RHEUMATOLOGY

Rheumatology 2015;54:1429–1434
doi:10.1093/rheumatology/kev005
Advance Access publication 3 March 2015

Concise report

Treatment with belimumab restores B cell subsets and their expression of B cell activating factor receptor in patients with primary Sjogren's syndrome

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Abstract

Objective. The aim of this study was to investigate the biological effects of belimumab on B cells in the first phase II open-label trial with belimumab in patients with primary SS (pSS) (BELISS).

Methods. Peripheral blood B cell subsets and their B cell activating factor-receptor (BAFF-R) expression were analysed by multicolour flow cytometry in 10 pSS patients either before or after 24 and 52 weeks of therapy with belimumab. Serum BAFF levels were analysed by ELISA.

Results. At baseline, pSS patients showed a significant increase in circulating B cells compared with healthy donors matched for age and sex, with a predominant expansion of transitional and naive B cell subsets. pSS patients also showed higher serum BAFF levels and lower B cell BAFF-R expression. Therapy with belimumab in pSS patients induced a significant reduction in transitional and naive B cell subsets to levels similar to those observed in healthy donors. Furthermore, belimumab normalized BAFF-R expression in all B subsets comprised within the memory compartment. The restoration of B cell frequency and subset composition in response to belimumab was also associated with a decrease in serum levels of Ig, RF, ANAs, and with an increase in the C4 complement fraction. All of these belimumab-mediated effects were observed after 24 weeks of therapy and maintained until the end of the therapeutic protocol.

Conclusion. Taken together, our findings show that targeting BAFF with belimumab is successful in normalizing B cell frequency, phenotype and functions in pSS.

Trial registration: clinicaltrials.gov; https://clinicaltrials.gov/; NCT01008982.

Key words: Sjogren's syndrome, belimumab, BAFF-receptor, B lymphocytes, transitional B lymphocytes.

Rheumatology key messages

- pSS patients have increased circulating levels of transitional and naive B cells and B cell activating factor.
- B cells in pSS patients have increased expression of B cell activating factor receptor.
- Belimumab acts on pSS patients by normalizing transitional and naive B cells and their B cell activating factor-receptor expression.

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Submitted 25 June 2014; revised version accepted 13 January 2015

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Introduction

Primary SS (pSS) is a chronic autoimmune disease characterized by lymphocytic infiltration of the salivary and lacrimal glands that progressively leads to tissue damage and dysfunction. Several extraglandular and systemic manifestations often characterize the clinical outcome of pSS as well. B cells are major players in pSS pathogenesis, as B cell hyperactivity and B cell lymphoproliferation are distinctive hallmarks of this disease [1, 2]. As a result of this, pSS patients are at higher risk of developing malignant lymphomas within the salivary gland mucosa–associated lymphoid tissue (MALT) [3].

B-cell activating factor (BAFF) is a key factor for B cell survival and proliferation, and it is increased in several autoimmune diseases, with pSS showing the highest levels [4, 5]. Produced by multiple cellular types, including epithelial cells and T and B lymphocytes, it is highly expressed in the serum and salivary glands of pSS patients, where it has been implicated in the formation of salivary ectopic germinal centres [6]. The pathogenic role of BAFF in pSS-associated lymphoproliferation has been demonstrated, in both the pre-lymphomatous stage and overt MALT lymphomas [7], and it is further supported by the observation that pSS patients co-affected by severe clinical manifestations associated with B-cell lymphoproliferation have higher BAFF serum levels than patients without co-morbidities [8]. High levels of salivary BAFF have also been involved in resistance to biologic therapies targeting B cells in pSS patients with MALT lymphoma. Together these findings have identified BAFF as a promising therapeutic target in pSS. BELISS is the first open-label phase II study conducted in pSS patients for investigating the efficacy and safety of belimumab, a human mAb neutralizing soluble BAFF [9]. BELISS provided a great opportunity to investigate the biological effects of belimumab on peripheral B cell subsets, BAFF and BAFF-receptor (BAFF-R) in pSS.

