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Immunopathology of primary Sjögren's syndrome (role of B lymphocyte, FLT3 ligand and BAFF) and the clinical consequences

Gabriel J. Tobon

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Gabriel J. TOBON

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Immunopathology of Primary Sjögren's Syndrome (Role of B lymphocyte, FLT3-Ligand and BAFF) and the clinical consequences

Thèse soutenue le 4 Juin 2012

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UNIVERSITE DE BRETAGNE OCCIDENTALE

FACULTE DE MEDICINE

THESE DE DOCTORAT

**IMMUNOPATHOLOGY OF PRIMARY SJÖGREN'S
SYNDROME (ROLE OF B LYMPHOCYTE, FLT3 LIGAND
AND BAFF) AND THE CLINICAL CONSEQUENCES**

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**Pour l'obtention du titre
du Docteur de l'Université de Bretagne Occidentale**

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ABSTRACT

Primary Sjögren's Syndrome (pSS) is a systemic autoimmune disease characterized by sicca symptoms and a broad variety of systemic clinical manifestations. The most severe complication of the disease is the development of non-Hodgkin's lymphoma in 5% of patients. Although many progresses have been done in basic research of pSS, the etiology of this disease remains unknown. The classic immunological concept ascribed a key role to the T-cell. However, recent evidence indicates a major contribution of B cells. In this work, we developed clinical and basic research subjects, related to the role of B-cell in the pathogenesis of pSS. **In the first section**, we evaluated the contribution of skin biopsy in the diagnosis of pSS. In this study, we showed that memory B-cell infiltrates are present in pSS and may be used as an additional diagnostic and follow-up tool. **In the second section**, prompted by the reports on the effects of the cytokine FLT3-L on lymphocyte development, lymphoid malignancies, B-lymphocyte alterations, and the risk of developing lymphoma, we evaluated the role of FLT3-L in pSS. In these studies, we found that serum levels of FLT3-L are high in pSS and these levels are associated with abnormal B-cell distribution, characteristic of pSS; and disease clinical activity. In addition, this cytokine may explain the development of lymphoma in some patients. FLT3-L proliferation effect may explain also survival of infiltrating B cells in exocrine glands of pSS patients. Thus this cytokine can be use as marker of activity, severity and association with lymphoma in pSS patients. **In the third section**, we studied the role of a new variant of BAFF, called Δ 4BAFF (due to the alternative splicing of exon 4) which has been recently described in our laboratory. Interestingly, this new variant is mainly detected in autoimmune diseases (including mononuclear and epithelial cells from pSS patients). Thus, our objective was to understand the role of this new variant and the study of the alternative splicing of the exon 4. We confirmed that Δ 4BAFF acts in association with p50 from the NF- κ B pathway as a transcription factor for its own parent gene. Then, to study the mechanisms implicated in the alternative splicing of *BAFF* exon 4, we used a minigene construction, and coding plasmids for splicing proteins (SR and hnRNP proteins). We found that one of the SR proteins (SC35) is essential to full-length BAFF mRNA splicing decreasing Δ 4BAFF expression. Subsequently, we analyzed the mechanisms of induction and regulation of Δ 4BAFF and the effects of IFN- γ (a cytokine known as a positive regulator of BAFF) on Δ 4BAFF expression. We demonstrated that IFN- γ induces Δ 4BAFF, due to an abnormal function of SC35 leading to an increase of BAFF. This finding provides an expanded conceptual view of *BAFF* gene regulation in autoimmune diseases, and contributes to a better understanding of the mechanisms involved in BAFF up-regulation in autoimmunity. Collectively, these results are of clinical and fundamental basic interest in pSS, in the diagnostic, physiopathology and therapeutic contexts.

Key-words: primary Sjögren's syndrome, B-cell, cytokines, FLT3-Ligand, BAFF, Δ 4BAFF, SC35, IFN- γ .

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ABBREVIATIONS

| | |
|--------------|---|
| Abs | Antibodies |
| ACh | Acetylcholine |
| AECG | American-European Community Study Group |
| AIDs | Autoimmune diseases |
| AIRE | Autoimmune regulator |
| ALL | Acute lymphoblastic leukemia |
| AML | Acute myeloid leukemia |
| ANAs | Antinuclear antibodies |
| APC | Antigen presenting cell |
| APRIL | A proliferation-inducing ligand |
| ATF | Activating transcription factor |
| BAFF | B-lymphocyte Activator Factor belonging to the TNF family |
| BCA-1 | B-cell-attracting chemokine 1 |
| Bcl-2 | B-cell lymphoma 2 |
| BCMA | B-cell maturation antigen |
| BCR | B-cell receptor |
| BLyS | B-lymphocyte stimulator |
| BM | Bone marrow |
| Breg | Regulatory B cells |
| CCP | Cyclic citrullinated peptide |
| CML | Chronic myeloid leukemia |
| CREB | cAMP response element binding protein |
| CRP | C-reactive protein |
| DCs | Dendritic cells |
| DHEA | Dehydroepiandrosterone |

| | |
|---------------|---|
| DLBCL | Diffuse large B cell lymphoma |
| EBV | Epstein-Barr virus |
| ECs | Epithelial cells |
| ELISA | Enzyme-linked immunosorbent assay |
| EMSA | Electrophoresis Mobility Shift Assay |
| ENAs | Extractable nuclear antigens |
| ER | Estrogen receptor |
| ERK | Extracellular-signal regulated kinase |
| ESEs | Exonic splicing enhancers |
| ESR | Erythrocyte sedimentation rate |
| ESSs | Exonic splicing silencers |
| ESSDAI | EULAR SS Disease Activity Index |
| ESSPRI | EULAR SS Patient Reported Index |
| EULAR | European League Against Rheumatism |
| FDC | Follicular Dendritic Cells |
| FDR | First-degree relatives |
| Fik-2 | Fetal liver kinase-2 |
| FLT3-L | Fms-like tyrosine kinase 3-Ligand |
| GAS | Gamma-Activated Sequences |
| GC | Germinal center |
| GR | Glucocorticoid receptor |
| GWA | Genome-wide association |
| GWB | GW bodies |
| HLA | Human Leukocyte Antigen |
| hnRNP | Heterogeneous nuclear ribonucleoprotein |
| HSC | Hematopoietic stem cells |
| HSG | Human salivary gland |
| HTLV-1 | Human T-lymphotropic virus-1 |

| | |
|---------------|--------------------------------------|
| ICAM-1 | Intracellular Adhesion Molecule-1 |
| IFI | Indirect immunofluorescence |
| IFN | Interferon |
| IGCs | Interchromatin granule clusters |
| Igs | Immunoglobulins |
| IL | Interleukin |
| IP3 | Inositol 1,4,5-triphosphate |
| IRF | Interferon-responsive factors |
| ISGs | IFN-stimulated genes |
| ISEs | Intronic splicing enhancers |
| ISREs | IFN-stimulated regulatory elements |
| ISSs | Intronic splicing silencers |
| ITDs | Internal tandem duplications |
| JAK | Janus activated kinase |
| KCS | Keratoconjunctivitis sicca |
| LPS | Lypopolysaccharide |
| LT | Lymphotoxin |
| mAbs | Monoclonal antibodies |
| MALT | Mucosa-associated lymphoid tissue |
| MAPK | Mitogen-activated protein kinase |
| M-CSF | Macrophage colony-stimulating factor |
| MSG | Minor salivary gland |
| MZ | Marginal zone |
| NEMO | NF-κB essential modulator |
| NFAT | Nuclear factor of activated T cells |
| NHL | Non- Hodgkin's lymphoma |
| NIK | NF-κB-inducing kinase |

| | |
|---------------|--|
| NK | Natural killer |
| NLS | Nuclear localization sequence |
| NMD | Nonsense-mediated decay |
| NOD | Non-obese diabetic |
| OSC | Ocular Sicca Score |
| PBMCs | Peripheral blood mononuclear cells |
| pDCs | Plasmacytoid dendritic cells |
| PI3K | Phosphatidylinositol 3'-kinase |
| pSS | Primary Sjögren's syndrome |
| PTCs | Premature termination codons |
| PTP | Protein tyrosine phosphatases |
| RA | Rheumatoid Arthritis |
| RbAp48 | Retinoblastoma-associated protein 48 |
| RBD | RNA-binding domains |
| RF | Rheumatoid Factor |
| RRM | RNA recognition motif |
| RTX | Rituximab |
| SCAI | Sjögren's Systemic Clinical Activity Index |
| SCF | Stem cell factor |
| SGs | Salivary glands |
| SGUS | Salivary glands ultrasound |
| SHIP | Src-homology 2 containing inositol phosphatase |
| SHP2 | Src-homology 2 containing protein tyrosine phosphate |
| SLE | Systemic lupus erythematosus |
| SLO | Secondary lymphoid organs |
| SNPs | Single nucleotide polymorphism |
| SRPKs | SR protein kinases |
| ss | Splice sites |

| | |
|--------------------------------|--|
| SS | Sjögren's syndrome |
| SSA | Sicca-syndrome A |
| SSB | Sicca-syndrome B |
| SSDAI | SS Disease Activity Index |
| SSDDI | SS Disease Damage Index |
| SSDI | SS Damage Index |
| STAT | Signal transducer and activator of transcription |
| T1D | Type 1 diabetes |
| TACI | Transmembrane activator and calcium modulator and cyclophilin ligand interactor |
| TCR | T-cell receptor |
| TGF-β | Transforming-growth factor beta |
| TLR | Toll-like receptor |
| TNF-α | Tumor necrosis factor-alpha |
| TRAF3 | TNF receptor associated factor 3 |
| Treg | Regulatory T cells |
| TrpRS | Tryptophanyl-tRNA synthetase |
| TSLP | Thymic stromal lymphopoietin |
| TWEAK | TNF-like weak inducer of apoptosis |
| U2AF | U2 snRNP auxiliary factor |
| UTR | Untranslated region |
| VCAM-1 | Vascular Cell Adhesion Molecule 1 |

INTRODUCTION

1. Primary Sjögren's Syndrome

1.1. Introduction

Autoimmune epithelitis which is also known as Sjögren's Syndrome (SS), occurs either alone, as primary SS (pSS), or associated with other autoimmune diseases, as secondary SS (Moutsopoulos, 1994). Even though keratoconjunctivitis sicca (KCS) (resulting from the involvement of lacrimal glands), and xerostomia (resulting from that of salivary glands-SGs) are usually prominent, SS presents as a multifaceted and systemic condition with a broad variety of clinical manifestations. The spectrum of SS extends from an organ-specific autoimmune disorder (referred to as an autoimmune exocrinopathy) to a systemic process that may involve the musculoskeletal system, nervous system, lungs, kidneys and blood vessels (Tzioufas et al., 2007). Biological abnormalities associated with B lymphocytes in SS are also a hallmark of the disease and these abnormalities are characterized by the presence of rheumatoid factor (RF), hypergammaglobulinemia, anti-sicca syndrome A/Ro (SSA) and anti-sicca syndrome B/La (SSB) antibodies (Sheldon, 2004), abnormal distribution of mature B lymphocytes in peripheral blood (Binard et al., 2009), in addition to an increased risk of non-Hodgkin's lymphoma (NHL) in 5% of patients (Voulgarelis et al., 1999).

1.2. History of pSS

The first description of SS is generally credited to Mikulicz, who in 1892 described a 42-year old man with bilateral enlargement of the parotid and lacrimal glands associated with a small-round-cell infiltrate (Mikulicz, 1892). However, the term Mikulicz's syndrome could comprise many different entities, including tuberculosis, other infections, sarcoidosis, and lymphoma, and it fell into disuse because it did not provide sufficient prognostic or therapeutic information. The term is still used occasionally to describe the histological appearance of focal lymphocytic infiltrates on SG biopsy samples (Morgan et al., 1953) and more recently to describe a subtype of IgG4-related disease (Yamamoto et al., 2004). In 1933, the Danish ophthalmologist Sjögren described the clinical and histological findings in 19 women, 13 of whom had probable rheumatoid arthritis (RA), with dry mouth and eyes. Sjögren introduced the term KCS for this syndrome, to distinguish it from dry eyes caused by lack of vitamin A (xerophthalmia). This work was included in his thesis called "Zur kenntniss der keratoconjunctivitis sicca". In 1953, Morgan and Castleman presented a case study of

a patient with SS in a clinical pathological conference and rekindled interest in the disorder originally known as Mikulicz's disease; subsequently this disorder been termed SS. The clinical features, as the syndrome in its florid form is currently recognized, were outlined in 1956 by Bloch and colleagues (Bloch et al., 1956).

1.3. Epidemiology of pSS

pSS is described all around the world. The disease predominantly affects females (female/male ratio 9/1) during the fourth and fifth decades of life. The few epidemiological studies of pSS in the general population yielded highly heterogeneous results, probably because of differences in diagnostic criteria set and study design. As a result, the prevalence and incidence of pSS in the general population are unclear (Binard et al., 2007). The prevalence estimates varied widely from 0.02% in the general Japanese population (Miyasaka et al., 1995) to 3.59% in United Kingdom (Thomas et al., 1998). These wide variations may partly reflect methodological differences among studies. However, these extreme variations are not likely to be due only to methodological differences among studies and ethnic and environmental differences may explain these variations. In Europe, the prevalence can be estimated at 0.5% to 1% (Binard et al., 2007). Concerning the incidence, three studies evaluated this item in the general population, ranging from 3.9 to 5.3 cases per 100.000 inhabitants. **Table 1** summarizes the incidence in pSS. Epidemiological evolution of pSS and others autoimmune diseases (AIDs) is reviewed in the **appendix 1 (évolution épidémiologique des maladies ostéoarticulaires. *La Lettre du Rhumatologue* 2010;361:4-6).**

1.4. Clinical picture

pSS has usually a slow and benign course, characterized by clinical non-specific manifestations and approximately 6 years elapse from the initial symptoms to the diagnosis (Pavlidis et al., 1982). Exocrine tissues, most notably lacrymal and SGs, exhibit structural lesions characterized by focal lymphocytic infiltrates surrounding the tubular epithelium. Glandular dysfunction leads to main clinical manifestations (sicca syndrome).

