Journal of Autoimmunity 77 (2017) 89–95

Contents lists available at ScienceDirect

Journal of Autoimmunity

journal homepage: www.elsevier.com/locate/jautimm

B cell depleting therapy regulates splenic and circulating T follicular helper cells in immune thrombocytopenia

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ARTICLE INFO

Article history:
Received 17 March 2016
Received in revised form
9 November 2016
Accepted 9 November 2016
Available online 15 November 2016

Keywords:
Immune thrombocytopenia
T follicular helper cells
Rituximab

ABSTRACT

B cells are involved in immune thrombocytopenia (ITP) pathophysiology by producing antiplatelet auto-antibodies. However more than a half of ITP patients do not respond to B cell depletion induced by rituximab (RTX). The persistence of splenic T follicular helper cells (TFH) that we demonstrated to be expanded during ITP and to support B cell differentiation and antiplatelet antibody-production may participate to RTX inefficiency. Whereas it is well established that the survival of TFH depends on B cells in animal models, nothing is known in humans yet. To determine the effect of B cell depletion on human TFH, we quantified B cells and TFH in the spleen and in the blood from ITP patients treated or not with RTX. We showed that B cell depletion led to a dramatic decrease in splenic TFH and in CXCL13 and IL-21, two cytokines predominantly produced by TFH. The absolute count of circulating TFH and serum CXCL13 also decreased after RTX treatment, whatever the therapeutic response. Therefore, we showed that the maintenance of TFH required B cells and that TFH are not involved in the inefficiency of RTX in ITP.

1. Introduction

B cells participate in immune thrombocytopenia (ITP) pathophysiology by producing auto-antibodies against glycoproteins expressed on platelets, thus leading to their phagocytosis by splenic macrophages [1]. T Follicular Helper cells (TFH) represent a major T cell subset involved in B cell differentiation and proliferation within lymphoid organs [2]. Their role in autoimmunity has been demonstrated in the sanroque mouse model [3]. These mice do not express *Roquin,* a negative regulator of ICOS which results in the expansion of TFH that stimulate germinal center (GC) formation and the production of autoantibodies leading to a lupus-like phenotype. Interestingly, the adoptive transfer of TFH from sanroque mice into naïve mice triggers similar autoimmune manifestations. On the contrary, the disruption of TFH and B cell interaction abrogates autoimmune manifestations and leads to a decrease in TFH frequency, thus underlining the bidirectional crosstalk between TFH and B cells, and the requirement of B cells for TFH survival. Indeed, it has been shown that GC B cell are particularly involved in the maintenance of TFH within secondary lymphoid organs in mice [4,5]. These cognate B-T cell interactions sustain TFH response by presenting antigen and *via* the ICOS-L/ICOS and CD40/CD154 signaling pathways [4].

Abbreviations: ADCC, antibody-dependent cell-mediated cytotoxicity; AID, autoimmune diseases; FC, fold change; GC, germinal center; ITP, immune thrombocytopenia; PBMC, peripheral blood mononuclear cells; PBL, peripheral blood lymphocytes; RTX, rituximab; TFH, T follicular helper cells.

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http://dx.doi.org/10.1016/j.jaut.2016.11.002
0896-8411/

We recently showed that splenic TFH are expanded during ITP and support B cell differentiation and antiplatelet antibody-production [6]. We speculated that rituximab (RTX), a B cell depleting therapy that is increasingly used to treat autoimmune diseases (AID) could regulate TFH numbers, in the blood and in lymphoid organs. Recent reports showed that the expansion of circulating TFH is reversed after RTX treatment in type 1 diabetes [7] and granulomatosis with polyangeitis [8]. Similarly, the level of a key chemokine produced by TFH, CXCL13, that binds to CXCR5 and participates to the recruitment of $CXCR5^+$ B cells within GC, decreases after RTX treatment [9,10]. Surprisingly, a combination of low dose RTX associated with mycophenolate mofetil and tacrolimus used to prevent kidney rejection, was reported to induce GC B cell ($CD19^+CD38^+Bcl6^+$) depletion without affecting TFH [11], thus contradicting what is observed in mice [4,5]. As both RTX and splenectomy are used to treat ITP [12,13], we aimed to clarify whether the survival of TFH depends on B cells in humans by investigating the effect of B cell depletion on circulating and splenic TFH in ITP patients treated with RTX.

2. Material and methods

2.1. Patients

Forty-three primary persistent/chronic ITP patients were included in the study for either blood or spleen analyses (blood and spleen samples were both available for only two patients). Blood samples, obtained from a cohort of 23 patients, were used to investigate the effect of RTX on circulating B cells and TFH, and to attempt to determine predictive factors of response by comparing responders to non-responders to RTX (Table 1). Peripheral blood mononuclear cells (PBMC) were isolated by gradient centrifugation and stored in liquid nitrogen at the Biological Resource Center Ferdinand Cabanne (BB-0033-00044) until needed.

