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The T-cell response to SARS-CoV- 2 vaccination persists beyond six months in rheumatoid arthritis patients treated with rituximab

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Abstract

Background The spike protein-specific humoral response observed after SARS-CoV- 2 vaccination is decreased in rheumatoid arthritis (RA) patients treated with rituximab (RTX). However, when analyzed immediately after vaccination, the spike-specific T-cell immune response appears to be preserved. The possible persistence of specific T cells over the long term is underexplored and could be a useful decision-making tool for deciding when to perform revaccination. This study aimed to assess the persistence of T-cell-mediated immunity after the last SARS-CoV- 2 vaccination or infection (named "SARS-CoV- 2 boost" in this study) in RA patients treated with RTX. Clinical and biological parameters that can influence this immune system were also explored.

Methods Our observational study cohort included 51 RA patients treated with RTX and 24 RA patients treated with other disease-modifying antirheumatic drugs (DMARDs) who had received at least one dose of the SARS-CoV- 2 mRNA vaccine. The T-cell immune response was assessed by flow cytometry, which focused on antigen-specific T-cell characterization between 3 and 18 months after the last SARS-CoV- 2 boost. T-cell activation was assessed by measuring CD69, CD154, CD137 and CD107a surface expression.

Results As expected, even if a lower mean antibody titer was measured in RA patients receiving RTX (RA RTX) than in RA patients treated with therapies other than RTX ($p = 0.034$), all patients exhibited CD4 + and CD8 + T-cell spike protein-specific responses, with an even greater spike-specific CD8 + T-cell response in RA RTX patients ($p < 0.001$). The main finding of our study was that the T-cell response remarkably persisted up to 18 months after the last SARS-CoV- 2 boost and no difference was found in COVID- 19 severity between RTX- and non-RTX-treated patients ($p = 0.770$).

Conclusions Even if RTX treatment prevented the SARS-CoV- 2 vaccine-dependent antibody response in RA patients, a strong spike protein-specific T-cell-mediated response that persisted for up to 18 months after the last SARS-CoV- 2 boost was found in RA RTX patients. With respect to personalized medicine, analyzing the spike protein-specific T-cell response might be a valuable strategy for deciding when revaccination is necessary.

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Keywords COVID- 19, Rheumatoid arthritis, Rituximab, MRNA vaccine, T-cell response, Immune response

Background

Since late 2019, severe acute respiratory syndrome coronavirus 2 (SARS-CoV- 2) has spread rapidly worldwide, causing coronavirus disease 2019 (COVID- 19) with symptoms ranging from mild to severe. Messenger ribonucleic acid (mRNA) vaccines have been developed to protect at-risk groups, including those with autoimmune diseases, such as rheumatoid arthritis (RA) [1–4]. While treatments like tumor necrosis factor alpha (TNFα) inhibitors or anti-interleukin- 6 (IL- 6) antibodies do not increase their risk of severe COVID- 19 [5–7], patients on corticosteroids or rituximab, a monoclonal antibody that targets CD20 on B cells [8], required multiple vaccine doses and strict isolation due to impaired immune responses [9–14].

mRNA vaccines may also induce a T-cell response, which is considered an essential component of the antiviral defense arsenal and confers protection against severe virus infections [15]. Several groups of investigators have reported that patients with autoimmune diseases treated with RTX and vaccinated with COVID- 19 mRNA vaccines have a cellular immune response even in the absence of a serological response [16–19]. However, the persistence of this spike protein-specific T-cell response has never been explored in RA patients beyond 6 months. Furthermore, long-term analysis of the spike protein-specific T-cell response could be a useful decision-making tool for determining whether booster shots are necessary in patients treated with RTX without an antibody response.

The spike protein-specific T-cell response is not routinely quantified in clinical practice. Classical methods for measuring cell proliferation, cytolytic activity and/or cytokine production assess the average response of highly heterogeneous cell populations [20]. Although these methods provide useful information, they do not allow in-depth characterization of the nature of the response and cell populations involved. Furthermore, they often require specific sample preparation (peripheral blood mononuclear cell (PBMC) isolation or specific cell type purification), long incubation times, multiple steps and thus trained technical staff. To determine complex signatures of immune responses, flow cytometry has been widely employed for high-throughput analysis of specific protein expression within complex cell populations, but its use in clinical practice remains complicated [21]. Cartagena et al. [22] demonstrated that a whole blood-based flow cytometry approach can be easily implemented for RA patient characterization, stratification and monitoring and can be used concomitantly for antigen-specific T-cell analysis. A new procedure relying on the study of activation-induced markers (AIMs) from whole blood [23] demonstrated its potential to accurately establish T-cell phenotypes with only three antigen-specific T-cell biomarkers, namely, *i)* CD154, also called CD40-ligand (CD40L), a protein primarily expressed on activated T cells that acts as a costimulatory molecule [24]; *ii)* CD107a, also called lysosomal-associated membrane protein 1 (LAMP- 1), a marker of degranulation of lymphocytes such as $CD8^+$ and NK cells; and *iii)* CD137, also called tumor necrosis factor receptor superfamily member 9 (TNFRSF9), which is induced upon lymphocyte activation at the surface of most leucocytes and nonimmune cells [25].

This study applied a modified version of the aforementioned protocol to explore the persistence of T-cell-mediated immunity for 3 to 18 months after the last SARS-CoV- 2 boost in RA patients treated with RTX. Clinical and biological parameters that can influence this immune system were also examined. The clinical outcomes of COVID- 19 patients treated with or without RTX after SARS-CoV- 2 vaccination were also compared to understand the clinical impact of this spike protein-specific T-cell response.

Patients and methods

Patients

Our observational study cohort included anti-citrullinated peptide antibody (ACPA)-positive RA patients (> 18 years old) who visited the rheumatology department of Sainte Marguerite Hospital (Marseille, France) between April 26 th, 2022, and October 15 th, 2022. All of them fulfilled the 2010 American College of Rheumatology/European Union League Against Rheumatism (ACR/EULAR) criteria [26] and had received at least one dose of the Pfizer-BioNTech mRNA anti-SARS-CoV- 2 vaccine. Patients with other rheumatic inflammatory diseases or patients who were not vaccinated were excluded.

In line with the recommendations to space out treatments for patients in remission, the included patients received rituximab according to the following protocol: 1) Initial treatment: administration of 1000 mg of rituximab on days D1 and D15 (two infusions 15 days apart) and the same protocol at month 6; 2) Consolidation: if CD19 < 0 (indicating the absence of detectable B lymphocytes) and the DAS28 score < 2.6 (indicating remission or low disease activity), then a single dose is administered at month 12 (M12 after the first infusion, D1 only); 3) Spacing

based on biological and clinical criteria: If CD19 remains < 0 and the patient is still in remission, the interval between infusions is progressively extended to 9 months, then 12 months, and potentially up to 18 months. During the spacing period, patients do not receive other biotherapies (no other treatments targeting specific mechanisms of inflammation).

