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ORIGINAL RESEARCH

Efficacy of BAFF inhibition and B-cell depletion in non-obese diabetic mice as a spontaneous model for Sjögren's disease

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ABSTRACT

Introduction The therapeutic interest of targeting B-cell activating factor (BAFF) in Sjögren's disease (SjD) can be suspected from the results of two phase II clinical trials but has not been evaluated in an animal model of the disease. We aimed to evaluate the therapeutic efficacy of this strategy on dryness and salivary gland (SG) infiltrates in the NOD mouse model of SjD.

Material and methods Female NOD mice between ages 10 and 18 weeks were treated with a BAFF-blocking monoclonal antibody, Sandy-2 or an isotype control. Dryness was measured by the stimulated salivary flow. Salivary lymphocytic infiltrates were assessed by immunohistochemistry. Blood, SGs, spleen and lymph-node lymphocyte subpopulations were analysed by flow cytometry. SG mRNA expression was analysed by transcriptomic analysis.

Results BAFF inhibition significantly decreased SG lymphocytic infiltrates, which was inversely correlated with salivary flow. The treatment markedly decreased B-cell number in SGs, blood, lymph nodes and spleen and increased Foxp3$^+$ regulatory and CD3$^+$CD4$^-$CD8$^-$ double negative T-cell numbers in SGs.

Conclusion A monoclonal antibody blocking BAFF and depleting B cells had therapeutic effectiveness in the NOD mouse model of SjD. The increase in regulatory T-lymphocyte populations might underlie the efficacy of this treatment.

WHAT IS ALREADY KNOWN ON THIS TOPIC

⇒ The therapeutic interest of targeting B-cell activating factor (BAFF) in Sjögren's disease (SjD) is suspected from the results of two phase II clinical trials.

WHAT THIS STUDY ADDS

⇒ A monoclonal antibody that blocks BAFF and consequently depletes B cells markedly improved salivary flow and salivary gland (SG) lymphocytic infiltrates in the NOD model of SjD.

⇒ The significant increase in Foxp3$^+$ regulatory and CD3$^+$CD4$^-$CD8$^-$ double negative T cell number in SGs might underlie the efficacy of B-cell depletion and BAFF inhibition.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

⇒ This study strengthens the rationale to target BAFF in patients with SjD.

INTRODUCTION

Sjögren's disease (SjD) is an auto-immune epithelitis leading to ocular and mucosal dryness, pain and various systemic manifestations.[1] SjD is characterised by lymphoid infiltration of the salivary glands (SGs) and lacrymal glands responsible for dryness and the secretion of autoantibodies. B lymphocytes play a key pathogenic role in SjD by their secretion of autoantibodies, proinflammatory cytokines and antigen presentation. B-cell activating factor (BAFF) is crucial for the activation and survival of autoreactive B cells and for autoantibody secretion. Systemic lupus erythematosus-like and SS-like symptoms spontaneously develop in BAFF transgenic mice.[2 3] In patients with SjD, BAFF levels are increased in serum, saliva and SGs.[4 5] In SjD, BAFF is expressed by dendritic cells and monocytes, salivary and conjunctival epithelial cells, immunofibroblasts and some autoreactive B and T cells.[5 6] An open-label phase II study suggested the efficacy of belimumab, an inhibitor of BAFF, in nearly two-thirds of SjD patients, as assessed by an alleviation of disease indicators, including dryness, pain, fatigue, systemic activity and B-cell biomarkers.[7] Two randomised controlled trials—a trial combining rituximab and belimumab, and a trial using a B cell-depleting, BAFF receptor-blocking monoclonal antibody, ianalumab—showed a decrease in systemic disease activity and an improvement in salivary dryness.[8 9]

The efficacy of BAFF specific inhibition has not been assessed in animal models of SjD.

