletely understood. This raises concerns about the vulnerability of these patients to emerging infections and following vaccination.

Methods In the current study, we investigated the impact of MTX treatment in patients with immune-mediated inflammatory disease on B and CD4 T cell SARS-CoV-2 vaccination responses. Eighteen patients with RA and two patients with psoriatic arthritis on MTX monotherapy were included, as well as 10 patients with RA without immunosuppressive treatment, and 29 healthy controls. CD4 T and B cell responses were analysed 7 days and 3–6 months after two SARS-CoV-2 messenger RNA vaccinations. High-dimensional flow cytometry analysis was used to analyse fresh whole blood, an activation-induced marker assay to measure antigen-specific CD4 T cells, and spike probes to study antigen-specific B cells.

Results Seven days following two SARS-CoV-2 vaccinations, total B and T cell counts were similar between MTX-treated patients and controls. In addition, spike-specific B cell frequencies were unaffected. Remarkably, the frequency of antigen-specific CD4 T cells was reduced in patients using MTX and correlated strongly with anti-RBD IgG antibodies. These results suggest that decreased CD4 T cell activity may result in slower vaccination antibody responses in MTX-treated patients.

Conclusion Taken together, MTX treatment reduces vaccine-induced CD4 T cell activation, which correlates with lower antibody responses.

Trial registration number NL8900.

WHAT IS ALREADY KNOWN ON THIS SUBJECT

⇒ Patients with immune-mediated inflammatory disease (IMID) treated with methotrexate (MTX) display delayed formation of newly formed SARS-CoV-2 antibody responses following vaccination; however, the cellular immune response is highly relevant for conferring long-term immune protection.
⇒ Data on antigen-specific CD4 T and B cell responses in MTX-treated patients following vaccination are scarce.

WHAT THIS STUDY ADDS

⇒ We conducted deep immune profiling of spike-specific B and T cells following messenger RNA vaccination in both MTX-treated and MTX-untreated patients with IMID, a level of analysis that has not been performed to this extent before.
⇒ The spike-specific B cell compartment revealed very similar B cell frequencies and phenotype between MTX-treated patients and controls.
⇒ In contrast, MTX-treated patients showed a reduced frequency of spike-specific CD4 T cells in comparison with MTX-untreated patients, which correlated strongly with anti-RBD antibody titres.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

⇒ In addition to humoral immune responses, it is wise to consider that cellular immune responses against novel pathogens may be less strong in MTX-treated patients with IMID.
⇒ Adaptation of vaccination strategies, such as temporarily pausing MTX treatment during vaccination, should be considered.

INTRODUCTION

Methotrexate (MTX) is considered the gold standard for treating rheumatoid arthritis (RA) and is also often used in the treatment of other immune-mediated inflammatory diseases (IMID) such as psoriatic arthritis

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(PsA) and spondyloarthritis (SpA). MTX is primarily known for its inhibitory effect on purine and pyrimidine synthesis required for cell division, making it a key anticancer treatment. While the exact anti-inflammatory mechanism of MTX in IMID treatment is still unknown, proposed modes of action include increased adenosine release, inhibition of nuclear factor-κB (NF-κB) signalling, increased expression of long intergenic non-coding RNA regulating inflammatory processes and promotion of nitric oxide synthase uncoupling leading to T cell apoptosis.[1 2]

Considering the high frequency of MTX medication used in IMID treatment, we have only limited knowledge about its effects on B and T cell responses. This raises the question how well MTX-treated patients can mount an immune response during infection or vaccination. Different studies on influenza or pneumococcal vaccine responses suggest that MTX treatment reduces antibody formation following vaccination, but the precise effect on B and T cell responses has not yet been established.[3–6] The SARS-CoV-2 pandemic provided an unique opportunity to systemically investigate newly developing antigen-specific immune responses in these patients. Studies on antibody responses following SARS-CoV-2 vaccination in MTX-treated patients revealed delayed antibody kinetics and lower antibody titres compared with controls after the first vaccine dose.[7–9] Results on antibody levels after two vaccine doses are conflicting, with some studies showing similar antibody titres to controls,[7 8 10] while others demonstrated decreased antibody titres.[9 11–14] Interestingly, pausing MTX treatment around the time of COVID-19 booster vaccination largely prevented the impaired humoral immune response.[15–17] The long-term effects of MTX on antibody responses have been investigated by Habermann *et al* and our study group, with both studies demonstrating no difference in seroconversion rates 3–6 months after vaccination when compared with controls.[18 19] Effective immune responses require both B and CD4 T cells. Previous studies on antigen-specific B and CD4 T cell vaccination responses in MTX-treated versus MTX-untreated patients with IMID reported similar induction of spike-specific B cells and activated (Ki67+CD38+) CD4 T cells, and similar levels of cytokine-secreting T cells.[8 12] In contrast, Klebanoff *et al* reported lower antigen-specific CD4 T cell induction post-SARS-CoV-2 vaccination in MTX-treated patients.[20] Nevertheless, a detailed description of the effects of MTX treatment on the induction and maintenance of antigen-specific B and CD4 T cell responses following vaccination is limited.

In the present study, we investigated the impact of MTX treatment on spike-specific B cell and CD4 T cell responses using high-dimensional flow cytometry following messenger RNA (mRNA)-1273 (Moderna) vaccination. We observed significantly lower antigen-specific CD4 T cell induction and minor changes in antigen-specific B cell phenotype compared with controls. This research will contribute to our understanding of the impact of MTX treatment on B and CD4 T cell immune responses.

