Short title: BAFF receptors in RA patients after Rituximab

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

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Objectives: Serum levels of B cell activating factor (BAFF) rise following Rituximab (RTX) therapy in patients with Rheumatoid arthritis (RA). Initiation of naïve 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).

Methods: The relationships between serum BAFF and BBR expression (BAFFR, TACI and BCMA) was determined on B cell subsets, defined using IgD/CD38. 20 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 (≥5 B cells/μL) and to serum BAFF levels.

Results: After B cell return, B cell populations from relapsing patients had significantly lower BAFFR+ expression compared to HC and to pre-RTX patients. %BAFFR+ B cells increased with time after B cell return and was inversely correlated with serum BAFF levels. BAFFR expression remained reduced. %TACI+ memory B cells were lower in RA patients after RTX compared with HC. BCMA expression (% and expression) did not differ between patients and HC.

Conclusion: Relapse following B cell return appeared largely independent of the %BAFFR or %BCMA positive B cells or serum BAFF levels. The lower %TACI+ memory B cells may reduce inhibitory signaling 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.

- Relapse close to B cell return appears independent of the BAFF/BAFFR system
- Relapse at longer periods after B cell return associated with more regeneration of the B cell pool
- BAFF-R expression was low at repopulation and increased with time similar to ontogeny

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 differentially expressed 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 pro-survival signals, particularly to early naïve 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 largely restricted to mature plasma cells in the BM and secondary lymphoid organs. TACI is expressed on a sub-population of activated naïve 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 naïve 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 previously shown that BAFFR expression (MFI) in naïve 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 ADAMTS-13.

The present study extends our previous observations in patients with RA to include transitional naïve 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 3 BBRs as well as 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 5 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 (DAS28>5.1), who were studied pre-rituximab (pre-RTX patients), including 4 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 2 infusions of 1g 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 regularly seen on 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 2 groups depending on the time relapse was noted after first documented repopulation. Mean age for RA patients was 58 (range 27-79) and mean disease duration was 12.7 years (range 1-39). There were 5 male patients. 3 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 2 (range 1-7).

Assessment of B cell depletion

The normal range for CD19+ B cells used by the local pathology laboratory was 0.03-0.40x10^9/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 ≥ 5/µl).

Peripheral blood mononuclear cells (PBMC): isolation and staining

PBMC isolated from 10 ml heparinised whole blood by density gradient centrifugation (Ficoll-Paque^{TM} Plus; GE Healthcare, Sweden) were stained on same day of collection. PBMC (1x10^6/sample) were incubated with appropriate conjugated antibodies for 20 minutes at 4ºC in the dark. Cells were washed and fixed with 2% paraformaldehyde for 5 minutes and kept at 4^0C in the dark until analysed the following day by flow cytometry. 300,000 events per sample gated on total lymphocytes were acquired with a FACSCaliBur (Becton Dickinson, New Jersey, USA). Data were analysed with FlowJo (TreeStar, Stanford University, CA). 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: naïve transitional (IgD+CD38++), naïve 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, USA), eBioscience (San Diego, USA) or R&D Systems (Minneapolis, 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, Minneapolis, USA). Mean+3SD for normal sera (n=36) given in this batch of kits was given as 1.17±0.78 ng/ml. Serum BAFF values above 1.95ng/ml were therefore regarded as elevated.

Statistical analysis

Frequencies of B cell subsets were compared using nonparametric Mann-Whitney U test. Correlation statistics (Spearman's Rank) were used to determine any relationship between serum BAFF levels and expression (percentage 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, USA.

RESULTS

B cell subpopulations in patients relapsing after RTX. Figure 1 (A-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/ul) after adequate depletion as defined and good clinical response (ΔDAS28>1.2) (C) 3 months later and (D) 6 months later and patient undergoing flare (DAS28>2.6). B cell return clearly occurs with transitional naïve B cells, with a decreasing percentage over time corresponding to an increase in the mature naïve compartment. This trend was also apparent in data from 18 RA patients studied post RTX (Figure 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 Figure 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 (ie 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 naïve B cells showed the same trend although was not significant (10.5 x10^6 (range

0.5-36 $x10^6$) vs 5.9 $x10^6$ (range 0.1-19 $x10^6$, respectively). Patients analysed ≥ 4 months after repopulation presented significantly higher median absolute numbers of mature naïve B cells compared to those analysed 0-3 months after repopulation 87.8$x10^6$ (range 7.2-331.0 $x10^6$) vs 6.4 $x10^6$ (range 0.1-349.7 $x10^6$), $p<0.05$). 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

a) BAFFR

Figure 2A-C are 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 (CB) and 3 months after B cell return (with sustained clinical response) (C). Figure 2A shows that virtually all CD19 gated B cells express BAFFR before RTX, although the patient had active disease. The %BAFFR+ B cells was greatly reduced after RTX at B cell return (Figure 2B), but showed some signs of recovery 3 months later (Figure 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 3 other patients followed longitudinally (data not shown). Figure 2D (a-f) compares %BAFFR+ B cells in the 6 B cell subpopulations defined by IgD/CD38 in HC and RA patients. %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 %BAFFR+ B cells in patients relapsing within 3 months of B cell return, compared with patients relapsing ≥4 months after repopulation. %BAFFR+ B cells remained significantly reduced in post-RTX patients studied ≥4 months after repopulation in transitional naïve, mature naïve and post-GC subpopulations compared with both HC and pre-RTX patients, but %BAFFR+ IgD-resting memory B cells were similar to HC (Figure 2D(e)).