Splenocytes were obtained from a second cohort of 22 patients (Table 2) as previously described [14] and stored in liquid nitrogen until needed. Splenocytes were used to determine the effect of RTX on the immune response *in vivo* by comparing RTX-untreated patients (n = 13) to patients treated with RTX prior to splenectomy (n = 9). Of note, all RTX-treated patients underwent splenectomy because a response was not achieved. Splenocytes from RTX-untreated patients were also used for certain experiments to determine the effect of RTX on splenocytes *in vitro*.

All patients gave an informed consent in accordance with the declaration of Helsinki. The study was approved by the institutional review board and the ethics committee of our University Hospital. Response to RTX was defined as an increase in platelet count above 30 G/L with at least a doubling of the baseline level 8 weeks after RTX was started. Patients not achieving a platelet count above 30 G/L with at least a doubling of the pretreatment baseline level or who needed rescue therapy after the 8 weeks following the first RTX infusion were considered as non-responders, as recommended [15].

2.2. Flow cytometry

The following antibodies were used for flow cytometry (FCM): anti-CD19 Allophycocyanin (APC), anti-CD38 PhycoErythrin (PE), anti-IgD Fluorescein isothiocyanate (FITC), anti-CD3 Brillant Violet 510, anti-CD4 PE-Cyanin7, anti-CXCR5 PerCP-Cy5.5, anti-ICOS APC, anti-PD-1 FITC. Data were acquired on a BD Biosciences LSRII cytometer (Flow cytometry department, IFR 100, Burgundy University) and analyzed with FlowJo® software.

2.3. In vitro B cell depletion

To assess the effect of RTX on TFH *in vitro*, splenocytes (1.10^6/mL) from RTX-untreated ITP patients were cultured in RPMI supplemented with 5% heat inactivated fetal bovine serum and 1% glutamine and incubated with RTX (rituximab, Roche). RTX was used at 10 μg/ml, a concentration that allows the saturation of CD20 on cell surface [16] and corresponds to residual concentration of RTX during its therapeutic use [17]. To test the role of complement in B cell depletion, 5% of rabbit serum was added to culture. Cells were harvested after 1, 3 and 6 days for flow cytometry staining. B cells were identified by CD19 expression. The efficient binding of RTX to B cells was checked by using an anti-CD20 antibody binding the same epitope of RTX: in this condition, B cells exposed to RTX were $CD19^+CD20^-$. TFH were defined as $CD3^+CD4^+CXCR5^+ICOS^+PD\text{-}1^{hi}$.

2.4. Cytokine measurement

For cytokine measurement, serum was isolated within four hours following blood collection and was stored in liquid nitrogen at the Biological Resource Center Ferdinand Cabanne (BB-0033-

Table 1
Characteristics of primary chronic ITP patients (blood analyses).

	Responders to rituximab n = 12	Non-responders to rituximab n = 11	*p*-value
Age (*years*), *median [IQ range]*	55.8 [37.4–71.5]	63.1 [47.2–79.3]	0.25
Gender ratio (female/male)	6/6	2/9	0.2
Disease duration (*months*), *median [IQ range]*	7.4 [2.6–30]	52.5 [3.5–166]	0.09
Nadir of platelet count within the 2 months prior to RTX ($\times 10^9$/L), *median [IQ range]*	11 [7–19]	10 [4–19]	0.08
Previous treatments, n (%)			
Steroids	12 (100)	11 (100)	–
IVIg	6 (50)	7 (64)	0.7
Dapsone	5 (42)	4 (36)	1
TPO-RA	1(8)	1 (9)	1
Hydroxychloroquine	1 (8)	1 (9)	1
Splenectomy	1 (8)	0	1
Treatment within the 2 weeks prior to rituximab, n (%)			
Steroids	6 (50)	7 (64)	0.7
IVIg	1 (8)	3 (27)	0.3
Dapsone	1 (8)	0	1
TPO-RA	1 (8)	1 (9)	1
None	2 (16)	0	0.5

ITP: immune thrombocytopenia; IVIg: intravenous immunoglobulins; TPO-RA: thrombopoietin receptor agonists.

Table 2
Characteristics of splenectomized primary ITP patients.