METHODS

Study design

This is a substudy of a prospective multicentre multi-arm cohort study on SARS-CoV-2 vaccination in patients with various IMIDs (Target-to-B!). Details on the full study design have previously been described and can be found in online supplemental file 7 .[13] This study was registered at the Dutch Trial Register, trial ID: NL8900. Participants were vaccinated between April 2021 and October 2021 with the mRNA-1273 (Moderna) vaccine, in accordance with the Dutch national vaccination campaign guidelines. The primary vaccination was given at a 6-week interval, and a third vaccination was provided 3 months after the primary vaccination. Peripheral blood was collected by venipuncture 7–13 days and 3–6 months post-second vaccination, as well as 7–13 days post-third vaccination. Peripheral blood mononuclear cells (PBMCs) were isolated within 12 hours and frozen at −80°C.

Patient inclusion

Adult participants were actively recruited between 16 February 2021 and 20 August 2021 at the Academic Medical Centre Amsterdam and the Reade Center of Rheumatology in Amsterdam, the Netherlands. Patients diagnosed with RA or PsA and actively treated with MTX monotherapy were included, as well as patients with RA without systemic immunosuppressive treatment. All patients were asked to recruit their own sex-matched and age-matched healthy control (HC) subject who were not diagnosed with IMID nor did they receive any immunosuppressive treatment. Patients with RA and PsA were diagnosed by a certified rheumatologist. Active MTX treatment was defined as treatment that was started >3 months before primary vaccination. MTX treatment was not paused during first, second or third SARS-CoV-2 vaccination. RA controls did not receive any systemic immunosuppressive maintenance therapy at time of vaccination, most often due to minimal disease activity. RA controls did receive non-steroidal anti-inflammatory drugs, painkillers or local corticosteroid injections if needed. RAPID3 scores from electronic patient files, scored within 4 months before and 3 months post-primary vaccination, were used to remotely assess RA disease activity during the pandemic.[21] Exclusion criteria were concomitant treatment with other systemic immunosuppressants, pregnancy and previous SARS-CoV-2 infection (as evidenced by self-reported positive PCR and/or positive anti-receptor binding domain (RBD) antibodies before first vaccination and/or positive anti-nucleocapsid protein antibodies). If changes in immunosuppressive treatment were made or participants became infected with SARS-CoV-2 during the study, they were excluded from further analysis.

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Patient and public involvement

1. At what stage in the research process were patients/the public first involved in the research and how? *Answer:* Patients and patient groups have been involved throughout the whole study process. Target-to-B! consortium is a partnership between researchers, clinicians and various patient groups in which patient-representatives are involved in a dedicated Work Package. This contributes to new research ideas, determines priorities and guarantees maximal outreach of our results to the community. This study is a substudy of one of these research lines, namely T2B-COVID, that was highly prioritised given the uncertainty of efficacy of vaccines in patients treated with immunosuppressants. During the study, participants were notified about their SARS-CoV-2 antibody results. The study results were also shared during multiple patient-centred update meetings and mailing with patient groups, who further disseminated our results. Our data were shared with the Dutch national health institute and greatly shaped the national vaccine campaign for patients with IMID.
2. How were the research question(s) and outcome measures developed and informed by their priorities, experience and preferences? *Answer:* Aside from the humoral and cellular vaccine responses in patients with IMID to determine vaccine efficacy, the T2B-COVID study also focused on the concerns of patients that vaccines might worsen their underlying diseases. To this end, we introduced various disease-specific patient-reported outcome measures to assess disease activity of underlying disease after vaccination and COVID-19 infections.
3. How were patients/the public involved in the design of this study? *Answer*: As described above, patients were directly involved in both conceptualisation of the T2B-COVID study as well as members of the review committee of the National Grand Agency (ZonMw).
4. How were they involved in the recruitment to and conduct of the study? *Answer:* Patients were involved in various ways. For example, patients at Reade Center for Rheumatology research were asked to introduce an age-matched and gender-matched acquaintance to participate as an HC in our study. This led to active participation from both patients and HCs who were invested in the proper conduct of the study. Various new research questions were added during the course of the study, often based on growing or new concerns of patients during the course of the pandemic.
5. Were they asked to assess the burden of the intervention and time required to participate in the research? *Answer*: The burden of participation was limited to few time points of blood sampling (many of these combined with vaccination time points) and filling in online questionnaires.
6. How were (or will) they be involved in your plans to disseminate the study results to participants and relevant wider patient communities (eg, by choosing what information/results to share, when and in what format)? *Answer:* See above, our dissemination strategy included sharing of individual results with participants as soon as results were known. In addition, patient groups were separately informed on our main study findings and we organised seminars for patients to disseminate our results.

A detailed description of all technical methods, including the anti-RBD ELISA, whole blood flow cytometry, B cell spectral flow cytometry, activation-induced marker (AIM) assay and the statistical analysis can be found in the online supplemental materials and methods.