Table 1. Incidence rates (and 95% CIs) of pSS in studies carried out in adult general populations (cases/100,000).

| Study | Population size | Study design | Classification criteria | Country | Incidence (95% CI) |
|--------------------------------|---|------------------|---------------------------------|----------|--------------------|
| Pillemer et al., 2001 | ~ 100,000 (total population of Olmsted County, Minnesota) | Population-based | Physician Diagnosis | USA | 3.9 (2.8–4.9) |
| Plesivcnik et al., 2004 | ~ 600,000 (total population of Ljubljana region) | Referral center | European (Vitali et al., 1993). | Slovenia | 3.9 (1.1–10.2) |
| Alamanos et al., 2006. | ~ 500,000 (total population of NW Greece) | Population-based | AECC (Vitali et al., 2002). | Greece | 5.3 (4.5–6.1) |

1.4.1. Sicca syndrome and glandular manifestations

Diminished tear production leads to the destruction of both corneal and bulbar conjunctival epithelium and the clinical findings of KCS. Patients' complains are burning, sandy or scratchy sensation under the lids, itchiness, redness and photosensitivity. Decreased production of saliva by the SGs is responsible of xerostomia and secondary manifestations such as dental caries, oral candidiasis, bacterial sialadenitis, and oral ulcers. Patients report difficulty to swallow food, inability to speak continuously, changes in sense of taste and burning sensation in the mouth. Dryness can affect the upper respiratory tract as well as the pulmonary tissue, causing hoarseness, recurrent bronchitis and pneumonitis. Patients may also experience skin dryness and loss of vaginal secretions. Although sicca symptoms are the main clinical manifestations in pSS, these symptoms are also common in other pathologies, and even in elderly individuals (ranging from 20 to 30%). Parotid or major SGs enlargement occurs in 60% of patients with pSS. In the majority of cases, this enlargement is episodic and bilateral, whereas others patients present chronic persistent enlargement. Some authors have described that persistent enlargement of SGs is a factor of high risk to NHL development (Sutcliffe et al., 1998; Anaya et al., 1996).

1.4.2. Extraglandular manifestations

Systemic manifestations occur in approximately 50% of patients. They include general constitutional symptoms, such as fatigability, low-grade fever, myalgias, arthralgias and other organ involvement. Fatigue is often one of the most important complains and occurs in around 50% of cases. Systemic manifestations can be subdivided into non-visceral (skin, arthralgia, myalgia) and visceral (lung, heart, kidney, gastrointestinal, endocrine, central and peripheral nervous system) manifestations. These manifestations can also be classified according to the localization in periepithelial and extraepithelial organ involvement. The first group includes interstitial nephritis, liver involvement and obstructive bronchiolitis, and usually they appear in the first years of disease. In the second group (extraepithelial involvement), the presence of palpable purpura, glomerulonephritis and peripheral neuropathy are produced by immune complex deposition as a result of B-cell hyperactivity. These last manifestations are usually observed late in the disease and are associated with increased morbidity and risk of lymphoma (Tzioufas et al, 2007). **Table 2** summarizes the frequency of systemic manifestations in pSS patients. Neurological involvement is widely reviewed in the **appendix 2 (neurological manifestations in primary Sjögren's syndrome. *Autoimmune Dis* 2012:645967).**

Table 2. Frequency of systemic manifestations in primary Sjögren's syndrome.

| Manifestation | Frequency (%) |
|---------------------------|---------------|
| Arthralgia/arthritis | 75 |
| Raynaud's phenomenon | 50 |
| Purpura | 11 |
| Pulmonary involvement | 23 |
| Peripheral neuropathy | 20 |
| Primary biliary cirrhosis | 4 |
| Interstitial nephritis | 9 |
| Glomerulonephritis | 2 |
| Myositis | 1 |
| Lymphoma | 5 |

1.5. Diagnosis

The clinical polymorphism accounts for the delay in the diagnosis around 6 years after the initial symptoms. Although classification criteria of the disease have been defined by the American-European Community Study Group (AECG) on diagnostic criteria for

SS (Vitali et al., 2002); the diagnosis in some patients may be difficult and delayed (**Table 3**). This set of criteria is based in two subjective items of sicca phenomenon and four objectives, including histopathology and autoantibodies (auto-Abs). To define pSS, 4 of 6 items must be positive with at least the item IV (histopathology) or VI (auto-Abs). This classification is more rigorous than some others previously used because it requires the demonstration of an autoimmune involvement of the SGs. In the initial cohort of 180 patients with pre-defined conditions (76 patients with clinically diagnosed pSS, 41 with connective tissue disease other than SS, and 63 with sicca symptoms but no SS), the AECG showed good diagnostic accuracy compared to a clinical diagnosis of pSS made by an expert, with 89.5% sensitivity and 95.2% specificity.

Among the objective parameters, the most useful in clinical practice are the minor SGs (MSG) biopsy, unstimulated salivary flow, ocular evaluation and auto-Abs determination (described in biologic abnormalities).

1.5.1. Minor salivary gland biopsy

The accuracy of SG biopsy is well established and focal sialoadenitis in MSG biopsy is one of the main criteria of the revised classification criteria proposed by the AECG group (Colella et al., 2010). The histopathologic characteristics of MSG biopsy include: focal aggregates of at least 50 lymphocytes, plasma cells and macrophages adjacent to and replacing the normal acini and the consistent presence of these foci in all or most of the glands in the specimen. In 1984, Daniels evaluating 362 lower lip biopsies concluded that a focus score ≥ 1 per 4 mm² tissue sample was indicative of SS (Daniels, 1984). The focus score correlates directly with the presence of KCS and inversely with the parotid flow rate (Daniels and Whitcher, 1994). Histopathologic report can be done in terms of focus score ≥ 1 (more than 50 lymphocytes per 4 mm² of glandular tissue) or using the score proposed by Chisholm and Masson in 1968 (Chisholm and Masson, 1968) (**Table 4**). Often associated with such inflammation are acinar epithelial cells (EC) atrophy, progressive fibrosis and the presence of adipocytes in the SGs. However it must be remembered that focal sialadenitis can occur in conditions other than pSS. Focal or diffuse inflammatory infiltration has been reported in cases of sialolithiasis (Segeberg-Konttinen, 1988), other AIDs, in the elderly, chronic hepatitis C virus infections, and sarcoidosis. Moreover, a focus score ≥ 1 has been found in 15% of healthy asymptomatic volunteers without correlation with age, smoking, serologic findings, or salivary flow (Radfar et al., 2002). This infiltrate

has also been reported between 7 to 35% of biopsies in postmortem studies of submandibular glands from subjects who had no clinical evidence of pSS during life (Al-Hashimi et al., 2001; Segerberg-Konttinen et al., 1986; Takeda et al., 1986).

Table 3. American-European classification criteria for Sjögren’s syndrome. (Vitali et coll., 2002). Exclusion criteria: Past head and neck radiation treatment, hepatitis C infection, AIDS, pre-existing lymphoma, sarcoidosis, graft versus host disease, and use of anticholinergic drugs.

I. Ocular symptoms: (Positive answer to at least one of three questions : dry eyes for more than 3 months, recurrent sensation of sand in the eyes, and use of tear substitutes more than three times a day).

II. Oral symptoms: (Positive answer to at least one of three questions : daily feeling of dry mouth for more than 3 months, recurrent of persistent swollen SGs, frequent need to drink liquids to aid in swallowing dry food)

III. Ocular signs: positive result for at least one of the following two tests: Schirmer’s test, performed without anesthesia (\square 5 mm in 5 minutes), and Rose Bengal score or other ocular dye score ($>$ 4 according to van Bijsterveld’s scoring system).

IV. Histopathology: In minor SGs obtained through normal-appearin mucosa, focal lymphocytic sialoadenitis with a focus score \geq 1, defined as a number of lymphocytic foci (which are adjacent to normal-appearing mucous acini and contain more than 50 lymphocytes) per 4 mm² of glandular tissue.

V. Test for SGs involvement: a positive result for at least one do the following diagnostic tests: unstimulated whole salivary flow (\leq 1.5 mL in 15 minutes); parotid sialography revealing diffuse sialectasias; salivary scintigraphy showing delayed uptake, reduced concentration and/or delayed excretion of tracer.

VI. Auto-Abs: Serum Abs to SSA (Ro) and SSB (La) antigens, or both.

Table 4. Chisholm and Masson grading score for salivary glands biopsy.

| Grade | Lymphocytes per 4 mm ² of Salivary Tissue |
|-------|--|
| 0 | Absent |
| 1 | Slight infiltrate |
| 2 | Moderate infiltrate or less than one focus |
| 3 | One focus |
| 4 | More than one focus |

Focus: an aggregate of 50 or more lymphocytes.

1.5.2. Whole Saliva Flow Collection

This is the simplest test in the determination of salivary flow. Flow rates ≥ 0.3 mL/min are considered normal (Speight et al., 1992). The collection may be performed with or without stimulus. Stimulated parotid flow may not correlate with the symptoms and may be normal even if basal secretion is reduced. On the other hand, the unstimulated salivary flow reflects the basal flow from all glands taking place during most of the day. It is easy to measure and reproducible. The unstimulated salivary flow is measured by the spitting method. Subjects are instructed to spit into a graduated test tube every minute. Saliva is collected for a period of 15 min and the measured volume expressed in mL/min (1 mg = 1 ml) (Navazesh et al., 1993). A shortened version measures the volume in a 5 min period. A value inferior to 1.5 mL/15 min is considered abnormal in the AECG criteria (Vitali et al., 2002). This method has a sensitivity of 56.1%–64% and a specificity of 80.7% for SS (Vitali et al., 1994).

1.5.3. Ocular tests

The tests used most frequently to assess the integrity of the corneal surface and the volume and quality of tear film are the Schirmer test, Rose Bengal staining, fluorescein or lissamine green staining, and the tear break-up time (Goren and Goren, 1988; Vitali et al., 1994). All tests for evaluation are sensitive but not specific for pSS, as KCS can occur in a number of other conditions. **Table 5** summarizes the main ocular test and the abnormal results suggestive of pSS. Recently, Whitcher et al. (Whitcher et al., 2010) described the Ocular Sicca Score (OSS), a new simplified quantitative grading system for KCS; including the Schirmer's test, tear break-up time, ocular surface staining and an external eye examination at the slit lamp. Using statistical analysis, an abnormal OSS >3 is statistically associated with positive serology and positive MSGs.

1.5.4. Other diagnostic test in evaluation

Recently, convincing data have been published on the diagnostic value of tests for pSS that are not included in the AECG criteria, such as **B-cell distribution** in peripheral blood, **skin biopsy**, **salivary biomarkers** and **SGs ultrasonography (SGUS)** abnormalities. The first item (B-cell distribution) will be discussed in pathophysiology chapter.

Table 5. Methods for evaluating xerophthalmia

| Test | Characteristics of evaluation | Abnormal result | Sensitivity for pSS diagnosis | Specificity for pSS diagnosis |
|---|--|---|-------------------------------|-------------------------------|
| Schirmer's test | Evaluation of tear secretion | ≤ 5 mm/5 min | 76% | 72% |
| Vital stains : Rose Bengal Fluorescein Lissamine green | Stain the ocular surface to discover abnormalities | Grading van Bijsterveld score ≥ 4 (van Bijsterveld, 1974) | 78% | 67% |
| Break-up time | Index of tear film stability (normal > 25 s) | ≤ 10 s | 77% | 38% |

1.5.4.1. SGs ultrasonography

Concerning SGUS, several studies proved valuable for assessing major SGs involvement in pSS and seemed to exhibit good diagnostic accuracy (Tzioufas and Moutsopoulos, 2008). SGUS is simple, noninvasive, widely available and less expensive than other imaging techniques. Its reproducibility is comparable to that of sialography (Takagi et al., 2010). Echogenicity and structure of both parotid and submandibular glands are scanned, and their homogeneity is assessed using the score proposed by De Vita et al (De Vita et al., 1992) (**Table 6**), from grade 0 to 4.

Table 6. De Vita classification for the SGs abnormalities determined by ultrasonography (De Vita et al. 1992).

| Grade | Description |
|-------|---|
| 0 | Normal glands |
| 1 | Regular contour, small hypoechogenic areas without echogenic bands, normal or increased gland size (mean 20 ± 3 mm for the parotids and 13 ± 2 mm for the submandibular glands), and definite echogenic border. |
| 2 | Regular contour, multiple hypoechogenic areas usually ≤ 2 mm without echogenic bands. |
| 3 | Regular or ill-defined contour, multiple hypoechogenic areas and hyperechogenic bands, and no visible posterior glandular border. |
| 4 | Irregular contour, multiple hypoechogenic areas >6 mm or multiple calcifications with echogenic bands, decreased gland size, and no visible posterior glandular border. |

This score is based in parenchymal homogeneity, echogenicity, gland size, and posterior glandular border. Ultrasonography abnormalities suggestive of pSS are more than two SGs with grade 2 or more. Gritzmann et al. (Gritzmann et al., 2003) showed that patients with grade 2 or more have an evolution of 7.8 years.