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 ≥4months after initiation of reconstitution in all except IgD+ resting memory B cells and plasmablasts.

b) 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 Figure 2 for BAFFR expression. The proportion of %CD19+TACI+ B cells in the lymphocyte gate was greatly reduced at B cell return, decreasing from nearly 10% pre-RTX to less than 1% (Figure 3B), but the %TACI+ B cells increased from <1% to 3% by 3 months after B cell return (Figure 3B and C). Figure 3D(a-f) shows that the % TACI+ B cells was low in naïve but present on the majority of memory B cell populations, with lower levels than HC in all memory B cell subpopulations in patients relapsing ≥ 4 months after B cell return compared with HC. The %TACI+ post-GC and resting memory B cell compartments were also significantly decreased in those relapsing closest to B cell return (0-3 months) compared with HC, but no significant difference between % TACI+ B cells in RTX-treated patients analyzed either 0-3 or ≥4 months after B cell return.

c) 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). %BCMA+ B cells were low within the CD19+ population and similar among all B cell sub-populations 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 ($p<0.01$) and in IgD+ resting memory B cells in patients relapsing ≥4 months after RTX compared to pre-RTX patients ($p<0.05$).

Serum BAFF levels: relationships with B cell phenotype and BBR expression. As shown in Figure 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; $p<0.05$), 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 significantly raised compared to HC and pre-RTX patients. We have previously found that BAFFR expression was significantly reduced on both naive and memory B cells (defined using IgD/CD27) in RA patients at relapse, regardless of serum BAFF levels (29). 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 time after repopulation (Figure 5B). However BAFF levels overall clearly tended to decrease with time after B cell return (Figure 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 %CD19+BAFFR+ B cells in transitional and mature naïve subpopulations showed an inverse correlation with serum BAFF levels ($r^2=0.41$ for both), as did post-GC B cells ($r^2=0.54$; $p<0.001$ (Figure 5C). We also confirmed a lack of any strong correlations between BAFFR expression (MFI) and BAFF levels (18) *(data not shown).*

Neither the % 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 Figure 6A, expression of BAFFR (MFI) in the transitional but not mature naïve subset showed a notable increase with time after repopulation ($r^2= 0.53$, $p<0.001$). Less significant was the increased expression with time in post-GC ($r^2=0.33$ $p<0.05$), IgD- resting memory ($r^2=0.34$, $p<0.05$) and plasmablasts ($r^2=0.41$, $p<0.01$). 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) (Figure 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 sub populations up to 7 months after B cell return (Figure 6A).

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 hypothesised 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 naïve 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 increased 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, 30, 31). It was clear however that at the higher levels of serum BAFF following RTX, the percentages of naïve and of post-GC B cells positive for BAFFR were lower, with a significant negative correlation.

As we did not however 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 in-appropriate signaling BAFF was not responsible for driving relapse.

Following BCDT, B cells repopulate primarily from bone marrow-derived naïve B cells, with regeneration of the memory B cell pool often delayed (15, 32, 33). 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 % transitional naïve B cells accompanied by an increase into the mature naïve compartment, confirmed in longitudinal 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 (34), systemic lupus erythematosus (SLE) (35) 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 (36, 37).

In conventional B cell development it is thought that apoptosis is the ‘default’ programme for transitional naïve B cells unless rescued by productive BAFFR (BR3) mediated signalling (38). Both the % and expression (MFI) of BAFFR on B cells returning post RTX was reduced compared to Pre-RTX values in transitional and mature naïve 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 sub-populations over 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, perhaps due to internalisation or shedding, independently of exposure to B cell agonists (24, 39). 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 (40). They are also expanding into an environment of high serum BAFF levels.

Some RA patients however relapsed soon after B cell return with predominantly early naïve (transitional) B cells in the circulation, suggesting that in these patients, the disadvantages of low BAFFR expression/signaling were not limiting the onset of relapse (18). Of the other 2 BBR, BCMA expression was low and relatively unchanged by RTX with the %TACI+ memory B cells remaining significantly lower than HC following RTX.

Patients with RA showed reduced %TACI+ B cells both in those with active disease pre-RTX and at relapse after RTX in all 3 memory B cell compartments. This suggests that disadvantages of low TACI expression are not translated into a lack of pro-inflammatory signaling. Whether this reflects a constitutive lack of up-regulation of TACI in RA patients or down-regulation due to internalisation 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 (41). Co-ligation of TACI and proteoglycans is thought to be essential for class switch recombination (42). This has been attributed to the accompanying loss of inhibitory signals through TACI to BAFFR and CD40 mediated signalling. Reduced %TACI+ memory B cell subsets may thus render B cells less responsive to inhibitory signaling through TACI.

