



## Comparison of follicular T helper cells, monocytes, and T cells priming between newly diagnosed and rituximab-treated MS patients and healthy controls

Sheida Yahyazadeh<sup>1</sup>, Nafiseh Esmaeil<sup>1,2,\*</sup>, Vahid Shaygannejad<sup>3,4</sup>, and Omid Mirmosayyeb<sup>3,5</sup>

<sup>1</sup>Department of Immunology, School of Medicine, Isfahan University of Medical Sciences, Isfahan, I.R. Iran.

<sup>2</sup>Research Institute for Primordial Prevention of Non-Communicable Disease, Isfahan University of Medical Sciences, Isfahan, I.R. Iran.

<sup>3</sup>Isfahan Neurosciences Research Center, Isfahan University of Medical Sciences, Isfahan, I.R. Iran.

<sup>4</sup>Department of Neurology, School of Medicine, Isfahan University of Medical Sciences, Isfahan, I.R. Iran.

<sup>5</sup>Universal Council of Epidemiology, Universal Scientific Education and Research Network, Tehran University of Medical Sciences, Tehran, I.R. Iran.

### Abstract

**Background and purpose:** The use of anti-CD20 monoclonal antibodies like rituximab (RTX) to deplete B cells has practical therapeutic implications in multiple sclerosis (MS) patients. However, the therapy's impact on other immune cells is also important. Therefore, in this study, we assessed the effects of RTX therapy on Tfh cells, T cells, T cells priming, and monocytes in MS patients compared to newly-diagnosed MS patients and healthy subjects.

**Experimental approach:** Thirty newly-diagnosed and RTX-treated MS patients and healthy control were included. Peripheral blood mononuclear cells were isolated from whole blood for assessment of Tfh cells, CD4<sup>+</sup>, CD8<sup>+</sup>, CD4<sup>+</sup>CD45RA<sup>+</sup>, CD3<sup>+</sup>HLA-DR<sup>+</sup>, and CD3<sup>+</sup>CD4<sup>+</sup>CD25<sup>+</sup> T cells by flow cytometry. Whole blood was lysed by lysis solution to assess CD45<sup>+</sup>CD14<sup>+</sup> monocytes by flow cytometry. Also, the serum level of interleukin 21 was measured by the ELISA method.

**Findings / Results:** We showed that RTX treatment led to a decrease in Tfh cells and their predominant cytokine, interleukin 21. Also, we found a statistically significant reduction in CD3<sup>+</sup>HLA-DR<sup>+</sup> and CD3<sup>+</sup>CD4<sup>+</sup>CD25<sup>+</sup> T cells in RTX-treated patients compared to new cases and healthy control. Moreover, we found a decrease in the CD45<sup>+</sup>CD14<sup>+</sup> monocyte population in the RTX-treated group compared to the healthy control.

**Conclusion and implications:** Our data suggest that following treatment with RTX, Tfh cells, monocytes, and T cells priming declined happened, and fewer T cells were activated. Also, due to the interaction between B cells and Tfh cells, Tfh targeting could be assessed as a therapeutic strategy in MS.

**Keywords:** Follicular; Multiple sclerosis; Rituximab; T cells priming; T helper cells.

### INTRODUCTION

Multiple sclerosis (MS) is a demyelinating and neurodegenerative disease characterized by auto-inflammatory immune responses involving both adaptive and innate immune systems in its pathogenesis (1). The main pro-inflammatory CD4<sup>+</sup> T-cell subsets related to autoimmune diseases including MS are Th1 and Th17 cells (2). B and T cells interaction usually happens in secondary lymphoid tissues to create an optimal

immune response. Interleukin 21(IL-21), produced by follicular T helper (Tfh) cells, induces B cell responses in the germinal center and B cells switching to IgG<sup>+</sup> subsets or antibody-producing plasmablasts/plasma cells (3,4). In MS, this crosstalk between B and T cells is probably agitated, which finally leads to unwanted immunopathogenic reactions rather than protection (5).

