Clinical & Experimental Immunology
The Journal of Translational Immunology

Clinical and Experimental Immunology ORIGINAL ARTICLE doi:10.1111/cei.13024

B cell phenotypes in patients with rheumatoid arthritis relapsing after rituximab: expression of B cell-activating factor-binding receptors on B cell subsets

E. Becerra, I. De La Torre, M. J. Leandro and G. Cambridge
Department of Rheumatology, University College London, London, UK

Accepted for publication 14 July 2017
Correspondence: E. Becerra, Division of Medicine, Department of Rheumatology, University College London, Rayne Building, 5 University Street, London WC1E 6JF, UK.
E-mail: elenabecerrafernandez@yahoo.es
and
G. Cambridge, Division of Medicine, Department of Rheumatology, University College London, Rayne Building, 5 University Street, London WC1E 6JF, UK.
E-mail:g.cambridge@ucl.ac.uk

Summary

Serum levels of B cell-activating factor (BAFF) rise following rituximab (RTX) therapy in patients with rheumatoid arthritis (RA). Initiation of naive B cell return to the periphery and autoreactive B cell expansion leading to relapse after RTX may therefore be linked to interactions between BAFF and BAFF-binding receptors (BBR). Relationships between serum BAFF and BBR expression [(BAFFR, calcium signal modulating cyclophilic ligand interactor (TACI) and B cell maturation antigen (BCMA)] were determined on B cell subsets, defined using immunoglobulin (Ig)D/CD38. Twenty pre-RTX and 18 RA patients relapsing after B cell depletion were included. Results were analysed with respect to timing of relapse up to 7 months after peripheral B cell return ($\geq$ 5 B cells/μl) and to serum BAFF levels. After B cell return, B cell populations from relapsing patients had significantly lower BAFFR$^+$ expression compared to HC and pre-RTX patients. The percentage of BAFFR$^+$ B cells increased with time after B cell return and was correlated inversely with serum BAFF levels. BAFFR expression remained reduced. The percentage of TACI$^+$ memory B cells were lower in RA patients after RTX compared with healthy controls (HC). BCMA expression (% and expression) did not differ between patients and HC. Relapse following B cell return appeared largely independent of the percentage of BAFFR$^+$ or percentage of BCMA$^+$ B cells or serum BAFF levels. The lower percentage of TACI$^+$ memory B cells may reduce inhibitory signalling for B cell differentiation. In patients relapsing at longer periods after B cell return, recovery of the B cell pool was more complete, suggesting that selection or expansion of autoreactive B cells may be needed to precipitate relapse.

Keywords: B lymphocytes, rheumatoid arthritis, rituximab

Introduction

The B cell maturation process is the result of complex interactions related to the antigen specificity and strength of signal through the B cell receptor (BCR) and B cell survival factors, in particular the B cell activating factor (BAFF)/APRIL (a proliferation-inducing ligand) axis. Results from murine studies have shown that co-ordinated expression of BAFF-binding receptors (BBRs); namely, BAFFR (BR3), transmembrane activator and calcium signal modulating cyclophilic ligand interactor (TACI) and B cell maturation antigen (BCMA), are key to the differentiation of B cells into immunoglobulin-secreting cells (ISC) [1]. The three BBRs are expressed differentially on B cells during development and also respond in context-dependent ways to BAFF/APRIL ligation [2]. In humans, BAFFR is expressed on the majority of B cells in the peripheral blood following exit from the bone marrow (BM) [3]. BAFFR binds only BAFF [4]. Occupation of BAFFR by BAFF in resting B cells is relatively constant and delivers prosurvival signals, particularly to early naive B cell populations [2,5]. BAFFR expression decreases when B cells differentiate into ISC, and loss of BAFFR is thought to be necessary for the expression of BCMA on late-stage plasmablasts, although short-lived plasmablasts can express both BAFFR and BCMA [2]. Expression of BCMA seems to be restricted largely to mature plasma cells in the BM

and secondary lymphoid organs. TACI is expressed on a subpopulation of activated naive B cells (< 25%), and increases with activation through T-dependent and -independent stimuli. TACI is expressed on virtually all memory B cells and variably on plasma cells. Signalling through TACI can result in both positive and negative regulation of antibody responses, but seems necessary for class-switch recombination [6]. Alternatively, signalling by agonists through TACI can attenuate CD40- and BAFFR-induced maturation to immunoglobulin (Ig) production through both T-dependent and -independent pathways [7].

Following its first reported success in the treatment of patients with rheumatoid arthritis (RA) in 2001 [8],B cell depletion therapy (BCDT) based on the anti-CD20 agent rituximab (RTX) has proved to be both effective and relatively safe [9,10]. Seropositive patients respond most favourably to BCDT [11], supporting the hypothesis that both B cells and their daughter plasma cells play a role in the pathogenesis of RA [12–14]. When adequate levels of B cell depletion are attained with RTX, clinical benefit to RA patients can last for months or, in some cases, years. Return of naive B cells to the circulation seems necessary for clinical relapse in responding patients, but it is not necessarily linked to concurrent relapse [13,15], with flare delayed for many months after B cell reconstitution begins. The pattern of relapse on repeat treatment, whether occurring close to B cell reconstitution or later, tends to be constant in individual patients [16]. Onset of relapse is associated with differentiation towards ISC, as shown by rises in autoantibodies [17], the $CD27^+$ memory phenotype [18,19] and the presence of circulating plasmablasts [20,21]. Results thus suggest that factors promoting B cell maturation either from newly generated immature B cells or the expansion of memory cells into ISC may be key to understanding mechanisms underlying clinical relapse [22,23].

Due to its importance for B cell survival and homeostasis, BAFF and expression of its receptors are considered to be key regulators of B cell recovery after rituximab [24,25]. Serum BAFF levels increase after BCDT in patients with RA, sometimes remaining raised even after B cell return. We have shown previously that BAFFR expression [mean fluorescence intensity (MFI)] in naive and memory B cells, as defined using IgD/CD27, was reduced compared with baseline in B cells returning after BCDT in patients with RA and in patients with thrombotic thrombocytopaenic purpura (TTP) [18,26]. In patients with TTP, long-term remission (> 60 months) was associated with persistently low BAFFR expression, limited differentiation to memory B cell phenotype and negative tests for autoantibodies to ADAM metallopeptidase with thrombospondin type 1 motif 13 (ADAMTS-13).

The present study extends our previous observations in patients with RA to include transitional naive B cells and plasmablasts in phenotype analyses, using the IgD/CD38 classification [3,27]. Four patients with RA were followed longitudinally and we also conducted a cross-sectional analysis of B cell phenotype and BBR expression in patients at key time-points within treatment cycles of RTX-based B cell depletion therapy. The relationship between serum BAFF levels at relapse and expression of each of the three BBRs as well as the B cell phenotype was determined. Results were also analysed on the basis of timing of clinical relapse after B cell return.

Materials and methods

Patients

Blood samples were obtained from five healthy controls (HC) and a total of 38 patients with a diagnosis of RA who fulfilled the 1987 revised American College of Rheumatology (ACR) criteria [28]. The cohort of patients with RA included 20 with severe active disease [disease activity score (DAS)28 > 5·1], who were studied pre-rituximab (pre-RTX patients), including four patients followed longitudinally with sampling pre-RTX, at B cell return and up to 6 months post-B cell return, and 18 patients undergoing clinical relapse after response (ΔDAS > 1·2) to one or more courses of BCDT (post-RTX patients). Clinical relapse was defined by (i) any return or increase of signs and symptoms caused by inflammation owing to RA (ii) with or without a rise in CRP [17]. Patients were all attending the Department of Rheumatology at University College London Hospital (UCLH) and treated on the basis of clinical need. BCDT consisted of two infusions of 1 g RTX. The study was approved by the UCLH Ethics Committee (08/H0715/18), and all patients gave informed consent before entering the study.

RA patients on RTX at UCLH are seen regularly at a dedicated clinic, where $CD19^+$ B cells are determined every 2–3 months, allowing us to distinguish depletion and repopulation time-points accurately. Post-RTX relapsing patients were divided into two groups depending on the time that relapse was noted after the first documented repopulation. Mean age for RA patients was 58 years (range = 27–79) and mean disease duration was 12·7 years (range 1 = 39). There were five male patients. Three pre-RTX patients were seronegative for both rheumatoid factor (RhF) and anti-citrullinated protein antibodies (ACPA); all post-RTX patients were seropositive either for RhF or ACPA. Median number of cycles for post-RTX relapsing patients was two (range = 1–7).