RESULTS

Cohort description

This substudy of Target to B (T2B!) included patients with IMID treated with MTX (n=20, including 18 patients with RA and 2 patients with PsA), RA controls (n=10) and HC (n=29) (table 1). Groups were similar in age and gender and were SARS-CoV-2-naïve before vaccination. Among MTX-treated patients, 19 used ≥15 mg MTX per week, while 1 patient used 7.5 mg/week. Disease activity before primary vaccination as measured with RAPID3 was similar between MTX-treated patients (mean 2.76, SD 1.88, n=18/20) and RA controls (mean 3.45, SD 2.77, n=6/10) (table 1). Information on individual participants can be found in online supplemental table 1. PBMCs were collected before SARS-CoV-2 mRNA vaccination (V1pre), 7 days after the second vaccination (V2D7) and 3–6 months after the second vaccination (V2M3-6; including eight MTX donors sampled at 3 months; and four MTX, seven RA controls and nine HC at 6 months) (figure 1A). In addition, serum samples were collected 28 days after each vaccination. Two SARS-CoV-2 vaccinations induced anti-RBD antibodies in all participants (figure 1B). MTX-treated patients had lower antibody titres after one, but not two vaccinations compared with RA controls, indicating slower induction of humoral vaccine responses in MTX-treated patients, consistent with previous findings.[7–9]

Whole blood flow cytometry reveals differences in CTLA4 expression on CD4 T cells

As humoral immunity is tightly regulated by B and CD4 T cell responses, we continued to analyse the effect of MTX on B cell and CD4 T cell kinetics post-SARS-CoV-2 vaccination. First, fresh whole blood flow cytometry analysis was performed at baseline and V2D7 in 18 MTX-treated patients and 24 HCs, to investigate the effect of MTX treatment on B and CD4 T cell vaccination responses. Fresh whole blood was used to study plasmablasts and plasma cells, as these cell types are affected by freezing. Using a 38-marker panel and FlowSOM clustering, eight major B cell and antibody secreting cell (ASC) clusters were identified including plasmablasts, plasma cells, memory B cells and atypical B cell subsets (figure 1C, online supplemental figure S1A). A second panel defined memory T

Table 1 Baseline characteristics of study participants

	Patients with IMID		Healthy controls (n=29)
	Methotrexate (n=20)	RA controls (n=10)	
Age in years, mean (SD)	56 (9)	53 (7)	51 (8)
Female sex, n (%)	18 (90%)	10 (100%)	21 (72%)
Disease, n (%)			
RA	18 (90%)	10 (100%)	–
Psoriatic arthritis	2 (10%)	0	–
Methotrexate dose (mg/week), n (%)			
7.5	1 (5%)	–	–
15–17.5	9 (45%)	–	–
20–25	10 (50%)	–	–
Time since IMID diagnosis in years, median (IQR)	8.5 (10.25)	10 (10)	–
RAPID3 score, mean (SD)	2.76 (1.88)	3.45 (2.77)	–
Days between second vaccination and V2D7 PBMC collection, median (IQR)	7 (0)	7.5 (4)	7 (0)
Days between second vaccination and V2M3-6 PBMC collection, median (IQR)	113 (38)	182 (10)	185 (5)
Seroconversion after second vaccination, n (%)	20 (100%)	10 (100%)	29 (100%)
Anti-RBD IgG titre, median (IQR)	198 (206)	292 (133)	222 (106)
Participants included per analysis, n			
Fresh whole blood	18	7	24
Spike-specific B cell	16	10	10
Spike-specific CD4 T cell	16	10	10

IMID, immune-mediated inflammatory disease; PBMC, peripheral blood mononuclear cell; RA, rheumatoid arthritis; RBD, receptor binding domain; V2D7, 7 days after second vaccination; V2M3-6, 3 to 6 months after second vaccination.

cell subsets, including T helper (Th) and circulating T follicular helper cells (cTfh) (figure 1D, online supplemental figure S1B,C). Counts per microlitre of blood of B and CD4 T cell populations were compared before and after two vaccinations and between MTX-treated patients and HC. B and CD4 T cell counts were similar between MTX-treated patients and HCs, although slight differences in CXCR3+Th2 and CD4 effector memory cells (EM) at baseline and CXCR3+Th17 cells at V2D7 were observed (figure 1E). Importantly, ASCs expanded in both MTX-treated patients and controls following SARS-CoV-2 vaccination (figure 1F).

Because our whole blood analysis lacks antigen-specificity, we studied activated CD4 T cells, which indicate antigen reactivity, using expression of PD1, CD40L, CD137, CD38/HLA-DR co-expression, CTLA4, TIGIT and TIM3. SARS-CoV-2 vaccination did not induce CD40L, CD137 or CD38/HLA-DR CD4 T cell expression, which are the most acclaimed CD4 T cell activation markers (figure 1G). On the other hand, PD1, CTLA4, TIGIT and TIM3 expressions were upregulated on vaccination in several CD4 T cell subsets (figure 1G), but were not differently expressed between MTX-treated patients and HCs at V2D7 (figure 1H). At baseline though, CD4 T cells of MTX-treated patients expressed significantly less CTLA4 and more PD1, compared with HCs (figure 1H). Validation in a small cohort of seven RA control patients demonstrated that at baseline CTLA4 expression, but not PD1 expression, differed between MTX-treated and MTX-untreated patients with RA, indicating that the reduced CTLA4 expression was attributed to MTX treatment and not RA disease itself (online supplemental figure S1D). Remarkably, vaccination restored CTLA4+ CD4T cell frequencies in MTX-treated patients to a similar level as controls (online supplemental figure S1E).