\*Correspondence author: N. Esmaeil  
Tel: +98-3137929097, Fax: +98-3137929031  
Email: n\_esmaeil@med.mui.ac.ir

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Successful results of B cells depletion therapy by different anti-CD20 monoclonal antibodies such as rituximab (RTX), ocrelizumab, ublituximab, and ofatumumab in relapsing MS patients and primary progressive MS indicate B cells' critical role in MS pathogenesis (6). B cells contribute to central nervous system (CNS) injury in MS by both antibody-dependent and independent mechanisms. In addition to antibody secretion by plasmablasts and plasma cells, B cells have the robust capacity to present antigen by their MHCII molecules and interact with T cells (7). Moreover, B cells are abundant producers of both pro-inflammatory (interferon- $\gamma$  and IL-6) and regulatory (IL-10) cytokines and soluble toxic factors contributing to oligodendrocyte and neuronal injury and also providing a reservoir for Epstein-Barr (EBV) virus infection (8). In addition, B cells can contribute to the formation of form ectopic lymphoid constructions or GCs, which have been found in autoimmune diseases such as rheumatoid arthritis and MS (9,10). Accordingly, targeting B cells with anti-CD20 monoclonal antibodies in MS treatment has highly protective effects on new relapsing disease activity. However, this therapy does not directly target plasma cells and has not significantly impacted the abnormal cerebrospinal fluid (CSF) antibody profile.

The interaction between Tfh cells and B cells is essential for an effective antibody response. Also, Tfh cells play critical roles in the function and organization of follicles in lymphatic tissues (11,12). Accordingly, the existence of Tfh in ectopic follicles and their interaction with B cells likely participate in MS pathogenesis (13). Deficiency of Tfh cells has been associated with decreased disease activity in the experimental autoimmune encephalomyelitis (EAE, an animal model of MS disease) (14,15). Highly expression of C-X-C chemokine receptor type 5 (CXCR5) by mature B cells and Tfh cells permits the localization of these cells in d C-X-C ligand 13 (CXCL13)-rich regions in follicles. Also, Tfh cells produce

IL-21 cytokine and promote B cells affinity maturation and differentiation into antibody-secreting cells (16,17). Anti-CD20 therapy, including RTX, has highly protective effects on MS patients. However, due to the presence of ectopic follicles and direct interaction between autoreactive B cells and Tfh cells, targeting autoantigens specific Tfh cells as critical cells in autoreactive B cells differentiation may also be valuable for MS treatment. Accordingly, this preliminary study assessed Tfh cells, T cells, monocytes, and T cells priming in newly-diagnosed MS who have not received any treatment, RTX-treated patients, and healthy controls.

## MATERIALS AND METHODS

### *Patients and sample collection*

Two groups of MS patients from the MS clinic of Kashani Hospital, Isfahan, Iran, were recruited to this study; one group (n = 30) was clinically defined as relapsing-remitting MS (RRMS) according to the McDonald criteria (18) who treated with RTX for at least 2 doses and the other group (n = 30) was newly-diagnosed RRMS patients, who did not receive any immunosuppressive drugs (Table 1). Patients who received anti-inflammatory drugs pregnant subjects and patients with other inflammatory disorders were eliminated from the study. Also, patients who received other immunosuppressive and immunomodulatory drugs such as interferon- $\beta$ , fingolimod and natalizumab were also excluded from the study. Thirty age-and-sex-matched healthy controls were enrolled in this study. Blood samples were collected from all participants, and peripheral blood mononuclear cells were obtained after Ficoll density gradient centrifugation and processed within 3 h at room temperature. Serum samples were collected from all participants and stored at -20 °C until analysis. Also, blood was lysed by lysis buffer for assessment of the monocytes.

**Table 1.** Baseline features of participants (30 newly-diagnosed and 30 rituximab-treated multiple sclerosis patients and 30 healthy control)

	RTX-treated	New cases	Healthy control
Averaged age (years)	33 (20-48)	32 (20-47)	33 (22-45)
Sex, female (%)	30 (86%)	30 (83.3%)	30 (83.3%)
Averaged of disease duration (years)	14 (2-37)	0	0

### Flow cytometry analysis

Peripheral blood mononuclear cells were stained with fluorescein isothiocyanate (FITC)-anti-CD4 (Sina Biotech, Iran), phycoerythrin (PE)-anti-CD45RA (Biolegend, USA), PerCP-anti-CXCR5 (BD Bioscience, USA), FITC/PE-anti-CD4/8 (BD Bioscience, USA), FITC/PE-anti-CD14/45 (BD Bioscience, USA), FITC/PE-anti-CD3/HLA-DR (BD Bioscience, USA), FITC/PE/PerCP-anti-CD4/25/3 (BD Bioscience, USA), FITC-anti-CD19 antibodies (Biolegend, USA) and incubated 20 min at 4 °C in the dark place. Flow cytometry was carried out on a BD FACSCalibur cytometer (Becton Dickinson, San Jose CA, USA), and data were analyzed using Flowjo software 10.