Methods

Patients and controls

This study was performed on the pSS patients enrolled in BELISS [9] by the Rheumatology Unit of the University of Udine. All patients who completed the clinical trial and had peripheral blood mononuclear cells (PBMCs) available at all the time points ($n = 10$) were included. All patients fulfilled the American–European Consensus Group criteria for pSS classification [10]. All patients were female with a median age of 49 years (range 20–66) and a median disease duration of 8 years (range 1–16). All were positive for ANAs, anti-SSA/Ro and anti-SSB/La antibodies, while RF was detected in 6 of 10 patients at baseline. The median baseline European League Against Rheumatism (EULAR) SS Disease Activity Index (ESSDAI) score was 7 (range 2–27), indicating moderate systemic disease activity in this pSS population; the median baseline EULAR SS Patient-Reported Index score was 5.8 (range 2.3–8.4), in line with baseline EULAR SS Patient-Reported Index scores reported in other clinical trials in pSS [11]. Stable concomitant medications were prednisone 5 mg/day in one patient and HCQ 6 mg/kg/day in another two patients. Before enrolment, none of the patients had received B cell–targeted therapy in the previous 12 months, CYC during the previous 6 months, biologic therapies during the previous 3 months, or immunosuppressive/immunomodulatory or antimalarial drugs during the previous 2 months. Severe renal or haematological failure, cancer (with the exception of pSS-associated lymphoma), evidence of systemic or local infections, severe diabetes or any other chronic disease were considered exclusion criteria. All patients were infused with 10 mg/kg belimumab at weeks 0, 2 and 4, and then every 4 weeks until week 48, with a final evaluation scheduled at week 52. The study was approved by the Institutional Review Board of the University of Udine and registered to clinicaltrials.gov (NCT01008982). Signed informed consent was obtained from all participants. Nine age- and sex-matched blood donors were enrolled as controls.

Laboratory analyses

Igs, RF, C3, C4, ANA and ENA were measured by routinely employed methods at the Central Laboratory of the University Hospital of Udine at weeks 0, 24 and 52. Serum BAFF levels were measured at weeks 0, 4, 24 and 52 by ELISA (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions. Sera were collected and immediately frozen at -80°C until analysis.

Flow-cytometric analyses

B cell subsets were analysed at a single time point in all control subjects and at weeks 0, 24 and 52 in all patients. They were identified by flow cytometry as previously described [12], with minor modifications. Briefly, thawed PBMCs were incubated with Fc-blocking solution (BioLegend, San Diego, CA, USA) to prevent specific binding, and then stained with LIVE/DEAD Fixable Aqua Dead Cell Stain (Invitrogen, Paisley, UK) to discriminate viable cells. PBMCs were labelled with the following mAbs: PB-conjugated anti-CD20, FITC-conjugated anti-IgD, APC-Cy7-conjugated anti-CD27 (all from BD Biosciences, San Jose, CA, USA); PE-conjugated anti-IgM, PE-Cy7-conjugated anti-CD21, PerCP-Cy5.5-conjugated anti-CD10, APC-conjugated anti-BAFF-R (all from Biolegend). Seven-colour data acquisition and analysis were performed on a FACSCantoII flow cytometer using FACSDiva software version 6.01 for Windows (BD Biosciences). As isotype controls were not used, those cells that did not express a certain marker were considered as negative controls for positive cells [12]. Lymphocytes were gated on forward (FSC) *vs* side scatter (SSC) plots. At least 100 000 lymphocyte-gated cells were routinely collected for each sample. As shown in supplementary Fig. S1, available at *Rheumatology* Online, B cells were identified in peripheral blood samples as $CD20^{+}$. Gated on B cells, $CD27^{-}$ and $CD27^{+}$ B cells were defined.