1.5.4.2. Skin biopsy

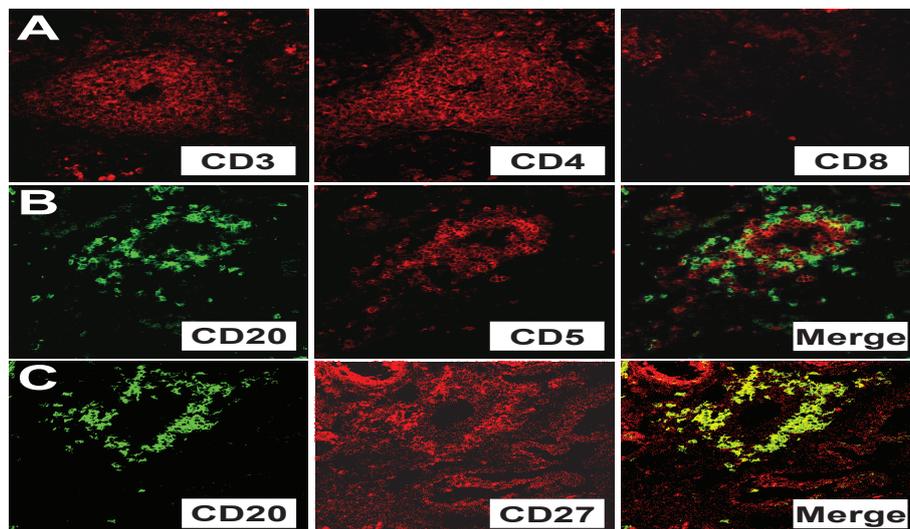
Xerosis is a common manifestation in pSS patients. Taking into account that in some cases MSGs biopsies may be non-conclusive to the initial diagnosis, in addition to the possibility to take serial samples, skin biopsy surges as a new tool for pSS diagnosis and follow-up. In the **article 1 (Memory B-cell aggregates in skin biopsy are diagnostic for primary Sjögren's syndrome, *J Autoimmun* 2010;35:241-247)** our objective was to evaluate the frequency of xerosis in patients with pSS, and compare histopathology findings on cutaneous sweat glands and MSGs, with respect to their contribution to the diagnosis. The skin was dryer (VAS: 58.3 ± 10.1 versus 38.9 ± 7.6 , $p < 0.01$), and xerosis more frequent in the patients than in the controls. In axilla skin biopsy, we documented lymphocytic infiltration in 8 of 12 patients tested. Interestingly, 2 patients with abnormal skin biopsy had normal MSGs biopsy. Most interestingly, B cell infiltrates were identified in patients' skin infiltrates, so that their presence might be a clue to the diagnosis of pSS. These cell aggregates associated memory CD10-/CD20+/CD27+/IgD- B lymphocytes and immature CD20+/CD24+ lymphocytes (**Figure 1**). In the **appendix 3, (Skin biopsy as a routine diagnostic tool for primary Sjögren's syndrome. *Int J Clin Rheumatol* 2011;6:291-296)** we proposed to add the question of skin dryness to the symptoms questionnaire designed for patients presenting clinical features of SS and to perform skin biopsy as a routine tool in the diagnosis of pSS, especially in patients with non-conclusive MSG biopsy. A flow chart according to symptoms, skin biopsy results and MSGs is proposed. In addition, the feasibility of skin biopsy, may allow the follow-up in pSS treated with the new biological treatments, most notably anti-B lymphocyte monoclonal antibodies. This question needs to be addressed in prospective series of patients.

1.5.4.3. Salivary biomarkers

In order to differentiate patients with pSS from patients with sicca symptoms, some authors have proposed the evaluation of candidate salivary biomarkers. Hu et al. (Hu

et al, 2010) have recently shown that the presence of three salivary biomarkers (cathepsin D, α enolase, β -2 microglobulin) measured by Western blotting or enzyme-linked immunosorbent assay (ELISA) is a good method to classify patients having pSS compared to healthy controls and other AIDs. These new biomarkers need to be validated in larger cohorts.

Figure 1. Lymphocytes phenotypes in the skin from patients with primary Sjögren's syndrome.



A. T cells Express CD3 and CD4 markers but not CD8. **B.** B cells (defined as CD20 positive cells) are negative for CD5. **C.** CD20-positive cells express CD27 (x 10).

1.6. Biologic abnormalities

1.6.1. Non-specific biologic abnormalities

Patients with pSS may present with a broad spectrum of non-specific laboratory abnormalities. Most of these laboratory abnormalities have been described more frequently in patients with positive immunological markers. The most frequent cytopenias are normocytic anemia, leukopenia, and thrombocytopenia. Cytopenias are

usually asymptomatic, but may be clinically important in some patients. Erythrocyte sedimentation rate (ESR) correlate closely with the percentage of circulating gammaglobulins (hypergammaglobulinemia)– frequently elevated in pSS– but serum C-reactive protein (CRP) concentrations are usually normal. Polyclonal hypergammaglobulinemia is one of the most characteristic laboratory abnormalities in pSS. **Table 7** summarizes the main laboratory findings in pSS patients and their association with other laboratory and clinical manifestations.

Table 7. Main laboratory findings in pSS patients.

| Laboratory finding | Prevalence of abnormality in pSS patients | Association with extraglandular manifestations | Association with laboratory and/or immunological markers | Association with lymphoma | References |
|-----------------------------------|---|--|---|---|--|
| ESR | 25% (more than 50 mm/h) | Yes | Yes. Association with anemia, anti-Ro, Anti-La and RF. | Not described. | <i>Ramos-Casals et al., 2002.</i> |
| CRP | 22% (minimal increase) | No | No | No | <i>Moutsopoulos et al., 1983.</i> |
| Polyclonal hypergammaglobulinemia | 6%-100% (according to the series and cutoffs) | Yes | Yes. Association with ANAs, anti-Ro, Anti-La and RF. | No | <i>Ramos-Casals et al., 2002. Alexander et al., 1983. Skopouli et al., 2000. Baimpa et al., 2009</i> |
| Hypogammaglobulinemia | 6%-15% | Yes. Association with palpable purpura, vasculitis and splenomegaly. | Yes. Lower levels of ANAs, anti-Ro and Anti-La. | Yes. Half of patients with lymphoma had hypogammaglobulinemia | <i>Ramos-Casals et al., 2002. Baimpa et al., 2009</i> |
| Monoclonal Igs | 8%-25%. (IgG: 60% of cases, IgM: 30%) | Yes. Lung involvement. | Yes. Anemia, hypergammaglobulinemia, elevated ESR, and cryoglobulins. | Yes. Mainly IgMκ | <i>Baimpa et al., 2009. Sibilia et al., 1999. Brito-Zeron et al., 2005.</i> |
| β2-microglobulin | 15%-30% | Yes. Lung and renal involvement. | Yes. Anti-Ro and anti-La. | Yes. 70% of patients with lymphoma. | <i>Davidson et al., 1999. Michalski et al., 1975. Gottenberg et al., 2005. Hatron et al., 1987. Pertovaara et al., 2001.</i> |
| Normocytic anemia | 11%-34% | Yes. Palpable purpura, lymphadenopathy, peripheral neuropathy | Yes. ANAs, RF, anti-Ro and anti-La. | No | <i>Ramos-Casals et al., 2002. Baimpa et al., 2009. Ramakrishna et al., 1992.</i> |
| Lymphopenia | 5% | Yes. Lymphoproliferation | Yes. Anti-Ro Abs | Yes. CD4+ lymphopenia and low CD4+/CD8+ ratio. | <i>Theander et al., 2006. Baimpa et al., 2009.</i> |
| Thrombocytopenia | 5%-15% | Not described. | Yes. Anti-Ro and anti-La. | No | <i>Baimpa et al., 2009. Ramos-Casals et al., 2008</i> |

1.6.2. Immunological abnormalities

Patients with pSS present a wide variety of auto-Abs directed against specific nuclear or cytoplasmic antigens. B-cell hyperactivity, the most typical etiopathogenic abnormality of pSS, accounts for the synthesis of auto-Abs. Although many auto-Abs may be found in pSS patients, only anti-SSA (Ro) and anti-SSB (La) Abs are important for the diagnosis and they are included in the AECG criteria (item VI) (Vitali et al., 2002). **Table 8** summarizes the main immunological abnormalities present in pSS patients and their clinical implications.

Table 8. Immunological abnormalities in pSS patients.

| Laboratory finding | Prevalence of abnormality in pSS patients | Association with extraglandular manifestations | Association with laboratory and/or immunological markers | Comments | References |
|--------------------------------|--|---|--|--|--|
| ANAs | 80% (half of patients with titers $\geq 1/320$) | Yes | Yes. Hypergamma-globulinemia, ESR and anti-ENAs. | Important role to differentiate between sicca syndrome and pSS | <i>Asmussen et al., 1996.</i> <i>Shah et al., 1990.</i> |
| Anti-SSA (Ro) Anti-SSB (La) | 50%-80% 30%-50% | Yes. Parotid gland enlargement, Lymphadenopathy, cutaneous vasculitis, peripheral neuropathy. Also neonatal lupus | Yes. Hypergamma-globulinemia, RF, cryoglobulins. | Anti-La is positive always with anti-Ro Abs. Anti-La Abs without anti-Ro are extremely rare reported | <i>Moutsopoulos et al., 1990.</i> <i>Alexander et al., 1993.</i> <i>Harley et al., 1986.</i> <i>Sheldom et al., 2004.</i> |
| RF | 50% | Yes. Joint and cutaneous involvement. | Yes. ANAs, anti-Ro, cryoglobulins. | | <i>Muller et al., 1988, 1989.</i> |
| Type II/III cryoglobulinemia | 20% | Yes. Vasculitis. | Yes. Anti-Ro, Anti-La, low C3 and C4 levels. | Risk factor for lymphoma. | <i>Tzioufas et al., 1996.</i> <i>Ramos-Casals et al., 1998.</i> <i>Ioannidis et al., 2002.</i> |
| Hypocomplementemia | 10%-25% | Yes. Joint, cutaneous, renal and neurological involvement. | Yes. RF, cryoglobulinemia | Risk factor for lymphoma and death. | <i>Theander et al., 2004.</i> <i>Ioannidis et al., 2002.</i> <i>Ramos-Casals et al., 2005.</i> |

ENAs: extractable nuclear antigens.

1.6.3. Anti-SSA(Ro) and anti-SSB (La) Abs

Anti-Ro and anti-La auto-Abs are the most useful Abs for the diagnosis of pSS. However, these Abs are not specific for pSS, and they can be found in patients with other AIDs, especially in systemic lupus erythematosus (SLE) in 25% of cases (**Table 8**). Anti-Ro Ab is responsible for neonatal lupus.

1.6.4. Other auto-Abs

Anti- α fodrin auto-Abs are common in pSS patients, and they correlate with anti-Ro and anti-La Abs (Haneji et al., 1997). The auto-antigen is a 120kD protein, derived from the cytoskeleton. An abnormal degradation of this protein has been characterized in the NFS/sld mouse model of human pSS. This autoantigen induces proliferative T-cell response and production of IL-2 and IFN- γ and may have a physiopathological role in pSS. In the initial description, sera from pSS reacted positively with purified antigen whereas those from patients with SLE and RA not. However, in recent studies, this auto-Ab seems to be not specific of pSS and it is found in other AIDs characterized by increased apoptosis, such as SLE, RA and multiple sclerosis (Locht et al., 2008).

Anti-muscarinic receptor 3 Abs have been described by some groups in up to 90% of pSS patients whereas others authors are unable to detect it by immunological methods. These Abs are of special interest on research because they may have a functional implication in decreasing glandular secretions (Dawson et al., 2006). Data from in vitro studies indicate that patients with pSS have IgG anti-muscarinic Abs that are capable of inhibiting both agonist- and nerve-evoked contractions in isolated mouse bladder or colon muscle strips, showing that these auto-Abs may have a pathogenic role (Waterman et al, 2000). Recently, the same group of investigators, (Park et al., 2011) have shown that these auto-Abs have the potential to mediate multiple dysfunctions of the gastrointestinal tract in pSS, ranging from reduced esophageal motor activity to altered colonic motility. These findings may explain common gastrointestinal symptoms in pSS patients. In addition, passive transfer of pSS patient IgG to mice alters muscarinic 3 receptor expression and cholinergic responses in bladder smooth muscle (Wang et al., 2004). However, due to the lack of an effective screening assay, the interpretation of these results in pathology is difficult.

Abs to **anti-carbonic anhydrase II** are classically described in autoimmune pancreatitis. Recently, they have also been described in patients with other AIDs, particularly those with pulmonary involvement (Caccavo et al., 2008) and pSS with renal involvement (Takemoto et al., 2005). These auto-Abs may have a pathophysiological role in renal tubular acidosis, because soluble carbonic anhydrase II is found in the cytosol of both proximal and distal renal tubular cells (Lonnerholm et al., 1980). In addition, mice immunized with this antigen develop systemic exocrine gland inflammation similar to SS (Nishimori et al., 1995). However, these findings have to be confirmed in others studies.

Anti-cyclic citrullinated peptides (CCP) Abs, a specific serological marker for RA diagnosis, have been also been described in pSS (between 7.5% to 9%), even in patients without articular involvement. The importance of these auto-Abs in pSS remains to be elucidated (Gottenberg et al., 2005; Tobón et al., 2005). Recently, IgA anti-CCP autoAbs have been described in 8.1% of pSS patients compared to 26.7% in RA patients (Haga et al., 2011). This isotype was associated with more cutaneous vasculitis but not with arthritis or other clinical manifestations in pSS patients.

In an attempt to identify new target antigens of ANAs, van de Bergh et al (van de Bergh et al., 2009) applied an immunoproteomics approach using serum from one patient with high ANAs titer, but with no reactivity to SSA, SSB, U1-RNP, Sm, Scl-70, or Jo-1. The antigen was then determined by MALDI-TOF/TOF analysis, showing that the serum reacted against heterogeneous nuclear ribonucleoprotein (hnRNP) H1. hnRNP H1 is a common nuclear matrix, implicated in splicing of mRNA. The presence of **anti-hnRNP H1** was confirmed by Western blotting in AIDs, mainly in pSS (48% in pSS vs. 5% in other AIDs). Interestingly, 5 of 11 pSS patients without anti-SSA/Ro or anti-SSB/La had anti-hnRNP H1 auto-Abs. In a recent analyses (De Beeck et al., 2012), in order to detect auto-Abs against other hnRNPs, multiple recombinant hnRNPs were tested in AIDs. This study confirmed that hnRNPs may be a new group of auto-antigens, mainly in pSS, because high reactivity against at least one hnRNPs among B1, E1, F, and H1 was seen in around 45% of pSS compared to 2.8% of controls. Reactivity with at least 3 of the 4 antigens was observed in 0% of controls, 3% of SLE patients and 15% of pSS.