### Cytokine assays

The concentration of the IL-21 cytokine in serum samples was measured using an ELISA kit (Biolegend, USA) in duplicate according to the manufacturer's instructions. The absorbance was read at 450 nm and the standard curve was created according to

the absorbance of standards. The sensitivity of the ELISA kit was > 4.2 pg/mL.

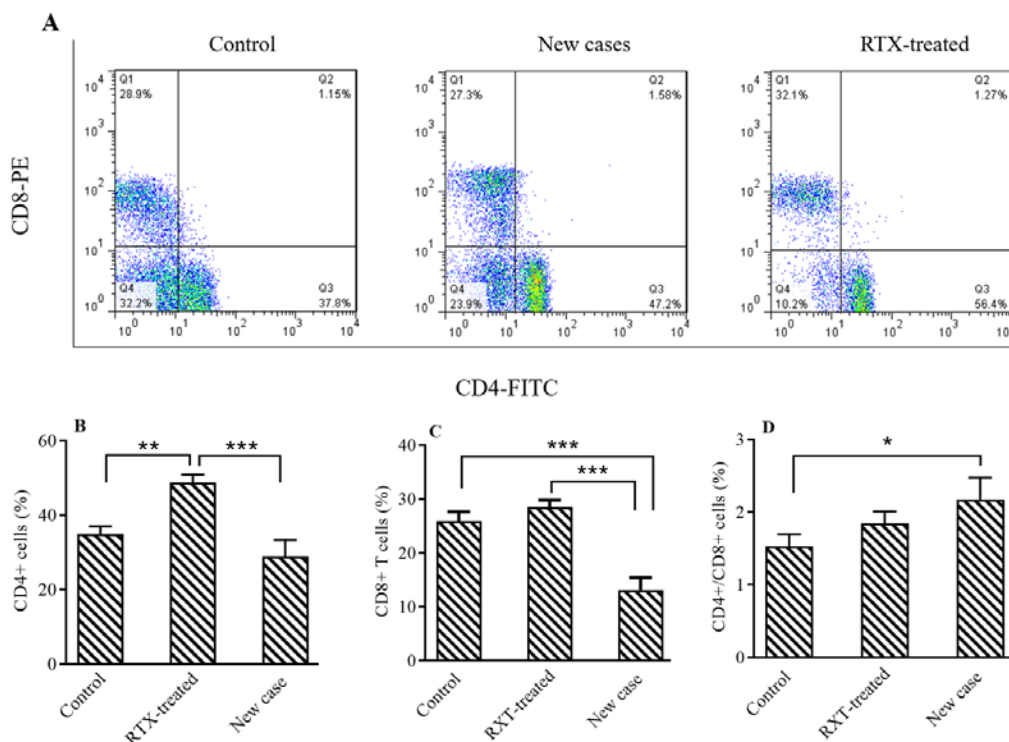
### Statistical analysis

GraphPad Prism version 6.0 (GraphPad Software, Inc., San Diego, CA) was used for statistical analyses. Statistical differences between control and experimental groups were evaluated by one-way analysis of variance (ANOVA) and multivariate analysis of variance (MANOVA) with post-Tukey's multiple comparisons test. Data were accounted as mean  $\pm$  SEM for replicate values.

## RESULTS

### Impacts of RTX treatment on T cells population

We assessed the impact of RTX treatment on the CD4<sup>+</sup> cell population, and we found a statistically significant increase in the CD4<sup>+</sup> T cells population in the RTX-treated group in comparison with the control group and newly-diagnosed group (Fig. 1A and B).