	RTX-untreated ITP n = 13	RTX-treated ITP n = 9	*p*-value
Age (*years*), *median [IQ range]*	27.5 [22–52]	71 [47–79]	0.002
Gender ratio (female/male)	11/2	6/3	0.6
Disease duration (*months*), *median [IQ range]*	20 [12–42]	16 [10–30]	0.5
Platelet count within the 2 months prior to splenectomy ($\times 10^9$/L), *median [IQ range]*	22.5 [17–27]	15 [10–22]	0.2
Previous treatments, n (%)			
Steroids	13 (100)	8 (89)	0.4
IVIg	9 (69)	9 (100)	0.1
Dapsone	8 (62)	5 (56)	1
Rituximab	0	9 (100)	<0.0001
TPO-RA	0	2 (22)	0.2
Vincristine	0	1 (11)	0.4
Treatment within the 2 weeks prior to splenectomy, n (%)			
IVIg	8 (61)	7 (78)	0.6
Steroids	3 (23)	1 (11)	0.6
Dapsone	1 (8)	0	1
TPO-RA	0	1 (11)	0.4
None	1 (8)	0	1
Responder to splenectomy, n (%)	12 (92)	6 (67)	0.3
Duration between rituximab and splenectomy *(months), median [IQ range]*	–	7.2 [5.6–9.3]	–

ITP: immune thrombocytopenia; IVIg: intravenous immunoglobulins; TPO-RA: thrombopoietin receptor agonists.

00044). Samples were thawed only once and centrifuged at 16,000 rpm for 5 min to remove clots or precipitates prior to cytokine measurement. A multiplex bead immunoassay (eBioscience) was used to simultaneously measure CXCL13 and IL-21, following manufacturer's instructions. Data were acquired and analyzed on a Bio-Plex® 200 system for multiplex analysis (Bio-Rad; Flow cytometry department, IFR 100, Burgundy University).

2.5. $CD4^+$ T cell separation

Splenocytes or PBMC were incubated with anti-CD4 microbeads (Miltenyi), following fabricant's instructions. Separations were performed with an AutoMACS device (Miltenyi). Purity, assessed by FCM, was >97%.

2.6. Gene expression quantification

RNA extraction was performed by using the RNeasy RNA-DNA-miRNA Universal kit (Qiagen) as recommended by the manufacturer. RNA was retrotranscribed by using the iScript (Qiagen) and the expression of CXCR5, CXCL13, IL-21 and GAPDH mRNA was quantified by quantitative PCR (qPCR) by using the following specific primer pairs: CXCR5: fw CTGGAGGACC-TGTTCTGGGA, rev AGGAGGAAGATGAGGCTGTAG, IL-21: fw GCCACATGATTAG-AATGCGTC, rev TTCAGGGACCAAGTCATTCAC, CXCL13: fw GCTCAAGTCTGAACTCTACCTC, rev TCTCTTGGACACATCTACACCT, GAPDH: fw ATGGGGAAGGTGAAGGTCG, rev GGGGTCATTGATGG-CAACAATA. qPCR was performed on a Quantstudio 12K real-time PCR system (Life technologies) using the following thermal cycle conditions: 50 °C for 2 min, 95 °C for 10 min, 40 cycles at 95 °C for 1 s and 60 °C for 30 s in the presence of the Sybr Select mastermix (Life technologies) and 500 nM forward and reverse primers. Gene expression values were calculated according to the comparative threshold cycle method [18] using the stable expressed GAPDH as endogenous control. The value of the lowest expressed sample was set as one and used to calculate fold change (FC) of target genes.

2.7. Immunohistochemistry

Anti-Bcl6 (Dako) and anti-PD-1 (Abcam) antibodies were used. Staining was performed using a BenchMark Ultra instrument (Ventana Medical Systems, Roche diagnostic). Visualization was based on enzymatic conversion of diaminobenzidine (DAB) into a brown-colored precipitate by horseradish peroxidase at the site of antigen localization. Ultra Red (Ventana Medical Systems) and DAB were used successively to perform double staining.

2.8. Statistical analysis

Data are given by median [interquartile range], excepted when specified. Fisher's exact test was used to compare qualitative data. Quantitative values from RTX-untreated and RTX-treated patients are represented as scatter plots and were compared by using Mann-Whitney test. Sequential data from responders and non-responders to RTX are represented by before/after plots and were compared by using the Wilcoxon matched-pairs test. The paired *t*-test was used to compare the average of cell depletion by RTX *in vitro*. $P < 0.05$ was considered significant.

3. Results

3.1. Rituximab induces a dramatic decrease in splenic TFH in vivo

As TFH are known to support B cell differentiation within lymphoid organs, immunohistochemistry was used to localize them within the spleen of ITP patients. In RTX-untreated patients, follicles were clearly identified and displayed a prominent germinal center (GC). TFH were localized within GC, as demonstrated by the PD-1 and Bcl6 staining (Fig. 1A). On the contrary, nor follicles nor TFH were observed in RTX-treated patients (data not shown).