1.7. Measurement of chronicity and activity in pSS patients

Different scales are now available to test the activity and severity of the disease such as the SS Disease Damage Index (SSDDI) and SS Disease Activity Index (SSDAI) elaborated in Italy (Vitali et al., 2007). However, these scales were built from a limited series of patients recruited in a single country and the validity and reliability of these indices need to be verified in larger studies in different countries. Similarly, two scales have been proposed in the United Kingdom: The Sjögren's Syndrome Damage Index (SSDI) (Barry et al., 2008) and the Sjögren's Systemic Clinical Activity Index (SCAI) (Bowman et al., 2007). The initial evaluation of both tools supported their utility for systemic activity assessment and longitudinal damage in pSS patients. In a multi-center approach, the European League Against Rheumatism (EULAR) proposed that two indices are needed to correctly defined activity in pSS, taking into account that one group of patients are characterized by a stable or slowly progressive disorder limited to epithelial regions with sicca complaints and constitutional symptoms such as fatigue and musculoskeletal pain; and a second group of patients with a more aggressive and systemic disorder, accompanied by extra-epithelial features and serological signs of autoimmune activation. The first index, termed the EULAR SS Patient Reported Index (ESSPRI) (Seror et al., 2011), is devoted to the assessment of sicca complaints and subjective symptoms such as dryness, fatigue and pain from the perspective of the patient. The second index, the EULAR SS Disease Activity Index (ESSDAI) (Seror et al., 2010), addresses global activity related to the systemic features of the disease. Nowadays, final validation studies of both the ESSDAI and ESSPRI in a large series of real patients are under way, and several publications start to report results with these indexes.

1.8. Lymphoma in pSS patients

1.8.1. Epidemiology and clinical characteristics

Although pSS is considered a benign disease, 5% of patients may present a NHL, usually after several years between pSS diagnosis and lymphoma (7.5-14 years) (Voulgarelis et al., 1999). This complication is associated with an excess in the overall mortality rate in pSS patients. The first description of NHL in pSS was reported by Talal and Bunim in 1964 (Talal and Bunim., 1964). After the initial publication, several series have shown the high risk of lymphoproliferation in pSS patients. Among all the AIDs, pSS displays the highest incidence of malignant lymphoproliferative disorders.

Recent meta-analysis have shown that the standardized incidence risk (SIR) to develop NHL is high in pSS (SIR: 9-48), moderate in SLE (SIR:3-7) and low in RA (RR: 1.5-4) (Zintzaras et al., 2005; Smedby et al., 2006). Various histological subtypes of lymphoma have been described, including follicular lymphoma, diffuse large B cell lymphoma (DLBCL) (30% of cases), and especially mucosa-associated lymphoid tissue (MALT) lymphoma (70%), preferentially extranodal marginal-zone (MZ), located mainly in the major SGs (80% of cases) (Baimpa et al., 2009; Voulgarelis et al., 1999; Ioannidis et al., 2002; Kassan et al., 1978). MZ lymphomas of the MALT type are primary low-grade and localized (stages I and II) with extranodal manifestations and few systemic symptoms (B symptoms in only 15% of cases) (Voulgarelis., 1999). In some patients, NHL in pSS tend to evolve toward a less differentiated cell type, from benign chronic lymphoepithelial sialadenitis to indolent extranodal MZ lymphomas of the MALT type and then to DLBCL. Thus, lymphoproliferation in pSS can be divided in two categories: the first relating to the majority of patients who present an extranodal MZ lymphoma, and the second one, less common, relating to patients developing de novo or secondary DLBCL. The type of lymphoma is also related with prognosis, being DLBCL cases associated with poor prognosis (Voulgarelis., 1999).

1.8.2. Risk factors for lymphoproliferation

Given that the risk of lymphoproliferation in pSS is higher compared to other AIDs, it is important to identify which patients present high risk of lymphoma determined by predictive markers for this complication. Several clinical and serological markers have been reported to predict the development of NHL in pSS in different series. Among these parameters, patients with pSS and splenomegaly, persistent enlargement of parotid glands, lymphadenopathy (Kassan et al., 1978; Anaya et al., 1996; Sutcliffe et al., 1998), palpable purpura (Ioannidis et al., 2002), cryoglobulinemia (Tzioufas et al., 1996), low levels of C4 (Ramos-Casals et al., 2005), neutropenia (Baimpa et al., 2009) or lymphocytopenia (Theander et al., 2006) have more than 5-fold increased risk of NHL compared to patients without risk factors. Thus, patients with high risk of NHL, have more extraglandular and immunological abnormalities, showing B-cell hyperactivity. Recently, Zhang et al. (Zhang et al., 2010), showed in a Chinese cohort of pSS patients that the risk of developing NHL is higher in patients with two or more risk factors [relative risk –RR- of lymphoma: 3.3 (CI95%:0.7-15.9) in patients with only one risk factor compared to RR: 12.3 (2.7-54.8) in patients with two risk factor ($p \leq 0.001$) and RR: 23.4 (4.17-132.2) in patients with three or more risk factor ($p \leq 0.001$).

Recently, Baldini et al, in a retrospective cohort (Baldini et al., 2012) have developed a propensity score for discrimination between pSS patients at risk of NHL and those not at risk. In a multivariate analysis of 318 patients, salivary gland enlargement (X_1 : OR 10.20), low C4 levels (X_2 : OR 11.53), and disease duration (X_3 : OR 1.14) were identified as independent risk factors for NHL in pSS. The corresponding β coefficients were used to calculate the linear predictor $Y = 2.32X_1 + 2.446X_2 + 0.132X_3$, ranging from 0 to 6.5. The optimal cutoff point of Y to determine patients at risk was 4.26 (sensitivity 78%, specificity 95%).

In conclusion, patients with such risk factors constitute a sub-group that must be monitored and managed closer than other patients with pSS with no risk factors. In addition, the analysis of new risk factors associated with B-cell hyperactivity (e.g. FLT3-L), in prospective cohorts, will be essential to understand the evolution from pSS towards NHL.

2. Pathophysiology of primary Sjögren's syndrome

2.1. Introduction

pSS is a complex disease of unknown etiology. Similar to all the AIDs, genetic, environmental and hormonal factors are implicated in the phenotype. However, pSS is an ideal model for dissecting the pathogenic mechanism of AIDs, because the MSGs are easily accessible for study. Two major characteristics are implicated in the autoimmune nature of pSS: the peri-epithelial lymphocytic infiltration of affected tissues and B-cell hyperactivity. Several studies have pointed the key role of ECs in the pathogenesis, suggesting that the disease is actually an "autoimmune epithelitis". The development of pSS may be simplified in three phases: (1) Autoimmunity is triggered by environmental factors (several bacteria and viruses have been implicated as the trigger mechanism) in a genetic susceptible individual; followed by (2) activation of immune system (innate and acquire) and an increased of chronic autoimmune response, and (3) finally tissue damage. The unknown environmental factor (i.e. cytomegalovirus, Epstein-Barr virus-EBV) is probably capable to transform the ECs from exocrine glands into nonprofessional Antigen Presenting Cell (APC) expressing HLA-I and HLA-II (Moutsopoulos et al., 1986) and co-stimulatory molecules such as B7.1, B7.2 (Manoussakis et al., 1999), CD40 (Dimitriou et al., 2002) and adhesion molecules such as Intracellular Adhesion Molecule-1 (ICAM-1) (Kapsogeorgou et al.,

2001). Then, the auto-antigen may be presented for the ECs to T-cells, where are recognized by the T-cell receptor (TCR) or B-cells. These T-cells are recruited by chemokines (Amft and Bowman, 2001). T-cell infiltrating exocrine glands represent 75%-80% of all the cells. Another important issue is that inflammation and dysfunction (due to neuro-exocrine junction abnormalities) may be discordant, and in some patients, severe glandular dysfunction is found without severe immunological infiltrates. In the next sections, the main genetic, epigenetic, environmental, hormonal, neuroexocrine and immunological factors implicated in the pathophysiology of pSS will be discussed. The updates in pathophysiology of pSS derived from the 10th International Symposium on Sjögren's Syndrome celebrated in Brest in 2009 are summarize in the **appendix 4 (Conference scene: Updating the highlights of Sjögren's syndrome. *Immunotherapy* 2010;2:13-20).**

2.2. Etiology

2.2.1. Genetic influence

In contrast to the genetic studies in other AIDs such as SLE and RA, the genetics of pSS is understudied, and to date, there is no specific gene for pSS. Similar to related AIDs, the evidence for a genetic component is derived from studies demonstrating increased concordance rates among monozygotic twins and familial aggregation. Several case reports of twins who presented SS have been published, but consistent twin concordance has not been estimated (Scofield et al., 1997; Bolstad et al., 2000). Taking into account the inter-relationships between pSS and SLE and RA, twin concordance could be expected to be between those of RA (15%) and SLE (25%). Familial aggregation, which includes not only cases of a single AID, but also several different AIDs, is also more frequent in first-degree relatives (FDR) of pSS patients (38%) compared to FDR of controls (22%), indicating that autoimmune phenotypes could represent the pleiotropic outcome of nonspecific disease genes underlying similar immunogenetic mechanism (Anaya et al., 2006). In the absence of large-scale genetic studies identifying strong associations, increased concordance rates among monozygotic twins and the familial aggregation are two parameters that evidence the genetic component in pSS. The study of genetics in pSS has been centered on candidate genes, focusing on those genes with biological plausibility for a role in pSS etiology, such as the Human Leukocyte Antigen (HLA) loci or genes demonstrating association in other AIDs. However, most of these associations should be interpreted with caution, due to lack of replication in independent cohorts and large patient cohorts.

Among the candidate genes, HLA alleles are associated with pSS in various populations. The HLA genes found to be associated with pSS vary in different ethnic groups. In general, studies have primarily focused on alleles at the Class II HLA-DR and –DQ loci. The most consistent associations to date have been with DR2 and DR3 alleles at the DRB1 locus in Caucasian populations. HLA-DR3 has been associated with pSS in Caucasian populations (Fei et al., 1991; Foster et al., 1992; Guggenbuhl et al., 1998) but not in other ethnic groups (Kang et al., 1993; Migagawa et al., 1998). Frequencies of ancestral haplotype 8.1-HLA-A1-B8-DR3-DQ2 are increased in Caucasian pSS patients. However, significant positive associations with HLA Class II markers may be restricted to the formation of different anti-Ro (HLA-DRB1*15) and anti-La (HLA-DRB1*03) auto-Abs, associated with DRB1*03-DQB1*02-DQA1*0501 haplotype (Bolstad et al., 2001). In Latin-America population, HLA- polymorphisms were not associated with the disease (Anaya et al., 2002). However, the HLA-DRB1*0301-DQB1*0201 haplotype was associated with more severe histopathologic disease (odds ratio [OR], 15.5; 95% confidence interval [CI], 1.9-129; $p < 0.001$) and the presence of anti-Ro (OR, 3.8; 95% CI, 1-15; $p < 0.04$) and anti-La antibodies (OR, 4.3; 95% CI, 1.3-14; $p < 0.01$). Another HLA allele, the HLA DQA1*0501 is more frequent in pSS compared to controls, independent of racial and ethnic differences (Reveille et al., 1991), suggesting that this allele may be a determining factor in the genetic susceptibility to pSS.

Additionally, a growing number of non-HLA genes have shown associations with pSS, particularly those that influence the type-I interferon (IFN)- response. Among the most important genes *IRF5* (Miceli-Richard et al, 2005), Signal transducer and activator of transcription-4 (*STAT4*) (Korman et al., 2008; Nordmark et al., 2009) and *EBF1*, *BKL* and *TNFSF4* have been recently described (Nordmark et al., 2011). *IRF5* is associated with type I IFN- (IFN- α and β) response following innate immune stimulation and *STAT4* is a transcription factor induced by IL-12, that increases type II IFN- synthesis (IFN- γ). Thus, the polymorphisms of these genes may be related to IFN- signature present in pSS patients. The main genetic non-HLA associations in pSS (evaluated at least in 100 cases) are described in **Table 9**. The future of genetic studies in pSS seems promissory, due to rapid technologic advances and the expectation of genome-wide association (GWA) scans. Based on the results of other GWA scans published in other complex phenotypes, the study on pSS has the potential to identify many variants that convey risk.

Table 9. Significant genetic non-HLA associations in pSS

| Gene | SNP | Population | N (patients /controls) | OR | 95%CI | Reference |
|---------------|----------------------------|---------------|------------------------|------|--------------|------------------------------------|
| STAT 4 | rs7574865 | USA | 124/1112 | 1,46 | 1.09-1.97 | <i>Korman et al., 2008</i> |
| | rs7582694 | Sweden/Norway | 368/711 | 1,41 | 1.14-1.73 | <i>Nordmark et al., 2009</i> |
| | rs7582694 | France | 378/635 | 1,57 | 1.27-1.93 | <i>Geterman et al., 2010</i> |
| IRF5 | CGGGGindel | Sweden/Norway | 368/711 | 1,49 | 1.24-1.79 | <i>Nordmark et al., 2009</i> |
| | CGGGGindel | France | 385/409 | 2 | 1.5-2.7 | <i>Miceli-Richard et al., 2009</i> |
| | rs10488631 | Sweden/Norway | 368/711 | 1,57 | 1.23-1.99 | <i>Nordmark et al., 2009</i> |
| | rs2004640 | France | 212/162 | 1,36 | 1.01-1.83 | <i>Miceli-Richard et al., 2007</i> |
| CHMR3 | rs7548522 | Sweden/Norway | 530/532 | 1.93 | 1.24 to 3.01 | <i>Appel et al., 2009</i> |
| FCGR3B | >2 copies of FCGR3B | Colombia | 61/409 | 2.26 | 1.01-5.05 | <i>Mamtani et al., 2010</i> |
| CTLA-4 | +49G/A CT60A/G 3'UTR | Australian | 111/156 | 1.78 | 1.08-2.93 | <i>Downie-Doyle et al., 2006</i> |
| FAM167A – BLK | rs12549796 | Sweden/Norway | 540/532 | 1.37 | 1.15-1.63 | <i>Nordmark et al, 2011</i> |
| EBF1 | rs3843489 | Sweden/Norway | 540/532 | 1.68 | 1.29-2.18 | <i>Nordmark et al, 2011</i> |
| TNFSF4 | rs1234315 | Sweden/Norway | 540/532 | 1.34 | 1.14-1.64 | <i>Nordmark et al, 2011</i> |
| MBL | Codon 54 (exon 1) | Japan | 104/143 | 1.93 | 1.14-3.27 | <i>Wang et al., 2001</i> |
| MECP2 | rs17435 | USA/European | 417/1279 | 2,17 | 1.30-3.62 | <i>Cobb et al., 2010</i> |
| IL-2-IL-21 | rs6822844 | Colombia | 94/368 | 0,46 | 0.23-0.95 | <i>Maiti et al., 2010</i> |

CHMR3: Muscarin receptor 3 gene; EBF1: Early B-cell factor; FAM167A-BLK: family with sequence similarity 167 member A–B-lymphoid tyrosine kinase; TNFSF4 (OX40L): Tumor necrosis factor superfamily ; MBL: Mannose binding lectin; MECP2: Methyl-CpG binding protein 2.