NOD mice are mostly used as an animal model for type 1 diabetes but also exhibit relevant features of SjD. Female NOD mice have focal infiltrates in SGs typically between ages 8 and 12 weeks, which increase over time.[10] NOD mice show the same progressive decrease in salivary flow as SjD patients.[11] Analyses of lymphocyte populations infiltrating the exocrine tissues have revealed a similar profile in NOD islets and human SS, with a predominance of T-cell receptor $\alpha\beta^+$ and $CD4^+$ T cells and significantly fewer $CD8^+$ T or B cells.[12] Anti-Ro/SSA and autoantibodies against the muscarinic 3 receptor, the acetylcholine receptor responsible for fluid secretion from the SGs, can be detected in sera from NOD mice.[12 13]

Sandy-2, a mouse monoclonal antibody that inhibits BAFF, was developed.[14] Sandy-2 blocks mouse BAFF and prevents it from binding to BAFF receptor, transmembrane activator and CAML interactor (TACI) and B-cell maturation antigen (BCMA). B-cell depletion by administration of Sandy-2 in vivo phenocopies BAFF-knockout mice. Switched memory B cells are more abundant in humans than in mice and have much higher resistance to BAFF deprivation than naïve B cells.[15] This difference in B-cell subset repartition between mice and men explains the higher depletion observed in mice with Sandy-2 than in patients treated with belimumab. Reduction in B cell numbers with Sandy-2 occurs through inhibition of BAFF. Fc-mediated depletion of cells bearing a BAFFR/BAFF complex seems unlikely as Sandy-2, when bound to BAFF, prevents BAFF binding to its receptors.

We investigated the potential effect of this monoclonal antibody that blocks BAFF and depletes B cells in the NOD mouse model of SjD.

MATERIALS AND METHODS

Mice and treatment

NOD mice were bred in the animal facility of Hôpital Necker-Enfants Malades, INSERM U1013-CNRS UMR 8147, under specific pathogen-free conditions. Substrains of NOD mice derived from the inbred strain NOD/Shi, have been bred all over the world. Our colony at Hôpital Necker in Paris (NOD/Nck) was then started in 1986.[16]

Untreated female and male NOD mice were used to determine the timing of onset of symptoms of dryness and sialadenitis. The SGs were sampled and stimulated salivary flow performed at different ages (3, 7, 10, 15 and 20 weeks).

The B-cell depleting, mouse BAFF blocking monoclonal antibody (Sandy-2) and its isotype control (EctoD1, a mouse IgG1 anti-ectodysplasin) were used as previously described.[14]

Sandy-2 or its isotype control, formulated in phosphate-buffered saline (PBS), was injected intraperitoneally, at 2 mg/kg, every other week from age 10 to 18 weeks. In total, 31 mice received Sandy-2 and 30 the isotype control.

The measure of stimulated salivary flow was performed throughout the study and the other experiments were performed at sacrifice, between ages 18 and 20 weeks, in mice that had not become diabetic. As not all the analyses were carried out on all the mice due to a lack of material to perform all the analyses, we indicate the number of mice from each group for each experiment.

Stimulated salivary flow

Mice were anaesthetised with a mild anaesthesia induced by 100 µL/10 g body weight of a solution of ketamine (30 µL; Centravet, Maison-Alfort, France) and xylazine (100 µL at 2%; Centravet) with 1 mL sterile water, given intraperitoneally. Saliva secretion was induced by subcutaneous injection of pilocarpine (0.5 mg/kg body weight; Sigma-Aldrich, St. Louis, MO, USA). Stimulated whole saliva was collected for 20 min from the oral cavity with haematocrit tubes and placed into preweighed 1.2 mL microcentrifuge tubes. Volume was determined gravimetrically. To smooth out the differences that may be related to the dose and injection of pilocarpine according to the experiments, we present the variation in salivary flow in delta (difference between the value at 20 weeks and the baseline value (10 weeks)). Delta of stimulated salivary flow was assessed in 27 mice (16 receiving Sandy-2 and 11 the isotype control).

Histology

Sections from the submandibular glands of NOD mice at age 18–20 weeks were examined histologically. SGs were embedded in paraffin. Sections were cut at 7 µm, and six non-consecutive sections were stained with H&E. The focus score (FS) was determined,[17] one focus is defined as an aggregate of ≥50 lymphocytes and the FS is defined as the average number of foci per 4 mm^2 SG tissue. Submandibular glands were also graded according to the scale of Chisholm and Mason[18]: 0=absent infiltrates, 1=slight infiltrates, 2=moderate infiltrates or <1 focus per 4 mm^2, 3=1 focus per 4 mm^2, 4=>1 focus per 4 mm^2.