Within CD27$^-$ B cells, transitional B cells were identified as CD10$^+$ cells [13], unswitched naive B cells as IgD$^+$/IgM$^+$ cells and CD27$^-$ memory B cells as IgD$^-$/IgM$^-$ cells. Within CD27$^+$ B cells, switched memory B cells were identified as IgD$^-$/IgM$^-$ cells, IgM-only memory B cells as IgD$^-$/IgM$^+$ cells and marginal zone-like B cells as IgD^{low}/IgM$^+$ cells. Estimates of the absolute numbers of B cells and their subsets were calculated from the proportion of cells recorded by flow cytometry in the lymphocyte gate multiplied by the absolute lymphocyte count measured using a standard haemocytometer. The expression of BAFF-R on B cells and B cell subsets was expressed as mean fluorescence intensity. A representative flow cytometry analysis of BAFF-R expression is shown in supplementary Fig. S2, available at *Rheumatology* Online.

Statistical analysis

Statistical analyses were performed with GraphPad Prism 5.01 for Windows (GraphPad Software, San Diego, CA, USA). The Mann–Whitney test was used for comparison of groups, the Wilcoxon test for comparison of paired variables, and Spearman's test was used to investigate for possible correlations. $P < 0.05$ was considered statistically significant.

Results

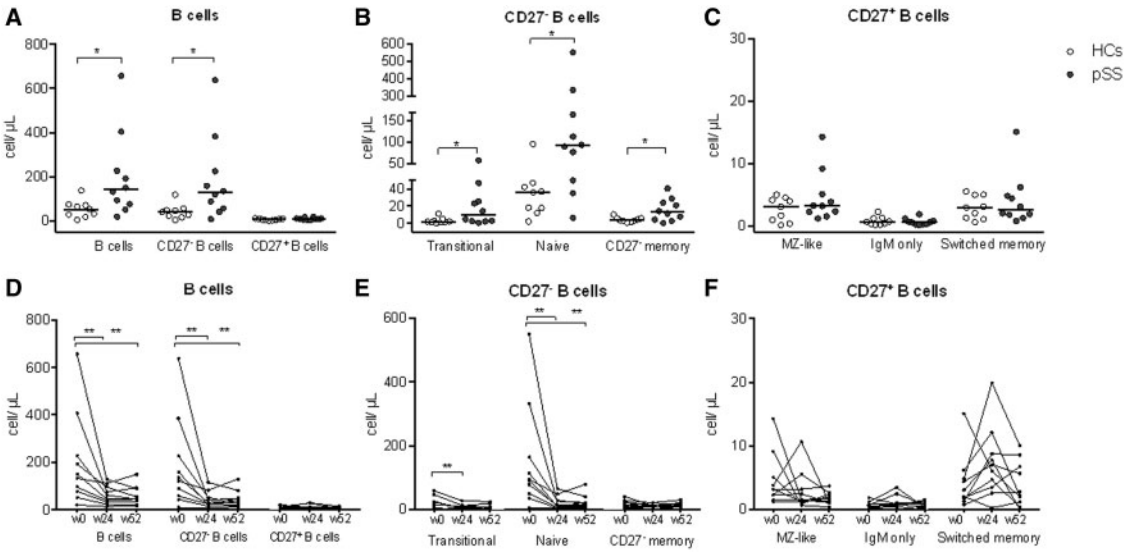
CD27$^-$ transitional and naive B cells are expanded in pSS patients

Flow-cytometric analysis of B cells and B cell subsets performed before belimumab treatment demonstrated a significant expansion of total B cells in pSS patients compared with controls (Fig. 1A), mainly due to expansion of the CD27$^-$ B cell compartment (Fig. 1B). Within the CD27$^-$ subpopulations, an increased number of transitional, naive and CD27$^-$ memory B cells was observed (Fig. 1B). No significant differences between patients and controls were observed in the CD27$^+$ compartment (Fig. 1C).