2.2.2. Epigenetics

Epigenetic can be defined as stable and heritable changes in gene expression that do not involve alterations in DNA sequence. Thus, the epigenetic process is important for controlling patterns of gene expression during the cell cycle, development, and in response to environmental or biological modifications. Epigenetic states change with age and can become disrupted by environmental influences, providing an explanation for the empirical link observed between environmental factors, aging and the development of AIDs. Three main epigenetic checkpoints exist in a normally regulated genome, DNA methylation, histone adjustments and micro-RNA. Several articles show that epigenetics modifications are present in AIDs (Ballestar E., 2011), mainly in SLE and RA (Richardson et al., 1990; Kawano et al., 2011). The most studied epigenetic mechanism in pSS is the role of microRNAs. Using Agilent microRNA microarrays (Alevizos et al., 2011), the authors showed that microRNA expression profiles can separate glands of pSS patients from healthy controls and also distinguish subsets of pSS patients with low or high grade inflammation. Among the microRNAs with different

levels of expression, miR-17-92 cluster (encoding miR-17, miR-18a, miR-19a, miR-20a, miR-19b-1 and miR 92-1) was significantly down regulated in patients with high focus score. Decrease of mir-17-92 cluster is associated with accumulation of pro-B cells with a marked reduction of pre-B and more mature B cells, and overexpression is linked to lymphoproliferative and AIDs (Xiao et al., 2008). The presence of microRNAs from salivary exosomes is another approach to use microRNAs as a non-invasive biomarker in pSS (Michael et al., 2010). These preliminary results showed that in the next years, microRNAs expression may help to the diagnosis of pSS, and also to better understand pathogenic mechanisms such as the effect of microRNAs controlling saliva production, or regulation of the inflammatory response in exocrine glands.

In addition, the microRNAs machinery in humans constitutes a target of autoimmune responses. Thus, auto-Abs against the GW bodies (GWB), where all proteins required for the processing of miRNAs (such as Ago2, GW182 and Rck/p54) are localized, have been detected in patients with AIDs (including 33% of pSS patients) (Bhanji et al., 2007).

2.2.3. Environmental factors (Viruses)

Although the implication of an infectious agent has long been suspected for the induction of pSS, no study has been conclusive so far. Several viruses have been implicated in the pathophysiology of the disease as the triggering factor. For instance, some infections by viruses with high affinity for glandular tissue, such as hepatitis C virus infection, are associated with type II cryoglobulinemia and SGs infiltrates. Other viruses such as cytomegalovirus and EBV have been implicated, but their responsibility has not been well established. **EBV** genome and proteins were detected in SGs from pSS patients more frequently than in controls (Mariette et al., 1991). In mice models, EBV-mediated activation of caspases leads to the accumulation of the 120kD α -fodrin auto-antigen, and EBV reactivation is associated with anti- α -fodrin auto-Abs (Inoue et al., 2001). In addition, EBV-encoded small RNA (EBER), a double-stranded RNA, complexes with SSB protein to induce TLR3-related secretion of type I IFN- (Iwakiri et al., 2009). Thus, EBV might be associated with innate immune activation. Although some experiments are interesting, the link between EBV infection and pathology is difficult to establish, and the presence of EBV may be a consequence of exocrine glands destruction. Recently, **Coxsackie** virus has been linked to pSS because some nucleotides sequences on the 5' non-coding region of Coxsackie virus B4 P2A gene are present in SGs of patients. Immunohistochemistry for the enteroviral capsid protein VP1 (indicating active viral replication) showed positive staining in epithelial cells and

lymphocytic infiltrates in pSS samples. Once again, it is difficult to demonstrate the real link between this infection and the pathology (Triantafyllopoulou et al., 2004). **Retroviruses** have been also implicated in pSS. In one study in Japanese population, positive serology for the human T-lymphotropic virus-1 (HTLV-1) was 23% in pSS patients compared to 3% of the controls from the same endemic region (Terada et al., 1994).

2.2.4. Hormonal factors

The high female/male ratio (9:1) and the fact that pSS primarily affects post-menopausal women, suggest a role for sex hormones in the prevention of this disease. Estrogen receptor (ER) and ER mRNA are detected in salivary tissue (Kassi et al., 2003; Tsinti et al., 2009). Healthy background mice models develop SS after ovariectomy (Ishimaru et al., 2003), showing the effect of estrogen to maintain normal exocrine secretions. Estrogen deficiency induces over-expression of the TF called retinoblastoma-associated protein 48 (*RbAP48*) (Ishimaru et al., 2008). Mice over-expressing RbAp48 show ECs apoptosis, lymphocytic infiltrates on exocrine glands and auto-Abs (anti-Ro, anti-La, anti- α fodrin). Secretion by ECs of IL-18 and IFN- γ is also increased. This cytokine synthesis is associated with the expression of HLA-II and co-stimulatory molecules that allow ECs to act as APC. Additional evidence of the role of estrogen is observed from mice lacking the aromatase gene that encodes the enzyme necessary to the production of estrogen. In this model, mice develop a lymphocytic exocrinopathy resembling pSS (Shim et al., 2004). Estrogens can diminish T-cell recruitment in SGs (Carlsten et al., 1992) and prevent apoptosis in the lacrimal glands (Ishimaru et al., 1999). In addition, the administration of estrogen in MRL/lpr mice model of secondary SS prevents the development of sialoadenitis.

Others studies propose that the hormonal factor implicated in pSS is the change in the androgen/estrogen ratio rather than absolute levels of estrogens. Low systemic and salivary dehydroepiandrosterone (DHEA) levels are found in pSS patients (Laine et al., 2007). They found that cysteine-rich secretory protein 3 (CRISP-3), an androgen responsive salivary protein that is upregulated by DHEA is diminished in SGs of pSS patients, and it has lost its polarized organization in the acini, even in regions without inflammatory cells. The conversion of DHEA to testosterone but not dihydrotestosterone in SGs is due to decreased expression and abnormal localization of key steroidogenic enzymes (Porola et al., 2008). The hypothesis of local and systemic androgen deficiency is also supported by decreased levels of the active metabolites of DHEA in the serum and saliva of pSS patients (Forsblad d'Elia et al.,

2009). All these findings may explain why women are particularly vulnerable to pSS, as their local production of dihydrotestosterone is completely dependent on local conversion of DHEA, whereas in men, systemic androgens may supply the local milieu requirement.

2.3. Immunopathology of primary Sjögren' syndrome

2.3.1. Immunological findings and histopathology in exocrine glands

The pathognomonic histological finding in exocrine glands is a progressive focal infiltration of mononuclear lymphoid cells, replacing glandular epithelium. The infiltrates extend from mild, focal infiltrates that do not significantly affect the gland organization to diffuse, severe lesions associated with concomitant loss of epithelial structures and tissue architecture. However, the mechanism leading to attraction and accumulation of infiltrating cells remains undefined. These cells interfere with glandular secretion altering the normal glandular structure, secreting cytokines that activate pathways related to IFN- and producing local auto-Abs. Despite systemic B-cell hyperactivity, analysis of infiltrates in SGs shows a predominance of T-lymphocytes surrounding ductal ECs. The majority of these T-lymphocytes is CD4+ cells (80%) and shows an activated phenotype (Xanthou et al., 1999). CD8+ T-lymphocytes with cytotoxic activity, characterized by their expression of granzymes constitute around 10% of infiltrating cells. B cells, plasma cells and macrophages are the remaining infiltrating cells in SGs tissues (Bodeutsch et al., 1993; Tapinos et al., 1998; Stott et al., 1998). The presence of T and B cells, macrophages and dendritic cells (DCs) varies according to the severity of the lesion (Christodoulou et al., 2010). T cells predominate in mild lesions, whereas B cells predominate in the advanced ones. The number of macrophages increases, whereas DCs decrease with lesion severity. The decline of T-cell population is due to a decrease of CD4+ T cells since infiltrating CD8+ T cells remain stable.

The invasion of exocrine glands by inflammatory cells requires the expression of adhesion molecules by endothelial cells, ECs and lymphocytic cells. Among these adhesion molecules, ECs derived E-cadherin levels are increased in serum from pSS patients (Jonsson et al., 2005).

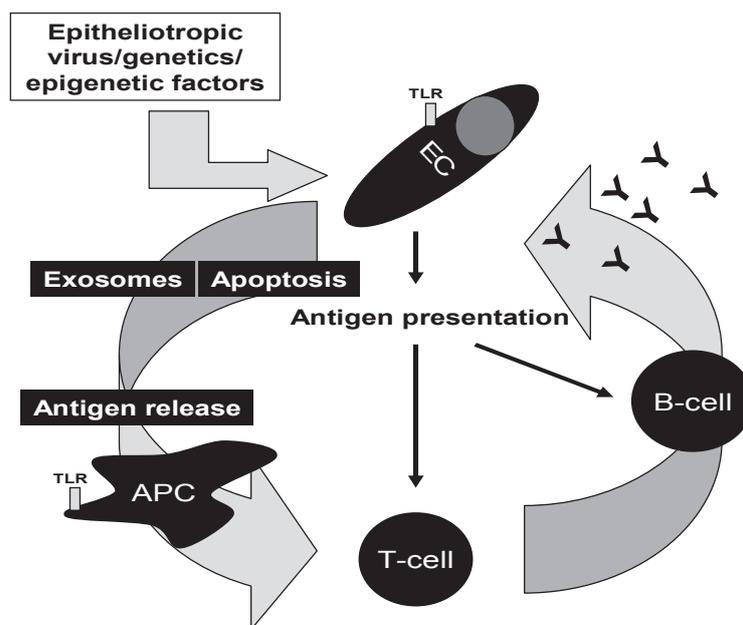
2.3.2. Epithelial cells as key regulators of autoimmune epithelitis

ECs apoptosis, induced by lymphocytic infiltrates in exocrine glands, is considered as a key factor of decreased glandular production. Fas-FasL mechanisms may explain the increased ECs apoptosis (Kong et al., 1997). Kong et al. demonstrated that the acinar ECs from pSS express Fas and FasL, and die by apoptosis. In contrast, the majority of infiltrating lymphocytes in SS were Fas⁺ and bcl-2⁺, but FasL negative, showing only minimal dead, particularly in dense periductal foci. However, according to other authors, ECs apoptosis induced by Fas/FasL pathway remains controversial (Ohlsson et al., 2001). Recently, our group evaluated the role of B-cell to induce ECs apoptosis. Using co-culture experiments with human salivary gland (HSG) cell line cells and tonsillar B lymphocytes, the direct HSG cell-B lymphocyte contacts were able to induce apoptosis in ECs. This B lymphocyte-mediated cell death could not be ascribed to Fas–Fas ligand interactions but required translocation of protein kinase C delta (PKC δ) into the nucleus of ECs. These results suggest that B lymphocytes infiltrating the SGs of pSS patients could induce ECs apoptosis (Varin et al., 2012).

In addition, non-apoptotic mechanisms may explain also the glandular dysfunction. ECs can secrete pro-inflammatory cytokines mediated at the beginning by Toll-like receptors (TLR) activation. Several TLRs are expressed by ECs in SGs tissues (TLR2, TLR3 and TLR4) (Kawakani et al., 2007; Spachidou et al., 2007). In cultured HSG cells, a similar expression pattern has been observed, and TLR ligands increased ICAM-1 expression and IL-6 production. In another study, cultured ECs from pSS patients showed functional expression of TLR2, TLR3 and TLR4 in a dose-dependent up-regulation of surface ICAM-1, CD40 and MHC-I expression (Spachidou et al., 2007), showing activation of ECs in pSS. This activation may be related with viral infection on the exocrine tissues. The link between a viral infection and TLR activation may be explained by findings in mice NZB/NZW F1 model (see animal models section) that show reversible hyposalivation after TLR3 stimulation. The saliva production is recovered if the stimulus is absent suggesting that a chronic stimulus (chronic and persistent infection) is related to the maintenance of this phenomenon. Under the effect of the environmental trigger, ECs are modified into nonprofessional APC expressing HLA-I and HLA-II and co-stimulatory molecules such as B7.1, B7.2, CD40 and adhesion molecules. ECs inappropriately express oncogenes (c-Myc and p53). Additionally, nuclear auto-antigens, such as Ro/SSA and La/SSB ribonucleoprotein may translocate from nucleus into the ECs membrane (Yannopoulos et al., 1992).

These ribonucleoproteins are present in apoptotic bodies increased in ECs from patients. Thus, ECs can present antigens to T and B cells. ECs also produce chemokines such as BCA-1 (CXCL13), ECC and SLC that promote lymphocyte migration into the SGs (Xanthou et al., 2001). As a consequence, by virtue of aberrant expression of various immunoactive factors, the ECs appear to have the potential to actively participate and modulate immune reaction in the inflammatory lesions.