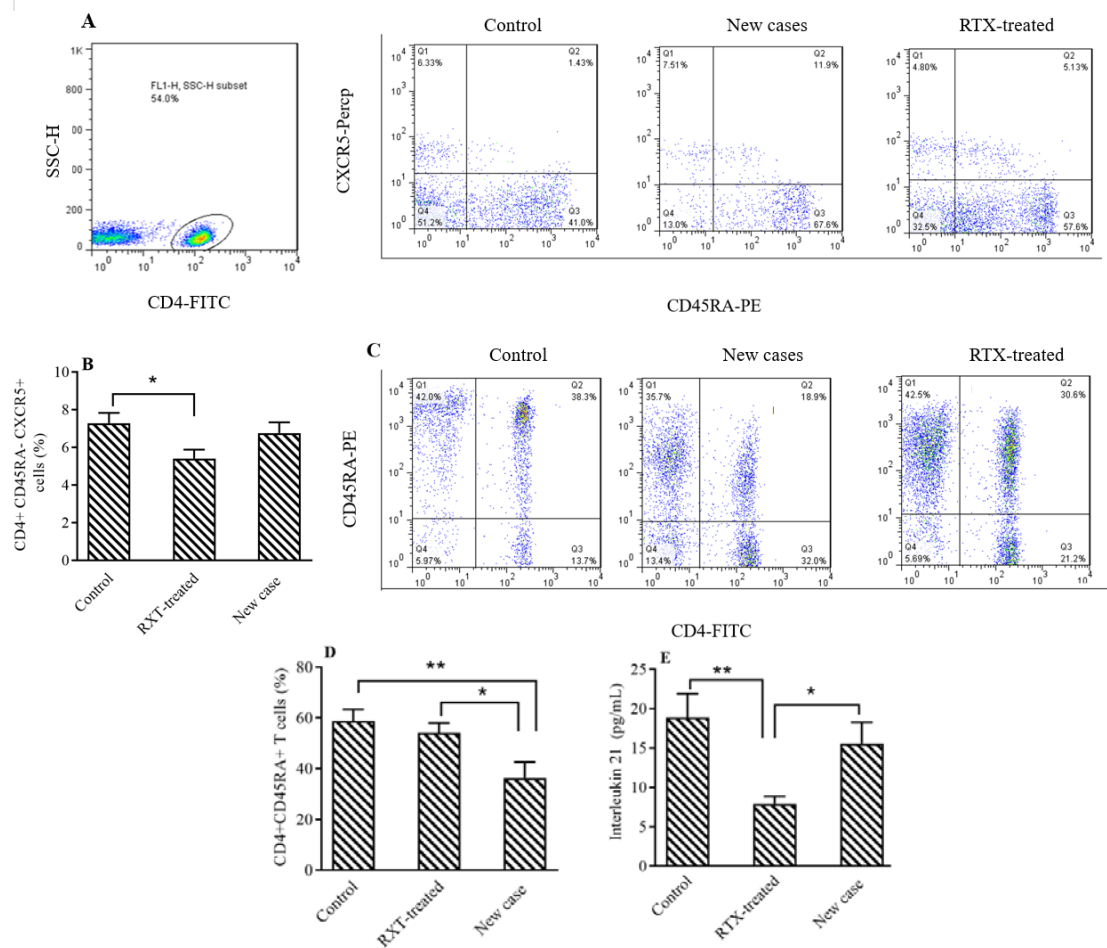


**Fig. 1.** Peripheral blood mononuclear cells from newly-diagnosed and RTX-treated multiple sclerosis patients (n = 30 in both groups) and healthy controls (n = 30) were stained with labeled antibodies. (A) Representative dot plots of CD4<sup>+</sup> and CD8<sup>+</sup> T cells in different groups of samples. At least about 30,000 events were analyzed for each sample; (B) CD4<sup>+</sup> T cells, (C) CD8<sup>+</sup> T cells, and (D) CD4<sup>+</sup>/CD8<sup>+</sup> T cells were compared between healthy controls, new cases, and RTX-treated multiple sclerosis patients. \**P* < 0.05, \*\**P* < 0.01, and \*\*\**P* < 0.001 indicate significant differences between defined groups. RTX, Rituximab.

Interestingly, CD8<sup>+</sup> cells were significantly increased after RTX treatment compared to newly-diagnosed patients, while this population was decreased in newly-diagnosed MS patients in comparison with healthy control (Fig. 1A and C). Moreover, we assessed the CD4<sup>+</sup>/CD8<sup>+</sup> ratio in three groups and we found that it was significantly increased in newly-diagnosed patients compared to the healthy control group. Also, this ratio decreased in RTX-treated patients compared to newly-diagnosed groups, but it was not statistically significant (Fig. 1D).

**Impacts of RTX treatment on T cells CD4<sup>+</sup>CD45RA<sup>-</sup>CXCR5<sup>+</sup> population**

To determine the effect of RTX treatment on the Tfh population, CD4<sup>+</sup>CD45RA<sup>-</sup>CXCR5<sup>+</sup> populations were assessed by flow cytometry analysis (Fig. 2A). We found that the Tfh cells' population decreased in RTX-treated patients compared to newly-diagnosed MS patients and healthy subjects. However, this decrease was statistically significant compared to healthy subjects (Fig. 2B). Also, we compared CD4<sup>+</sup>CD45RA<sup>+</sup>T cells between groups and we found that this population was increased in healthy subjects and RTX-treated patients compared to newly-diagnosed patients (Fig. 2C and D).