Ethics

All patients provided informed written consent for this study in accordance with the Helsinki Declaration [27]. Sample collection was approved by the National Ethics Committee under the number DC- 2008–327. Some bioresources were provided by the Biological Resources Center of the Assistance Publique – Hôpitaux de Marseille (CRB AP-HM, certified NF S96 - 900 & ISO 9001 v2015) from the CRB-TBM component (BB- 0033–00097). Patient data were pseudoanonymized.

Routine sample testing and clinical data collection

A single heparin-anticoagulated blood sample was collected from each patient and sent to the Immunology Laboratory as part of the standard routine clinical tests.

First, the serum ACPA concentration was confirmed with an ELISA IMMUNOSCAN CCPlus® kit (Euro Diagnostica, Arnhem, The Netherlands) according to the manufacturer's recommendations. Immunoglobulin G (IgG) antibody titers to cyclic citrullinated peptide (CCP) were expressed in arbitrary units (AUs)/mL and were considered positive when they were greater than 25 AU/mL. IgM rheumatoid factor (RF) levels were also assessed with an ELISA RF IgM kit (ORGENTEC Diagnostika GmbH, Mainz, Germany), and the results were considered positive when more than 20 international units (IU)/mL were detected.

Disease activity was measured using the Disease Activity Score calculated with the level of C-reactive protein (DAS28-CRP) within the day of sample collection [28, 29].

Other RA characteristics were collected from patients' medical files, including age at RA diagnosis and at blood collection, sex, prednisone use, and ongoing therapies such as biologics or conventional synthetic disease-modifying anti-rheumatic drugs (bDMARDs or csDMARDs).

Second, patient personal history of COVID- 19 and vaccination (number of doses, vaccination and/or infection dates) was retrospectively recorded by interviewing the patient and reading medical records on the day of sample collection to determine the number of "SARS-CoV- 2 boosts" and delay since the last vaccine dose or infection. The vaccine-induced antibody response was evaluated with the Liaison® immunoluminometric assay (DiaSorin, Saluggia, Italy). A value above 33,8 binding antibody units (BAU)/mL was indicative of positive SARS-CoV- 2 serology according to the manufacturer's instructions.

For patients specifically treated with RTX, specific data such as the number of previous RTX infusions and the associated cumulative dose of RTX were collected. The percentage of remaining CD19 +B cells was measured using flow cytometry.

Whole blood-based functional assay

A single heparin-anticoagulated blood sample was collected from each patient and sent to the Immunology Laboratory as part of the standard routine clinical tests.

First, the serum ACPA concentration was confirmed with an ELISA IMMUNOSCAN CCPlus® kit (Euro Diagnostica, Arnhem, The Netherlands) according to the manufacturer's recommendations. Immunoglobulin G (IgG) antibody titers to cyclic citrullinated peptide (CCP) were expressed in arbitrary units (AUs)/mL and were considered positive when they were greater than 25 AU/mL. IgM rheumatoid factor (RF) levels were also assessed with an ELISA RF IgM kit (ORGENTEC Diagnostika GmbH, Mainz, Germany), and the results were considered positive when more than 20 international units (IU)/mL were detected.

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Flow cytometry analysis

All the data were collected on a 3-laser, 13-color Cytoflex flow cytometer and analyzed using Kaluza Software

version 2.1 (both from Beckman Coulter Inc.). Compensations between channels were made using samples labeled with conjugated antibody alone to remove residual spectral overlap. Flow-Set beads (Beckman Coulter Inc.) were used daily to control potential variability in device performance, but no harmonization between the measured values over the study period was necessary.

Lymphocytes were first gated on their typical side (SS) and forward (FS) scatter characteristics. Then, T, B, natural killer (NK) and NKT lymphocytes were gated on their CD3 and CD56 phenotypes. T cells were divided into $CD4^+$ T cells or $CD8^+$ T cells depending on their CD8 expression. CD69, CD154, CD137 and CD107a expression was monitored to characterize $CD4^+$ or $CD8^+$ T-cell activation phenotypes. For in-depth characterization of activated cells, CCR7 and CD45RA were used to divide lymphocyte subsets into naïve ($CD45RA^+CCR7^+$), terminally differentiated effector memory (TEMRA) ($CD45RA^+CCR7^-$), central memory ($CD45RA^-CCR7^+$) or effector memory ($CD45RA^-CCR7^-$) subsets. An example of the gating strategy is given in supplementary Fig. 1.

Flow cytometry parameters

The flow cytometry results were measured through six different percentages of either $CD69^+CD154^+$ or $CD69^+CD137^+$ or $CD69^+CD107a^+$ or $CD154^+CD137^+$ or CD154 + CD107a + or CD137 + CD107a + CD4 + or $CD8^+$ T cells related to the entire $CD4^+$ or $CD8^+$ T-cell population (a total of 12 parameters/activator).

Flow cytometry raw percentages were normalized as stimulation indexes (Stim Index) and calculated either as ratios between the percentages of cells in the CEFX-positive condition and those in the negative condition or as ratios between the percentages of cells in the spike condition and those in the negative condition for each of the 6 parameters measured for each of both the $CD4^+$ and $CD8^+$ T-cell subsets (a total of 12 parameters/activator).

The 12 Stim Index generated between the CEFX and negative control conditions were only used to validate the flow cytometry data. The 12 Stim Index generated between the spike and negative conditions were first individually compared among patient cohorts to obtain a detailed overview of lymphocyte subset activation. Then, as all the parameters of the T subsets evolved in an equivalent manner when individually considered, the 6 $CD4^+$ T-cell Stim Index and the 6 $CD8^+$ T-cell Stim Index were summed to determine the global activation of $CD4^+$ T cells and the global activation of $CD8^+$ T cells, respectively, to obtain a global overview of lymphocyte activation (a total of 12 vs 2 parameters).

Data analysis

The RA patient cohort was divided into two groups: those treated with RTX (tested group) and those treated with any other DMARDs (control group). JMP software version 14.2.0 (SAS Institute Inc., NC, USA) was used for statistical analysis. For all tests, p values less than 0.05 were considered significant.

Quantitative clinical features are expressed as the means ± standard deviations (SDs) or medians with interquartile ranges (IQRs) and were compared using Student's t tests or nonparametric Wilcoxon tests. Qualitative variables are expressed as frequencies with percentages and were compared using χ^2 or Fischer's exact tests.

Receiver operating characteristic (ROC) analysis was performed to discriminate patient groups according to flow cytometry positivity. ROC analysis was based on the area under the curve (AUC), sensitivity (true positives/positives [TP/P]) and specificity (true negatives/negatives [TN/N]). All values are expressed as ranges (between 0 and 100%) with 95% confidence intervals.