Figure 2. Pathogenic model of lymphoepithelial lesions in the SGs of pSS patients. A vicious circle of aberrant activation of EC, persistent antigen presentation to T and B cells, and EC apoptosis may explain the induction and/or maintenance of focal lymphocytic aggregates and destruction of epithelia. The activated EC produce either physiologically (exosomes) or by apoptosis vesicles that contain intracellular antigens. These vesicles may be captured by antigen-presenting cells, and subsequently the activation of T- and B-cell. An exogenous infectious agent, such as an epitheliotropic virus may be responsible for the chronic activation of epithelia.



Disturbances in cytokines and cytokines receptors have been also been described in exocrine glands in pSS. For example, FLT3-L, a cytokine implicated in B-cell ontogenesis, is expressed by ECs (**article 2**) and may favor B-cell survival in SGs infiltrates. Furthermore, the expression of CXCL12 and IL-6 by ECs favors the survival of infiltrating auto-Abs producing plasma cells (Szyszko et al., 2011). **Figure 2** summarizes the relationship between environmental and genetics factors, with ECs activation, antigen presentation to T and B cells and induction and maintenance of inflammation in the pathophysiological model of pSS.

2.3.3. Neuro-exocrine junction

Lack of glandular function in many patients with pSS is the result of a perturbation of acinar function, followed by atrophy. These findings are not always related to lymphocytic infiltrates and ECs apoptosis, suggesting that other mechanisms, such as neuro-exocrine factors are associated. Exocrine secretions depend from parasympathetic nervous system, induced by acetylcholine (ACh) stimulation on type 3 muscarinic receptor, and subsequently activation of inositol 1,4,5-triphosphate (IP3) and increased cytoplasmic Ca^{2+} . For example, in salivary acinar cells isolated from pSS patients, the remaining tissue is functional *in vitro*, but with a reduced sensitivity to threshold levels of muscarinic stimulation (Dawson et al., 2001). This phenomenon may be explained by inhibitory auto-Abs directed against muscarinic receptors. Some authors have shown that aquaporin 5, which is a water channel protein and plays a role in the generation of saliva, tears and pulmonary secretions, has an abnormal basal localization in the acinar ECs and not in the apical region in pSS. This translocation is also induced by anti-M3 muscarinic receptor auto-Abs (Tsubota et al., 2001). The interaction between the immune system and the secretory process that could lead to glandular hypofunction may be summarized as (1) inhibition of ACh release induced by cytokines; (2) enhanced breakdown of ACh by increased levels of cholinesterase; (3) blockade of muscarinic type 3 receptor by anti-muscarinic auto-Abs; (4) altered nitric oxide synthesis; (5) perturbation of calcium-induced calcium release and (6) altered distribution of aquaporin 5 (reviewed by Dawson et al, 2006). These mechanisms have important implications to better understand the pathophysiology and to develop new treatments in pSS. The ECs apoptotic model and the non-apoptotic exocrine gland dysfunction model are not exclusive.

2.3.4. Innate immunity

Transcriptome analyses from mononuclear cells from SLE patients have shown the increased expression of IFN- induced genes, a phenomenon which is called in the literature as “IFN-signature”. This “IFN-signature” is related to disease activity in SLE patients. Recently, the same findings have been documented in SGs and mononuclear cells from pSS patients (Hjelmervik et al., 2005; Gottenberg et al., 2006; Emanian et al., 2009). Plasmacytoid DCs (pDCs), the main cell type to produce type I IFN-cytokines, are recruited in SGs from pSS patients and not in controls. IFN- production by pDCs may be induced by infection stimulus or immune-complex containing anti-Ro or anti-La auto-Abs and their respective auto-antigen. In addition to increased

expression of IFN-related genes, *IRF-5* polymorphisms have been described in association with pSS (see genetics), showing together that IFN- induction in pSS play a role in the immunological activation both in peripheral blood and exocrine glands. One of the most relevant IFN-induced genes is B-cell activating factor belonging to the TNF family (BAFF), binding the IFN- signature with BAFF overexpression in pSS (Ittah et al., 2006).

TLRs stimulation in the innate immune plays also a role in the pathogenesis. This affirmation is based in findings in the NZB/NZW F1 mice model (see animal models section), demonstrating that the activation of innate immunity might be a triggering event of pSS. In addition, DCs infiltrates are mainly observed in the early stage of the disease, suggesting that innate immunity is involved in the induction of the disease, but it is not capable to the maintenance (Ozaki et al., 2010).

Other findings supported the importance of the innate immunity creating a proinflammatory microenvironment in the target organ prior to disease onset. One study published by Bulosan et al. (Bulosan et al, 2009) showed increased caspase-11 in macrophages, STAT-1 and caspase-1 in apoptotic ECs and elevated salivary levels of IL-18 before the development of the disease in the C57BL/6.NOD-Aec1Aec2 mice model. In the non-obese diabetic (NOD) model, SG inflammation was preceded by accumulation of DCs lacking the innate immunity scavenger chemokine (C-C motif) receptor 5 (CCR5) associated with increased IL-12 levels. Similar downregulation of CCR5 on peripheral blood mononuclear cells (PBMCs) and increased levels of serum IL-12 has been found in pSS (Wildenberg et al., 2008).

2.3.5. T cells

Infiltrating T-cells in the exocrine glands are activated cells, with high expression of HLA class II molecules, co-stimulatory molecules such as B7.1 and B7.2, in association with adhesion molecules such ICAM-1 and Vascular Cell Adhesion Molecule 1 (VCAM-1). T-cells are characterized by production of Th1 type cytokines (IL-2, IFN- γ , IL-6) and also the expression of IL-2 receptor (Fox et al., 1994; Ohyama et al., 1996; Mitsias et al., 2002). CD45RO characteristic of memory T-cells is also more expressed by infiltrating T-cells. Th1 cytokines are important to perpetuate the inflammatory medium in the exocrine tissues (Amft et al., 2001). CD4+ cells present a restriction of junction region, suggesting that the auto-antigen recognized by T cells is a common one. Supporting this hypothesis, T-cell repertory is similar in lacrimal and SGs from pSS

patients. In addition, certain TCR variable (V) region genes ($V\alpha 2$, $V\alpha 11.1$, $V\alpha 17.1$, $V\beta 2$ and $V\beta 13$) are predominantly expressed, suggesting limited heterogeneity of the infiltrating T cells (Sumida et al., 1994; Ohyama et al., 1995; Matsumoto et al., 1996). These findings support an antigen-driven T-cell proliferation in exocrine glands. Other T-cell populations are also present in exocrine glands. Presence of regulatory T cells (Treg) in SGs is associated with severity of the disease, suggesting that they are not able to suppress the autoimmune inflammatory process (Christodoulou et al., 2008). LTh17 have been also described in SGs from pSS patients, and their presence in T-cells infiltrates may be related to the pathogenesis of the disease (Sakai et al., 2008).

2.3.6. B cells

2.3.6.1. Introduction

The classic concept ascribes a key role to the T cell, because their predominance in exocrine glands. However, recent evidence indicates a major contribution of B cells (Hansen et al., 2002; Daridon et al., 2007). B cells produce not only auto-Abs, but also several cytokines, and act as APC. B-cell infiltrates may constitute GCs in the salivary glands (Le Pottier et al., 2009). In addition, BAFF, also known as B-lymphocyte stimulator (BLyS), is a cytokine that prevents apoptosis of autoreactive B cells (D'Arbonne et al., 2006). Mice transgenic for BAFF develop autoimmune traits reminiscent of pSS (Groom et al., 2002). In patients with pSS, circulating BAFF levels are increased and correlate with autoantibody production (Pers et al., 2005). B cells may differentiate into two types of effector cells, Be1 producing Th1 cytokines and Be2 producing Th2 cytokines (Daridon et al., 2007). Moreover, the distribution of mature B-cell subsets from Bm1 through Bm5 in peripheral blood of patients with pSS differs from that in patients with other autoimmune diseases and in controls, being characterized by higher percentages of Bm2 and Bm2' cells (Binard et al., 2009). Thus, B cells are involved in the development of SS and therefore hold promise as therapeutic targets. In the next sections a detailed description of B cells in SGs, peripheral blood from pSS patients, and mice models will be described. The role of BAFF will be described extensively in section 5 (Introduction).

2.3.6.2. B-cell classification according to their ontogenic state

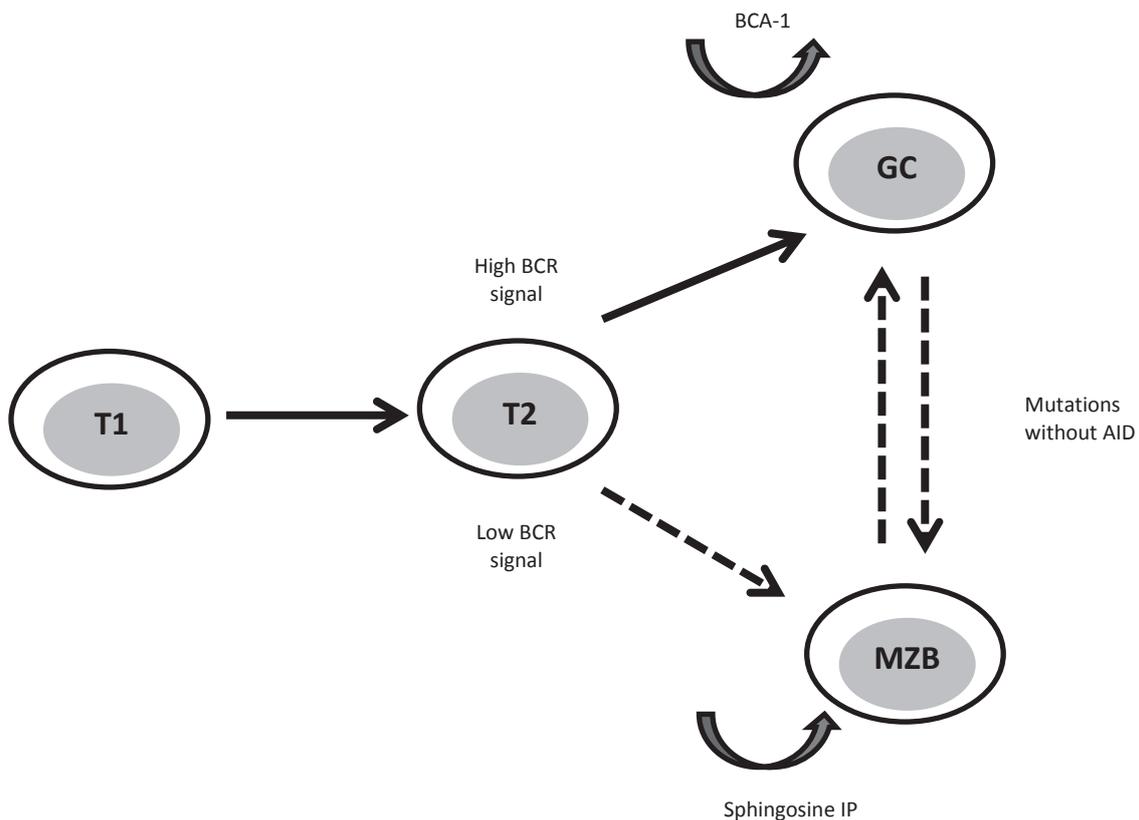
B cells originate in the bone marrow (BM). As soon as they have productively rearranged their Ig genes, pro-B-cells proceed to the pre-B-cell stage. On their arrival to the spleen, immature B cells give rise to type-1 (BT1), type-2 (BT2) and possibly type-3 (Palanichamy et al., 2009) **transitional B cells**. As transitional B cells they are pushed into migrating from the BM to secondary lymphoid organs (SLO). There, they carry on maturing, and are further-selected by antigens. As BT1, they present as CD20+CD5+CD10+/-CD21+/-CD23+/-IgM+IgD+/- and CD38+, but once they have evolved to type 2 (BT2), they become CD20+CD5+/-CD21++CD23+/-IgM++IgD++ and CD38+/- . In pSS, T2 B cells accumulate in SGs (Daridon et al., 2006). T2 B cells differentiate either to circulating lymphocytes that get organized as **GCs**, or to non-circulating lymphocytes that populate the **MZ**. Progression of T2 B-cells towards MZ or GCs may be determined by the quality of B Cell Receptor (BCR)-evoked signals and the subsequent expression of the Notch proteins (Saito et al., 2003). Alternatively, MZ B-cells with mutated Ig genes, but without activation-induced cytidine deaminase, may have passed a germinal center (GC) response (Willenbrock et al., 2005). Finally, the expression of sphingosine 1-phosphate receptor 1 on the B-cells may overcome the recruiting activity of the B-cell-attracting chemokine (BCA)-1 to the GCs (Reif et al., 2002), and thereby retain B-cells within the MZ (Cinamon et al., 2004). BCA-1 (CXCL13) is expressed within lymphoid follicles (Amft et al., 2001), and its receptor upregulated by B cells (Hansen et al., 2005) of pSS patients (**Figure 3**).

2.3.6.3. B lymphocytes in SGs

Together with a decrease of memory B cells in peripheral blood, memory B cells (CD20+, CD27+) are observed in SGs of pSS, whereas the infiltrating B cells are hyperreactive, as indicated by the expression of elevated Ig levels with auto-Ab activity (Hansen et al., 2002). Plasma cells producing the different Ig isotypes are altered in the inflammatory lesions, where the IgG-and IgM-producing plasma cell predominates, comparing to normal SGs, where the main plasma cell isotype is IgA (Speight et al., 1990; Matthews et al., 1993). B lymphocytes infiltrating SGs may be driven oligoclonal or monoclonal (Pablos et al., 1994; Jordan et al., 1995; Stott et al., 1998;) and locally produce RF and anti-SSA/Ro and anti-SSB/La auto-Abs (Halse et al., 1999; Salomonsson et al., 2003). B-cell clustering into aggregates and GC-like structures may be identified in up to 1/3 of SGs samples and coincided with elevated titres of RF, increased IgG levels and higher focus score (Jonsson et al., 2007). The local elevated

expression of BAFF by infiltrating mononuclear cells and ECs has been implicated in the expansion of autorreactive B lymphocytes, the altered B-cell differentiation and distribution; GC-like structures formation and lymphoma transformation (see Chapter 4). In a retrospective study, it has been proposed that the presence of GC-like structures in diagnostic SG biopsy might be used as a highly predictive marker for NHL development (Theander et al., 2011).