Assessment of B cell depletion

The normal range for $CD19^+$ B cells used by the local pathology laboratory was $0{\cdot}03–0{\cdot}40 \times 10^9$/l. Adequate depletion of B cells in the peripheral blood was deemed to have occurred when $CD19^+$ cells were < 5/μl. B cell return

(repopulation) was defined as when B cells were again readily detectable in the peripheral blood (when the $CD19^+$ cell count was $\geq 5/\mu l$).

Peripheral blood mononuclear cells (PBMC): isolation and staining

PBMC isolated from 10 ml heparinized whole blood by density gradient centrifugation (Ficoll-Paque^{TM} Plus; GE Healthcare, Uppsala, Sweden) were stained on the same day of collection. PBMC (1×10^6/sample) were incubated with appropriate conjugated antibodies for 20 min at 4°C in the dark. Cells were washed and fixed with 2% paraformaldehyde for 5 min and kept at 4°C in the dark until analysed the following day by flow cytometry; 300 000 events per sample gated on total lymphocytes were acquired with a fluorescence activated cell sorter (FACS)-CaliBur (Becton Dickinson, Franklin Lakes, NJ, USA). Data were analysed with FlowJo (TreeStar, Stanford University, CA, USA). Absolute cell counts for RA patients were calculated from routine lymphocyte counts at each time-point.

B cell phenotype analysis

Immunophenotyping of PBMC was performed using matched combinations of anti-human murine monoclonal antibodies conjugated to fluorescein isothiocyanate (FITC), phycoerythrin (PE), peridinin chlorophyll protein cyanin (PerCP-Cy5.5) or allophycocyanin (APC). For B cell analysis, combinations of anti-CD19 PerCP-Cy5.5, anti-IgD-FITC and anti-CD38-APC were used to define B cell ($CD19^+$) subsets; namely, naive transitional (IgD^+CD38^{++}), naive mature (IgD^+CD38^+), IgD^- resting memory (IgD^-CD38^-), IgD^+ resting memory (IgD^+CD38^-), post-germinal centre (GC) B cells (IgD^-CD38^+) and plasmablasts ($IgD^-CD38^{++/+++}$). Expression of BBRs on each subset was analysed using anti-BAFFR-PE (11C1), anti-TACI-biotin with streptavidin PE and anti-BCMA-PE. All antibodies were purchased from BD Biosciences (San Jose, CA, USA), eBioscience (San Diego, CA, USA) or R&D Systems (Minneapolis, MN, USA).

Measurement of BAFF

Serum BAFF levels were quantified in healthy controls and RA patients, using Human Quantikine BAFF/BLyS Immunoassay ELISA kit (R&D Systems). Mean ± 3 standard deviations (SD) for normal sera ($n = 36$) in this batch of kits was given as $1{\cdot}17 \pm 0{\cdot}78$ ng/ml. Serum BAFF values above 1·95 ng/ml were therefore regarded as elevated.

Statistical analysis

Frequencies of B cell subsets were compared using non-parametric Mann–Whitney *U*-test. Spearman's rank correlation statistics were used to determine any relationship between serum BAFF levels and expression (percentage of positive cells and MFI) of BBRs, length of time after repopulation or after RTX or between B cell repopulation and disease relapse. All analyses were with GraphPad Prism (San Diego, CA, USA).

Results

B cell subpopulations in patients relapsing after RTX

Figure 1a–d shows flow cytometry plots of B cell phenotypes defined by IgD/CD38 expression in an RA patient followed longitudinally; (a) pre-RTX, (b) after RTX and remaining in remission with B cells detectable (CD19 $>5/\mu l$) after adequate depletion as defined and good clinical response ($\Delta DAS28 > 1{\cdot}2$), (c) 3 months later and (d) 6 months later and patient undergoing flare ($DAS28 > 2{\cdot}6$). B cell return occurs clearly with transitional naive B cells, with a decreasing percentage over time corresponding to an increase in the mature naive compartment. This trend was also apparent in data from 18 RA patients studied post-RTX (Fig. 1e), where the changes in percentages of $CD19^+$ B cell populations as defined by IgD/CD38 against time after B cell return are shown. In Fig. 1f, absolute numbers of $CD19^+$ B cell subpopulations are shown for RA patients either pre-RTX ($n = 20$) or at relapse, grouped into patients relapsing either concurrent or close to B cell return (i.e. within 3 months; $n = 10$) and those with a more prolonged remission period (≥ 4 months after B cell return; $n = 8$). B cell regeneration after RTX mimics ontogeny, and this was again evident from analysing patients relapsing closer to repopulation. Median absolute numbers of transitional naive B cells showed the same trend, although this was not significant ($10{\cdot}5 \times 10^6$ (range = $0{\cdot}5$–36×10^6) *versus* $5{\cdot}9 \times 10^6$ (range = $0{\cdot}1$–19×10^6, respectively). Patients analysed ≥ 4 months after repopulation presented significantly higher median absolute numbers of mature naive B cells compared to those analysed 0–3 months after repopulation $87{\cdot}8 \times 10^6$ (range = $7{\cdot}2$–$331{\cdot}0 \times 10^6$) *versus* $6{\cdot}4 \times 10^6$ (range = $0{\cdot}1$–$349{\cdot}7 \times 10^6$, $P < 0{\cdot}05$). Median percentages (data not shown) and absolute values of IgD^+ resting memory B cells and median absolute values of post-GC B cells were significantly higher in patients ≥ 4 months after repopulation. Absolute numbers of IgD-resting memory B cells and plasmablasts were low in both groups, with no statistically significance differences seen.

BAFF-binding receptor expression in RA patients pre-RTX and at relapse after RTX

BAFFR. Figure 2a–c shows flow cytometry plots and associated histograms showing BAFFR expression on $CD19^+$ gated B cells from an individual RA patient studied pre-RTX (a), at B cell return (b) and 3 months after B cell return (with sustained clinical response) (c). Figure 2a