The frequencies of $CD19^+$ B cells and TFH, defined as $CD3^+CD4^+CXCR5^+ICOS^+PD\text{-}1^{hi}$, were determined among splenocytes by FCM and compared between RTX-untreated and RTX-treated patients (Fig. 1B). As expected, we observed a complete B cell depletion in almost all RTX-treated patients as compared to untreated ones (0.6% [0.3–4] *vs.* 58.2% [52–63] of total lymphocytes, $P = 0.0003$; Fig. 1C). B cell depletion was associated with a profound decrease in $CXCR5^+CD4^+$ T cells and most particularly in TFH (0.1% [0.05–0.23] *vs.* 2% [1.4–3.2], $P = 0.001$; Fig. 1D). Confirming these results, CXCR5 expression in splenic $CD4^+$ T cells was decreased (Fig. 1E). Furthermore, the expression of CXCL13 and IL-21, two cytokines produced by TFH [2,6] were also diminished in RTX-treated patients compared to untreated ones (Fig. 1E).

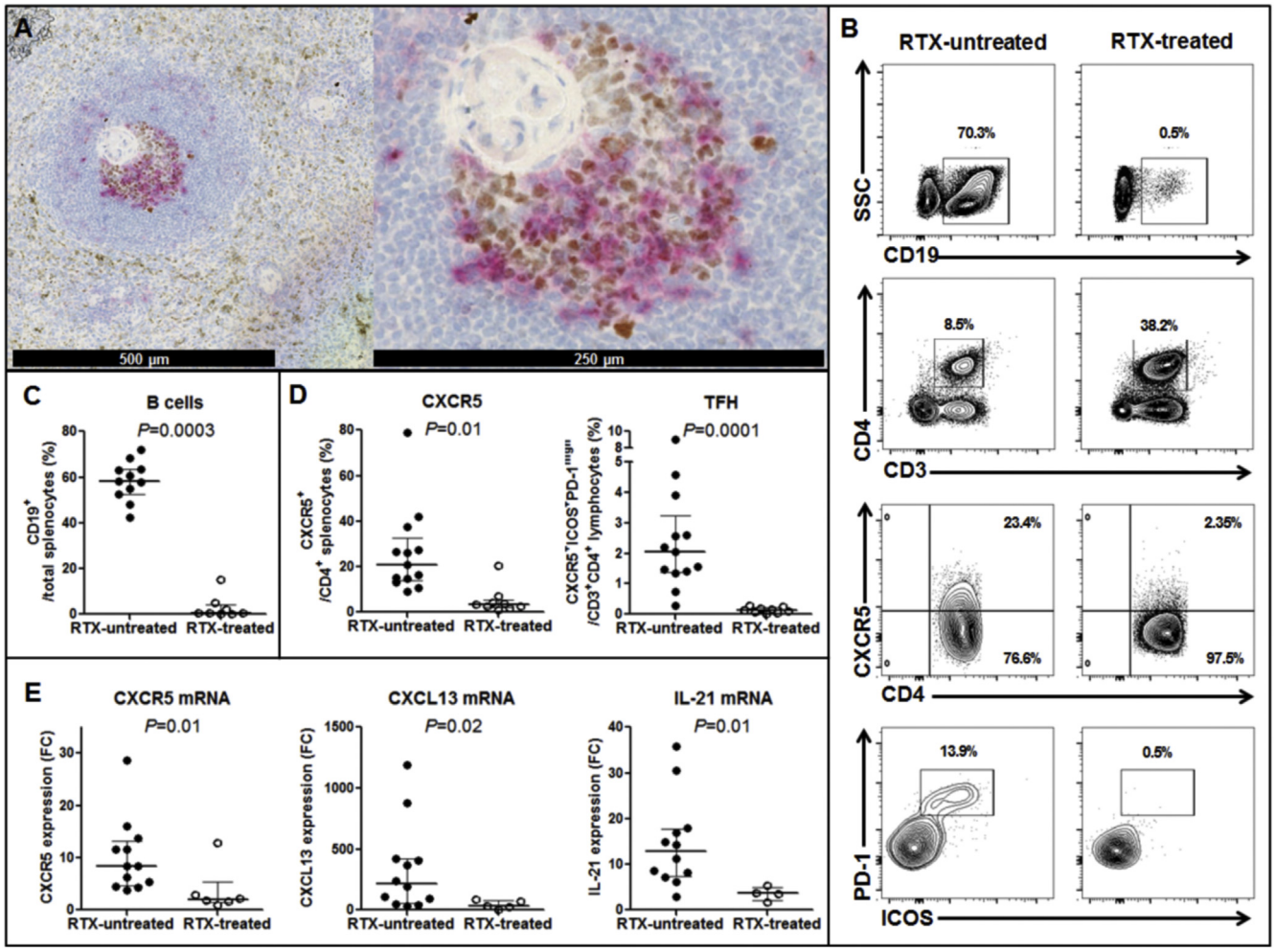

Fig. 1. Effect of rituximab on splenic cells *in vivo*. **A**) PD-1 (red) and Bcl6 (brown) staining was used to localized TFH within the spleens by immunohistochemistry. Representative spleen of one RTX-untreated ITP patient showing a follicle with germinal center (magnification ×10 and ×35). **B**) Frequencies of splenic B cells and TFH were determined by flow cytometry. Representative dot plots of one rituximab-untreated and one rituximab-treated ITP patient. **C)** Splenocytes from RTX-untreated ITP patients (black circles) or treated with rituximab prior to splenectomy (open circles) were compared for B cell frequency, **D**) $CXCR5^+$ and TFH ($CXCR5^+ICOS^+PD\text{-}1^{hi}$) frequencies, measured by flow cytometry, and for **E**) the expression of CXCR5, CXCL13 or IL-21, measured by qPCR in $CD4^+$ T cells. The horizontal bar represents the median with interquartile range, FC: fold changes. *P*-value derived by Mann-Whitney test.

3.2. B cell depletion by rituximab induces a decrease in TFH in vitro