Automated analysis using JMP's response screening platform was used to identify the most discriminative clinical/biological parameters based on Student's t test or ANOVA and false discovery rate (FDR)-corrected p values. The correlation rate was calculated to analyze the correlation between the T-cell response and the most discriminative clinical/biological parameter.

Results

Patient characteristics

Fifty-one patients with ACPA-positive rheumatoid arthritis treated with RTX (RTX-treated RA patients) and 24 RA patients treated with therapies other than RTX (non-RTX-treated RA patients, control group) were enrolled. The clinical characteristics of both groups are detailed in Table 1.

As expected for RA patients, the final population comprised more women than men. Patients in the RTX group were older ($p = 0.022$) and had a longer RA duration ($p < 0.001$), suggesting that they may have a more depleted immune system than did those in the control group.

Regarding therapy, as most of the RA patients in the control group were newly diagnosed, most of them were treated with conventional DMARDs. As expected, the RTX group showed an increase in the Ig titer and in the CD19 B-cell number as a function of time since the last RTX infusion (supplementary Fig. 2).

As the RTX group may have been considered at risk of more severe COVID- 19, it was not surprising that they had a greater number of vaccine doses ($p = 0.034$).

Table 1 Study cohort characteristics

	RTX-treated RA patients (*n*=51)	Non-RTX-treated RA patients (*n*=24)	*p value*
Age, years (median, IQR)	67 (57-76)	59 (51-63)	***0.022****
Gender: Female/Male	45 (88%)/6 (12%)	15 (62.5%)/9 (37.5%)	***0.014****
RA duration, years (median, IQR)	22 (13-29)	5 (2-14)	***<0.001****
Treatments:			
bDMARDS:			
- **RTX**	100%	0	
- **Others (Etanercept, Abatacept, Tocilizumab**	0	7 (29%)	0.850
o **Monotherapy**	-	4 (16.7%)	
Concomitant csDMARDS:			
- **Methotrexate**	20 (39.2%)	11 (45.8%)	
- **Leflunomide**	6 (11.8%)	2 (8.3%)	
- **Others (Sulfasalazine, Hydroxychloroquine)**	6 (11.8%)	0	
- **None**	19 (37.3%)	11 (45.8%)	
Prednisone use:			*0.710*
- **No**	38 (74.5%)	17 (70.8%)	
- **5 mg/day**	7 (13.7%)	5 (20.8%)	
- **5 mg/day**	6 (11.8%)	2 (8.3%)	
DAS 28 (mean, SD)	2.96 (1.26)	3.48 (1.55)	*0.150*
Number of vaccine doses	Median: 3	Median: 3	***0.034****
- **1 or 2**	3 (5.9%)	7 (29.2%)	
- **3**	34 (66.7%)	13 (54.2%)	
- **4**	14 (27.5%)	4 (16.7%)	
History of SARS-CoV-2 infection	25 (49%)	14 (58.3%)	*0.770*
- **Mild symptoms**	19 (37.3%)	10 (41.7%)	
- **Hospitalization/O2**	5 (9.8%)	3 (12.5%)	
- **Intensive care**	1 (2%)	1 (4.2%)	
Number of "SARS-CoV-2 boost" (cumulative number of SARS-CoV-2 vaccine doses and infections)	Median: 4	Median: 3	0.099
- **< 3**	0 (0%)	2 (8.3%)	
- **3**	51 (100%)	22 (91.7%)	
SARS-CoV-2 serology level (BAU/mL) (mean, SD)	562 (768)	839 (645)	***0.034****
SARS-CoV-2 serology positivity	30 (59%)	13/14 (93%)#	***0.024****
Time between sample collection and last SARS-CoV-2 boost, months (median, IQR)	5 (3-8)	4 (2-9.75)	0.490
- **0-6 months**	30 (58.8%)	15 (62.5%)	
- **6-12 months**	18 (35.3%)	7 (29.1%)	
- **12-18 months**	3 (5.9%)	2 (8.3%)	
Number of RTX infusions (median, IQR)	10 (6-16)	NA	NA
Cumulative dose of RTX, g (median, IQR)	11 (6-20)	NA	NA
Time between sample collection and last RTX infusion, months (median, IQR)	9.3 (6.5-13.1)	NA	NA
Time between last RTX infusion and last vaccine dose, months (median, IQR)	5.6 (3.2-7.8)	NA	NA
CD19 B-cell count, % (mean, SD)	6.84 (9.54)	NA	NA
Patients with CD19 B-cell count < 6% (clinical criteria)	30 (59%)	NA	NA
IgG titer, g/L (mean, SD)	8.75 (3.35)	NA	NA

RA Rheumatoid arthritis, *RTX* Rituximab, *IQR* Interquartile range, *SD* Standard deviation, *DMARDS* Disease-modifying anti-rheumatic drugs, *DAS 28* Disease activity score, *SARS-CoV-2* Severe acute respiratory syndrome coronavirus-2, *O2* Oxygen, *IgG* Immunoglobulin G, *NA* Not applicable, #: SARS-CoV-2 Serology data were available for only 14 out of 24 non-RTX-treated RA patients

However, the interval between sample collection and the last SARS-CoV- 2 boost was not significantly different between patients treated with or without RTX ($p=0.4899$). The number of patients with a history of COVID- 19 was also not significantly different among the groups ($p=0.770$). Surprisingly, no difference in COVID-19 severity was found between RTX-treated patients and non-RTX-treated patients ($p=0.770$). Most patients in both groups had mild symptoms (19/25 (76%) RTX-treated RA patients versus 10/14 (71%) non-RTX-treated patients), whereas more severe symptoms were expected in the RTX group.

As expected, the RTX group exhibited a decreased antibody titer (562 ± 768 BAU/mL versus 839 ± 645 BAU/mL, $p=0.034$). However, a spike protein-specific antibody response was observed in only 30/51 (59%) patients treated with RTX versus 13/14 (93%) patients treated with other therapies ($p=0.024$), indicating that the T response of RTX-treated RA patients should be compared with the response of the control group not only as a whole but also by dividing the RTX group into two different subgroups (negative or positive serology).

Interestingly, supplementary Fig. 3 shows that the antibody response was maintained in both patient groups up to at least 10 months after the last SARS-CoV- 2 boost and depended on the time of the last RTX infusion.

Spike protein-specific T-cell response

CD154, CD137 and CD107a expression on $CD4^+$ and $CD8^+$ T cells was individually monitored and compared in 51 patients with ACPA-positive rheumatoid arthritis patients treated with RTX and 24 RA patients treated with other therapies to characterize T-cell activation levels and associated phenotypes upon spike activation (Fig. 1).