In summary, fresh whole blood analysis revealed similar dynamics of multiple B and CD4 T cell populations on SARS-CoV-2 vaccination, even though fewer CTLA4+ CD4T cells were observed in MTX-treated patients at baseline. To better understand the effect of MTX treatment on antigen-specific vaccine responses, SARS-CoV-2-specific CD4 T and B cells were further examined.

Methotrexate treatment causes minor differences in spike-specific B cell phenotype

To study the effect of MTX treatment on antigen-specific B cell responses, cryopreserved PBMCs were stained with a 31-marker spectral flow panel including fluorescently labelled spike and RBD probes (online supplemental figure S2A,B, online supplemental tables 4 and

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Figure 1 SARS-CoV-2 antibody responses and fresh whole blood analysis. (A) Study design with timing of SARS-CoV-2 mRNA-1273 vaccination and peripheral blood collection. (B) Dynamics of anti-RBD antibody titres over time, with comparisons between MTX and RA control, as well as HC versus RA control at V1D28 (MTX n=19, RA control n=10, HC n=26) and at V2D28 (MTX n=20, RA control n=9, HC n=27). Horizontal bars represent the median. (C–D) Fit-SNE map and cluster identification of B cell subsets, and (D) CD4 T cell subsets in whole blood using FlowSOM analysis, including data from both MTX-treated patients (n=18) and HCs (n=24) at baseline and V2D7. (E–F) Volcano plot displaying differentially expressed B cell and CD4 T cell populations in counts per µL, with a log2 fold change >0.5 and p value <0.5 (E) between HC and MTX and (F) between V1pre and V2D7. Numbers in the top left and right corner of volcano plots indicate the number of differentially expressed populations. (G–H) Volcano plot illustrating percentage of CD4 T cells expressing PD1, CTLA4, TIM3, TIGIT, CD137, CD40L or CD38/HLA-DR, with a log2 fold change >0.5 and p value <0.5 (G) between V1pre and V2D7 and (H) between MTX and HC. Statistical significance was determined using the Wilcoxon ranked sum test for unpaired comparisons and the Wilcoxon signed rank test for paired comparisons. Bonferroni-Holm correction for multiple comparisons was used to adjust for multiple testing. HC, healthy control; MTX, methotrexate; ns, not significant; PBMC, peripheral blood mononuclear cell; RA, rheumatoid arthritis; RBD, receptor binding domain.

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5). Following vaccination, MTX-treated patients and RA controls generated similar percentages of spike-specific and RBD-specific B cells, although a trend towards lower induction in MTX-treated patients compared with RA controls was observed (spike median (IQR): MTX: 0.26 (0.08–0.44); RA control: 0.38 (0.17–0.59); figure 2A). We showed in whole blood that total number of CD19+ B cells is unaffected by MTX treatment (online supplemental figure S1D). Next, unsupervised FlowSOM clustering revealed 16 different B cell clusters (online supplemental figure S2C–E). These clusters included naïve B cells (IgD+CD27−), IgG/IgA/IgM memory B cells (MBC; CD27+CD71+CD21+CD38−), IgG/IgA/IgM ASC (CD27hiCD38hi), IgG/IgA activated B cells (ActBC; CD27+CD71+CD21−CD38−) and atypical B cells (IgD−CD27−CD71−CD11c+/−). We observed similar spike-specific B cell dynamics as other studies,[22–24] demonstrating an expansion of IgG ActBCs, IgG ASC, IgA ASC and IgG double negatives 2 (DN2) at day 7, while IgG MBCs dominated 3–6 months later (figure 2B). The frequencies of the 16 populations were compared between our patient groups. MTX-treated patients had slightly lower spike-specific IgG MBC and naïve B cells compared with RA controls at V2D7 (figure 2C), even though frequencies of spike-specific B cells were similar (figure 2A). Furthermore, RA controls displayed increased IgG ASC when compared with HC at V2M3-6. However, IgG MBCs contributed only very little to the initial spike-specific B cell response at V2D7, and IgG ASC frequencies had a minimal contribution to the long-term spike-specific response (figure 2B,D). Remarkably, spike- and RBD-specific B cells correlated positively with anti-RBD IgG antibodies in MTX-treated patients, but not in controls (figure 2E, online supplemental figure S2F). This effect was strongest at V2D7 and mainly associated with IgG ActBC and IgG DN2, but not with IgG MBC suggesting that the lower frequency of IgM MBC in MTX-treated patients at V2D7 is probably not a driver of the slower antibody response (figure 2F).

Taken together, spike-specific B cell phenotype was slightly affected by MTX treatment in patients with RA, but observed phenotypic differences did not correlate with the induction of specific antibodies. Overall, total specific B cell frequencies correlated with RBD-specific antibodies in MTX-treated patients only.