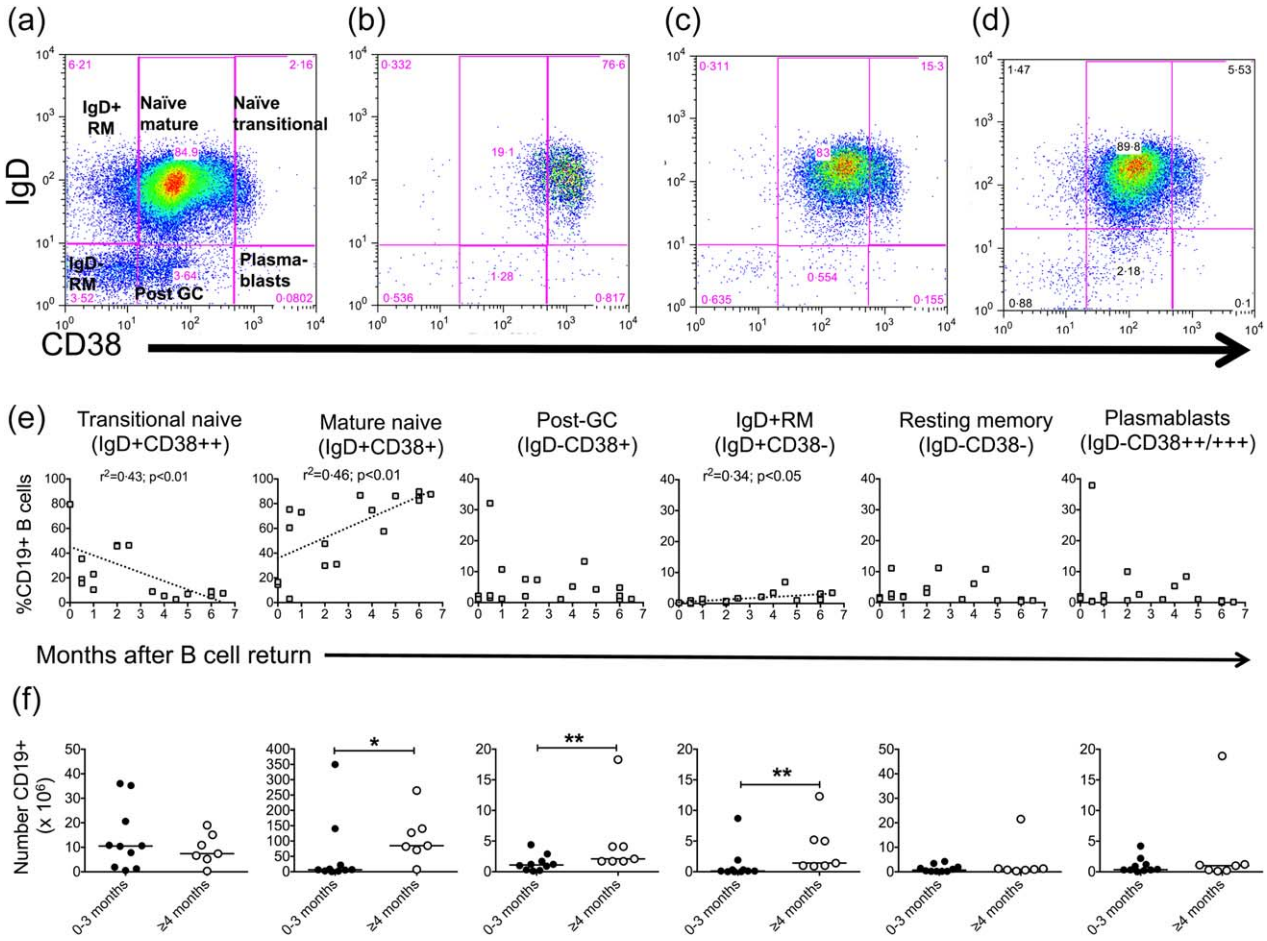

Fig. 1. Example of a flow cytometry plot showing B cell phenotypes defined on the basis of immunoglobulin (Ig)D/CD38 expression for CD19-gated cells in a rheumatoid arthritis (RA) patient pre-rituximab (RTX) (a), at first documented B cell return (b), 3 months post-B cell return (c) and (d) undergoing clinical relapse 6 months post-B cell return. B cell subpopulations are indicated as follows: transitional naive, mature naive, resting memory (IgD-RM), IgD$^+$ resting memory (IgD $^+$ RM), post-germinal centre (post-GC) and plasmablasts. In panels (e) and (f) results of the cross-sectional study of B cell phenotypes of 20 RA patients studied pre-RTX and 18 relapsing post-RTX, either within 0–3 months first documented B cell return or $\geq$ 4 months later, are shown. In panel (e) The relationship between percentage of CD19$^+$ B cells within each subpopulation as defined by IgD/CD38 and time (months) to relapse after the first documented sign of B cell return ($>$ 5 CD19$^+$ B cells/μl) after RTX. Linear regression was used to calculate Spearman's rank coefficients, which are shown for those with $r^2 > 0{\cdot}4$ and giving $P \leq 0{\cdot}05$. (f) absolute numbers CD19$^+$ B cells from patients post-RTX are shown. Statistical analysis compared values in post-RTX patients relapsing close to (0–3 months) or $\geq$ 4 months after B cell return. Horizontal lines on graphs represent median values and results were analysed using the Mann–Whitney *U*-test; $^*P < 0{\cdot}05$; $^{**}P < 0{\cdot}01$; $^{***}P < 0{\cdot}001$. [Colour figure can be viewed at wileyonlinelibrary.com]

shows that virtually all CD19 gated B cells express BAFFR before RTX, although the patient had active disease. The percentage of BAFFR$^+$ B cells was reduced greatly after RTX at B cell return (Fig. 2b), but showed some signs of recovery 3 months later (Fig. 2c). In the histograms for each FACS plot in the series, BAFFR expression (MFI) in the CD19 population can be seen to remain reduced even at 3 months post-RTX. Results were similar in the three other patients followed longitudinally (data not shown). Figure 2d(1–6) compares percentage of BAFFR$^+$ B cells in the six B cell subpopulations defined by IgD/CD38 in HC and RA patients. The percentage of BAFFR$^+$ B cells were similar in patients pre-RTX and HC, but significantly decreased percentages of BAFFR$^+$ B cells in all B cell subpopulations (except for plasmablasts) were present in both post-RTX groups compared to both HC and pre-RTX patients. There was a greater decrease in percentage of BAFFR$^+$ B cells in patients relapsing within 3 months of B cell return, compared with patients relapsing $\geq$ 4 months after repopulation. The percentage of BAFFR$^+$ B cells remained reduced significantly in post-RTX patients studied $\geq$ 4 months after repopulation in transitional naive, mature naive and post-GC subpopulations compared with both HC and pre-RTX patients, but the percentage of

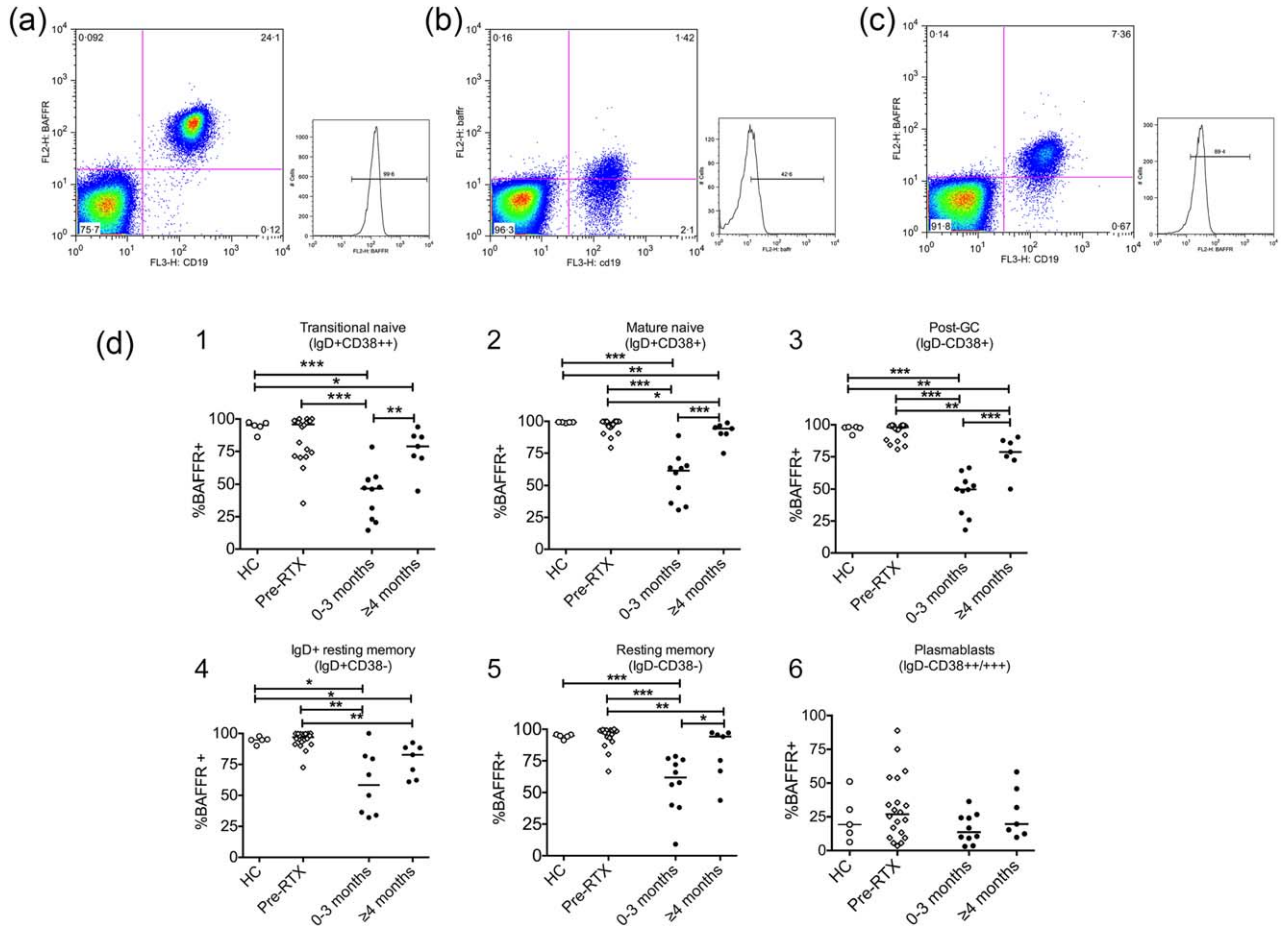

Fig. 2. (a) Flow cytometry plot of B cell activating factor receptor (BAFFR) expression on $CD19^+$ B cells within the lymphocyte gate in an RA patient studied pre-rituximab (RTX) and (b) at B cell return (which occurred 6 months after RTX), (c) at 3 months after B cell return. The associated histograms for (a–c) show relative expression of BAFFR within the gated $CD19^+$ population. The patient relapsed 3 months after the sample shown in (c). (d) (1–6): percentage of $BAFFR^+$ expression on $CD19^+$ B cell subpopulations (defined using immunoglobulin (Ig)D/CD38) in healthy controls (HC) and in pre- and post-RTX rheumatoid arthritis (RA) patients. Results for post-RTX patients were divided on the basis of proximity to first documented B cell return, as described in Fig. 1. Lines on graphs represent median values and results were analysed using the Mann–Whitney *U*-test; $*P < 0{\cdot}05$; $**P < 0{\cdot}01$; $***P < 0{\cdot}001$. [Colour figure can be viewed at wileyonlinelibrary.com]

$BAFFR^+IgD^-$resting memory B cells were similar to HC (Fig. 2d5).