There were no significant differences in individual $CD4^+$ T-cell responses between RTX-treated patients and the control group. However, a greater $CD8^+$ T-cell response was observed in the RTX-treated RA patients for the $CD69^+CD154^+$ ($p=0.015$), $CD137^+CD154^+$ ($p<0.001$), $CD137^+CD107a^+$ ($p<0.001$) and $CD107a^+CD154^+$ ($p<0.001$) $CD8^+$ T-cell stimulation indexes.

Interestingly, patients who presented significant activation for one individual parameter might not exhibit activation for another parameter. Individual activation patterns were thus indicative of spike-specifically activated subsets.

Similar results were obtained when RTX-treated RA patients were divided according to their SARS-CoV- 2 serology (supplementary Fig. 4).

These results were confirmed by the JMP response screen platform (supplementary Fig. 5), which revealed no discriminative spike protein-specific $CD4^+$ T-cell responses but several significant differences in $CD8^+$ T-cell-related features. The activation of three differentiated $CD8^+$ T cells (TEMRA, central memory, effector memory) was greater than that of $CD8^+$ naïve T cells, which contributed to the greater number of responses observed in the RTX-treated RA patients, whereas only the TEMRA $CD4^+$ T cells exhibited a greater $CD69^+CD154^+$ stimulation index (Supplementary Fig. 6).

Global spike-specific T-cell response

As our goal was rather to globally capture the T-cell response upon spike activation, we added individual activation parameters measured in $CD4^+$ or $CD8^+$ T cells to obtain $CD4^+$ and $CD8^+$ T-cell global spike-specific response parameters (% $CD4^+$ T-cell global activation = %$CD69^+CD154^+$ $CD4^+$ T cells + %$CD69^+CD137^+$ $CD4^+$ T cells + %$CD69^+CD107a^+$ $CD4^+$ T cells + %$CD154^+CD137^+$ $CD4^+$ T cells + %$CD154^+CD107a^+$ $CD4^+$ T cells + %$CD137^+CD107a^+$ $CD4^+$ T cells, and % $CD8^+$ T-cell global activation = %$CD69^+CD154^+$ $CD8^+$ T cells + %$CD69^+CD137^+$ $CD8^+$ T cells + %$CD69^+CD107a^+$ $CD8^+$ T cells + %$CD154^+CD137^+$ $CD8^+$ T cells + %$CD154^+CD107a^+$ $CD8^+$ T cells + %$CD137^+CD107a^+$ $CD8^+$ T cells).

Global $CD4^+$ and $CD8^+$ T-cell activation was then compared between the 51 RA patients treated with RTX and the 24 RA patients treated with other therapies (Fig. 2).

As shown by the individual T-cell analysis, no significant difference ($p=0.320$) in the global CD4 + T-cell response was found between the RTX-treated and non-RTX-treated RA patients. In contrast, compared with non-RTX-treated RA patients, RTX-treated RA patients exhibited a stronger $CD8^+$ T-cell response ($p<0.001$).

Corresponding ROC analyses were also performed to further visualize how each of these global responses could discriminate patient groups (Fig. 3).

When the cutoff point was greater than 1.26%, the global activation of CD4 + T cells exhibited an AUC of 0.57 with 80% specificity and 59% sensitivity, whereas when the cutoff point was greater than 1.10%, the global activation of CD8 + T cells exhibited an AUC of 0.81 with 90% sensitivity and 63% specificity. Interestingly, ROC analysis of global activation parameters better discriminated RA patients treated with or without RTX than did ROC analysis of each individual parameter (Supplementary Fig. 7).

Persistence of the global spike-specific T-cell response

The persistence of the T-cell response was further assessed in RTX-treated RA patients by studying the global spike protein-specific T-cell response as a function of the time since the last SARS-CoV- 2 boost (Fig. 4). Both the last anti-SARS-CoV- 2 vaccine and the last SARS-CoV- 2 infection were considered SARS-CoV- 2 boosts, as both participate in the reactivation of the immune system.