For immunofluorescence microscopy, paraffin sections were stained with primary anti-CD3 polyclonal rabbit antibody (DAKO) and anti-B220 biotin (clone RA3-6B2; eBioscience) and Streptavidin-Alexa-Fluor 546 (Molecular Probes) and goat anti-rabbit IgG Alexa-Fluor 647 (Life Technologies) as secondary antibodies. To determine the proportion of B cells in SG infiltrates, the proportion of B220-positive area to total lymphocytic infiltrates was assessed. Treatment and analyses were performed with Fiji (ImageJ) software. The FS was assessed in 23 mice (14 receiving Sandy-2 and 9 the isotype control) and immunostaining was performed in 15 mice (9 and 6 mice, respectively).

Flow cytometry

Blood cells, lymph node cells, SG cells and splenic cells were obtained from individual mice at sacrifice.

Submandibular glands were removed at sacrifice and one gland was used for flow cytometry. After removal, the digestive milieu (RPMI 2% BSA, Collagenase D: 1.5 mg/mL, DNAse I: 1 U/mL; RPMI, Gibco; BSA, Gibco; Collagenase, Roche, Boulogne-Billancourt, France; DNAse I,

Invitrogen, Carlsbad, CA, USA) was injected directly in situ and left for 5 min at room temperature. Then glands were mashed by using gentleMACS Dissociator (Miltenyi Biotec, Bergisch, Germany), digested again in the same digestive milieu for 30 min at 37°C and mashed again. The cellular suspension was passed through a 70 µm filter before staining.

After removal, the spleen was immersed in 3 mL PBS-2% FCS, and submandibular lymph node cells were immersed in 1 mL PBS-2% FCS, then mashed on a 100 µm filter. Cellular suspensions were centrifuged and incubated into 1 mL ammonium-chloride-potassium for 4 min at 4°C. Finally, cells were washed, counted and distributed in a 96-well plate.

An amount of 200 µL blood was collected by intracardiac puncture after anaesthesia with urethane (600–800 µL intraperitoneally at 75 mg/mL, Sigma-Aldrich) in a heparinised microtube (Sanofi-Aventis, Paris, France).

Cells were stained for 30 min at 4°C with the following antibodies/reagents: CD45 APC-Cy7 (clone 30-F11, BioLegend, San Diego, CA, USA), CD19 FITC (clone 1D3, BD Pharmingen, San Diego, CA, USA), CD3e PerCP (clone 145-2C11, BD Pharmingen), CD4 V450 (clone RM4-5, BD Pharmingen), CD8a/Ly-2 PE (clone 53-6.7, eBioscience, San Diego, CA, USA), Fixable Viability Dye EF506 (eBioscience), CD45.1 FITC (clone A20, BD Pharmingen), CD3e APC (clone 145-2C11, BD Pharmingen), CD4 V450 (clone RM4-5, BD Pharmingen), CD8a/Ly-2 PE (clone 53-6.7, eBioscience), CD19 PE-Cy7 (clone 1D3, BD Pharmingen), CD4 PerCP (clone RM4-5, BD Pharmingen) and FoxP3 APC (clone FJK-16S, eBioscience). Antibodies used for staining (volume per volume for a final dilution of 1/200 in 2% PBS-FCS, GIBCO) were incubated with cells for 30 min at 4°C. FoxP3 intracellular staining was performed after permeabilisation using a Foxp3/Transcription Factor staining buffer set (00-5523-00, eBioscience).

For blood cells, a red blood cell lysing solution (349202, BD Biosciences, San Jose, CA, USA) was added for 15 min at room temperature. Finally, cells were centrifuged for 5 min at 400 g and washed twice with PBS-2% FCS.

SGs were stained with fixable viability dye before other stainings and incubated for 10 min at 4°C in the dark. Cell acquisition involved a Canto II cytometer (BD Biosciences). Data were analysed with FlowJo (Tree Star).