**Fig. 2.** Peripheral blood mononuclear cells from newly-diagnosed and RTX-treated multiple sclerosis patients (n = 30 in both groups) and healthy controls (n = 30) were stained with labeled antibodies. (A) Representative dot plots of CD4<sup>+</sup>, CD45RA<sup>-</sup>, CXCR5<sup>+</sup> T cells as follicular T helper cells in different groups of samples. First CD4<sup>+</sup> T cells were gated and CD45RA<sup>-</sup> CXCR5<sup>+</sup> T cells in the upper left area of the quadrant were selected. At least about 30,000 events were analyzed for each sample; (B) CD4<sup>+</sup> CD45RA<sup>-</sup> CXCR5<sup>+</sup> T cells were compared between healthy controls, new cases and RTX treated MS patients; (C) representative dot plots of CD4<sup>+</sup> CD45RA<sup>+</sup> T cells in different groups of samples; (D) CD4<sup>+</sup> CD45RA<sup>+</sup> T cells and (E) interleukin 21 concentration were compared between healthy controls, new cases, and RTX-treated multiple sclerosis patients. \**P* < 0.05 and \*\* *P* < 0.01 indicate significant differences between defined groups. RTX, Rituximab.

**Effects of RTX treatment on IL-21 concentration**

IL-21 concentration in serum of RTX-treated MS patients was significantly decreased compared to new cases and healthy control (Fig. 2E). However, we did not find a significant difference between new cases and healthy control in IL-21 levels.

**Effects of RTX treatment on HLA-DR expression on lymphocytes**

To assess HLA-DR expression, we first gated total lymphocytes and evaluated HLA-DR expression in this population and lymphocytes; then we analyzed HLA-DR expression in CD3<sup>+</sup> lymphocytes. In patients who were treated with RTX, a significant decrease was found in HLA-DR<sup>+</sup> lymphocytes compared to the control group and newly-diagnosed patients. We also compared CD3<sup>+</sup>HLA-DR<sup>+</sup>T cells between groups. We found a statistically significant decrease in the HLA-DR<sup>+</sup>CD3<sup>+</sup>T cells population in RTX-

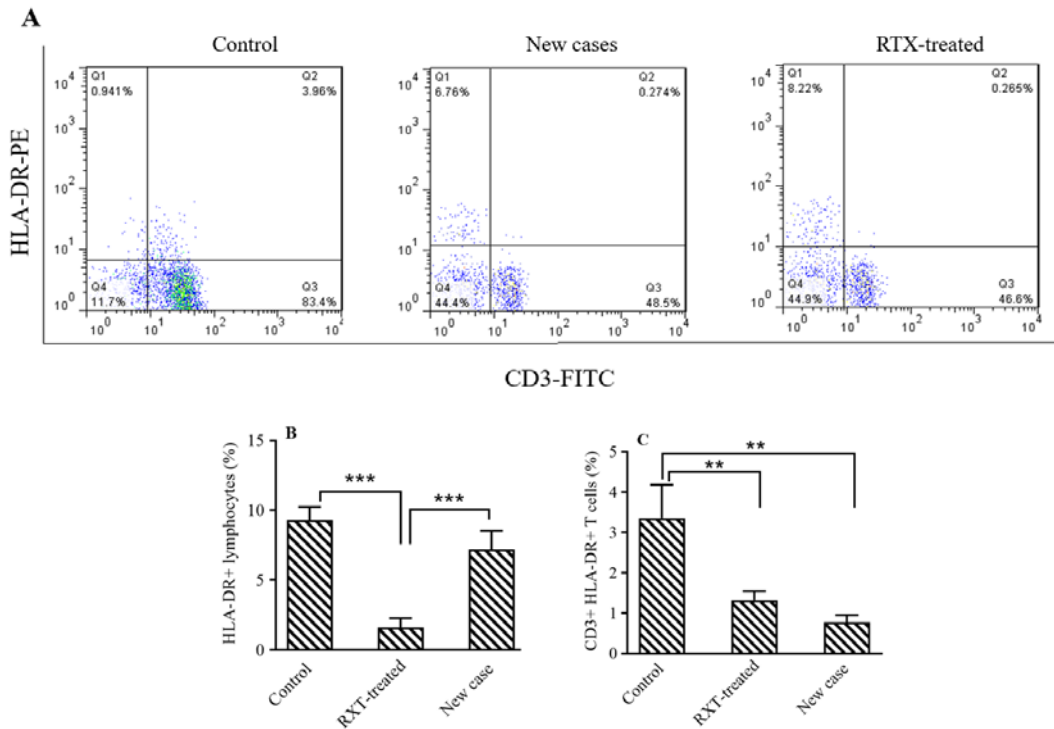
treated patients and new cases compared to healthy control (Fig. 3A-C).