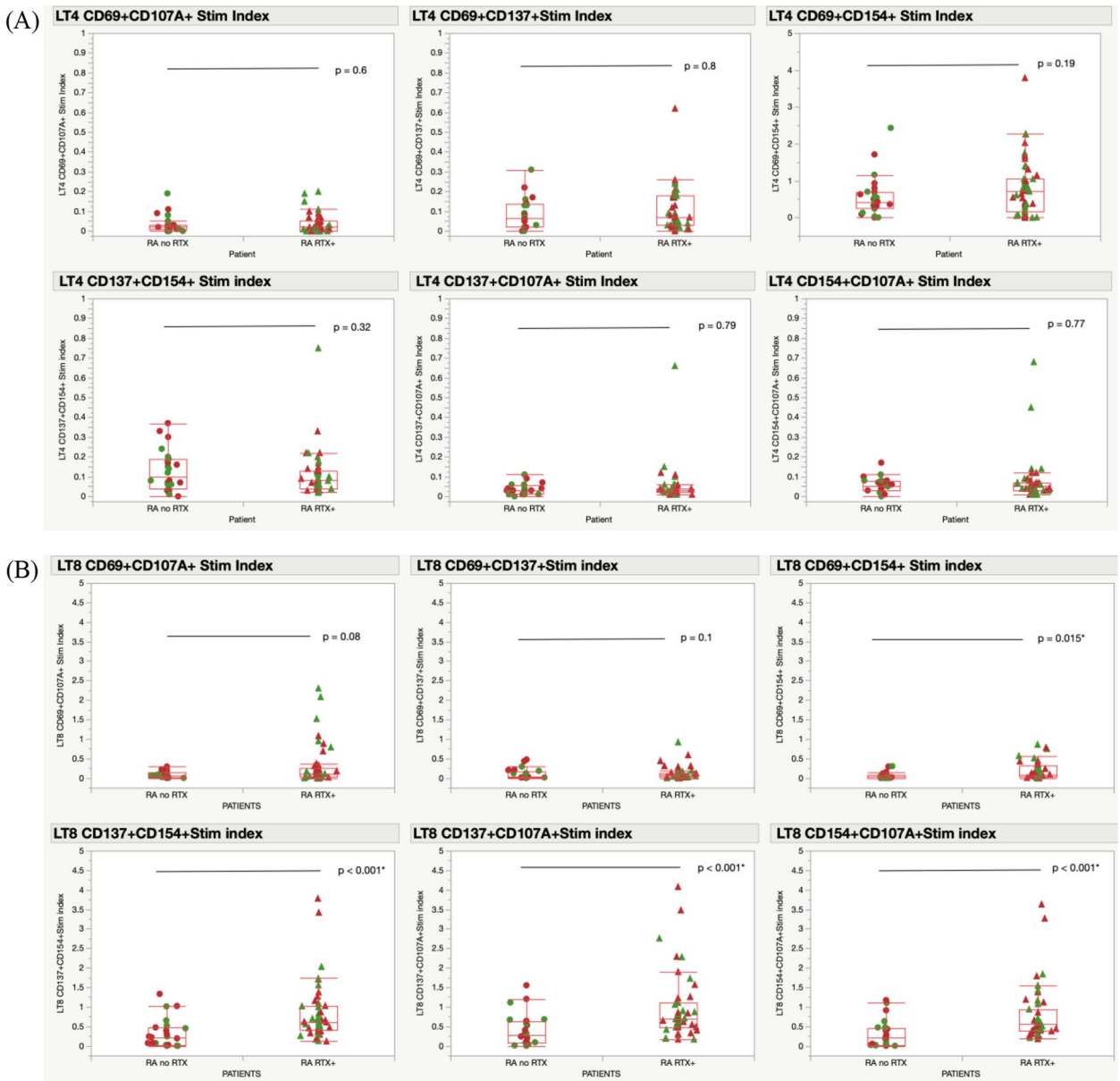

Fig. 1 Spike-specific T-cell response in the study cohort. Blood samples from patients with RA not treated with RTX (RA no RTX, n = 24) or treated with RTX (RA RTX +, n = 51) were analyzed by flow cytometry after spike peptide activation. Stimulation indexes (Stim Index) were calculated as ratios between spike activation and negative condition percentages. The data are given for either CD69 + CD154 + or CD69 + CD137 + or CD69 + CD107a + or CD154 + CD137 + or CD154 + CD107a + or CD137 + CD107a + $CD4^+$ T cells (A) or $CD8.^+$ T cells (B) and were compared between both groups. Green symbols (circles in the RA without RTX group and triangles in the RA RTX + group) refer to vaccinated patients who had no history of COVID- 19. Red symbols (circles in the RA non-RTX group and triangles in the RA RTX + group) refer to vaccinated patients who had a history of COVID- 19

In RTX-treated RA patients, both $CD4^+$ and $CD8^+$ T-cell global specific responses against spike peptides were stable for 22/51 patients (43%) for up to 6 months, including 7/51 (14%) patients whose response persisted between 9 and 12 months and 3/51 (6%) patients whose response persisted up to 18 months after the last SARS-CoV- 2 boost. Similar observations were made when all individual parameters were considered (supplementary Fig. 8).

Clinical and biological parameters impacting the global spike-specific T-cell response

Clinical and biological parameters that might influence the spike protein-specific T-cell response in

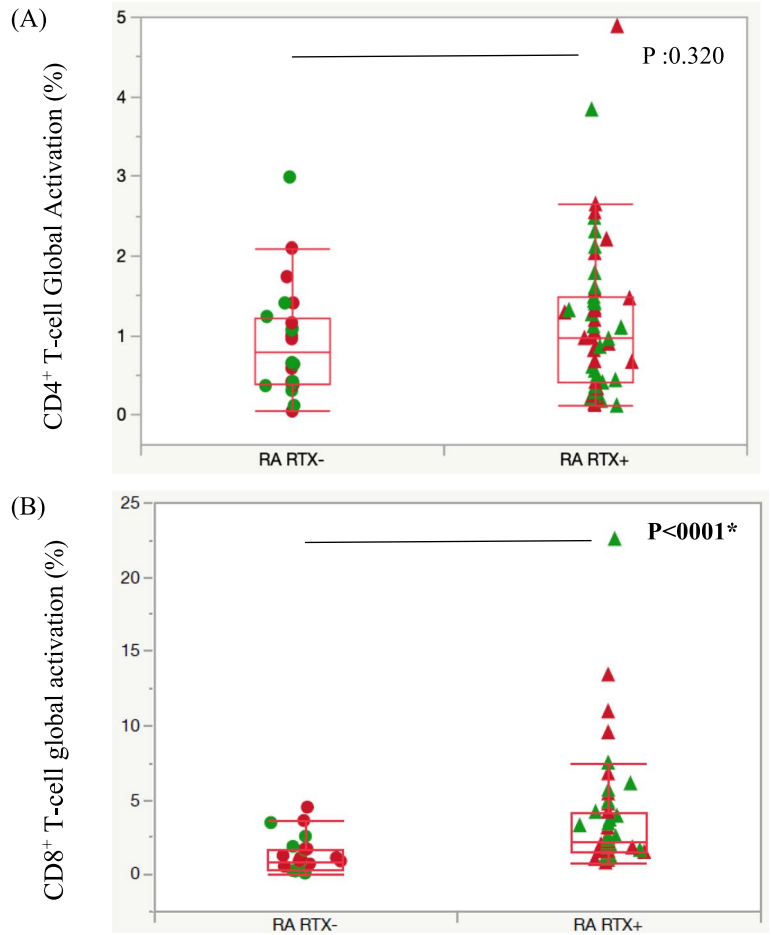

Fig. 2 Global spike-specific T-cell response in the study cohort. $CD4^+$ T-cell (A) or CD8 + T-cell (B) global activation parameters in RA patients treated (RA RTX +, n = 51) or not treated (RA no RTX, n = 24) with rituximab. Green symbols (circles in the RA without RTX group and triangles in the RA RTX + group) refer to vaccinated patients who had no history of COVID- 19. Red symbols (circles in the RA non-RTX group and triangles in the RA RTX + group) refer to vaccinated patients who had a history of COVID- 19

RA patients treated with RTX were then studied via automated response screen analysis (Supplementary Table 1). Features related to patient age, sex, RA (symptom duration, associated treatments (bDMARDS, csDMARDs, corticosteroid therapy), disease activity (DAS28)), SARS-CoV- 2 parameters (serology, number of months since the last SARS-CoV- 2 boost, number of vaccine doses) and RTX therapy (cumulative dose of RTX, number of months since last RTX infusion, level of $CD19^+$ B cells, IgG titer, time between last RTX infusion and last infection or vaccine dose) were considered. No relationship was found for the global spike protein-specific $CD4^+$ T-cell response or clinical features. In contrast, SARS-CoV- 2 serology was inversely correlated with the global $CD8^+$ T-cell spike protein-specific response. The correlation between both parameters was thus studied ($R^2 = 0.64$, Fig. 5).

Discussion

The aim of this study was to assess SARS-CoV- 2-specific T-cell-mediated immunity and T-cell retention up to 18 months after the last SARS-CoV- 2 boost (vaccine or infection) in RA patients treated with RTX. Our study included 51 RA patients treated with RTX and 24 patients treated with other DMARDs or without treatment, all with at least one dose of the anti-SARS-CoV- 2 mRNA vaccine (minimum of 3 SARS-CoV- 2 boosts).