Transcriptomic analysis

From one of the two SGs taken from mice receiving Sandy-2 (n=5) or the isotype control (n=4), total RNA was isolated and reverse transcribed to cDNA by using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems by Thermo Fisher Scientific, Foster City, CA, USA) according to the manufacturer's instructions. RNA quality was validated by using the RNA 6000 Nano Kit and the Bioanalyzer 2100 (Agilent Technologies). An amount of 75 ng total RNA was reverse transcribed by using the GeneChip WT Plus Reagent kit (ThermoFisher Scientific reference: 902281), without globin mRNA reduction. Briefly, the resulting double-stranded cDNA was used for in vitro transcription with T7 RNA polymerase. After purification, 5.5 µg of the resulting sense-strand cDNA was fragmented and labelled with biotin. These steps involved using the GeneChip WT Plus Reagent kit according to the manufacturer's instructions. After size analysis of fragmented sense-strand cDNA using the Bioanalyzer 2100 (Agilent Technologies), cDNA was then hybridised to the GeneChip Clariom S Human (Affymetrix) at 45°C for 17 hours. After hybridisation, chips were washed on the GeneChip Fluidics Station 450 (Affymetrix) according to the manufacturer's instructions and scanned by using the GeneChip Scanner 3000 7G system. The scanned images were then analysed by using Expression Console software (Affymetrix) to obtain raw data (.cel files) and quality control metrics.

Statistical analysis

Differences were assessed with the non-parametric Mann-Whitney test. Spearman rank correlation was used for correlation analysis. All the analyses were performed with GraphPadPrism V.8 (GraphPad Software, La Jolla, CA, USA). $P<0.05$ was considered statistically significant. Genes with adjusted absolute fold change in expression >2 and $p<0.05$ were identified as differentially expressed genes (DEGs). Gene Ontology (GO) annotation of DEGs involved using Enrichr[19] and GO[20] databases. Significantly enriched Kyoto Encyclopedia of Genes and Genomes pathways and GO terms were identified at $p<0.05$.

RESULTS

Anti-BAFF therapy significantly decreased SG lymphocytic infiltrates and increased salivary flow

Anti-BAFF antibody treatment significantly decreased SG infiltrates as compared with the isotype control (median FS 1.8 (IQR 1.1–2.5) vs 3.9 (3.5–4.8), p=0.002; figure 1A–C) and significantly increased salivary flow (median difference between sacrifice value and baseline value (10 weeks) 2.1 (0.8–4.2) vs 0.6 (−1.6 to 1.9), p=0.020; figure 1D). Salivary flow rate ratio was inversely correlated with the FS (rho=−0.553, r^2=0.31, p value=0.0093; figure 1E).

Anti-BAFF therapy markedly decreased B-cell number in blood, spleen, lymph nodes and SGs

Anti-BAFF antibody treatment markedly decreased B-cell number assessed by flow cytometry (online supplemental figure 1), as compared with the isotype control, in blood (proportion of B lymphocytes among total lymphocytes: median 12.0% (4.9–26.4) vs 29.2% (23.3–40.6), p=0.02), SGs (3.1% (1.1–61.1) vs 22.6% (16.4–38.3), p<0.0001), spleen (7.2% (6.7–13.5) vs 43.0% (42.1–46.2), p<0.0001) and submandibular lymph nodes (3.6% (1.1–5.5) vs 25.2% (22.4–39.6), p=0.06) (figure 2A). Anti-BAFF antibody treatment also decreased B-cell staining in SG tissue as compared with the isotype control (median stained area of $B220^+$ infiltrates: 5.1 (1.5–12.5) vs 34.0 (14.8–40.5), p=0.0028; figure 2B–D).

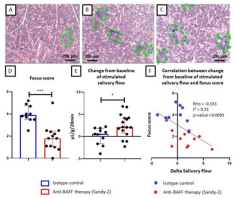

Figure 1 Sialadenitis and salivary flow. (A) Representative section of a salivary gland (SG) of an anti-B-cell activating factor (BAFF)-treated NOD mouse (H&E staining). One focus can be observed (highlighted by the dotted green line). (B) Representative section of an SG of an isotype control-treated NOD mouse. Three foci can be observed. (C) Representative section of an SG of a non-treated NOD mouse. Four foci can be observed. (D) Focus score (FS) of anti-BAFF-treated mice (n=13) and isotype control (n=10). One focus defined as an aggregate of ≥50 lymphocytes and the FS defined as the average number of foci per 4 mm^2 SG tissue. (E) Change from baseline of stimulated salivary flow (final–baseline) in anti-BAFF-treated NOD mice (n=16) and isotype control-treated NOD mice (n=11). (F) Correlation between change from baseline in stimulated salivary flow (final–baseline) and FS after treatment of NOD mice (n=21). Mann-Whitney and Spearman correlation: *p<0.05, ***p<0.001. Data are median (IQR).