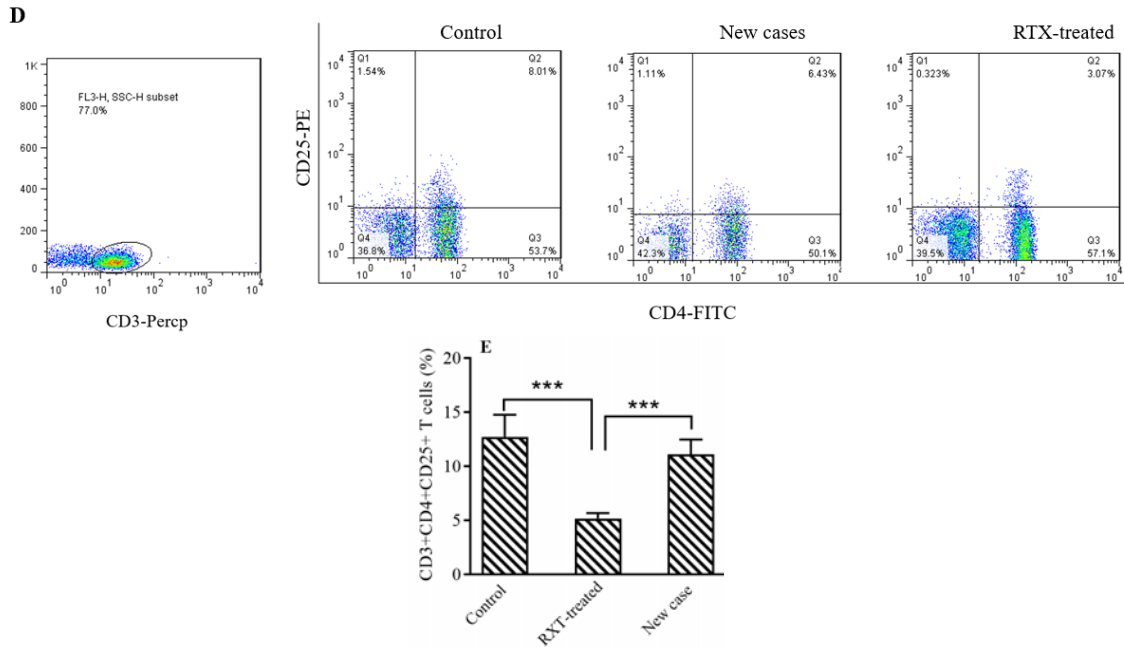
**Effects of RTX treatment on CD3<sup>+</sup>, CD4<sup>+</sup>, and CD25<sup>+</sup> cells**

CD3<sup>+</sup>CD4<sup>+</sup>CD25<sup>+</sup> T cells, also known as activated T cells, were compared between groups. A statistically significant decrease was found in these cells in RTX-treated patients in comparison with newly diagnosed and healthy control groups (Fig. 3D and E).

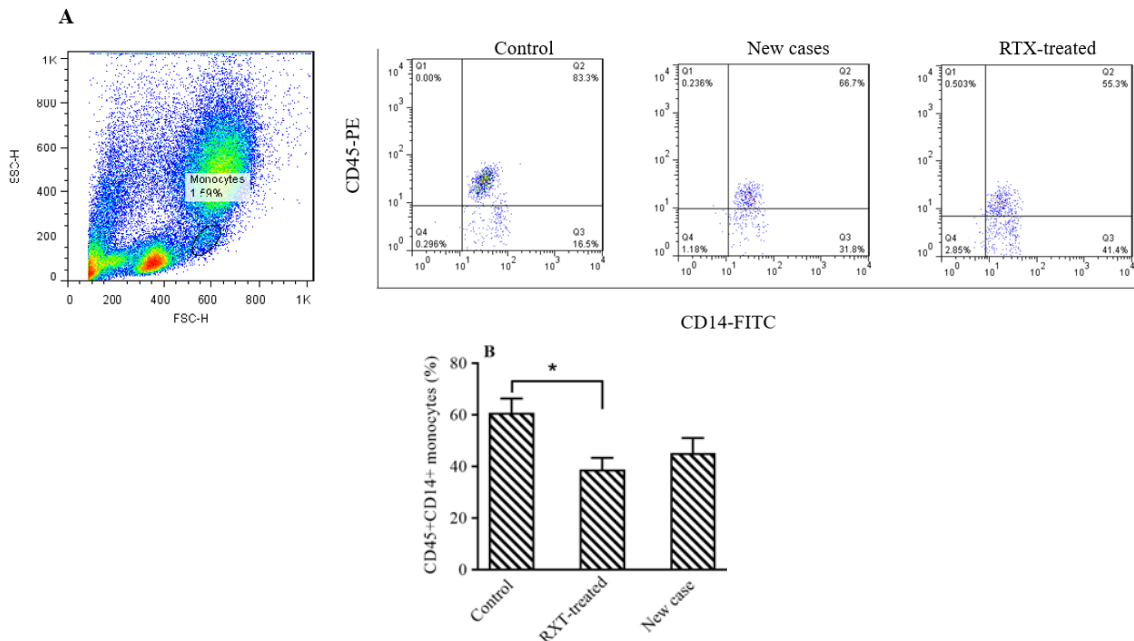
**Effects of RTX treatment on CD45<sup>+</sup>CD14<sup>+</sup> monocytes**