To determine whether RTX could deplete TFH, splenocytes from RTX-untreated patients were cultured *in vitro* with RTX and/or complement. B cell and TFH frequencies were determined by FCM as represented in Fig. 2A. It is established that the major mechanism of B cell depletion by RTX is antibody-dependent cell-mediated cytotoxicity (ADCC), but as a type 1 IgG monoclonal antibody, RTX also strongly activates complement that increases effector cell functions [19]. Consistent with these mechanisms of action, B cell depletion was around 50% at day 1, reaching 80% ($P = 0.05$) after 3 days and 90% ($P = 0.001$) at day 6 in presence of complement, whereas it represented only 35% ($P = 0.13$) without complement at day 6 (Fig. 2B). Contrary to the rapid B cell depletion induced by RTX with complement, TFH frequency decreased slowly overtime, representing 60% of baseline level at day 6 ($P = 0.007$). Without complement, only a partial B cell depletion was achieved and TFH frequency was nearly not affected, representing an average of 93% of baseline level at day 6 ($P = 0.2$). To confirm the absence of direct interaction between RTX and TFH, we measured CD20 expression on TFH by FCM. TFH did not express CD20, which was confirmed by adding RTX just before the staining, as the mean fluorescence intensity (MFI) was not affected, whereas it was completely abolished on B cells (Fig. 2C).

Altogether, these results rather argue for an indirect effect of RTX on TFH due to B cell depletion than for a direct effect of RTX on TFH.

3.3. Rituximab induces a decrease in the absolute number of circulating TFH

The effect of RTX on circulating cells was determined before and 3 months [range: 2.5–4.3] after RTX treatment. As observed in the spleen, B cell depletion was also evident in the blood of RTX-treated patients (Fig. 3A), while the frequency of circulating $CD4^+CXCR5^+$ was not affected (Fig. 3B). However, when TFH were specifically studied, their number diminished after RTX treatment (687 [280–914] *vs.* 239 cells/µL [106–366]; $P = 0.001$), whatever the therapeutic response, further arguing for a role of B cells to their maintenance (Fig. 3B). The pretreatment absolute counts of TFH were not different between responders and non-responders ($P = 0.9$, data not shown).