MFI for BAFFR expression was significantly lower in all subpopulations in post-RTX patients compared to HC and pre-RTX patients. BAFFR MFI was also significantly lower in patients 0–3 months after B cell return compared with those relapsing $\geq$ 4 months after initiation of reconstitution in all except IgD^+ resting memory B cells and plasmablasts.

TACI. Figure 3a–c shows flow cytometry plots and associated histograms of TACI expression on $CD19^+$ gated B cells from the same RA patient studied longitudinally, as shown in Fig. 2, for BAFFR expression. The proportion of percentage of $CD19^+TACI^+$ B cells in the lymphocyte gate was reduced greatly at B cell return, decreasing from nearly 10% pre-RTX to fewer than 1% (Fig. 3b), but the percentage of $TACI^+$ B cells increased from $<$ 1 to 3% by 3 months after B cell return (Fig. 3b,c). Figure 3d(1–6) shows that the % $TACI^+$ B cells was low in naive but present on the majority of memory B cell populations, with lower levels than HC in all memory B cell subpopulations in patients relapsing $\geq$ 4 months after B cell return compared with HC. The percentage of $TACI^+$ post-GC and resting memory B cell compartments were also decreased significantly in those relapsing closest to B cell return (0–3 months) compared with HC, but no significant difference between percentage of $TACI^+$ B cells in RTX-treated patients analysed either 0–3 or $\geq$ 4 months after B cell return.

BCMA. Figure 4a shows results for BCMA expression on $CD19^+$ B cells on 20 RA patients before and 18 after RTX therapy compared with HC. Figure 4b–d shows flow cytometry plots of BCMA expression on $CD19^+$ gated B cells from a patient followed longitudinally (as for BAFFR and TACI). The percentages of $BCMA^+$ B cells were low

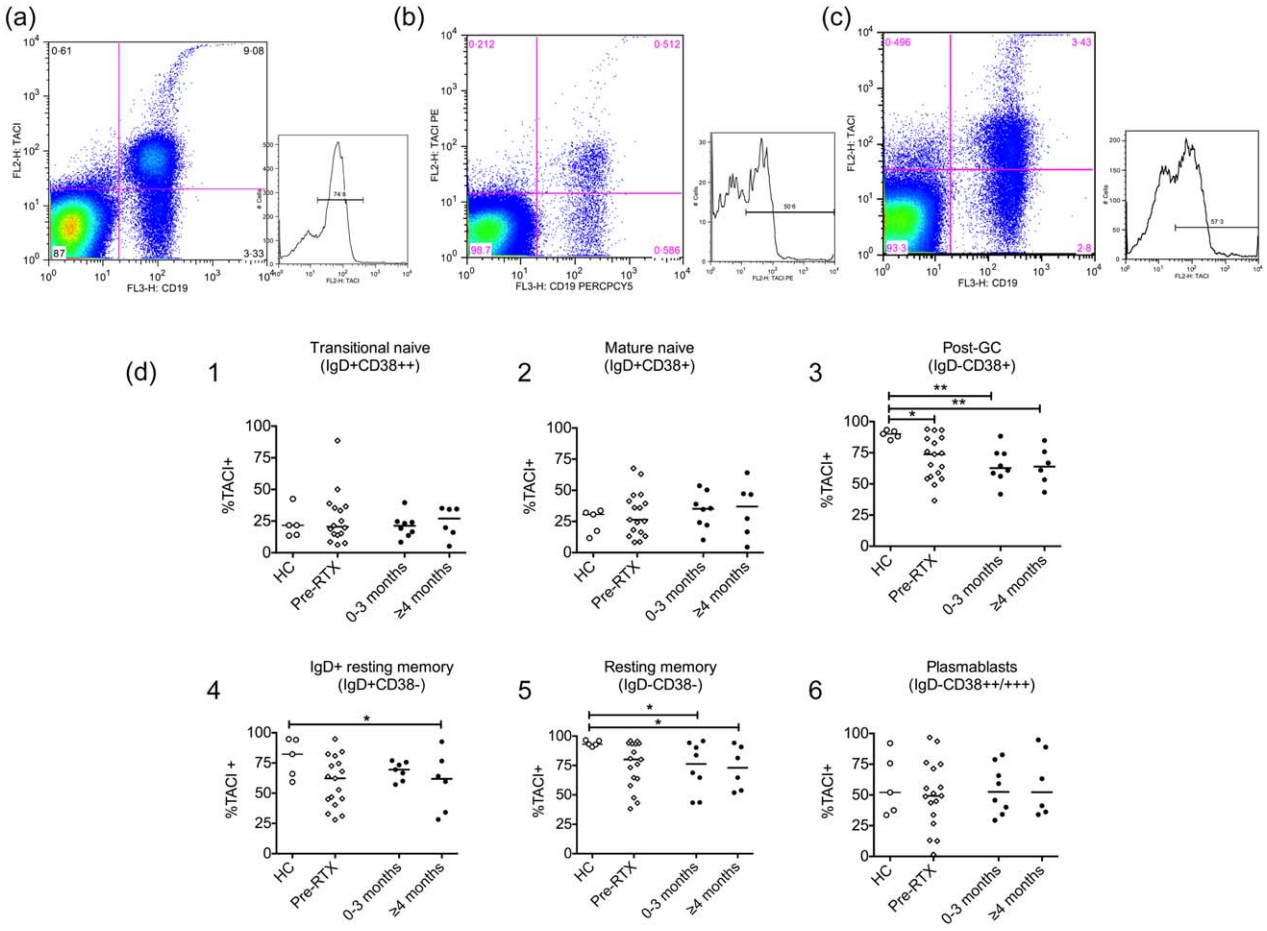

Fig. 3. Flow cytometry plot of transmembrane activator and calcium signal modulating cyclophilic ligand interactor (TACI) expression on CD19$^+$ B cells within the lymphocyte gate in the rheumatoid arthritis (RA) patient studied pre-rituximab (RTX) (a) and (b) at B cell return (which occurred 6 months after RTX), and (c) at 3 months after B cell return. The associated histograms for (a–c) show relative expression of TACI within the gated CD19$^+$ population. The patient relapsed 3 months after the sample shown in (c). (d) (1–6): percentage of TACI$^+$ expression on CD19$^+$ B cell subpopulations (defined using immunoglobulin (Ig)D/CD38) as in healthy controls (HC), pre-RTX patients and patients relapsing post-B cell return. Results for HC and pre-RTX patients were compared and results between the two patient groups relapsing at different intervals after B cell return were also compared. Lines on graphs represent median values and results were analysed using the Mann–Whitney *U*-test; $^*P < 0{\cdot}05$; $^{**}P < 0{\cdot}01$; $^{***}P < 0{\cdot}001$. [Colour figure can be viewed at wileyonlinelibrary.com]

within the CD19$^+$ population and similar among all B cell subpopulations in all HC and patient groups, including plasmablasts, except for a weak but significantly decreased expression in IgD$^-$ resting memory B cells in patients relapsing 0–3 months after repopulation compared to pre-RTX patients ($P < 0{\cdot}01$) and in IgD$^+$ resting memory B cells in patients relapsing $\geq$ 4 months after RTX compared to pre-RTX patients ($P < 0{\cdot}05$).