To determine the effects of RTX treatment on monocytes, whole blood cells were lysed by lysis buffer and was stained with anti-CD4 FITC and anti-CD45 PE antibodies. A statistically significant decrease was found in the CD45<sup>+</sup>CD14<sup>+</sup> monocyte population in the RTX-treated group compared to the control group (Fig. 4A and B).





**Fig. 3.** Peripheral blood mononuclear cells from newly-diagnosed and RTX-treated multiple sclerosis patients (n = 30 in both groups) and healthy controls (n = 30) were stained with labeled antibodies. (A) Representative dot plots of CD3<sup>+</sup> HLA-DR<sup>+</sup> T cells in different groups of samples. At least about 30,000 events were analyzed for each sample; (B) HLA-DR<sup>+</sup> lymphocytes and (C) CD3<sup>+</sup> HLA-DR<sup>+</sup> T cells were compared between healthy controls, new cases, and RTX-treated patients; (D) Representative dot plots of CD3<sup>+</sup> CD4<sup>+</sup> CD25<sup>+</sup> T cells as activated T cells in different groups of samples. First CD3<sup>+</sup> T cells were gated and then CD4<sup>+</sup> CD25<sup>+</sup> T cells were selected; and (E) CD3<sup>+</sup> CD4<sup>+</sup> CD25<sup>+</sup> T cells were compared between healthy controls, new cases, and RTX-treated patients. \*\**P* < 0.01 and \*\*\**P* < 0.001 indicate significant differences between defined groups. RTX, Rituximab.



**Fig. 4.** Whole blood cells were lysed by lysis buffer and white blood cells monocytes from newly-diagnosed and RTX-treated multiple sclerosis patients (n = 30 in both groups) and healthy controls (n = 30) were stained with labeled antibodies. (A) Representative dot plots of CD45<sup>+</sup> CD14<sup>+</sup> monocytes in different groups of samples. The first monocytes population was gated and then CD45<sup>+</sup> CD14<sup>+</sup> cells were assessed. At least about 30,000 events were analyzed for each sample; (B) CD45<sup>+</sup> CD14<sup>+</sup> monocytes cells were compared between healthy controls, new cases, and RTX-treated patients. \**P* < 0.05 indicates significant differences between defined groups. RTX, Rituximab.

## DISCUSSION

RTX affects different innate and specific immune cells in MS in addition to the decrease of B cells. In the present study, we found that CD4<sup>+</sup> T cells increased in the RTX-treated group compared to the control and newly-diagnosed groups. Also, CD8<sup>+</sup> cells significantly increased after RTX treatment compared to newly-diagnosed patients, while this population decreased in newly-diagnosed MS patients compared to healthy control. Moreover, we assessed the CD4<sup>+</sup>/CD8<sup>+</sup> ratio in three groups and found that it was significantly increased in newly-diagnosed patients relative to the healthy control group and decreased in RTX-treated patients compared to newly-diagnosed groups; it was not statistically significant. In a study, Ellrichmann *et al.* have compared B cells, CD4<sup>+</sup>T cells, CD8<sup>+</sup>T cells, and CD4<sup>+</sup>/CD8<sup>+</sup> in MS patients and neuromyelitis optica/neuromyelitis optica spectrum disorders at baseline, 3, 6, 12, and 15 months of RTX treatment. They have found the absolute cell count of CD4<sup>+</sup> and CD8<sup>+</sup> T cells increases after 6 months. Also, they have indicated CD4<sup>+</sup>/CD8<sup>+</sup> ratio did not change significantly compared to baseline after 6 months (19). In the present study, we found that CD4<sup>+</sup> CD45RA<sup>-</sup>CXCR5<sup>+</sup> percentage as Tfh cells population was decreased in RTX-treated patients compared to newly-diagnosed MS patients and healthy subjects. However, this decrease was statistically significant in comparison with healthy subjects.