Methotrexate dampens spike-specific CD4 T cell induction following vaccination

As our analysis of the spike-specific B cell compartment could not explain the slower antibody kinetics observed in MTX-treated patients, we next investigated the effect of MTX treatment on the spike-specific CD4 T cell response. CD4 T cells play an essential role in the generation of high-affinity antibodies, by aiding in class-switch recombination and B cell receptor affinity maturation. To capture the spike-specific CD4 T cell response, an AIM assay was performed using CD40L and CD137 co-expression as proxy for antigen-specific CD4 T cells following spike peptide stimulation *in vitro* (figure 3A and online supplemental figure S3A). Seven days after second vaccination, up to 0.9% of CD4 T cells were spike-specific, which decreased over time (figure 3B, online supplemental figure S3B). Strikingly, patients treated with MTX had lower induction of spike-specific CD4 T cells compared with RA controls at V2D7 (median (IQR): 0.17 (0.12–0.21) and 0.29 (0.18–0.39), respectively, p=0.017; figure 3B), while total number of CD4 T cells were not affected by MTX treatment as determined in fresh whole blood (online supplemental figure S1D). Furthermore, spike-specific CD4 T cells correlated with anti-RBD antibody titres, but only in the MTX-treated group, suggesting that the reduced number of spike-specific CD4 T cells may limit antibody responses (figure 3C, online supplemental figure S3C). Moreover, CD4 T cells strongly correlated with spike-specific B cells in MTX-treated patients (figure 3D). In this small cohort, smoking and years since IMID diagnosis were not associated with CD4 T cell frequencies (data not shown).

To validate our findings of reduced spike-specific CD4 T cells using CD40L+CD137+ expression, we measured spike peptide-induced co-expression of cytokines, including tumour necrosis factor (TNF)-α, interferon (IFN)-γ and interleukin (IL)-2, in CD4 T cells as another proxy for spike-specific T cell activation (online supplemental figure S3D). Indeed, CD4 T cells of MTX-treated patients also co-expressed fewer cytokines after spike stimulation (median (IQR); MTX: 0.06 (0.04–0.08); RA controls: 0.11 (0.07–0.14); p=0.046, figure 3E). Not surprisingly, these spike-induced cytokine co-expressing CD4 T cells correlated with CD40L+CD137+ CD4 T cells (figure 3F; online supplemental figure S3E). Furthermore, the percentage of CD40L+CD137+ CD4 T cells that (co-)expressed TNF-α, IFN-γ and IL-2 was similar between all groups (online supplemental figure S3F).

Next, we assessed whether spike-specific CD4 T cells were phenotypically similar between MTX-treated patients and controls. First, CD27 and CD45RA were used to identify central memory (CM) and EM spike-specific CD4 T cells (figure 3G). The majority of spike-specific CD4 T cells had a CM phenotype which expanded over time (mean; V2D7: 57%, V2M3-6: 72% in MTX-treated patients), while the EM compartment contracted (mean; V2D7: 29%, V2M3-6: 6.7%). No differences in CM or EM phenotype were observed between the groups (figure 3H). In addition, the expression of activation markers PD1 and ICOS was determined (figure 3I). The majority of spike-specific CD4 T cells expressed both PD1 and ICOS at V2D7, a phenotype that was lost over time (mean; V2D7: 70%, V2M3-6: 29%, in MTX-treated patients) (figure 3I). Again, PD1+ICOS+ expression in spike-specific CD4 T cells was similar between MTX-treated and MTX-untreated patients with RA (figure 3J).

A third vaccination was provided to MTX-treated patients (n=8) 109–113 days after the second vaccination (figure 4A). Anti-RBD antibody titres rose on a third vaccination but did not increase compared with after two

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Figure 2 Spike-specific B cell vaccination responses are not affected by MTX treatment. (A) Percentage of spike-specific and RBD-specific B cells of total CD19+B cells at V2D7 (MTX n=16, RA control n=10, HC n=10) and V2M3-6 (MTX n=12, RA control n=6, HC n=9). Horizontal bars represent the median. (B) Stacked bar graph illustrating the mean proportion of the 16 spike-specific B cell clusters as a percentage of total CD19+ B cells. (C) Heatmap displaying the differential cluster expression between MTX versus RA control and RA control versus HCs at V2D7 and V2M3-6. (D) Kinetics of significantly differentially expressed B cell clusters (as observed in 1C) over time. (E) Correlation analysis of %spike-specific B cells at V2D7 vs anti-RBD antibody titre at V2D7 or V2D28, in both MTX-treated patients and controls. (F) Correlation analysis of spike-specific IgG+ clusters at V2D7 with anti-RBD antibody titre at V2D7 in MTX-treated patients. Statistical significance was determined using the Wilcoxon ranked sum test for unpaired comparisons with Bonferroni-Holm correction for multiple comparisons. Correlations were calculated using Spearman's rank correlation. ASC, antibody secreting cell; HC, healthy control; MTX, methotrexate; ns, not significant; RA, rheumatoid arthritis; RBD, receptor binding domain.