Relapse coincident with or close to naïve B cell re-entry to the periphery was not associated with prominent memory or plasmablast populations, despite 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 %BCMA+ B cells. Coincident with differentiation into ISC, BCMA is acquired on mature B cells accompanied by loss of BAFFR expression (2, 43). BCMA expression can be increased on plasmablasts and memory B cells from patients with SLE (25, 44, 45), and also be expressed in transitional B cells, where it is associated with inappropriate naïve 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) however showed an incremental rise which correlated with time to flare after B cell return (19). In the current studies, precipitation of relapse in patients analyzed ≥4 months after B cell return would seem to depend on further recovery of the mature naïve B cell pool with % 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 into follicular or other secondary lymphoid organs or inflammatory sites.

In healthy individuals, further differentiation of B cells from transitional to mature naïve stage is prevented if they are exposed to uncomplexed antigen through their BCR, even in the presence of both T-dependent and T- 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 pre-immune repertoire due to defective central tolerance checkpoints. Further it was shown that there were also defects in peripheral checkpoints with continued persistence of autoreactive naïve B cells (46-48). Survival of these newly exiting B cells may thus favour those with autoreactive BCR. Based on our results therefore we propose that in-appropriate signaling 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 in which differ between human autoimmune diseases, thereby influencing the institution of effective therapies (49, 50). 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 neutralise soluble BAFF in patients with RA may also be explained by our findings.

Authors' 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. Drs Becerra, De La Torre, Leandro and Cambridge had full access to all of 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: Becerra, De La Torre, Leandro, Cambridge
Acquisition of data: Becerra, Leandro, Cambridge
Analysis and interpretation of data: Becerra, De La Torre, Leandro, Cambridge.

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Figure Legends:

Figure 1: Example of a flow cytometry plot showing B-cell phenotypes defined on the basis of IgD/CD38 expression for CD19-gated cells in an RA patient pre-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 sub-populations 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 ≥4 months later are shown. In panel E) The relationship between %CD19+ B cells within each sub-population 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 coefficient which are shown for those with r^2>0. 4 and giving p≤0.05. 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 ≥ 4 months after B cell return. Horizontal lines on graphs represent median values and results were analysed using Mann-Whitney U test; *p<0.05; ** p<0.01; ***p<0.001.

Figure 2: A) Flow cytometry plot of BAFFR expression on CD19+ B cells within the lymphocyte gate in an RA patient studied pre-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) (a-f): %BAFFR+ expression on CD19+ B cell subpopulations (defined using IgD/CD38) in HC, and in pre- and post-RTX RA patients. Results for post-RTX patients were divided on the basis of proximity to first documented B cell return, as described in Figure 1. Lines on graphs represent median values and results were analysed using Mann-Whitney U test; *p<0.05, **p<0.01, ***p<0.001.

Figure 3: Flow cytometry plot of TACI expression on CD19+ B cells within the lymphocyte gate in the RA patient studied pre-RTX A) 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 TACI within the gated CD19+ population. . The patient relapsed 3 months after the sample shown in C. D) (a-f): %TACI+ expression on CD19+ B cell subpopulations (defined using IgD/CD38) as in in HC, pre-RTX patients and patients relapsing post-B cell return. Results for HC and pre-RTX patients were compared and results between the 2 patient groups relapsing at different intervals after B cell return were also compared. Lines on graphs represent median values and results were analysed using Mann-Whitney U test; *p<0.05, **p<0.01, ***p<0.001.

Figure 4: A) Flow cytometry plot of BCMA expression on CD19+ B cells within the lymphocyte gate in an RA patient studied pre-RTX **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 BCMA within the gated CD19+ population. The patient relapsed 3 months after the sample shown in C). In **D)**, (a-f) %BCMA+ expression on CD19+ B cell subpopulations (defined using IgD) in HC, pre-RTX patients and patients relapsing after RTX. Results for HC and pre-RTX patients were compared and results between the 2 patient groups relapsing at different intervals after B cell return were also compared. Lines on graphs represent median values and results were analysed using Mann-Whitney U test; *$p<0.05$, **$p<0.01$, ***$p<0.001$.

Figure 5: A) Serum 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 manufacturers. B) Relationship between serum BAFF levels and time after B cell return in RA patients relapsing after RTX. C) The relationship between %BAFFR+ B cells and serum BAFF levels are shown for each B cell sub-population, defined using IgD/CD38. Linear regression analysis (Spearman’s correlation) and exact p values are shown for significant correlations ($p<0.05$) between serum BAFF levels and %BAFFR+ B cells.

Figure 6: In **A)** the relative expression (MFI) of BAFF-R and in **B)** expression of TACI expression on B cells was measured within each B cell sub-population, as defined using IgD/CD38, and plotted against time (months) after peripheral B cell return in RA patients relapsing after RTX. Linear regression analysis (Spearman’s correlation) and exact p values are shown for significant correlations ($p<0.05$) Shaded areas indicate the ranges for MFI of the two BBR+ given by the respective B cell subpopulations in 5 HC samples taken within the same time period.