Also, IL-21 concentration in serum of RTX-treated MS patients was significantly decreased compared to new cases and healthy control. However, we did not find any significant difference between new cases and healthy control groups in IL-21 levels. Zhao *et al.* have evaluated CD4<sup>+</sup>CXCR5<sup>+</sup>PD-1<sup>+</sup> cells as circulating Tfh (cTfh) cells in neuromyelitis optica spectrum disorder, and consistent with our finding, they have indicated cTfh cells decreased after RTX treatment (20). They have also have found the frequencies of cTfh cells were up-regulated in relapsing neuromyelitis optica spectrum disorder patients compared to healthy control. In comparison, they have not detected significant differences between the

remission group and healthy control. We assessed newly-diagnosed RRMS patients while they evaluated patients with more than 3 months' disease duration (20). Therefore, this discrepancy may be due to differences in the disease duration and the frequency of relapses in patients. Besides, Christensen *et al.* have reported that ectopic follicles in the CNS and Tfh cells in the blood are more dominant in secondary progressive MS patients than relapsing-remitting cases, and CXCR5<sup>+</sup> Tfh cell numbers correlate with the severity of disability (21). So, no differences between new cases and the control group in cTfh cells may be related to lower numbers of ectopic follicles in RRMS patients. However, more detailed studies are needed to assess the correlation between the severity of disease, Tfh cells, and ectopic follicles.

Previous studies have suggested RTX treatment excludes IL-6-producing B cells and prevents their direct contact with the cTfh cells, and as a result, cTfh cells expansion is inhibited (20,22). Also, another possible mechanism of RTX in MS treatment may be due to its interference in the development of Th17 cells (21). T follicular regulatory cells are a subpopulation of Tfh cells that dominantly express Foxp3<sup>+</sup> and CXCR5 (99% nTreg) (23). The functional mechanism of these cells is defined as inhibiting and eliminating autoreactive B cells during an autoimmune response (24). So, the removal of autoreactive B cells and decrease of Tfh cells by rituximab in MS treatment likely compensate for the function of T follicular regulatory cells.

We compared CD4<sup>+</sup> CD45RA<sup>+</sup> T cells between groups, and we found that this population was significantly increased in RTX-treated compared to newly-diagnosed patients. CD4<sup>+</sup> CD45RA<sup>+</sup> T cells are defined as unprimed T cells, and engagement of T cell receptors with their specific antigens is followed by loss of CD45RA (25). In our study, patients treated with RTX showed a significant decrease in HLA-DR<sup>+</sup> lymphocytes compared with the control group and newly-diagnosed patients. Furthermore, we assessed CD3<sup>+</sup>CD4<sup>+</sup>CD25<sup>+</sup> T cells as activated T cells. We found a statistically significant decrease in these cells in RTX-treated patients than

newly-diagnosed and healthy control groups. We also compared CD45<sup>+</sup> CD14<sup>+</sup> monocytes population between groups and we found a decrease in CD45<sup>+</sup> CD14<sup>+</sup> monocyte population in RTX-treated group and new cases, but it was only statistically significant in comparison between RTX and control group. The previous study has shown that a subset of memory B cells that produce GM-CSF is more prevalent in MS patients than in healthy subjects. They have indicated following B cells depletion therapy, an inflammatory response by myeloid cells is diminished (26). So, a decrease of monocytes following RTX therapy may be related to the depletion of GM-CSF producing memory B cells.

### CONCLUSION

B cells depletion therapy contribution to MS treatment is now widely appreciated. However, this treatment seems to affect different immune cells, including innate and specific cells. In fact, following B cells depletion by RTX in MS patients, a decrease of monocytes and an increase of unprimed T cells can help alleviate neuroinflammation. Moreover, due to the interaction between B cells and Tfh cells in ectopic follicles in MS patients, targeting auto-antigens-specific Tfh cells could be assessed as a therapeutic strategy in autoimmune disorders like MS.

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### Conflict of interest statement

The authors declared no conflicts of interest in this study.

### Authors' contributions

N. Esmaeil conceived of the presented idea; S. Yahyazadeh and O. Mirmosayyeb developed the theory and performed the research; S. Yahyazadeh performed the experiments and wrote some parts of the manuscript; V. Shaygannejad confirmed MS cases and clinical information. All authors discussed the results

and contributed to and approved the final version of the manuscript.

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