The expansion of CD27$^-$ transitional and naive B cells in pSS patients is reversed by treatment with belimumab

Belimumab administration to pSS patients induced a reduction in total B cell count down to levels similar to those observed in healthy controls. Belimumab-induced B cell reduction was evident at week 24 and maintained until the end of treatment, and it was mainly confined to the CD27$^-$ compartment (Fig. 1D). Within this compartment (Fig. 1E), only the transitional and naive B cell subsets were reduced by belimumab treatment, reaching

Fig. 1 Cell count of peripheral B cells and B cell subsets in pSS patients before and after therapy with belimumab



(**A**–**C**) Number of peripheral B cells and B cell subsets in pSS patients at baseline ($n = 10$) compared with healthy donors ($n = 9$). Each symbol represents a single sample. Median values represented by horizontal lines in each series. (**A**) The number of total peripheral B cells was higher in pSS patients than in controls. (**B**) pSS patients exibited an expansion of CD27$^-$ B cells, characterized by a marked increment of transitional, naive and CD27$^-$ memory B cells (*$P < 0.05$, **$P < 0.005$ Mann–Whitney test). (**C**) No significant differences between patients and controls were observed in CD27$^+$ B subsets. (**D**–**F**) Kinetics of peripheral B cell subsets in pSS patients during belimumab treatment (weeks 0, 24 and 52). (**D**) Belimumab induced a significant reduction in total peripheral B cells that was confined to the CD27$^-$ compartment. (**E**) Belimumab reduced transitional and naive B cells to levels similar to those observed in healthy controls. (**F**) CD27$^+$ B cell subsets were not significantly affected by belimumab (*$P < 0.05$, **$P < 0.005$, Wilcoxon test).

values similar to those observed in healthy controls. In contrast, $CD27^-$ memory B cells and all subsets in the $CD27^+$ compartment were not significantly affected by belimumab treatment (Fig. 1F).

The expression of BAFF-R on peripheral B cell subsets is markedly reduced in pSS patients

Total B cells obtained from pSS patients before belimumab treatment showed a marked and significant downregulation of BAFF-R expression compared with B cells from healthy controls (Fig. 2A). This downregulation of BAFF-R was observed in both $CD27^-$ and $CD27^+$ compartments (Fig. 2B and C), and it was observed to a similar and significant extent in all B cell subsets.

The expression of BAFF-R on peripheral B cells is normalized by treatment with belimumab

BAFF-R expression on B cells of pSS patients increased during treatment with belimumab. The increment of BAFF-R expression was more evident and statistically significant in the $CD27^+$ B cell compartment (Fig. 2D). Within the different B cell subsets, belimumab-induced upregulation of BAFF-R was restricted to memory B cell populations. In particular, BAFF-R upregulation was observed in $CD27^-$ memory B cells (Fig. 2E) and in all $CD27^+$ subsets, where belimumab treatment increased the surface expression of BAFF-R to levels similar to those observed in healthy controls (Fig. 2F).

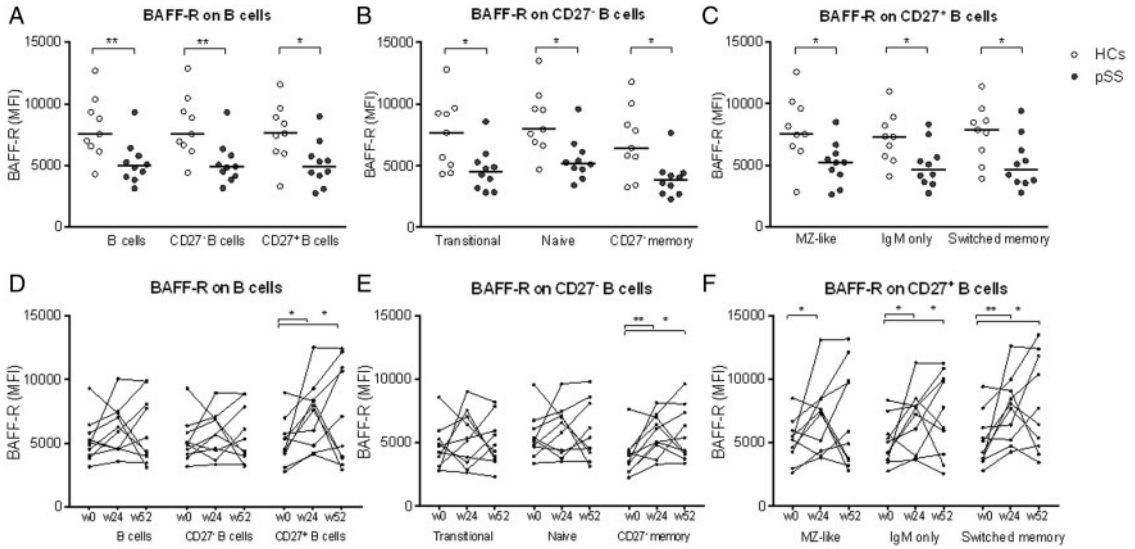
Serum BAFF levels in pSS patients during belimumab treatment