From the start of the SARS-CoV- 2 epidemic, it was shown that the humoral response was defective in patients treated with RTX. Numerous teams have rapidly demonstrated that the T response is preserved, but no study of the persistence of this T-cell response has been carried out in RA patients; however, this information could be valuable for improving the spacing of additional doses of SARS-CoV- 2 vaccines.

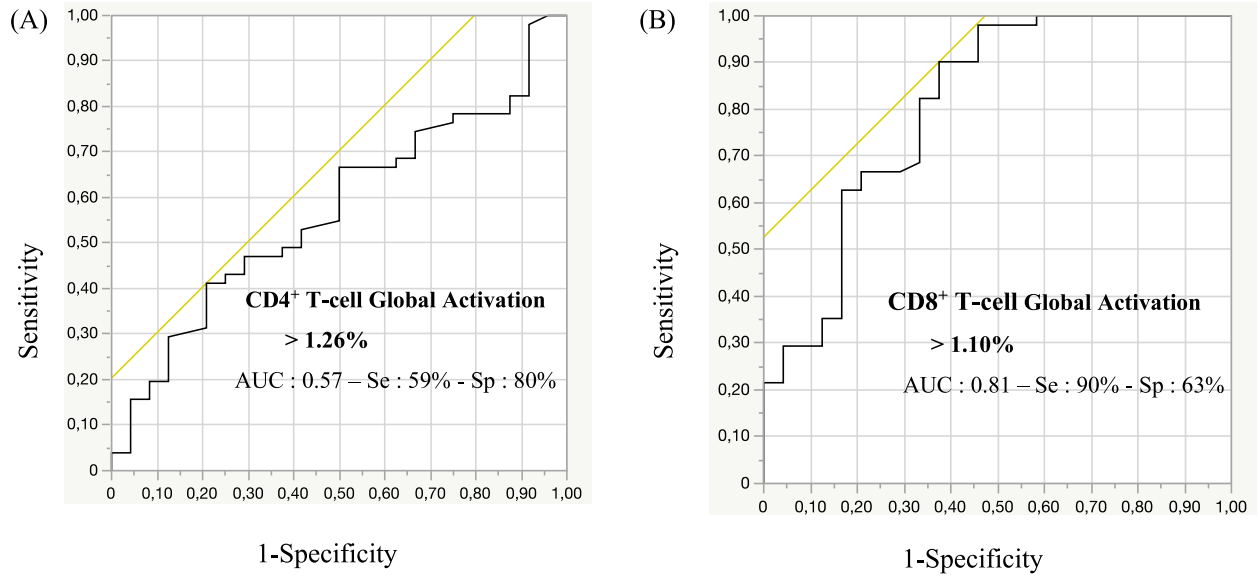

Fig. 3 ROC analysis of the global T-cell response. ROC curves for the discrimination of RTX-treated and non-RTX-treated RA patients were generated for global activation parameters of $CD4^+$ (A) and $CD8^+$ T cells (B). The area under the curve (AUC), sensitivity (Se) and specificity (Sp) are indicated

Humoral response in the study cohort

Since the beginning of the SARS-CoV- 2 pandemic, numerous studies have shown that anti-CD20 treatments such as RTX induce a decrease in the anti-spike antibody response following SARS-CoV- 2 vaccination [11, 12, 30]. This decrease in humoral response in RTX-treated patients was confirmed in our cohort: 59% of RTX-treated patients had positive SARS-CoV- 2 serology, whereas 93% of non-RTX-treated patients had positive SARS-CoV- 2 serology, with significantly lower antibody levels in RTX-treated patients. As other parameters reflecting the humoral response, such as the Ig titer or CD19 B-cell count, the observed decrease in SARS-CoV- 2 serology seemed to be directly linked to the time since the last RTX infusion. However, the percentage of patients with positive SARS-CoV- 2 serology tended to be greater in our study than in most previously published studies, such as Furer et al. [31] (40% after 3 doses) and Mrak et al. [32] (33% after 3 doses, 58% after 4 doses).

Two factors may influence the vaccine antibody response in these patients: the cumulative RTX dose and the delay between the last RTX infusion and the vaccination [9, 11, 33, 34].

The seropositivity rate clearly increased when patients were vaccinated more than 6 months after their last RTX infusion. In our cohort, half of the RTX-treated patients (49%) had a history of SARS-CoV- 2 infection in addition to vaccination, which could have enhanced anti-SARS-CoV- 2 immunity. This immune boost could have been a confounder, but the number of months since last COVID boost, the number of months since last vaccine dose, number of COVID vaccine doses and the number of months since last COVID infection, were not significantly impacting both CD4 + and CD8 + T-cell responses.

On the other hand, the median time between two RTX infusions was greater than 9 months in our study, while the usual infusion interval was six months. Indeed, the included patients received rituximab according to the recommendations to space out treatments for patients in remission. This long interval could have limited the interpretability of the results, however, the number of months since last RTX infusion, the number of months between last RTX infusion and last COVID infection, the RTX cumulative dose (g), the CD19 (%) and the number of months between last RTX infusion and last vaccine dose, were not significantly impacting both CD4 + and CD8 + T-cell responses.

This suggests that increasing the delay between consecutive RTX infusions in the context of COVID- 19 may have favored a better humoral response.

T-cell response in RA patients treated with RTX or other DMARDs

The goal of our study was to explore the cellular $CD4^+$ and $CD8^+$ T-cell responses in 51 RA patients treated with RTX and 24 RA patients treated with other DMARDs. $CD4^+$ and $CD8^+$ T-cell-activated subpopulations were examined using an early activation marker (CD69) and specific activation markers (CD107a for degranulation, CD154 for costimulation and CD137 for antigen-specific stimulation). Specific