Serum BAFF levels: relationships with B cell phenotype and BBR expression

As shown in Fig. 5a, median BAFF levels in pre-RTX patients (1·43 ng/ml, range = 0·84–2·39) were raised compared to HC (1·10 ng/ml, range = 0·89–1·24; $P < 0{\cdot}05$), although they remained largely within the normal range (< 1·95 ng/ml). Median BAFF levels in post-RTX patients (1·95 ng/ml; range = 0·96–6·47) were raised significantly compared to HC and pre-RTX patients. We have found previously that BAFFR expression was reduced significantly on both naive and memory B cells (defined using IgD/CD27) in RA patients at relapse, regardless of serum BAFF levels [18]. Using linear regression, we also found no correlation between serum BAFF levels and absolute numbers or percentage of B cell subpopulations based on IgD/CD38 expression (data not shown). There was no significant correlation between serum BAFF levels and number of RTX cycles received (data not shown), nor with

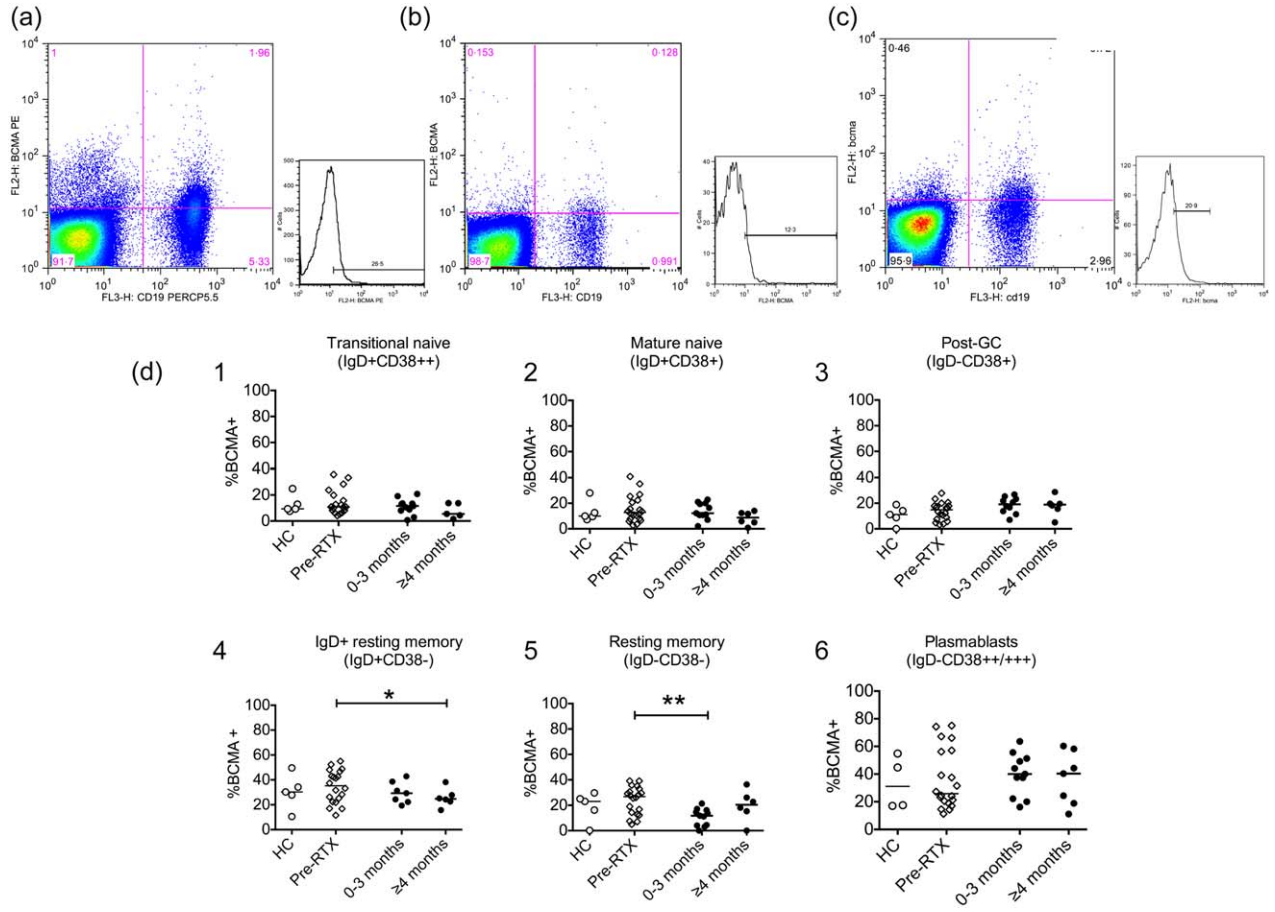

Fig. 4. (a) Flow cytometry plot of B cell maturation antigen (BCMA) expression on $CD19^+$ B cells within the lymphocyte gate in a rheumatoid arthritis (RA) patient studied pre-rituximab (RTX) (b) at B cell return (which occurred 6 months after RTX) and (c) at 3 months after B cell return. The associated histograms for (a–c) show relative expression of BCMA within the gated $CD19^+$ population. The patient relapsed 3 months after the sample shown in c). In (d), (1–6) percentage of $BCMA^+$ expression on $CD19^+$ B cell subpopulations [defined using immunoglobulin (Ig)D/CD38) in healthy controls (HC), pre-RTX patients and patients relapsing after RTX. Results for HC and pre-RTX patients were compared and results between the two patient groups relapsing at different intervals after B cell return were also compared. Lines on graphs represent median values and results were analysed using the Mann–Whitney *U*-test; $^*P < 0{\cdot}05$; $^{**}P < 0{\cdot}01$; $^{***}P < 0{\cdot}001$. [Colour figure can be viewed at wileyonlinelibrary.com]

time after repopulation (Fig. 5b). However, BAFF levels overall clearly tended to decrease with time after B cell return (Fig. 5b).

We next examined whether there was any correlation between circulating BAFF levels and percentage of $CD19^+$ B cells positive for each of the 3 BBRs. Post-RTX the percentage of $CD19^+BAFFR^+$ B cells in transitional and mature naive subpopulations showed an inverse correlation with serum BAFF levels ($r^2 = 0{\cdot}41$ for both), as did post-GC B cells ($r^2 = 0{\cdot}54$; $P < 0{\cdot}001$ (Fig. 5c). We also confirmed a lack of any strong correlations between BAFFR expression (MFI) and BAFF levels [18] (data not shown).

Neither the percentage of B cells positive for, nor expression of, TACI and BCMA showed any significant correlations with serum BAFF levels in any of the RA patients studied (data not shown).

Is there any correlation between BBR expression (MFI) and the time after B cell return in patients relapsing post-RTX?

As shown in Fig. 6a, expression of BAFFR (MFI) in the transitional but not mature naive subset showed a notable increase with time after repopulation ($r^2 = 0{\cdot}53$, $P < 0{\cdot}001$). Less significant was the increased expression with time in post-GC ($r^2 = 0{\cdot}33$, $P < 0{\cdot}05$), IgD^- resting memory ($r^2 = 0{\cdot}34$, $P < 0{\cdot}05$) and plasmablasts ($r^2 = 0{\cdot}41$, $P < 0{\cdot}01$). There was no correlation between the MFI of TACI expression and time after repopulation in any of the B cell subsets studied. Interestingly, however, TACI expression (MFI) (Fig. 6b) could be seen to remain largely within normal limits after RTX treatment, whereas BAFFR expression remained below the normal range in all B cell subpopulations up to 7 months after B cell return (Fig. 6a).