Treatment-induced increase in regulatory T-cell subsets in SGs and spleen

Total $CD4^+$ and $CD8^+$ subpopulations

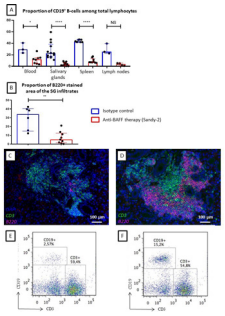

Figure 2 B-cell depletion. (A) The proportion of $CD19^+$ B-cells to total lymphocytes in blood, salivary glands, spleen and lymph nodes determined by flow cytometry in NOD mice treated with anti-B-cell activating factor (BAFF) antibody (n=8, 12, 9 and 4, respectively) and isotype control (n=3, 13, 8 and 3, respectively). (B) The area of the infiltrate delimited on microscopy slides. The proportion of this area occupied by $B220^+$ cells was determined in salivary glands of NOD mice treated with anti-BAFF antibody (n=9) and isotype control (n=6). (C) Representative photograph of a salivary gland embedded in paraffin and stained with DAPI (blue), CD3 (green) and B220 (pink) from an anti-BAFF-treated NOD mouse. (D) Representative photograph of a salivary gland embedded in paraffin and stained with DAPI (blue), CD3 (green) and B220 (pink) from an isotype control-treated NOD mouse. (E) Representative flow cytometry plots illustrating the proportion of B cells ($CD19^+$) among lymphocytes from a BAFF-treated NOD mouse. (F) Representative flow cytometry plots illustrating the proportion of B cells ($CD19^+$) among lymphocytes from an isotype control-treated NOD mouse. Mann-Whitney: NS: non-significant; *p<0.05, **p<0.01, ****p<0.0001. Data are median (IQR).

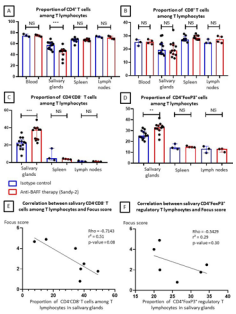

Figure 3 Modifications of T-cell subpopulations. (A) Proportion of $CD4^+$ T cells among all T cells in blood, salivary glands, spleen and lymph nodes of NOD mice treated with anti-B-cell activating factor (BAFF) antibody (n=4, 13, 8 and 4, respectively) or the isotype control (n=3, 12, 8 and 3, respectively). (B) Proportion of $CD8^+$ T cells among all T cells in blood, salivary glands, spleen and lymph nodes of NOD mice treated with anti-BAFF antibody (n=4, 13, 9 and 4, respectively) or the isotype control (n=3, 12, 8 and 3, respectively). (C) Proportion of $CD4^-CD8^-$ T cells among all T cells in salivary glands, spleen and lymph nodes of NOD mice treated with anti-BAFF antibody (n=13, 4 and 4, respectively) or the isotype control (n=12, 3 and 3, respectively). (D) Proportion of $CD4^+FoxP3^+$ T cells among all T cells in salivary glands, spleen and lymph nodes of NOD mice treated with anti-BAFF antibody (n=13, 4 and 3, respectively) or the isotype control (n=12, 3 and 3, respectively). Data are median (IQR). (E) Correlation between the proportion of salivary $CD4^-CD8^-$ T cells among all T cells and the focus score for NOD mice treated with anti-BAFF antibody (n=4) or the isotype control (n=3). (F) Correlation between the proportion of salivary $CD4^+FoxP3^+$ regulatory T cells among all T cells and the focus score for NOD mice treated with anti-BAFF antibody (n=4) or the isotype control (n=2). Mann-Whitney and Spearman correlation: **p<0.01, ***p<0.001. NS, not significant.

Anti-BAFF antibody treatment decreased the proportion of $CD4^+$ T cells in SGs but not blood, spleen or lymph nodes as compared with the isotype control (median proportion of $CD4^+$ T cells among total T cells in SGs: 46.8% (41.8–49.1) vs 57.4% (50.5–61.9), p=0.0007; blood: 74.6% (72.9–76.1) vs 74.7 (72.7–77.6), p=0.86; spleen: 66.8% (65.1–70.4) vs 67.6% (64.1–68.2), p=0.91; lymph nodes: 70.9% (68.4–73.7) vs 72.7% (70.6–73.7), p=0.86; figure 3A). The two treatments did not differ in proportion of $CD8^+$ T cells in SGs, blood, spleen or lymph nodes (median proportion of $CD8^+$ T cells among total T cells in SGs: 19.0% (15.8–23.5) vs 19.3% (16.5–24.3), p=0.55; blood: 25.4% (23.9–27.2) vs 25.3 (22.5–27.3), p=0.86; spleen: 28.5% (27.2–30.2) vs 27.2% (26.5–29.7), p=0.19; lymph nodes: 27.5% (24.9–29.8) vs 24.7% (24.6–27.5), p=0.46; figure 3B).