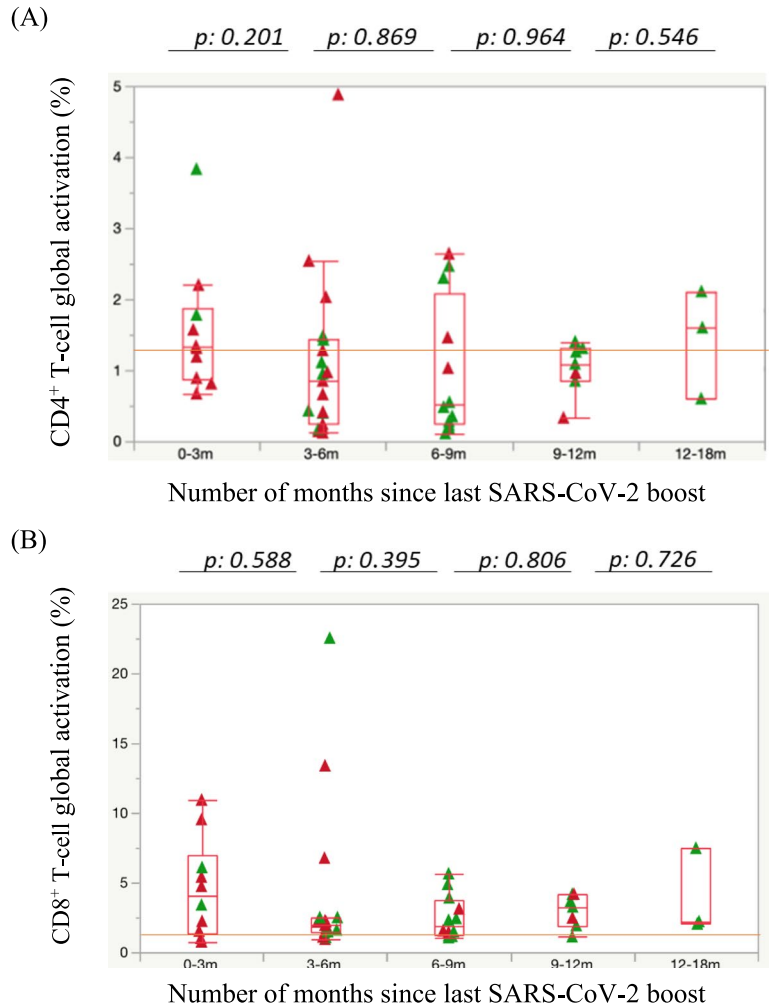

Fig. 4 Persistence of the global spike protein-specific T-cell response over time. $CD4^+$ and CD8 +T-cell global activation parameters were analyzed in blood samples from patients with RA treated with RTX (RA RTX +, n = 51). Samples were segregated according to the time between their last SARS-CoV- 2 boost (last vaccine or infection) and blood collection, with 3-month time intervals considered for each group. Green triangles refer to vaccinated patients who had no history of COVID- 19. Red triangles refer to vaccinated patients who had a history of COVID- 19. The orange line corresponds to the positivity threshold determined by ROC analysis

$CD4^+$ and $CD8^+$ T-cell responses against spike peptides were largely greater than the positivity threshold in both patient groups, with significantly greater $CD8^+$ T-cell-specific responses in RA patients treated with RTX than in those not treated with RTX, despite the older age, longer history of RA and elevated number of DMARDs. Spike-specific $CD4^+$ T-cell responses were not significantly different between the two groups.

These results are consistent with those of Zonozi et al. [18], who reported greater SARS-CoV- 2–specific $CD8^+$ T-cell responses in 33 individuals who received anti-CD20 therapy than in 44 nontreated controls.

Long-term persistence of the global spike-specific T-cell response

Both global $CD4^+$ and $CD8^+$ T-cell responses remained stable beyond 6 months in 43% of the patients for whom we had data available and even up to 18 months after the last SARS-CoV- 2 boost in 3 patients from our cohort of RTX-treated RA patients. The persistence of the T-cell response was studied in relation to the last SARS-CoV-2 boost (vaccine or infection) to consider potential new immune stimulation induced by SARS-CoV- 2 infection occurring between the last vaccine administration and blood sampling. In contrast to the present study, most previous investigations explored the T-cell response

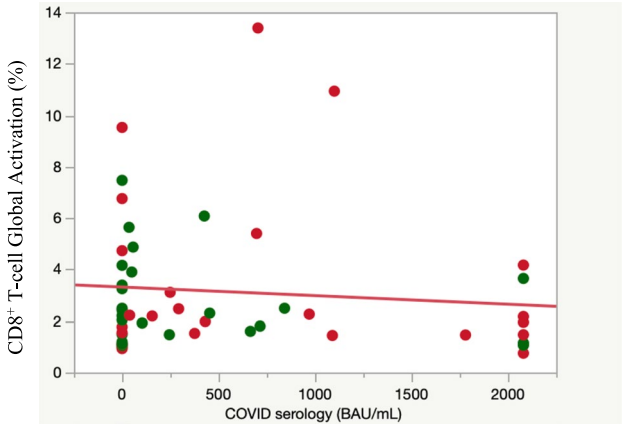

Fig. 5 Correlation between the global spike protein-specific response of CD8 + T cells and SARS-CoV- 2 serology. $CD8^+$ T-cell global activation parameters in patients with RA treated with RTX (n = 51) are expressed as a function of their SARS-CoV- 2 serology (BAU/mL). The green and red circles show patients who had no history or a history of COVID- 19, respectively, in addition to their vaccination. The red line indicates the linear regression analysis curve

until 1.5 months after the last vaccine dose [17, 35, 36]. Mrak et al. assessed T-cell response persistence in RTX-treated patients and showed no significant difference in the T-cell response between day 15 and day 42 after the second dose of the vaccine [37].

Our study is the first to investigate the T-cell-specific response up to 18 months after the last SARS-CoV- 2 boost in RA patients treated with RTX. The persistence of a spike-specific T-cell response after SARS-CoV- 2 vaccination was demonstrated up to 6 months after the last vaccine dose by Woopen et al. in multiple sclerosis patients treated with anti-CD20 therapies. In this study, only five patients were treated with RTX, and nonspecific interferon tests were used to assess the T-cell response [38].

Factors influencing the cellular response

We also sought to investigate the clinical or biological features that influence the T-cell response in RTX-treated patients. None of the studied factors influenced the CD4 + T-cell response. However, we found that the CD8 + T-cell response was inversely correlated with the SARS-CoV- 2 antibody response.

These results corroborate the observations of Madelon et al. [39]. It can be hypothesized that a stronger cellular response in RTX-treated RA patients counterbalanced the lower humoral response, as described by Apostolidis et al. [40]. One hypothesis to explain the greater T-cell activation in those patients could be the presence of more activated antigen-presenting cells (e.g., monocytes) at the time of vaccination as a result of B-cell depletion [41].

In contrast, Stefanski et al. [42] showed that the $CD8^+$ T-cell response was independent of the B-cell count and antibody response and that patients with a significantly lower B-cell count and an absence of an antibody response had a poorer $CD4^+$ T-cell response. The contrasting results between the current study and that of Stefanski et al. might rely on the small sample size (51 vs 19 RTX-treated patients, respectively) and the different samples used to analyze the T-cell response, which was performed by flow cytometry not on fresh whole blood but on cryopreserved PBMCs in the latest study.

Clinical consequences of immunological findings

The beneficial effect of the spike protein-specific T-cell response on the risk of severe COVID- 19 has been established by Zonozi et al. [18], who demonstrated that vaccinated patients receiving RTX had a 4.8-fold reduced risk of moderate/severe/critical/fatal COVID- 19 compared to nonvaccinated patients, despite the absence of an antibody response.