Serum BAFF levels were significantly higher in pSS compared with healthy donors [median: 1191 (range 875–2439) *vs* 672 (range 484–846) pg/ml, $P < 0.0001$]. Belimumab downregulated circulating BAFF levels in pSS patients at week 4 [696 (range 370–2048) *vs* 1191 (range 875–2439), $P = 0.027$]. However, this reduction was transient and was reversed at subsequent time points. In particular, at weeks 24 and 52, serum BAFF levels were similar to those observed before treatment [1443 (range 682–4536) and 1387 (range 667–3785) *vs* 1191 (range 875–2439)] (data not shown).

Serological markers of B cell activity improve during belimumab treatment

Next, we evaluated whether belimumab-induced changes in B cell subsets and BAFF-R expression were associated with restraint of B cell activity. As shown in supplementary Fig. S3, available at *Rheumatology* Online, belimumab indeed induced an overall reduction in circulating Ig levels, characterized by a marked and significant reduction in IgM levels at both week 24 and week 52, and a less pronounced reduction in IgG. Belimumab also induced a

Fig. 2 Expression of BAFF-R on peripheral B cells and B cell subsets in pSS patients before and after therapy with belimumab



(**A**–**C**) B cell activating factor-receptor (BAFF-R) expression on B cells and B cell subsets in pSS patients at baseline ($n = 10$) compared with healthy donors ($n = 9$). Data are expressed as mean fluorescence intensity (MFI). Each symbol represents a single sample. Median values are represented by horizontal lines in each series. Compared with healthy controls, pSS patients showed a significant downregulation of BAFF-R on total B cells (**A**), and all subsets in the $CD27^-$ (**B**) and $CD27^+$ (**C**) compartments (*$P < 0.05$, **$P < 0.005$ Mann–Whitney test). (**D**–**F**) Kinetics of B cell expression of BAFF-R in pSS patients during belimumab treatment (weeks 0, 24 and 52). Belimumab induced an increase in BAFF-R expression that was more evident in the $CD27^+$ compartment (**D**), and confined to B cell memory subsets (**E** and **F**) (*$P < 0.05$, **$P < 0.005$, Wilcoxon test).

significant decrease in RF levels and ANA titres, and a significant increase in serum C4. It did not affect SSA and SSB levels, which remained high over time (data not shown). No significant correlations associated with belimumab treatment were found between serological markers of B cell activity and B cell subsets or BAFF-R expression.

Discussion

This study aimed to fully characterize circulating B cells in pSS patients before and after 1-year treatment with belimumab. At baseline, pSS patients showed a marked increase in circulating B cells that was ascribable to a pre-eminent expansion of the $CD27^-$ compartment. The absolute count of $CD27^+$ B cells was similar in patients and controls, probably because the reduced frequency of $CD27^+$ cells that reflected the increased frequency of $CD27^-$ B cells in pSS was balanced by the increased number of circulating B cells. Altogether, these findings are in accordance with all previous studies reporting similar reciprocal changes in the frequency of $CD27^-$ and $CD27^+$ B cells in pSS [14–17], although they partially contrast with regard to the absolute number of B cells, which have been variously reported to be either unchanged or reduced in pSS [15, 16]. In our patients, we further observed that, within the $CD27^-$ compartment, all subsets (i.e. transitional, naive and $CD27^-$ memory B cells) were expanded. Particularly relevant to pSS pathogenesis was the marked increase in transitional B cells, new emigrant lymphocytes that after leaving the bone marrow must still undergo peripheral tolerance checks and therefore still contain high rates of autoreactive cells [18]. Moreover, an accumulation of transitional B cells in pSS salivary glands has been suggested to be pathogenic for the progression of local disease [6]. Our findings that pSS patients have an increased number of circulating transitional B cells is in accordance with previous studies reporting increased circulating Bm2′ cells in pSS, as defined based on the Bm1–Bm5 B cell classification [14, 17]. In fact, it has been reported that the circulating Bm2′ compartment partially overlaps with transitional B cells [13]. Also the finding of expanded $CD27^-$ memory B cells in our patients may be relevant to pSS pathogenesis, because these cells have been suggested to be enriched in autoreactive B cells, as well [19]. Altogether, the overall distribution of circulating B cell subsets observed in our pSS patients can be explained well by the increased serum levels of BAFF typical of pSS patients [5], and confirmed in our pSS cohort. In fact, high BAFF levels promote survival and expansion of transitional B cells, increase the cell lifespan of mature naive B cells and contribute to differentiation of $CD27^-$ memory B cells [4, 19].