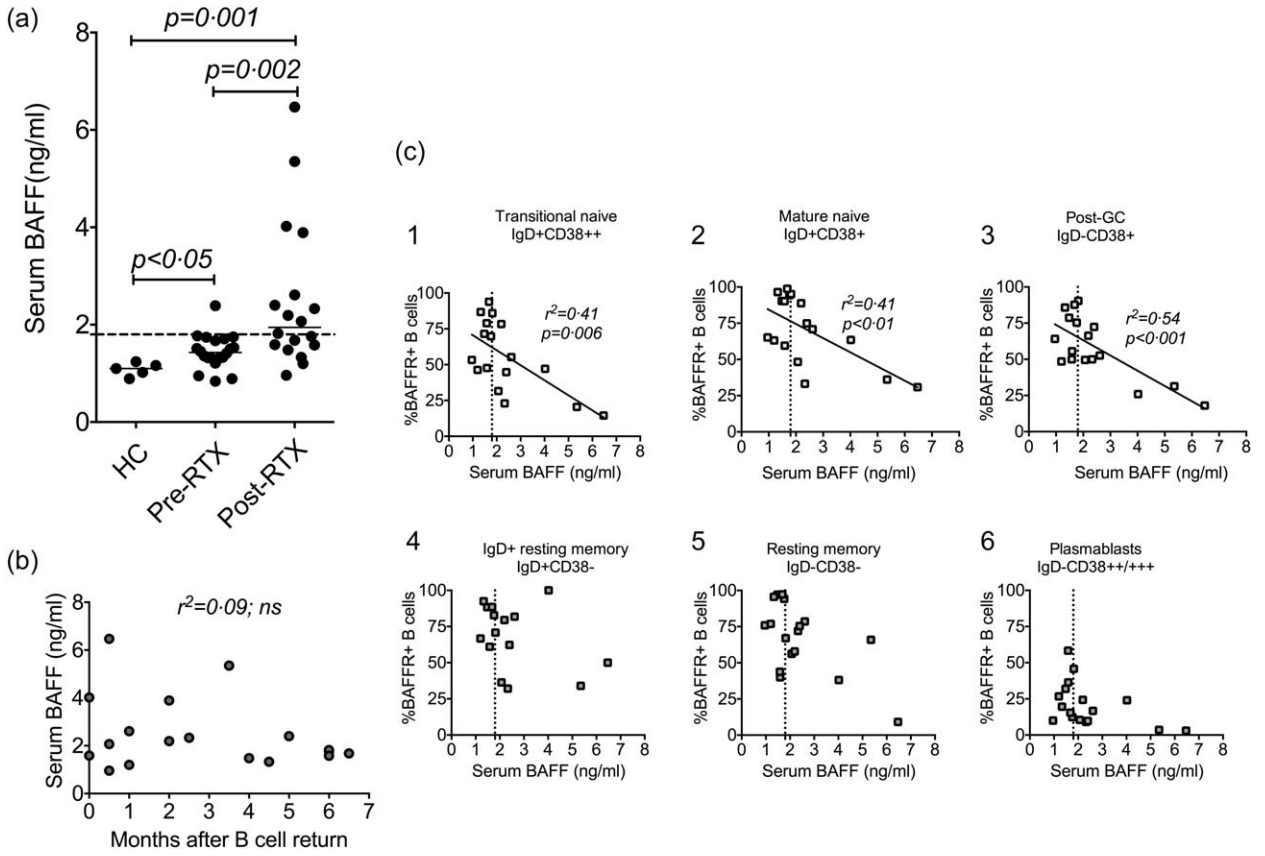

Fig. 5. (a) Serum B cell activating factor (BAFF) levels in 5 HC, pre-RTX patients ($n = 20$) and patients relapsing after RTX ($n = 18$). Dotted line indicates upper limit for normal control values as defined by the manufacturers. (b) Relationship between serum BAFF levels and time after B cell return in rheumatoid arthritis (RA) patients relapsing after RTX. (c) The relationship between percentage of $BAFFR^+$ B cells and serum BAFF levels are shown for each B cell subpopulation, defined using immunoglobulin (Ig)D/CD38. Linear regression analysis (Spearman's correlation) and exact *P*-values are shown for significant correlations ($P < 0{\cdot}05$) between serum BAFF levels and percentage of BAFF receptor $(BAFFR)^+$ B cells.

Discussion

Observations in other autoimmune rheumatic diseases and data from animal models have suggested that the consequences of raised BAFF levels in the circulation may include lowering the threshold for autoreactive B cell survival and expansion, with promotion of autoantibody production. We therefore hypothesized that the timing of re-establishment of symptoms following B cell return to the periphery in patients with RA treated with RTX may be related to the BAFF/BBR axis.

We found that in patients relapsing shortly after B cell repopulation, which is the situation where high BAFF levels are present due to the depletion of BAFF-binding B cells by RTX, returning B cells had very low levels of BAFFR. Repopulating naive B cells may therefore be unable to bind all the available BAFF until more B cells had exited the bone marrow and/or expanded into memory subsets or, as suggested in Ref. [2], through high BAFF levels inducing increasing recycling of BAFFR. BAFF levels in serum from RA patients before RTX were higher than HC but generally within the normal range, as shown in previous studies [18,29,30]. It was clear, however, that at the higher levels of serum BAFF following RTX, the percentages of naive and of post-GC B cells positive for BAFFR were lower, with a significant negative correlation. As we did not see a correlation between serum BAFF levels and patient relapse (some patients relapse with high BAFF levels, some do not); we interpreted this to indicate that inappropriate signalling mediated by soluble BAFF was not responsible for driving relapse.

Following BCDT, B cells repopulate primarily from bone marrow-derived naive B cells, with regeneration of the memory B cell pool often delayed [15,31,32]. The high percentage of transitional B cells in patients relapsing closest to B cell return thus reflected the initial repopulation process, and mimics ontogeny. Increasing time to relapse was associated with a significant fall in the percentage of transitional naive B cells accompanied by an increase into the mature naive compartment, confirmed in longitudinal

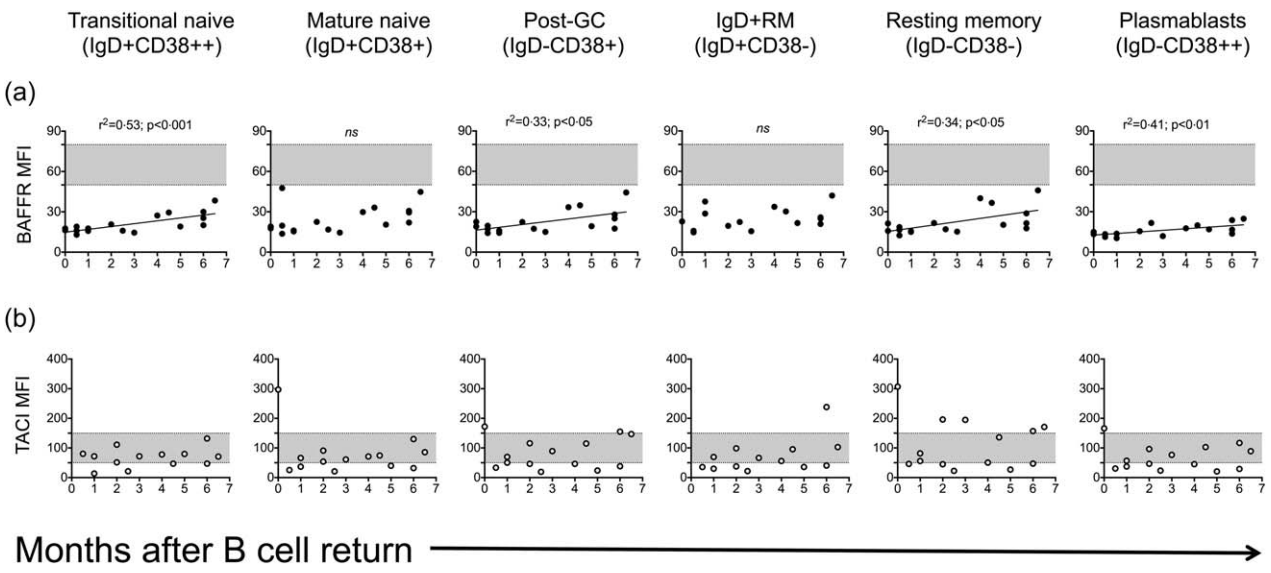

Fig. 6. In (a) the relative expression [mean fluorescence intensity (MFI)] of B cell activating factor receptor (BAFFR) and in (b) expression of transmembrane activator and calcium signal modulating cyclophilic ligand interactor (TACI) expression on B cells was measured within each B cell subpopulation, as defined using immunoglobulin (Ig)D/CD38, and plotted against time (months) after peripheral B cell return in rheumatoid arthritis (RA) patients relapsing after rituximab (RTX). Linear regression analysis (Spearman's correlation) and exact *P*-values are shown for significant correlations ($P < 0{\cdot}05$) Shaded areas indicate the ranges for MFI of the two BAFF-binding receptors $(BBR)^+$ given by the respective B cell subpopulations in five healthy control (HC) samples taken within the same time-period.

studies of individual patients. Absolute numbers of IgD^+ and post-GC memory B cells also increased with time after repopulation. We found that circulating memory B cell pools were low after RTX, even in patients relapsing ≥ 4 months after B cell return. It has been described that RTX induces a long-lasting effect on the memory cell subset in RA [20] and in lymphoma [33], systemic lupus erythematosus (SLE) [34] and TTP [26]. We found that relapse occurred despite low circulating memory B cells, suggesting that long-lived memory B cells (and possibly $CD20^-$ plasma cells) were the possible repository for disease memory for relapse after B cell return [23,35].