Figure 3. B-cell classification according to their ontogenic state. From the transitional type 1 (T1) and T2 B-cells, two options depend on the B-cell receptor (BCR)-evoked signal and the downstream Notch 2 proteins: germinal center (GC) B-cells driven by the B-cell-attracting (BCA)-1 chemokine (or CXCL13), and MZ B-cells with mutations but without activation-induced cytidine deaminase (AID). (Modified from Le Pottier et al., 2006).



However, our group has shown recently that real GCs are less common in pSS that was previously suggested. Notably, B-cell aggregates in SGs lack the GC B-cell associated CD10 and CD38 markers (Le Pottier et al., 2009). The transcripts for activation-induced cytidine deaminase in most of the microdissected GC-like cell aggregates from SGs of pSS patients were not found. Thus, the finding of B-cell aggregates in the SGs from patients does not necessarily imply that they are functional

GCs (Salomonsson et al., 2003). On the other hand, immature pre-GC B cells have been identified in these SGs aggregates (Daridon et al., 2006). BAFF produced in the nearby tissue favor the formation of these cell aggregates.

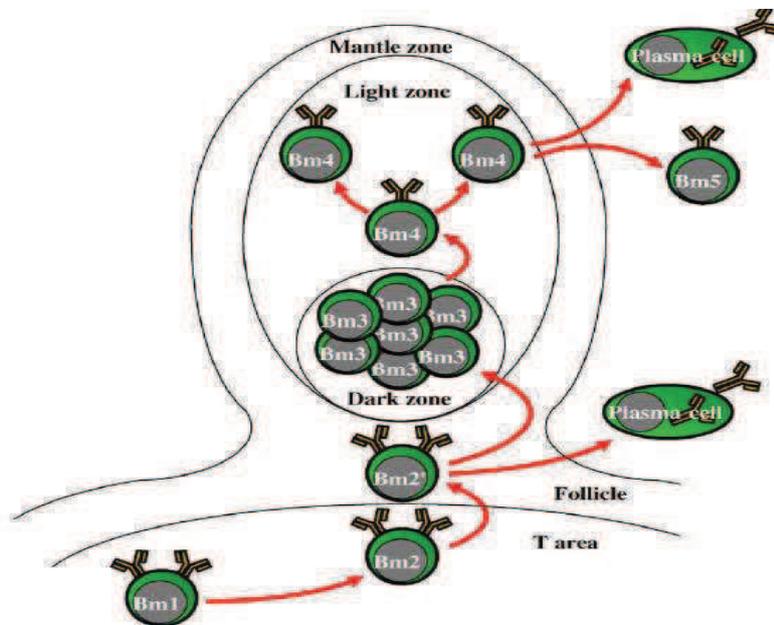
Immunohistological analyses of the primary effects after B-cell depletion by rituximab (RTX) in SGs have provided important information on the pathology of B-cell abnormalities in pSS. After treatment with RTX, lymphocytic infiltrates of SGs were reduced, with decrease of the lymphoepithelial lesion and partial disappearance of GC-like structures (Seror et al., 2007). Our group showed that while the peripheral blood repopulation after RTX-induced B-cell elimination first mimics a second round of B-cell ontogeny, later on, the characteristics of peripheral B-cell abnormality, with a predominance of naïve B cells and reduced amounts of memory B cells re-occurred (Pers et al., 2007). Memory B cells and transitional (T) 1 B cells were the first B cells which repopulated the SGs. This sequence resembles the pathophysiology of memory B-cell accumulation and provides evidence that the local microenvironment contributes to B-cell abnormalities in the disease.

2.3.6.4. B lymphocytes in peripheral blood

Increase of CD5+ B lymphocytes have been described in peripheral blood and SGs of pSS patients (Youinou et al., 1988). In addition, the distribution of peripheral blood B-cell subsets is markedly abnormal in patients with pSS. Memory B cells accumulate in epithelial organs, where they may form tertiary lymphoid structures, and their proportion in peripheral blood is decreased (Hansen et al., 2002; Daridon et al., 2006). In contrast, the proportion of transitional and naïve B cells is increased in peripheral blood (d'Arbonne et al., 2006). The development stages of GC B cells are based on the relative expression of IgD and CD38 on mature B (Bm) lymphocytes (Pascual et al., 1994) from naïve cells leaving the bone marrow (Bm1) to memory B cells activated and differentiated by their specific antigen (Bm5). The development starts with CD38-IgD+ naïve Bm1 that progress into CD38+IgD+ antigen activated Bm2, of which some become CD38++IgD+ Bm2' GC founder cells. These differentiate into CD38++IgD- Bm3 centroblasts and Bm4 centrocytes. Two types of B-cells arise from GC reactions : CD38+IgD- early memory B-cells that mature locally into CD38-IgD-Bm5 memory B cells, and CD38++IgD- plasmablasts which have first been described by Odendahl et al. (Odendahl et al., 2000) and return to the bone marrow where they differentiate into long-lived PCs (**Figure 4**). A few cells of each subset escape into the circulation from

GCs. For unknown reasons, distribution of Bm cells is abnormal in peripheral blood of pSS patients (Bohnhorst et al., 2001; Hansen et al., 2002). Recently, our group showed that a high $(Bm2+Bm2')/(eBm5 + Bm5)$ ratio (≥ 5) was strongly associated with a diagnosis of pSS compared to RA, SLE patients or healthy controls (Binard et al., 2009) (Figure 5).

Figure 4. Germinal center (GC) changing a primary lymphoid follicle (LF) into a secondary LF. The GC is surrounded by the mantle zone, comprised of the light and dark zone, and populated by mature B (Bm)-cells evolving from Bm1 in the T-cell area through plasma cells getting back to the bone marrow (From Le Pottier et al., 2006).

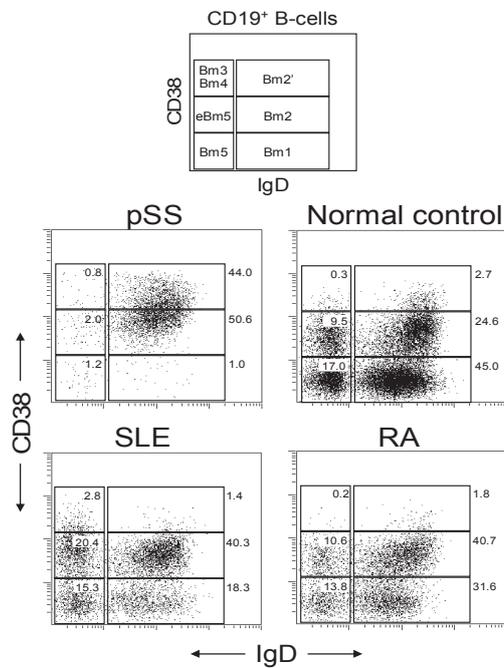


In addition to this abnormal distribution, the membrane expression of CD72, a transmembrane lectin expressed throughout the period of B-cell maturation that can both positively and negatively modulate BCR-mediated signaling, is up-regulated in B lymphocytes from pSS patients as compared with RA or SLE patients (Smith et al., 2004).

2.3.6.5. Regulatory B cells

Regulatory B cells (Breg), recently described in human, do exert regulatory effects through the production of cytokines (Jamin et al., 2008). Their role to regulate the inflammatory autoimmune process in pSS remains to be elucidated.

Figure 5. B-cell subsets in patients with primary Sjögren’s syndrome (pSS). Bm2 + Bm2^l/eBm5 + Bm5 ratio is increased in pSS patients compared to systemic lupus and rheumatoid arthritis patients and healthy controls. pSS: primary Sjögren’s syndrome; SLE: systemic lupus erythematosus; RA: rheumatoid arthritis (From Binard et al., 2009).



2.3.6.6. Memory B cells

Based on flow cytometric staining of memory B cells with CD27 and IgD, a significant reduction of peripheral CD27⁺IgD⁺IgM⁺ (memory B cells that have not undergone isotype switching) B cells along with an enhanced surface-expression of HLA-DR, CD38 and CD95, as well as a reduced surface-expression of CD21, CXCR5 and ICOS-L has been identified in pSS patients compared to controls (Hansen et al., 2009). This may reflect impaired differentiation and censoring mechanisms within SLO and/or ectopic “tertiary” lymphoid tissues in pSS. Classical memory B cells (CD27⁺IgD⁻IgM⁻) generated in GCs are markedly reduced in peripheral blood of pSS patients, whereas are accumulated in inflamed tissues. Chemokine receptor-ligand interactions, especially CXCL12-CXCR4 and CXCL13-CXCR5 interactions may explain B-cell abnormalities in pSS and decrease of peripheral blood memory B cells (Bohnhorst et al., 2001).

2.3.6.7. Intrinsic B-cell defects

The cause of B-cell hyperactivity in pSS is not known, but at least 3 different intrinsic abnormalities have been identified. First, pre-switch Ig transcripts are retained in circulating memory B cells, regardless of the advent of post-switch Ig transcripts (Hansen et al., 2004). Secondly, the kinetics of BCR translocation to lipid-rich membrane signaling microdomains (lipid rafts) is altered in pSS (d'Arbonne et al., 2006). There is evidence of prolonged translocation of the BCR into lipid rafts in B cells, resulting in inappropriately enhanced signaling. Third, elevated serum and salivary levels of BAFF in pSS are responsible for B-cell survival and B-cell hyperactivity.

2.3.6.8. Autoreactivity-driven B lymphocyte proliferation

Autoreactive transitional type 1 (BT1) and type 2 (BT2) B lymphocytes may accumulate in an area reminiscent of the splenic MZ, serving as a fast track to autoimmunity. In SGs, this event is likely to happen, since SG-cell aggregates appear to primarily include T2 B cells and MZ-like B cells (Daridon et al., 2006). These clusters of MZ-like B cells act as reservoirs for autoreactive B cells. Because of the permanent availability of autoantigens, a clone can be transformed and become dominant. BAFF transgenic mice that lack MZ B cells develop nephritis but not sialadenitis (Fletcher et al., 2006). These data show that the development of autoimmune epitheliitis requires the presence of MZ B cells.

The development of lymphoma may thus arise secondary to the autoimmune response. Sustained stimulation promotes the expansion of scarce clones and results in the outgrowth of monoclonal aggregates of B cells (Friedman et al., 1991). Thus, B-cell transformation from the naïve stage to a mature proliferating single clone, through to a nonmalignant pseudolymphoma stage represents a continuum. Risk factors associated with increased development of NHL in pSS are discussed in chapter 1.

2.3.6.9. B cell-derived cytokines

Recent discoveries have unveiled new insights into B cell-derived cytokines, including IFN- γ and IL-4 that modulate the response (Youinou et al., 2009). They are likely to serve as effectors of some B cells functions. Given the kinetics of B cell generation and

the cytokine profile of B lymphocytes, T helper (Th) 1 phenotype may be imprinted by B effectors (Be) 1 cells through their expression of IL-2 and IFN- γ by B cells. This is sustained by an IFN- γ /IFN- γ receptor autocrine loop. Conversely, Th2 cells induced naïve B-cell polarization into Be2, which produce IL-4 and IL-6 in the absence of GATA-3. In fact, the Th1/Th2 cytokine balance changes with the progress of the immunopathological lesions (Mitzias et al., 2002). Distinct populations of serum cytokines have also been found (Szodoray et al., 2004) to differentiate patients from controls, and from one patient to another, depending on the presence or absence of extra-glandular manifestations. B-cell produced cytokines may be classified as pro-inflammatory (IL-1, IL-6, TNF- α and lymphotoxin-LT- α); immunosuppressive cytokines (TGF- β and IL-10); or as hematopoietic growth factors (granulocyte/monocytes-colony stimulating factor and IL-17).

IL-14 α , a B-cell growth factor has been studied in mice models. Transgenic mice models for this cytokine develop clinical and immunological characteristics of pSS (see animal models). In humans, PMBCs from pSS patients express higher levels of IL-14 α compared to controls (Shen et al., 2009). Interestingly, IL-14 α transgenic mice develop clinical and immunological characteristic of SS in the same order that human pathology (from early hypergammaglobulinemia to B-cell lymphoma), showing that this cytokine may play an essential role in pSS pathogenesis.

2.3.6.10. IL-6

In pSS, IL-6 participates not only in the generation of Th17 cells but also activates local B cells in an autocrine manner. Th17 cells induced in the presence of IL-6 orchestrate the GCs development by auto-reactive lymphocytes (Hsu et al., 2008), such as those that our group has described in SGs from pSS patients (Le Pottier et al., 2009). IL-6 contributes to the expression of recombination-activating genes (*rag*). Results from our laboratory have shown that IL-6 signaling results in secondary Ab gene rearrangements, and thereby favors the Auto-Abs synthesis (Hillion et al., 2007).