Upon treatment with the anti-BAFF antibody belimumab, pSS patients showed a remarkable reduction in total B cells that was confined to transitional and naive B cells, the subsets characterized by BAFF-dependent survival. Similar results have been reported in previous studies assessing the effects of belimumab in patients affected by SLE who showed preferential depletion of transitional and naive B cells, while conventional memory B cells were quite resistant to anti-BAFF treatment [20]. As a possible consequence of these effects on B cells, we further observed that belimumab treatment resulted in sustained normalization of total Ig levels and C4 concentrations. Similar to SLE individuals, in pSS also, the reduction in Ig levels was mostly restricted to the IgM class, in line with the resistance of conventional memory cells to belimumab treatment. The reduction of RF in our pSS patients may be consistent with the expected preferential effect of belimumab on autoreactive B cells, as well.

Finally, in this study we demonstrated that at baseline pSS patients have significantly reduced expression of BAFF-R on all B cell subsets. According to a previous study describing similar results [16], BAFF-R downregulation may result from a negative feedback related to high serum BAFF levels. Consistent with this, treatment of our patients with belimumab induced an increase in BAFF-R expression that was evident after 24 weeks of treatment and was maintained for the entire duration of the study. It was more pronounced and significant in the memory B cell compartment, where BAFF-R expression was restored to levels similar to those of healthy controls. This is a completely novel finding, as BAFF-R expression on B cells has never been investigated in previous trials with belimumab. Together with our demonstration that, apart from an early and transient reduction after 4 weeks of therapy, treatment with belimumab did not affect serum BAFF levels in the long term, these results may suggest that belimumab, which acts by preventing the binding of soluble BAFF to its cognate receptors [20], may affect the feedback mechanisms regulating BAFF and BAFF-R interaction rather than directly countering the primary overproduction of BAFF occurring in pSS [21]. However, the complexity of BAFF quantification may suggest caution in the interpretation of the results [22].

This study provides evidence that patients with pSS have a profound perturbation of peripheral B cell homeostasis consistent with primary BAFF overproduction. By investigating for the first time the effects of belimumab on B cells in pSS, we demonstrated that belimumab restores peripheral B cell homeostasis and improves serological activity, possibly by regulating BAFF and BAFF-R interactions. These observations may represent the biological background to explain and support the preliminary results on the clinical efficacy of a BAFF-targeted therapy in pSS.

Acknowledgements

The authors thank the patients for their generosity and participation in this study. F.C. and A.R. are recipients of the Guglielmina Lucatello e Gino Mazzega fellowship from the Fondazione Italiana per la Ricerca sul Cancro.

Funding: This work has been supported by Associazione Italiana per la Ricerca sul Cancro (IG 9104 to D.M.), by the Italian Ministry of Health (Bando Giovani Ricercatori GR-2008-1135082 to D.M.) and by intramural research

and clinical funding programs of Humanitas Research Hospital assigned to D.M. E.P. is a recipient of the Luigi Tocco e Liliana Mirizio fellowship from the Fondazione Italiana per la Ricerca sul Cancro.

Disclosure statement: The authors have declared no conflicts of interest.

Supplementary data

Supplementary data are available at *Rheumatology* Online.

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