In conventional B cell development it is thought that apoptosis is the 'default' programme for transitional naive B cells unless rescued by productive BAFFR (BR3)-mediated signalling [36]. Both the percentage and expression (MFI) of BAFFR on B cells returning post RTX was reduced compared to pre-RTX values in transitional and mature naive B cells and in memory B cell subsets, although tending to increase with time to relapse after B cell return, but remaining below the normal range in all B cell subpopulations during the course of follow-up. In our previous studies of patients with TTP treated with RTX, serum BAFF levels returned to within normal limits close to onset of repopulation. BAFFR expression (MFI) on all IgD/CD27-defined B cell subpopulations, however, remained well below the normal values for more than 5 years in patients maintaining remission [26]. This was associated with the lack of recovery of ADAMTS13 autoantibody production, suggesting a prolonged lack of (pathogenic) B cell differentiation to ISC in patients with long-lasting remissions, with low expression of BAFFR contributing to the lack of survival and/or further differentiation of ADAMTS13-committed B cells. Chronically raised serum BAFF has been suggested to exert a negative feedback on BAFFR expression, due perhaps to internalization or shedding, independently of exposure to B cell agonists [24,37]. Following RTX, however, low BAFFR expression may be a repopulation phenomenon where newly exiting (from the bone marrow) B cells physiologically express less BAFFR, similar to what is seen in preterm neonates [38]. They are also expanding into an environment of high serum BAFF levels. Some RA patients relapsed soon after B cell return with predominantly early naive (transitional) B cells in the circulation, suggesting that in these patients, the disadvantages of low BAFFR expression/signalling were not limiting the onset of relapse [18]. Of the other two BBR, BCMA expression was low and relatively unchanged by RTX, with the percentage of $TACI^+$ memory B cells remaining significantly lower than HC following RTX.

Patients with RA showed a reduced percentage of $TACI^+$ B cells both in those with active disease pre-RTX and at relapse after RTX in all three memory B cell compartments. This suggests that disadvantages of low TACI expression are not translated into a lack of proinflammatory signalling. Whether this reflects a constitutive lack of up-regulation of TACI in RA patients or down-regulation due

to internalization or shedding of bound BAFF/APRIL heterodimers is not known. The role of TACI in B cell responses is complex, with contrasting functional outcomes depending on context, but mutation in TACI genes has been associated with some forms of common variable and IgA deficiency in man [39]. Co-ligation of TACI and proteoglycans is thought to be essential for class-switch recombination [40]. This has been attributed to the accompanying loss of inhibitory signals through TACI to BAFFR- and CD40-mediated signalling. Reduced percentage of $TACI^+$ memory B cell subsets may thus render B cells less responsive to inhibitory signalling through TACI.

Relapse coincident with or close to naive B cell re-entry to the periphery was not associated with prominent memory or plasmablast populations, and with greatly reduced percentages and expression of the main pro-survival BBR, namely BAFFR, by the newly exiting B cells. We also found no increases in percentage of $BCMA^+$ B cells. Coincident with differentiation into ISC, BCMA is acquired on mature B cells accompanied by loss of BAFFR expression, and has been described to be functional and responsible for mediating BAFF-induced survival of ISCs [2,41]. BCMA expression can be increased on plasmablasts and memory B cells from patients with systemic lupus erythematosus (SLE) [25,42,43] and can also be expressed in transitional B cells, where it is associated with inappropriate naive B cell activation to ISC [25]. We found no differences in BCMA expression (% and MFI) between HC and pre- and post-RTX RA patients, however, with the exception of a slight increase in memory resting populations. In our previous studies of autoantibody kinetics and serological markers of B cell differentiation, we found that most of the ACPA specificities rising coincident with relapse after RTX appeared to be derived from resident memory B cells, with few new specificities arising. Rises in RhF and levels of a soluble marker of B cell differentiation to memory phenotype (sCD23) showed an incremental rise, which correlated with time to flare after B cell return [19]. In the current studies, precipitation of relapse in patients analysed $\geq$ 4 months after B cell return would seem to depend upon further recovery of the mature naive B cell pool with percentage of $BAFFR^+$ B cells approaching those of HC over time and increasing BAFFR expression. This suggests that a 'critical mass' of autoreactive B cells may be needed to trigger flare. The mechanism of relapse may therefore follow a more conventional pathway, with T cell-mediated selection occurring in secondary lymphoid organs or inflammatory sites.

In healthy individuals, further differentiation of B cells from transitional to mature naive stage is prevented if they are exposed to uncomplexed antigen through their BCR, even in the presence of both T-dependent and -independent co-stimulation. B cells exiting the BM in RA patients have been reported to have a high proportion of B cells expressing autoreactive BCR specific for citrullinated antigens and Fc of IgG (rheumatoid factors), suggesting alterations in the preimmune repertoire due to defective central tolerance checkpoints. Further it was shown that there were also defects in peripheral checkpoints with continued persistence of autoreactive naive B cells [44–46]. Survival of these newly exiting B cells may thus favour those with autoreactive BCR. Based on our results, we therefore propose that inappropriate signalling through a potentially autoreactive BCR rather than through BAFF may drive relapse.

In conclusion, aberrant B cell selection, expansion and differentiation to autoantibody production, combined with inappropriate T cell responses, can occur at different points within B cell maturation pathways which differ between human autoimmune diseases, thereby influencing the institution of effective therapies [47,48]. Although we found changes over time in the expression of BBRs on the different B cell subpopulations in relation to timing of relapse in individual RA patients, most of the process appears independent of the BAFF/BBR system. The lack of clear success in clinical trials of agents to neutralize soluble BAFF in patients with RA may also be explained by our findings.

Author contributions

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be submitted for publication. E. B., I. D. L. T., M. J. L. and G. C. had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. Study conception and design: E. B., I. D. L. T., M. J. L. and G. C.; acquisition of data: E. B., M. J. L. and G. C.; analysis and interpretation of data: Becerra, I. D. L. T., M. J. L. and G. C.

Acknowledgements

E. B. and I. T. were supported by a grant from Alfonso Martín Escudero Foundation, Madrid, Spain. I. D. L. T. was also supported by a short term-grant from the Sociedad Española de Reumatología (Spanish Society of Rheumatology).

Disclosure

E. B., I. D. L. T. and G. C. have no conflicts of interest: M. J. L. has received honoraria for participating in meetings from Roche UK, Roche Brazil and Roche Portugal, support for attending conferences from Roche and Chugai UK.

References

1 Cancro MP. The BLyS/BAFF family of ligands and receptors: key targets in the therapy and understanding of autoimmunity. Ann Rheum Dis 2006; **65** (Suppl 3):iii34–6.

2 Darce JR, Arendt BK, Wu X, Jelinek DF. Regulated expression of BAFF-binding receptors during human B cell differentiation. J Immunol 2007; **179**:7276–86.

3 Sims GP, Ettinger R, Shirota Y, Yarboro CH, Illei GG, Lipsky PE. Identification and characterization of circulating human transitional B cells. Blood 2005; **105**:4390–8.

4 Rodig SJ, Shahsafaei A, Li B, Mackay CR, Dorfman DM. BAFF-R, the major B cell-activating factor receptor, is expressed on most mature B cells and B-cell lymphoproliferative disorders. Hum Pathol 2005; **36**:1113–9.

5 Darce JR, Arendt BK, Chang SK, Jelinek DF. Divergent effects of BAFF on human memory B cell differentiation into Ig-secreting cells. J Immunol 2007; **178**:5612–22.

6 He B, Santamaria R, Xu W *et al*. The transmembrane activator TACI triggers immunoglobulin class switching by activating B cells through the adaptor MyD88. Nat Immunol 2010; **11**: 836–45.

7 Sakurai D, Kanno Y, Hase H, Kojima H, Okumura K, Kobata T. TACI attenuates antibody production costimulated by BAFF-R and CD40. Eur J Immunol 2007; **37**:110–8.

8 Edwards JC, Cambridge G. Sustained improvement in rheumatoid arthritis following a protocol designed to deplete B lymphocytes. Rheumatology 2001; **40**:205–11.

9 Edwards JC, Szczepanski L, Szechinski J *et al*. Efficacy of B-cell-targeted therapy with rituximab in patients with rheumatoid arthritis. N Engl J Med 2004; **350**:2572–81.

10 Lee YH, Bae SC, Song GG. The efficacy and safety of rituximab for the treatment of active rheumatoid arthritis: a systematic review and meta-analysis of randomized controlled trials. Rheumatol Int 2011; **31**:1493–9.

11 Isaacs JD, Cohen SB, Emery P *et al*. Effect of baseline rheumatoid factor and anticitrullinated peptide antibody serotype on rituximab clinical response: a meta-analysis. Ann Rheum Dis 2013; **72**:329–36.