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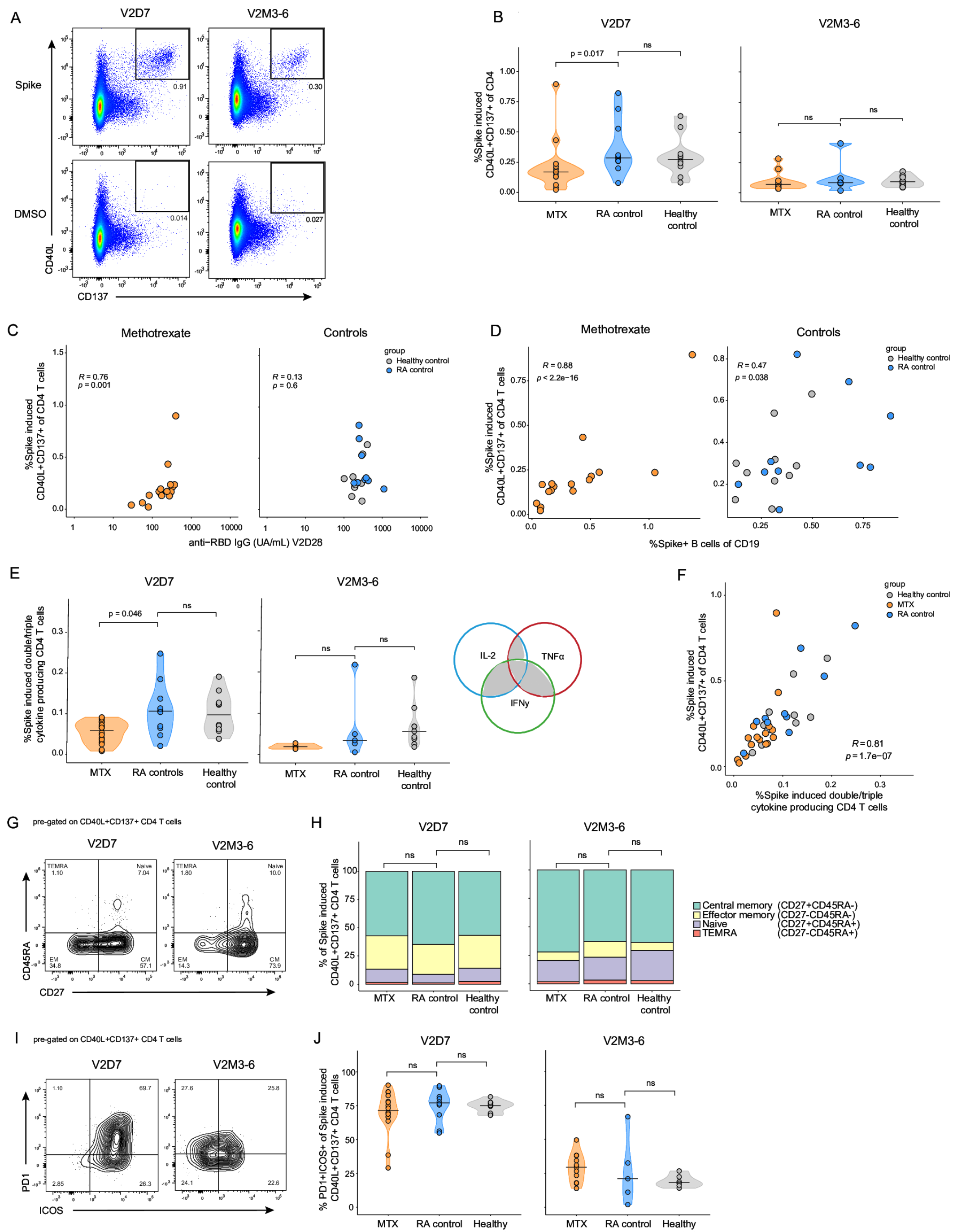

Figure 3 MTX reduces spike-induced CD40L+CD137+ CD4T cells post-SARS-CoV-2 vaccination. (A) Representative flow cytometry plots of spike-induced and DMSO-induced (background) CD40L+CD137+ CD4T cells at V2D7 (MTX n=16, RA control n=10, HC n=10) and at V2M3-6 (MTX n=12, RA control n=6, HC n=9). (B) Percentage of spike-induced CD40L+CD137+ CD4T cells per group. Background was subtracted. Horizontal bars represent the median. (C–D) Correlation analysis of spike-induced CD40L+CD137+ CD4T cells at V2D7 with (C) anti-RBD antibody titre at V2D28 or (D) spike-specific B cells at V2D7, in both MTX-treated patients and controls. (E) Percentage of spike-induced CD4 T cells expressing two or three of the following cytokines: interleukin-2, tumour necrosis factor-α and interferon-γ. (F) Correlation analysis of spike-induced CD40L+CD137+ CD4T cells and spike-induced double or triple cytokine-producing CD4 T cells, at V2D7. (G) Representative flow cytometry plots of CD27 and CD45RA expression on spike-induced CD40L+CD137+ CD4T cells. (H) Phenotypic distribution of central memory (CD27+CD45RA–), effector memory (CD27–CD45RA–), naïve (CD27+CD45RA+) and TEMRA (CD27–CD45RA+) spike-induced CD40L+CD137+ CD4T cells. (I) Representative flow cytometry plots of expression of PD1 and ICOS on spike-induced CD40L+CD137+ CD4T cells. (J) Percentage of PD1 and ICOS expressing spike-induced CD40L+CD137+ CD4T cells per time point. Statistical significance was determined using Wilcoxon ranked sum test for unpaired comparisons with Bonferroni-Holm correction for multiple comparisons. Correlations were calculated using Spearman's rank correlation. ICOS, inducible T-cell costimulator; MTX, methotrexate; ns, not significant; PD1, programmed cell death 1; RA, rheumatoid arthritis; RBD, receptor binding domain.