In our cohort of vaccinated patients, we observed no difference in the severity of SARS-CoV- 2 infection between RTX-treated (n = 51) and non-RTX-treated (n = 24) RA patients. These results suggest that after SARS-CoV- 2 vaccination, RA patients treated with RTX are not at greater risk of severe COVID- 19 than are RA patients receiving other therapies. This suggested that the presence of a T-cell response was sufficient to protect against severe forms of SARS-CoV- 2 infection even in the absence of an antibody response.

These data need to be confirmed in a larger RA cohort study [15].

A new method for assessing the T-cell response in whole blood

The method used herein relies on a flow cytometry-based functional assay performed on whole blood. It enables the triggering of immune cascades similar to what can be observed in vivo, prevents cell loss during sample preparation, requires minimal technical skills and laboratory material and can thus be used in clinical practice. Using this method, we performed the first *whole blood-based* study of antigen-specific T-cell responses in RTX-treated patients, whereas previous studies investigated T-cell activation in PBMCs or isolated cells using methods such as measurement of cytokine secretion (ELISPOT method) or immunostaining of intracellular cytokines. Another major advantage of the implemented procedure was the ability to simultaneously assess four activation biomarkers (CD69, CD154, CD107a and CD137) that, both individually and in combination, provide important information about spike-specific activation patterns of T cells. This new flow cytometry-based technology was thus more specific (use of multiple antigen-specific biomarkers) and more sensitive (multiplexed global activation parameters) than other commonly used techniques for assessing T-cell activation.

Strengths and limitations of this study

The strengths of this study include the use of a homogeneous group of RA patients treated with RTX, the evaluation of the T-cell response in all included patients thanks to a technology directly assessing the T-cell response in whole blood and the long follow-up (up to 18 months) of patients. Additionally, we think that the scope of our results is important because they could be extended to hematologic cancer patients treated with anti-CD20, where a greater number of CD8 + T cells has also been associated with improved COVID- 19 survival, despite impaired humoral immunity, and 77% of patients had detectable SARS-CoV- 2-specific T-cell responses [15].

However, this study has several limitations. Although this study was performed on the largest homogeneous group of RTX-treated RA patients described so far, the size of our cohort remains small, especially for the number of follow-ups at 18 months which was limited. It would be interesting to increase the number of patients recruited in future studies. Furthermore, it did not include healthy vaccinated controls, as we worked with anonymized patients for whom we had no clinical data. Since this was a retrospective study, it was not possible to determine their last vaccination or infection date, so we could not interpret those data. Moreover, information about COVID- 19 was collected retrospectively and may therefore be subject to recall bias. Indeed, due to the retrospective nature of the work, it's possible that, for instance, silent COVID- 19 infections occurred in these patients. However, our study goals constrained the possibility of follow-up within the recruitment period. Consequently, our study was conducted retrospectively, without longitudinal assessments. Finally, we do not have information on SARS-CoV- 2 infection in nonvaccinated RTX-treated patients to compare their clinical outcomes with those of vaccinated patients.

Conclusion

RA patients treated with RTX are known to have an impaired humoral immune response after receiving the SARS-CoV- 2 vaccine. Our results, similar to those of other studies, further confirmed that the anti-SARS-CoV- 2 T-cell response is rather strong, at least equivalent to that of $CD4^+$ T cells and even better than that of $CD8^+$ T cells, in patients with RA treated with other therapies. More interestingly, both $CD4^+$ and $CD8^+$ T-cell responses persisted for at least 6 and up to 18 months after the last SARS-CoV- 2 vaccine or infection, respectively.

Although larger studies are needed to understand and characterize the clinical protection conferred, the data from our cohort strongly suggest the ability of the SARS-CoV- 2 vaccine to drive T-cell immunity in RA patients treated with RTX over time. Finally, the simple method used for analyzing the specific T-cell response against the spike protein could be a valuable approach for guiding personalized medicine and objectively deciding when revaccination is necessary for each patient.

Abbreviations

AA750	APC Alexa Fluor™ 750
ACPA	Anti-citrullinated peptide antibody
ACR/EULAR	American College of Rheumatology/European Union League Against Rheumatism
AUs	Arbitrary units
AUC	Area under the curve
BAU	Binding antibody units
CCP	Cyclic citrullinated peptide
CD40L	CD40-ligand
COVID- 19	Coronavirus disease 2019
DAS28-CRP	Disease Activity Score calculated with the level of C-reactive protein
DMARDs	Disease-modifying antirheumatic drugs
ECD	Phycoerythrin-Texas Red®-X
FITC	Fluoroisothiocyanate
FS	Forward scatter
IL- 6	Anti-interleukin- 6
IQRs	Interquartile ranges
IU	International units
KRO	KromeOrange
LAMP- 1	Lysosomal-associated membrane protein 1
mRNA	Messenger ribonucleic acid
NK	Natural killer
PBE	Pacific Blue™ succinimidyl ester
PBMC	Peripheral blood mononuclear cell
PC7	Phycoerythrin-Cyanin7
PE	Phycoerythrin

RA	Rheumatoid arthritis
RA RTX	RA patients receiving RTX
RBCs	Red blood cells
RF	Rheumatoid factor
ROC	Receiver operating characteristic
RTX	Rituximab
SARS-CoV- 2	Severe acute respiratory syndrome coronavirus 2
SDs	Standard deviations
SS	Side scatter
Stim Index	Stimulation indexes
TEMRA	Terminally differentiated effector memory
TNFα	Tumor necrosis factor alpha
TNFRSF9	Tumor necrosis factor receptor superfamily member 9

Supplementary Information

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Supplementary Material 1.

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Authors' contributions
PL, JMB and NB contributed to the conception and design of the study. JM, PB, NS and CCG organized the database and performed the statistical analysis. JM and PB verified the underlying data reported in the manuscript. JM, PB, JMB and NB wrote the first draft of the manuscript. All authors contributed to manuscript revision and read and approved the submitted version. All authors confirmed that they had full access to all the data in the study and accept responsibility for its submission for publication.

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Data availability
The raw data that underlie the results reported in this article are available from the corresponding author following publication. Additional data are available upon reasonable request.

Declarations

Ethics approval and consent to participate
All patients provided informed written consent for this study in accordance with the Helsinki Declaration. Sample collection was approved by the National Ethics Committee under the number DC- 2008–327. Some bioresources were provided by the Biological Resources Center of the Assistance Publique – Hôpitaux de Marseille (CRB AP-HM, certified NF S96 - 900 & ISO 9001 v2015) from the CRB-TBM component (BB- 0033–00097). Patient data were pseudoanonymized.

Consent for publication
Not applicable.

Competing interests
Pénélope Bourgoin, Noémie Saverna, Celia Cartagena and Jean-Marc Busnel are Beckman Coulter employees.

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