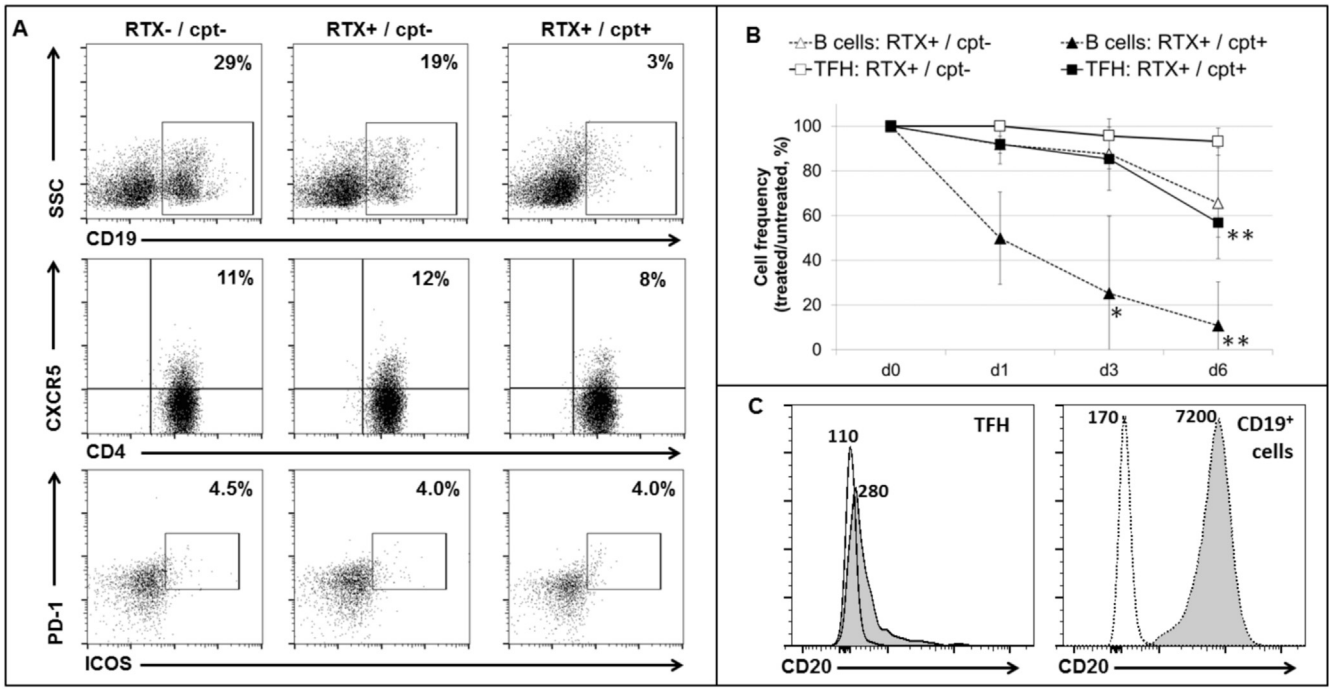

Fig. 2. Effect of rituximab on splenic cells *in vitro*. **A**) Splenocytes from 3 rituximab-untreated ITP patients were cultured in presence or not of rituximab (RTX: 10 μg/mL) and rabbit complement (cpt, 5%). The frequencies of B cells ($CD19^+$), $CD4^+CXCR5^+$ and TFH ($ICOS^+PD1^+$ in $CD4^+CXCR5^+$) were determined by flow cytometry. Representative dot plots of one spleen after 6 days of culture. **B**) B cell (triangles, dashed line) and TFH frequencies (squares, full line) were determined by flow cytometry at day 1 (d1), d3 and d6. Cell frequency is expressed as a percentage of untreated cells. *P*-value derived by paired *t*-test. Only significant differences with baseline level are represented: *: $P < 0.05$; **: $P < 0.01$ **C**) CD20 expression reported as mean fluorescence intensity (MFI) on TFH (left panel) and B cells (right panel) at baseline (grey shaded histogram) or after 30 min culture with rituximab (blank histogram). A representative histogram of one ITP patient is shown.