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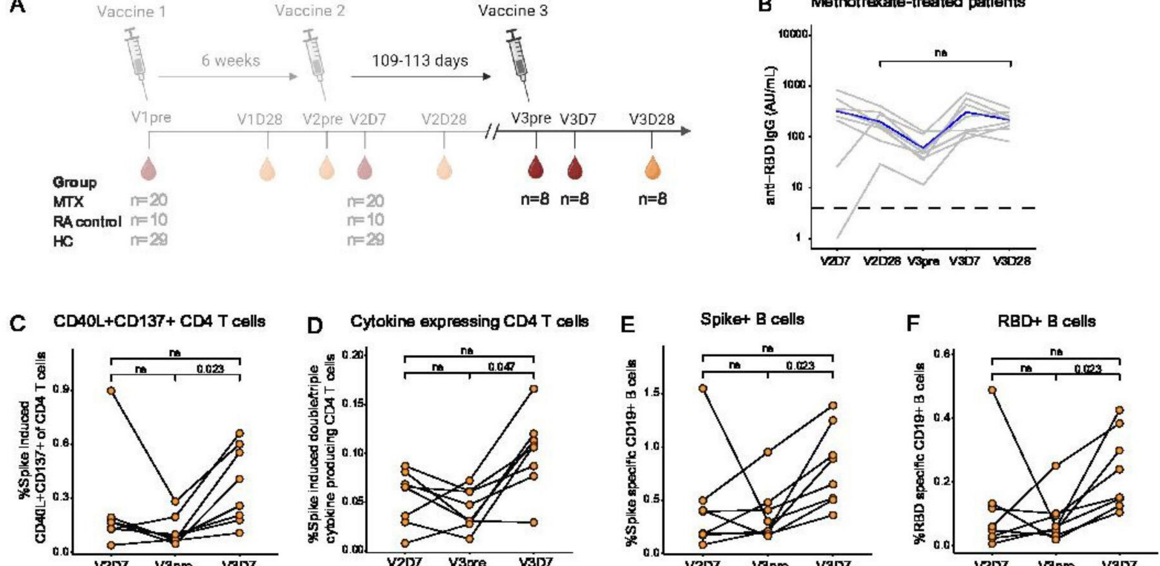

Figure 4 A third SARS-CoV-2 vaccination shows a trend of increased spike-specific B and CD4 T cell responses. (A) Schematic overview of third SARS-CoV-2 mRNA-1273 vaccination, provided 109–113 days post-second vaccination to MTX-treated patients (n=8). (B) Anti-RBD antibody titre dynamics in MTX-treated patients after second and third SARS-CoV-2 vaccination. (C) Percentage of spike-induced CD40L+CD137+ CD4 T cells, (D) spike-induced double or triple cytokine-producing CD4 T cells, (E) spike-specific B cells and (F) RBD-specific B cells 7 days post-second vaccination (V2D7), at the day of third vaccination (V3pre) and 7 days post-third vaccination (V3D7). Statistical significance was determined using Wilcoxon signed-rank test with Bonferroni-Holm correction for multiple comparisons. HC, healthy control; MTX, methotrexate; ns, not significant; RA, rheumatoid arthritis; RBD, receptor binding domain.

vaccinations (figure 4B). On the other hand, a trend was observed of an increased percentage of spike-specific CD4 T cells (figure 4C,D), and spike-specific and RBD-specific B cells (figure 4E,F) 7 days after a third vaccination as compared with after a second vaccination. However, these differences did not reach statistical significance.

In summary, MTX treatment affected the magnitude of spike-specific CD4 T cell induction following SARS-CoV-2 vaccination. Moreover, spike-specific CD4 T cells correlated with antibody titres and spike-specific B cells 7 days after second vaccination, but only in MTX-treated patients.

DISCUSSION

In the current study, the impact of MTX therapy in patients with IMID on B and CD4 T cell vaccination responses was investigated. This is of particular interest since MTX-treated patients have slower humoral kinetics following vaccination.[7–9] Our findings indicate that MTX treatment suppresses the induction of spike-specific CD4 T cells following SARS-CoV-2 mRNA vaccination, and causes minor changes in spike-specific B cell phenotype. A third vaccination may potentially enhance the formation of antigen-specific CD4 T and B cells, but further research in larger cohorts is necessary to substantiate this.

In-depth fresh whole blood analysis revealed no major differences in B cell and CD4 T cell counts following vaccination in MTX-treated patients versus HCs. However, we did observe fewer CTLA4+ CD4 T cells at baseline in MTX-treated patients compared with HC and RA controls. Previous studies have described MTX treatment to rescue CTLA4 expression on CD4 regulatory T cells (Tregs) in patients with RA.[25 26] In contrast, our data suggest the opposite: MTX-treated patients had lower frequencies of CTLA4+ CD4 T cells, although this effect disappeared following vaccination. This interesting observation warrants further research. Interestingly, Nived *et al* performed a similar study in MTX-treated patients with RA in the context of pneumococcal vaccination.[27] In agreement with our findings, no differences in B and T cell counts were observed between MTX-treated patients and controls at baseline. However, they reported lower plasmablasts and lower Th17 CD4 T cells in MTX-treated patients following pneumococcal vaccination. We did not observe plasmablasts to be affected by MTX treatment but did witness slightly lower numbers of CXCR3+Th17 cells following vaccination. Although MTX has been described to reduce Th17 frequencies,[28–30] it remains debatable whether this impacts cellular vaccination responses.