Anti-BAFF antibody treatment increased the proportion of $CD3^+CD4^-CD8^-$ double negative (DN) T cells in SGs but not spleen or lymph nodes as compared with the isotype control (proportion of $CD3^+CD4^-CD8^-$ DN T cells among total $CD3^+$ T lymphocytes in SGs: 37.5% (26.5–39.8) vs 22.7% (17.3–26.5), p=0.0007; spleen: 3.9% (3.5–4.6) vs 4.8% (2.8–16.2), p=0.63; lymph nodes: 1.1%

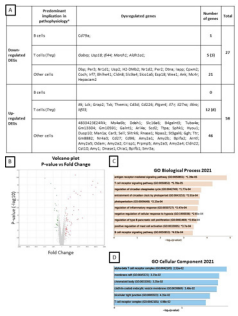

Figure 4 Transcriptomic analysis. (A) List of differentially expressed genes (DEGs) (absolute fold change in expression >2, p<0.05) and their known predominant implication in the pathophysiology of B cells, T cells, T regulatory cells (Tregs) and others. Among those involved in T-cell pathophysiology, those related to Tregs are in italics and their numbers are in brackets. *Some genes might be involved in different pathways but were mentioned only once. (B) Volcano plot. The log2 fold change indicates the mean expression level for each gene. Each dot represents one gene. After B-cell activating factor (BAFF) inhibition, grey dots represent no significant DEGs between the anti-BAFF therapy group and the isotype control group; the green dots represent downregulated genes and red dots upregulated genes. (C) Gene Ontology (GO) classifications of DEGs. With GO functional enrichment, 'biological process', 'molecular function' and 'cellular component' are ranked by p value (all p<0.05). (D) Kyoto Encyclopedia of Genes and Genomes pathway enrichment of DEGs.

(0.9–1.4) vs 1.3% (0.9–2.1), p=0.94; figure 3C). The proportion of salivary DN $CD4^{-}CD8^{-}$ T cells tended to be inversely correlated with the FS (rho=–0.7143, r^2=0.51, p value=0.08; figure 3E).

Regulatory T-cells

Anti-BAFF antibody treatment increased the proportion of $Foxp3^{+}$ regulatory T cells in SGs but not spleen or lymph nodes as compared with the isotype control (median proportion of $Foxp3^{+}$ regulatory T cells among total $CD4^{+}$ T cells in SGs: 33.0% (30.7–34.9) vs 25.4% (23.4–29.6), p=0.0014; spleen: 14.9% (14.7–15.8) vs 14.3% (13.0–17.9), p=0.63; lymph nodes: 13.1% (10.2–13.3) vs 13.3% (9.6–15.6), p=0.80; figure 3D). The proportion of salivary $Foxp3^{+}$ regulatory T cells was not significantly correlated with the FS (rho=–0.5429, r^2=0.29, p value=0.30; figure 3F).

Transcriptomics analysis of therapy-induced gene expression change in SGs

We used transcriptomic analysis to analyse mRNA SG expression. A total of 85 genes were significantly differentially expressed: 27 downregulated and 58 upregulated (figure 4A,B). On GO analysis, 'biological process' and 'cellular component' terms were identified (p<0.05; figure 4C,D). The antigen receptor-mediated and T-cell receptor signalling pathways were the two most enriched biological processes ($p=1.39\times10^{-5}$ and 5.78×10^{-5}, respectively). The B-cell receptor signalling pathway was also significantly enriched ($p=4.03\times10^{-4}$). 11 (13%) DEGs were related to the homeostasis and function of T regulatory cells (Tregs; figure 4A). Treg signature genes, Foxp3, Ctla4 and Il2ra were all upregulated after BAFF inhibition (Foxp3: adjusted fold-change=1.3, p value=0.022; Ctla4: adjusted fold-change=1.46, p value=0.035; Il2ra: adjusted fold-change=1.26, p value=0.71) although it did not reach the predefined definition of DEGs (adjusted fold change >2 and p value <0.05).