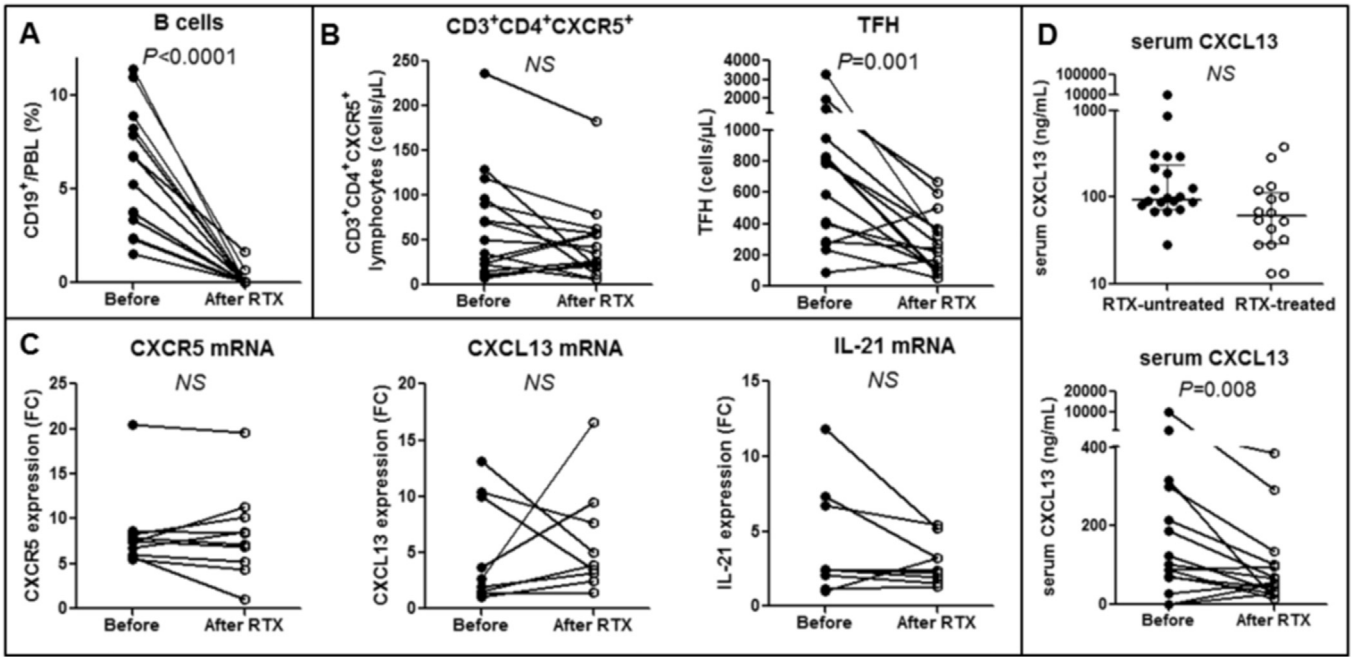

Fig. 3. Effect of rituximab on circulating cells and serum cytokines. ITP patients were compared before (black circles) and 3 months after rituximab treatment (open circles) for **A**) B cell frequency and **B**) $CXCR5^+$ T cell and TFH ($CXCR5^+ICOS^+PD\text{-}1^{hi}$) frequencies in the peripheral blood as determined by flow cytometry. **C**) The expression of CXCR5, CXCL13 and IL-21 was measured by quantitative PCR in circulating $CD4^+$ T cells. *P*-value derived by Wilcoxon matched-pairs test, FC: fold changes. **D**) CXCL13 was measured by multiplex immunoassay in the serum of ITP patients before (n = 22) and after (n = 16) RTX treatment (upper panel). The horizontal bar represents the median with interquartile range. *P*-value derived by Mann-Whitney test. Paired-data are depicted in a before-after graph for 16 patients (lower panel). *P*-value derived by Wilcoxon matched-pairs test. PBL: peripheral blood lymphocytes.

CXCL13 and IL-21 are two cytokines predominantly produced by TFH that are increased during several AID (ANCA-associated vasculitis [9], rheumatoid arthritis [20], type 1 diabetes [7], sicca syndrome [21]). As both CXCL13 [22] and IL-21 [23] serum concentrations have been reported to be increased in ITP, we measured their expressions in circulating $CD4^+$ T cells and in serum before and after RTX treatment. Probably due to the low proportion of TFH among circulating $CD4^+$ T cells, CXCR5, CXCL13 and IL-21 expressions were unchanged after RTX treatment (Fig. 3C). In our hands, serum IL-21 was not detected (data not shown). Interestingly, serum CXCL13 tended to be lower in patients treated with RTX than in untreated ones (60.7 ng/mL [29.4–114] *vs.* 94 [71.7–234.7], $P = 0.08$, Fig. 3D). When paired samples were considered, a significant decrease in CXCL13 was observed after RTX (100 [68.6–298.5] to 54.9 ng/mL [28.2–100.7]; $P = 0.008$), and most particularly in patients who responded to RTX (data not shown). Serum CXCL13 concentration was not correlated with circulating TFH frequency or absolute number (data not shown). Altogether these results show that B cell depletion is associated with a decrease in circulating TFH and serum CXCL13 concentration, whatever the therapeutic response to RTX.

The determination of predictive factors of response to RTX remains a critical issue to manage ITP patients. Thus, we compared age, sex, disease duration, serum CXCL13, absolute count of TFH and B cell subsets before RTX treatment, between responders and non-responders. Despite none of these parameters were significantly associated with therapeutic response to RTX (data not shown), it is worth noticing that non-responders tended to have a longer disease duration than responders (7.4 [2.6–30] *vs.* 52.5 [3.5–166] months, $P = 0.09$, Table 1).

4. Discussion

We here show that B cell depletion obtained by RTX *in vivo* is associated with a decrease in TFH both in the blood and in the spleen of ITP patients, a phenomenon that we confirmed *in vitro*, thus demonstrating that B cells participate to the maintenance of TFH in human secondary lymphoid organs, similarly to what is observed in animals [4,5]. As a complete B cell depletion was achieved following RTX, we were not able to determine whether a particular subset of B cells was specifically involved in TFH persistence. In animals, GC B cells support TFH survival [4,5], whereas in humans, their role seems less prominent, as showed by Wallin et al. [11]. In their study, they analyzed lymph nodes from patients treated with RTX in association with mycophenolate mofetil and tacrolimus before kidney engraftment, to avoid acute transplant rejection. Although patients were treated with 500 mg RTX one month before, only a partial B cell depletion within lymph nodes was achieved and preferentially affected GC B cells. The frequency of TFH that were defined as $CD4^+CXCR5^+CD57^+$ was not different from patients not treated with RTX. Different factors could explain the discrepancies between these results and our study. First, it has been shown in monkeys that B cell depletion was not similar between lymphoid organs, especially, that B cell depletion is more difficult to be achieved in lymph nodes than in the spleen [24]. Secondly, different dosages of RTX were used: 2 g in our study *vs.* 500 mg in the aforementioned paper, a dosage that could be insufficient to induce a total B cell depletion within lymphoid organs.