Since we did not observe significant alterations in B and CD4 T cell vaccination responses in whole blood, we moved on to antigen-specific analyses to enhance resolution. Our most striking finding was the reduction in spike-specific CD4 T vaccination responses in patients using MTX 7 days after vaccination. As we included both RA controls and HCs, we demonstrate that this effect is attributed to the MTX treatment and not the RA disease. These findings are corroborated by Klebanoff *et al*, who also detected lower spike-specific CD4 T cell induction

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following SARS-CoV-2 vaccination in MTX-treated patients with RA.[20] However, the question remains via which mechanism MTX affects antigen-specific CD4 T cell induction. Freeman *et al* demonstrated that MTX hampers CD4 T cell proliferation, but not activation, when added to PBMCs *in vitro*.[1] This suggests that the lower spike-specific CD4 T cell response observed in our study may be caused by lower CD4 T cell proliferation capacity. It is however very difficult to translate *in vitro* MTX culture data to *in vivo*, as MTX is a folic acid antagonist and therefore hampers the cell cycle in high doses. Importantly, we found a profound correlation between antigen-specific CD4 T cells and the humoral response in MTX-treated patients. As this correlation was not observed in controls, we propose that a minimal required threshold for the number of antigen-specific CD4 T cells is necessary to provide optimal B cell help. While there is currently no conclusive evidence linking MTX treatment in patients with IMID to an increased risk of SARS-CoV-2 breakthrough infection or severe COVID-19 following vaccination,[31–35] previous studies have shown that temporary MTX discontinuation around influenza or COVID-19 vaccination may improve humoral vaccine responses without substantial impact on disease flares.[15–17 36 37] Moreover, Martinéz-Fleta *et al* reported increased IFN-γ-secreting T cell frequencies when MTX was withheld for 2 weeks following SARS-CoV-2 vaccination.[16] These data suggest that temporarily suspending MTX 2 weeks following each vaccination could increase vaccine-specific CD4 T cell responses.

Interestingly, although antibody kinetics in MTX-treated individuals were slower and spike-specific CD4 T cells were reduced, the percentage and phenotype of spike-specific B cells following two SARS-CoV-2 vaccinations were similar to controls. We observed similar spike-specific B cell dynamics in MTX-treated patients as those previously described in healthy donors,[22–24] namely an early induction of ActBCs and ASCs, followed by MBC formation over time. Previous studies in patients with IMID treated with MTX reported reduced antibody responses following influenza, pneumococcal and SARS-CoV-2 vaccination,[3 5 6 11–14] but results on antigen-specific B cells are contradicting. While one study found reduced percentages of antigen-specific plasmablasts and memory B cells following vaccination,[5] others reported, similar to what we found, no effects.[4 20] Although all studies collected PBMCs around 1 week post-vaccination, the exact timing of PBMC collection may explain observed differences in ASC frequencies.[22] Subtle differences in antigen-specific B cell frequency and phenotype might as well be affected by sampling after either one or two subsequent vaccinations, type of vaccination and previous antigenic exposure. We hypothesise that our timing of antigen-specific B cell analysis is suboptimal as B cell frequencies after two vaccinations may already have been catching up and that sampling after one vaccination would have given more insight into the modulatory effects of MTX on B cell kinetics.

This study had some limitations. First, the number of study participants was relatively small. Second, we included patients with RA without systemic immunosuppressive treatment as disease controls. These patients are rare since most patients with RA are actively treated with disease-modifying antirheumatic drugs, biologicals or systemic corticosteroids to control disease activity. Therefore, our RA control group may have a bias towards lower disease activity, which could potentially confound the differential CD4 T cell frequencies observed. Furthermore, technical constraints prevented us from characterising spike-specific cTfh cells, which could provide further insights into the relationship between humoral and CD4 T cell vaccination responses. However, Klebanoff *et al* did not observe significant differences in Th or Tfh phenotype between MTX-treated patients and controls.[20] Also, the longitudinal time point (V2M3-6) included a spread in the timing of sampling; half of the MTX-treated donors were sampled at 3 months post-second vaccination, and all other donors, including the remaining MTX donors and all controls, at 6 months. Therefore, our results might overestimate the percentage of spike-specific CD4 and B cells in MTX-treated patients at this later time point after vaccination, as some donors were sampled earlier, introducing a possible underestimation of the effect of MTX on long-term vaccine responses.

Overall, these data present evidence of reduced vaccine-specific CD4 T cell responses in MTX-treated patients with RA, which is associated with slower antibody kinetics in these patients. Although it remains unclear to what extent lower CD4 T cell responses impact clinical outcomes in these patients, it is wise to consider that CD4 T cell immunity may be less robust in these patients during future vaccinations or emerging infections. Furthermore, these data potentially contribute to elucidating the anti-inflammatory mechanism of action of MTX in IMIDs.

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Contributors AtB led the study and is the guarantor. SMvH, TWK, CEvdS supervised the study. FE, TWK, SMvH, AtB conceptualised the study. AtB, CK, LFB, AB, LK, NJMV, CEvdS, TR, MC, JJG-V, MJvG and MS designed the methodology. LYLK, LFB, AB, LK, NJMV, VALK, MCD, CM, TJ, RRH and JvdD performed the experiments. LYLK, LFB, CK, SMvH and AtB verified the overall replication/reproducibility of the research output. LYLK, LFB, AB, LK, TA and NJMV analysed the data. LYLK, JvdD, EWS, LB, GW, SWT provided study materials. LYLK, JvdD, LW, VALK, EWS managed the research data. LYLK, JvdD, LW, EWS, CEvdS, TR, TWK, FE, SMvH and AtB managed and coordinated the research activity and planning. LW, TR, TWK, FE, SMvH, AB acquired the financial support for the project leading to this publication. LYLK, LK, MC, TA and NJMV designed and implemented computer codes. LYLK visualised the work. LYLK and AtB wrote the original draft. All authors reviewed and approved the manuscript.

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