DISCUSSION

The present results demonstrate a significant decrease in lymphocytic infiltration of SGs and a significant improvement in salivary flow with BAFF inhibition. They confirm the pathogenic role of BAFF and B cells in a model of SjD.

No previous data were available regarding the efficacy of specific BAFF inhibition in animal models of SjD. Of note, BAFF and APRIL inhibition using BCMA-Fc ameliorated insulitis in NOD mice,[21] but the effect of their inhibition on salivary infiltrates and salivary flow was not studied. TACI-Fc gene therapy to inhibit BAFF and APRIL, reduced SG lymphocytic infiltrates in NOD mice but had no effect on dryness.[22] Overexpression of ΔBAFF, a physiological inhibitor of BAFF, in SGs decreased SG infiltrates and improved salivary flow in the NOD mouse model,[23] with no marked decrease in BAFF expression.

In the present study, the efficacy of BAFF-specific inhibition was related to deep immunological changes affecting not only B but also T cells. First, the treatment with this mouse monoclonal antibody against BAFF markedly depleted B cells, as expected and previously reported.[14] The decrease in B-cell proportion was even more striking in SGs and lymph nodes rather than in blood or spleen, which might be related to the higher dependence on BAFF of auto-reactive B cells that infiltrate SGs than allo-reactive B cells, which are more abundant in blood and spleen.[24] In addition, transitional B cells, the stage for which B-cell differentiation requires BAFF, do not express CD62L, which allows more mature B cells to home to lymph nodes.[25] The decrease in dryness in the present study might result from both BAFF inhibition and B-cell depletion, as also observed in a trial combining rituximab and belimumab[8] and in one using a B cell-depleting BAFF receptor-blocking monoclonal antibody, ianalumab.[9 26]

Indeed, several arguments suggest that targeting BAFF and B cells could have a synergistic effect.[27] The ectopic expression of BAFF in inflamed tissues was found an important factor mediating the resistance to B-cell depletion by rituximab.[28 29] The upregulation of BAFF after B-cell depletion could contribute to the repopulation of autoreactive B cells.[30] Plasmablasts can persist in the peripheral blood after rituximab treatment because they do not express CD20 and because of BAFF-driven survival.[31] In addition, the increase in circulating memory B cells observed after treatment with belimumab, a BAFF-blocking antibody, likely renders B cells more susceptible

to rituximab-mediated depletion.[32] Although our study did not specifically investigate the impact of anti-BAFF treatment on memory B cells and plasmablasts, we do have some data from clinical trials on the use of sequential treatment combining belimumab and rituximab[8] or ianalumab.[9 26] In patients treated sequentially with rituximab and belimumab, total peripheral blood B cells, as well as other B cell subsets in the circulation, were almost completely depleted. Plasmablasts decreased. In SG, near total depletion of B cells was observed and memory B cells were depleted at week 24.[8] In patients treated by ianalumab, substantial depletion occurred within 24 hours in the peripheral blood mature, naive, memory and transitional B cell subsets (data not shown). By the end of the study, these B cell subpopulations had returned to baseline levels with the exception of memory B cells which were increased relative to naive B cells.[26]

The role of BAFF on T-cell function has been known for decades but is often sidelined by the choice to focus on the impact of BAFF on B cells.[33] In the present study, BAFF inhibition increased regulatory T-lymphocyte populations, which might contribute to the therapeutic efficacy observed. The increase in proportion of $Foxp3^+$ regulatory T cells agrees with three studies evaluating B-cell targeted therapies (anti-CD20,[34] anti-CD22[35] and BCMA-Fc[21]) in diabetes in NOD mice but not with another study evaluating BAFF inhibition.[36] However, the protective role of $Foxp3^+$ regulatory T cells remains uncertain in patients with SjD.[37–40] In humans, belimumab therapy could restore the Treg/T helper 17 cell balance in patients with refractory systemic lupus erythematosus and the number of Tregs increased after belimumab therapy and were fully functional.[41] Of note, the regulatory function of Tregs after treatment could not be analysed in the present study. In patients with SjD treated with rituximab and belimumab, at week 24, no change in the count of T cells ($CD3^+$) or their subsets ($CD3^+CD4^+$ $CD3^+CD8^+$) was observed.[8] At day 2 after ianalumab exposure, transient reductions in T cells and natural killer cells were reported without any additional precision.[26]