12 Edwards JC, Cambridge G. Rheumatoid arthritis: the predictable effect of small immune complexes in which antibody is also antigen. Br J Rheumatol 1998; **37**:126–30.

13 Edwards JC, Cambridge G. B-cell targeting in rheumatoid arthritis and other autoimmune diseases. Nat Rev Immunol 2006; **6**:394–403.

14 Sellam J, Hendel-Chavez H, Rouanet S *et al*. B cell activation biomarkers as predictive factors for the response to rituximab in rheumatoid arthritis: a six-month, national, multicenter, open-label study. Arthritis Rheum 2011; **63**:933–8.

15 Leandro MJ, Cambridge G, Ehrenstein MR, Edwards JC. Reconstitution of peripheral blood B cells after depletion with rituximab in patients with rheumatoid arthritis. Arthritis Rheum 2006; **54**:613–20.

16 Popa C, Leandro MJ, Cambridge G, Edwards JC. Repeated B lymphocyte depletion with rituximab in rheumatoid arthritis over 7 yrs. Rheumatology (Oxf) 2007; **46**:626–30.

17 Cambridge G, Leandro MJ, Edwards JC *et al*. Serologic changes following B lymphocyte depletion therapy for rheumatoid arthritis. Arthritis Rheum 2003; **48**:2146–54.

18 de la Torre I, Moura RA, Leandro MJ, Edwards J, Cambridge G. B-cell-activating factor receptor expression on naive and memory B cells: relationship with relapse in patients with rheumatoid arthritis following B-cell depletion therapy. Ann Rheum Dis 2010; **69**:2181–8.

19 Cambridge G, Perry HC, Nogueira L *et al*. The effect of B-cell depletion therapy on serological evidence of B-cell and plasmablast activation in patients with rheumatoid arthritis over multiple cycles of rituximab treatment. J Autoimmun 2014; **50**:67–76.

20 Roll P, Palanichamy A, Kneitz C, Dorner T, Tony HP. Regeneration of B cell subsets after transient B cell depletion using anti-CD20 antibodies in rheumatoid arthritis. Arthritis Rheum 2006; **54**:2377–86.

21 Moller B, Aeberli D, Eggli S *et al*. Class-switched B cells display response to therapeutic B-cell depletion in rheumatoid arthritis. Arthritis Res Ther 2009; **11**:R62.

22 Boumans MJ, Thurlings RM, Gerlag DM, Vos K, Tak PP. Response to rituximab in patients with rheumatoid arthritis in different compartments of the immune system. Arthritis Rheum 2011; **63**:3187–94.

23 Cambridge G, Leandro MJ, Lahey LJ, Fairhead T, Robinson WH, Sokolove J. B cell depletion with rituximab in patients with rheumatoid arthritis: multiplex bead array reveals the kinetics of IgG and IgA antibodies to citrullinated antigens. J Autoimmun 2016; **70**:22–30.

24 Sellam J, Miceli-Richard C, Gottenberg JE *et al*. Decreased B cell activating factor receptor expression on peripheral lymphocytes associated with increased disease activity in primary Sjogren's syndrome and systemic lupus erythematosus. Ann Rheum Dis 2007; **66**:790–7.

25 Kim J, Gross JA, Dillon SR, Min JK, Elkon KB. Increased BCMA expression in lupus marks activated B cells, and BCMA receptor engagement enhances the response to TLR9 stimulation. Autoimmunity 2011; **44**:69–81.

26 Becerra E, Scully MA, Leandro MJ *et al*. Effect of rituximab on B cell phenotype and serum B cell-activating factor levels in patients with thrombotic thrombocytopenic purpura. Clin Exp Immunol 2015; **179**:414–25.

27 Bohnhorst JO, Bjorgan MB, Thoen JE, Natvig JB, Thompson KM. Bm1-Bm5 classification of peripheral blood B cells reveals circulating germinal center founder cells in healthy individuals and disturbance in the B cell subpopulations in patients with primary Sjogren's syndrome. J Immunol 2001; **167**:3610–8.

28 Arnett FC, Edworthy SM, Bloch DA *et al*. The American Rheumatism Association 1987 revised criteria for the classification of rheumatoid arthritis. Arthritis Rheum 1988; **31**:315–24.

29 Sellam J, Rouanet S, Hendel-Chavez H *et al*. Blood memory B cells are disturbed and predict the response to rituximab in patients with rheumatoid arthritis. Arthritis Rheum 2011; **63**: 3692–701.

30 Cambridge G, Stohl W, Leandro MJ, Migone TS, Hilbert DM, Edwards JC. Circulating levels of B lymphocyte stimulator in patients with rheumatoid arthritis following rituximab treatment: relationships with B cell depletion, circulating antibodies, and clinical relapse. Arthritis Rheum 2006; **54**:723–32.

31 Muhammad K, Roll P, Einsele H, Dorner T, Tony HP. Delayed acquisition of somatic hypermutations in repopulated $IGD^{+}CD27^{+}$ memory B cell receptors after rituximab treatment. Arthritis Rheum 2009; **60**:2284–93.

32 Gremese E, Tolusso B, Fedele AL, Canestri S, Alivernini S, Ferraccioli G. ZAP-70^{+} B cell subset influences response to B cell depletion therapy and early repopulation in rheumatoid arthritis. J Rheumatol 2012; **39**:2276–85.

33 Anolik JH, Friedberg JW, Zheng B *et al.* B cell reconstitution after rituximab treatment of lymphoma recapitulates B cell ontogeny. Clin Immunol 2007; **122**:139–45.
34 Anolik JH, Barnard J, Owen T *et al.* Delayed memory B cell recovery in peripheral blood and lymphoid tissue in systemic lupus erythematosus after B cell depletion therapy. Arthritis Rheum 2007; **56**:3044–56.
35 Hiepe F, Dorner T, Hauser AE, Hoyer BF, Mei H, Radbruch A. Long-lived autoreactive plasma cells drive persistent autoimmune inflammation. Nat Rev Rheumatol 2011; **7**:170–8.
36 Cancro MP. Living in context with the survival factor BAFF. Immunity 2008; **28**:300–1.
37 Kreuzaler M, Rauch M, Salzer U *et al.* Soluble BAFF levels inversely correlate with peripheral B cell numbers and the expression of BAFF receptors. J Immunol 2012; **188**:497–503.
38 Kaur K, Chowdhury S, Greenspan NS, Schreiber JR. Decreased expression of tumor necrosis factor family receptors involved in humoral immune responses in preterm neonates. Blood 2007; **110**:2948–54.
39 Rachid R, Castigli E, Geha RS, Bonilla FA. TACI mutation in common variable immunodeficiency and IgA deficiency. Curr Allergy Asthma Rep 2006; **6**:357–62.
40 Sakurai D, Hase H, Kanno Y, Kojima H, Okumura K, Kobata T. TACI regulates IgA production by APRIL in collaboration with HSPG. Blood 2007; **109**:2961–7.
41 Avery DT, Kalled SL, Ellyard JI *et al.* BAFF selectively enhances the survival of plasmablasts generated from human memory B cells. J Clin Invest 2003; **112**:286–97.
42 Koarada S, Tada Y, Sohma Y *et al.* Autoantibody-producing RP105(-) B cells, from patients with systemic lupus erythematosus, showed more preferential expression of BCMA compared with BAFF-R than normal subjects. Rheumatology (Oxf) 2010; **49**:662–70.
43 Zhao LD, Li Y, Smith MF Jr *et al.* Expressions of BAFF/BAFF receptors and their correlation with disease activity in Chinese SLE patients. Lupus 2010; **19**:1534–49.
44 Samuels J, Ng YS, Coupillaud C, Paget D, Meffre E. Impaired early B cell tolerance in patients with rheumatoid arthritis. J Exp Med 2005; **201**:1659–67.
45 De La Torre I, Leandro MJ, Valor L, Becerra E, Edwards JC, Cambridge G. Total serum immunoglobulin levels in patients with RA after multiple B-cell depletion cycles based on rituximab: relationship with B-cell kinetics. Rheumatology (Oxf) 2012; **51**:833–40.
46 Rudnicka W, Burakowski T, Warnawin E *et al.* Functional TLR9 modulates bone marrow B cells from rheumatoid arthritis patients. Eur J Immunol 2009; **39**:1211–20.
47 Wahren-Herlenius M, Dorner T. Immunopathogenic mechanisms of systemic autoimmune disease. Lancet 2013; **382**:819–31.
48 Iwata S, Tanaka Y. B-cell subsets, signaling and their roles in secretion of autoantibodies. Lupus 2016; **25**:850–6.