Interestingly, we observed a progressive decrease in TFH frequency following B cell depletion *in vitro*. RTX mechanism of action relies on ADCC that is even more efficient with complement activation [25,26], a phenomenon we confirmed by showing an increase in B cell depletion in presence of complement. The direct effect of RTX on TFH was ruled out as they do not express CD20, supporting the fact that their progressive decline was due to the disruption of survival signal following B cell depletion, notably by the loss of stimulation through ICOS, as previously described [27,28]. However, as TFH were quantified by FCM, it cannot be excluded that their decrease was due to changes in their phenotype. In mice, in the absence of cognate antigen stimulation, TFH downregulate CXCR5, PD-1 and Bcl6 and survive in secondary lymphoid organs as memory cells [4,29]. After subsequent antigen stimulation, these quiescent memory TFH are activated, expressed CXCR5 and PD-1 again and provide efficient B cell costimulation [29]. Thus, in patients who relapse after a first response to RTX treatment, one can hypothesize that the persistence of TFH in such a memory state could support the differentiation of autoreactive B cells during the reconstitution of the B cell compartment. Unfortunately, such a hypothesis will be hardly demonstrated in humans, as lymphoid organs from responder patients could not obviously be assessed.

Whether the decrease in TFH following RTX therapy we observed is specific of ITP or represents a general mechanism cannot be fully answered. However, the literature reported a decrease in circulating TFH following B cell depletion during type 1 diabetes [7] and granulomatosis with polyangeitis [8]. Moreover, we also measured TFH within the spleen of a patient suffering from autoimmune hemolytic anemia, who was previously treated with RTX. No B cells were detected and splenic TFH frequency represented 0.06% of total $CD4^+$ T cells, a percentage that is similar to the one we measured in RTX-treated ITP patients. Thus, it argues for the fact that B cell depletion induced by RTX is responsible for a decrease in circulating and splenic TFH, whatever the disease and the response to treatment.

In ITP, the mechanisms that lead to thrombocytopenia rely on both an inappropriate bone marrow platelet production and a peripheral destruction of platelets due to autoantibodies produced by B cells [1] or to a $CD8^+$ T cell-mediated cytotoxicity [30]. At a patient level, one mechanism could be predominantly involved, thus leading to variable response to treatment, as observed in murine models [31]. To date, no predictive factors of response to RTX have clearly been established, even if RTX appeared to be more efficient in patients with disease duration of less than one year and who previously responded to steroids [32]. In line with this, we observed that non-responders to RTX tended to have a longer disease duration compared to responders. Biomarkers that will help identifying patients with a preferential B cell-mediated disease are thus required. Here, we cannot identify a clear predictive factor of response to RTX. As previously shown, in those non responder patients, platelet destruction can be due to the persistence of long-lived specific plasma cells in the spleen [33], or in the bone marrow where their survival do not rely on IL-21 produced by TFH [34], or due to cytotoxic T cell mediated cytotoxicity [30,35]. Thus, even if B cells are depleted and TFH no more present in secondary lymphoid organs, other mechanisms maintain the disease.

5. Conclusions

In conclusion, our data support a role for B cells in TFH survival as a significant decrease in TFH was observed both in the spleen and in the blood, whatever the response to RTX.

Authorship contributions

S.A. and B.B. were the principal investigators; S.A, T.R. and B.B. designed the study; S.A., M.S., S.B., V.L.-S., B.L. and B.B. recruited the patients; S.A., M.R., M.T., K.S., L.M., T.R. and B.B. designed the experiments; S.A., M.Z., M.T., M.S., K.S., A.G., C.B., M.B., L.M., M.C. and

N.J. performed the experiments; O.F., N.C. and P.O.-D. performed the splenectomies; S.A., M.R., T.R. and B.B. analyzed the results; S.A., T.R. and B.B. coordinated the research; S.A., M.R., N.J., P.S., T.R. and B.B. wrote the manuscript.

Conflict of interest disclosure

The authors declare no competing financial interests.

Acknowledgements

The study was supported by the clinical research department of the university hospital of Dijon (Direction de la Recherche Clinique, CHU de Dijon, PARI 2010-A00598-31), the Burgundy regional council (Conseil Régional de Bourgogne, PARI 2010-A00598-31, S.A. and B.B.). S.A. was supported by a grant from the Foundation for the Development of Internal Medicine in Europe (FDIME Research Project Grant 2013).

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