The proportion of another potentially regulatory T-cell population, $CD3^+CD4^-CD8^-$ cells, was increased after BAFF inhibition and B-cell depletion in SGs. In NOD mice, $CD4^+$ T cells converted to pancreatic islet B-cell antigen-specific DN T cells can prevent the development of autoimmune diabetes and promote islet allograft survival.[42] These splenic DN T cells from young NOD mice also provided long-lasting protection against diabetes transfer in NOD/Scid immunodeficient mice.[43] We found an increased proportion of $CD3^+CD4^-CD8^-$ cells in SGs, which tended to be inversely correlated with the FS which suggests a regulatory role of this population in mice. The expansion of these DN cells might also explain the decrease in total $CD4^+$ T cells in SGs that may be 'converted' within SGs (ie, lose their $CD4^+$ expression). In patients with SjD, a higher proportion of DN T cells in SGs was found associated with more severe tissue inflammation, presence of germinal centre-like structures and major xerostomia.[44] In addition, these cells have also been described as proinflammatory and interleukin (IL)-17 secreting cells.[45] This increase in proportion of DN T cells with disease severity can reflect a compensatory but insufficient regulatory mechanism in patients with SjD or a different role of DN T cells in humans and mice.

Our transcriptomic results complemented those of flow cytometry on the impact on T cells, BAFF inhibition and B-cell depletion. The antigen receptor-mediated, T-cell receptor and B-cell signalling pathways were the most modulated biological processes, in agreement with the fact that BAFF co-opts the B-cell receptor pathway through the PI3K pathways[46]; in addition, BAFF provides a complete costimulation effect into T cells.[47] Cd79a, also known as B-cell antigen receptor complex-associated protein alpha chain, was significantly downregulated. CD79a is expressed on the surface of B cells throughout their life cycle.[48] The association of the CD79a/b heterodimer with immunoglobulin heavy chain is required for surface expression of the B-cell receptor.[49] Differential expression of genes involved in Treg ontogeny might reflect the impact of BAFF-specific inhibition and B-cell depletion on Tregs. Thus, the IL-7 receptor, which supports the functional activity of effector Tregs by increasing their IL-2 sensitivity[50]; Ccl21b, increasing Treg function and migration[51]; and Il-27ra, essential for $Foxp3^+$ Treg suppressive function[52] were upregulated in treated mice in the present study. Nr4a3, expressed by Tregs and B regulatory cells, was upregulated after BAFF-specific inhibition. Of note, excess BAFF was recently found to decrease Nr4a3 expression and B regulatory cell function.[53] Therefore, the results of the transcriptomic analysis agree with those of flow cytometry regarding the increase in regulatory populations after BAFF blockade and B-cell depletion.

Our study, for the first time, provides valuable preclinical evidence supporting the efficacy of BAFF inhibition in a murine model of SjD. The significant reduction in SG infiltrates and improvement in salivary flow underscore the potential therapeutic impact. We present the immunological characterisation, encompassing the effects of anti-BAFF treatment on the main B and T-cell subsets. The inclusion of transcriptomic analyses adds to the understanding of the immune response.

Limitations of the present study include the absence of more in-depth blood and SG immunophenotyping (including regulatory B cells), of assessment of Treg function and of serum autoantibody levels. Of note, in the randomised trial evaluating the combination of rituximab and belimumab in patients with SjD, no significant reduction was observed in anti-SSA/SSB levels.[8] Regarding the potentially different effect of BAFF inhibition depending on B-cell subsets, BAFF inhibition decreased effector B cells while sparing regulatory B cells in a model of systemic sclerosis.[54] Future perspectives could also incorporate more detailed histological analyses, focusing on markers associated with

germinal centres, to unravel the dynamics of immune responses within the SGs after BAFF inhibition and B-cell depletion.

In conclusion, a monoclonal antibody that blocks BAFF and depletes B cells significantly improved dryness and markedly reduced SG lymphocytic infiltrates in the NOD mouse model of SjD. The increase in regulatory T-lymphocyte populations might underlie the efficacy of BAFF inhibition and B-cell depletion. This study strengthens the rationale to target BAFF and B cells in SjD.

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