2.3.7. Cytokines and chemokines other than B-cell cytokines

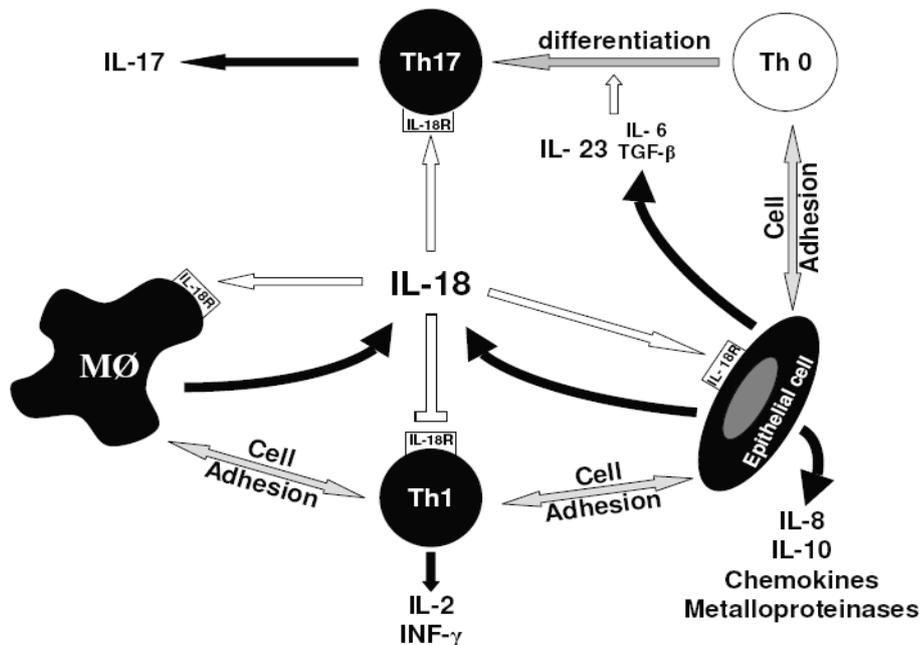
Although many cytokines may be implicated in pSS pathophysiological mechanisms, in the next sections, the role of FLT3-Ligand and BAFF (both cytokines implicated in ontogenesis and survival of B-cells respectively) will be deeply reviewed in the next

sections.

2.3.7.1. T-cell cytokines

Upon T-cell activation, the cytokine milieu dictates the Th cell polarization. Thus, IFN- γ and IL-12 induce the T box transcription factor (Tbet) and STAT4 leading to the differentiation of naïve CD4⁺ T cells to Th1 cells. Th1 cells produce IFN- γ and TNF- α . In contrast, IL-2 and IL-7 drive the binding of a specific transcription factor (TF) to the **WGATAR** nucleotide consensus sequence (GATA-3), leading to the differentiation into Th2 cells. Th2 cells synthesize IL-4, IL-5, IL-6, IL-10 and IL-13. In the case of pSS, patients have long been thought to suffer from a Th1-mediated condition, supported by the high levels of IFN- γ in serum (Hooks et al., 1979), and a predominance of Th1 cells in blood (Hagiwara et al., 1998). However, the importance of the Th1/Th2 balance in pSS seems to be more subtle than the initial data. Th1 activity is actually decreased in peripheral blood but increased in SGs of patients (van Woerkom et al., 2005). Furthermore, the cytokine pattern would be predicted to shift from Th1 to Th2 with the progression of SGs lesions. For example, IFN- γ expression is associated with high-grade infiltrates of the SGs, while a low-grade infiltrate is rather accompanied by a Th2 pattern (Mitzias et al., 2002). The following discovery of IL-23 explained the contradictory results about the role of Th1 and Th2 in pSS. This cytokine has been classified together with the IL-17 in the Th17 cells (Nguyen et al., 2008). IL-23 is required for the maintenance of Th17. High serum and saliva levels of IL-17 have been reported in pSS patients (Katsifis et al., 2009). In addition, MSGs biopsies have shown a predominance of IL-17-containing cells within the inflammatory lesions (Sakai et al., 2008). This finding is associated with the presence of transforming-growth factor beta (TGF- β), IL-6 and IL-23, all requisite for the Th17 differentiation. These results show that Th17 cells are central to the pathophysiology of the disease. On the other hand, Th17 cell homeostasis is regulated by Treg cells. Circulating Treg cells, defined as CD4⁺CD25⁺Foxp3⁺, correlate inversely with those Treg infiltrating SGs (Christodoulou et al., 2008). The fact that there are fewer Treg cells in the advanced than in the mild SGs infiltrates supports the view that DC-derived TGF- β induces Foxp3 in naïve T cells and switches T-cell differentiation from the default Treg-cell pathway to a Th17 differentiation in presence of IL-6. IL-18, a pro-inflammatory cytokine secreted by EC has been claimed to regulate the Th1 response and to amplify the effects on IL-17 synthesis (Sakai et al., 2008) (**Figure 6**).

Figure 6. The network of T helper (Th) cells gathers together Th0, Th1, Th2 and Th17 lymphocytes. Th0 differentiation into Th17 is induced by IL-23, IL-6 and TGF- β . IL-18 regulates the Th1 response and amplifies the IL-17 synthesis. (From Youinou and Pers, 2011).



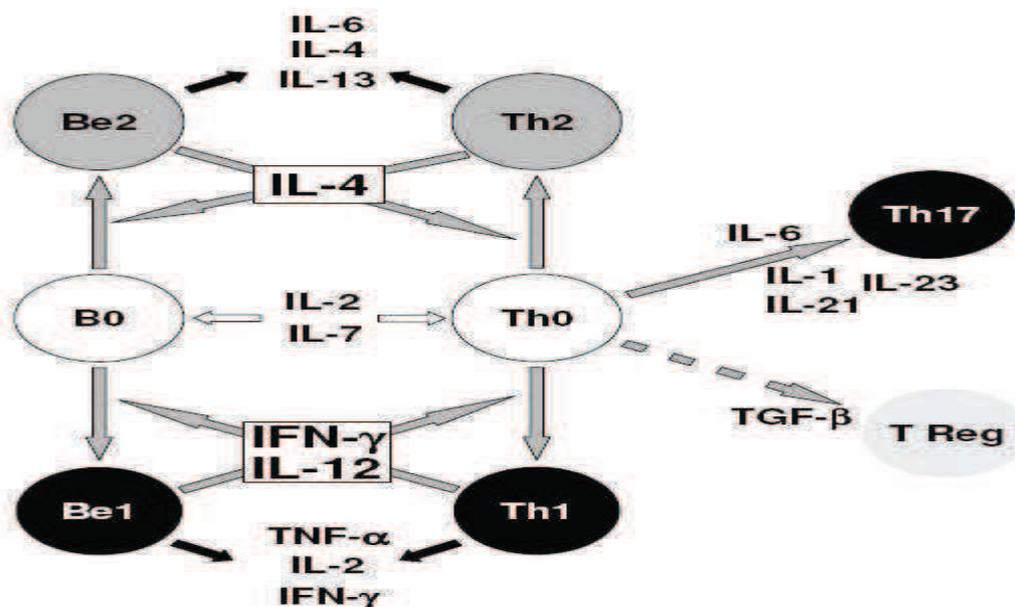
Fox et al. (Fox et al., 1994) reported that CD4⁺ T cells in SGs of pSS patients produce mainly IL-2, IFN- γ and IL-10, but not IL-4 or IL-5. IL-2, IFN- γ , IL-10, IL-6 and TGF- β are consistently detected in all patients, while IL-4 and IL-5 are detected in some patients in association with strong B-cell accumulation in SGs. These results suggest that Th1 cytokines are essential for the induction and/or maintenance of pSS, and that Th2 cytokines are involved in the progression of the disease, especially local B-cell activation (Ohyama et al., 1996).

Recently, other T-cell cytokines have been described in MSGs biopsies. Bikker et al (2011), show that IL-7R α is increasingly expressed by T cells from SGs from pSS and not by controls. This expression was correlated with severity of infiltrates and IL-7 expression. Considering the immunostimulatory role of IL-7/IL-7R stimulation, this cytokine deserves to be further studied in pSS pathogenesis.

2.3.7.2. Interconnections between B- and T-cell cytokine networks

The activated Th cells crosstalk with activated B cells to regulate their response. Conversely, Be cell modulate T-cell polarization. The factors that affect T-cell differentiation toward Th1 cells induce naïve B cells to produce IFN- γ via activation of STAT-3, the phosphorylation of which is initiated by IL-12 (Durali et al., 2003). Once B cells have been induced to produce IFN- γ , the presence of Th1 is no longer required to maintain polarized Be cells. Aside from promoting Th1 cell polarization, Be1 cells amplify IFN- γ production by T cells via a tumor necrosis factor (TNF)- α -mediated mechanism. In the other hand, IL-10 produced by B cells suppresses IL-12 production by DCs, thus blocking Th1 cell responses. **Figure 7** summarizes the cell and cytokines interaction in the development of pSS.

Figure 7. Polarization of T cells and B cells within the salivary gland inflammatory response. Naïve B cells (B0) polarized in the presence of T helper (Th)1 cells are designed B effector (Be)1 cells. Naïve T cells (Th0) polarized in the presence of Be2 cells are designated Th2 cells. Consequently, interconnections exist between the B-cell and T-cell cytokine networks. (From Youinou and Pers, 2011).



2.3.7.3. Type I IFN-s

Type I IFN-s in humans consist of 13 IFN- α molecules produced predominantly by pDCs, the IFN- β produced by fibroblasts and other less significant variants (such as IFN- κ). Although all nucleated cells can produce type I IFN-s in response to viral infections, the pDCs produce the highest amount (Cella et al., 1999). Type I IFN-signal through the receptor activates the inducible expression of hundreds of genes implicated in antiviral response. Signaling involves Janus activated kinase (JAK)-1, STAT, mitogen-activated protein kinase p38 and phosphatidylinositol 3 kinase (PI3) cascades. In addition, several immunomodulatory effects such as activation of immature DCs, B-cell activation and Ig class switching through induction of BAFF, enhancement of T-cell proliferation, and induction of chemokines have been described. Type I IFNs link together both innate and adaptive immune systems. Their effects in adaptive immunity are mediated through activated DCs and also by direct binding to IFN-R on B cells, T cells, neutrophils and natural killer (NK) cells. The “IFN- signature” characterized by overexpression of type I IFN-inducible genes in MSGs and peripheral blood of pSS patients is described above. While the initial triggers of the innate immune response in pSS remain elusive, preliminary evidence suggests the role of inappropriately expressed endogenous LINE-1 (L1) retroelements as potential triggers of type I IFNs activation in the disease, possible through TLR dependent pathways (Mavragani et al., 2010). Regulation of L1 is dependent of epigenetic mechanism (methylation), suggesting that under environmental influence (e.g. viral infections) the abnormal expression of endogenous nucleic acids (such as L1) induces the production of type I IFN in SGs. Under the influence of IFN, ECs could be activated expressing MHC-I and co-stimulatory molecules.

2.3.7.4. Type II IFN-s

IFN- γ is secreted by activated T cells, natural killer cells and macrophages. Mature IFN- γ is a protein of 143 amino acids with a molecular weight of 20-kDa. The IFN- γ receptor consists of two subunits, IFN- γ receptor (IFNGR) 1 and IFNGR2, and each molecule interacts with a member of the JAK family. IFN- γ responses are mainly mediated via JAK/STAT and sequence elements (IFN- γ -activated sequence or GAS for Gamma-Activated Sequence) in the promoters of IFN- γ targeted genes (Bach et al., 1997; Darnell et al., 1997; Ramana et al., 2000). However, several IFN- γ stimulated genes are regulated by IFN-stimulated regulatory elements (ISREs) instead of GAS

elements. ISREs can be activated either by IFN-stimulated genes (ISGs) F3, composed of IRF9 and Stat1-Stat2 dimer, formed in response to type I IFN-dependent signaling, or by IRF proteins, activated by IFNs or TLR signaling pathways. In addition, some ISGs require IKK β for their induction by IFN- γ . These IKK β dependent genes are activated synergistically by the combination of IFN- γ -dependent signaling and Nuclear Factor- κ B (NF- κ B) activation (Shultz et al., 2009).

Recently, Conzelmann et al. (Conzelman et al., 2010) showed that IFN- γ -induced JAK1 enhances CD40-induced IL12p70 release from DCs, involving STAT1 activation and *de novo* induction of interferon-responsive factors (IRF)-1 and IRF-8. Thus, the CD40- and JAK/STAT/IRF-signaling pathways are complementary for the induction of a pro-inflammatory cytokine profile in human DCs. However, the studies that have shown induction of BAFF by IFN- γ showed that JAK and STAT1 are not involved, and that PKA/CREB was actually the dominant pathway (Kim et al., 2008). Consistent with these results, it has been reported that IFN- γ activates the cAMP/PKA/CREB signaling pathway in murine peritoneal macrophages (Liu et al., 2004). In fact, there is considerable evidence for Stat1-independent pathways in IFN- γ signaling (Briscoe et al., 1996; Gil et al., 2001; Ramana et al., 2001). In normal immune response against pathogens, IFN- γ production by T cells is under the control expression of the proximal promoter -73 to -48 bp upstream of the transcription starting site. The cAMP response element binding protein/activating transcription factor (CREB/ATF) and AP-1 regulate the secretion by human T cells in response to infectious antigens (Samten et al., 2008).

In pSS, IFN- γ levels are increased. HSG cell lines exposed to IFN- γ inhibited calcium signaling (Meehan et al., 1997). IFN- γ increases HLA class II expression by ECs, playing an important role in the ECs transformation into APC. IFN- γ also induces, together with IL-1 β , the expression of CD40 by ECs in culture, showing that IFN- γ stimulation may contribute to an activated status of ECs in pSS (Dimitriou et al., 2002). Furthermore, experimental evidence indicates that ECs are particularly susceptible to Fas-mediated apoptosis after IFN- γ stimulation, probably via the downregulation of the apoptosis inhibitor protein c-FLIP (Abu-Helu et al., 2001).

2.3.7.5. Lymphotoxins

The lymphotoxins (LT α and LT β) and their receptors are members of the TNF superfamily. LT β R signaling pathway is important in the development of organized lymphoid structures (Alimzhanov et al., 1997). This signaling pathway is also crucial in lymphoid neogenesis at sites of inflammation (Kratz et al., 1996), showing the role of lymphotoxins in the pathogenesis of pSS. Inhibition of LT β pathway in NOD mice model shows ablation of lymphoid neogenesis in the SGs and an improvement in SGs function (Gatumu et al., 2008).

2.3.7.6. IL-1

IL-1 is a proinflammatory cytokine produced by many cell types, including mucosal ECs. Increased levels of IL-1 α and IL-1 β have been identified in tear fluid and conjunctival epithelium of pSS (Solomon et al., 2001). In a recent study, Chen et al. (Chen et al., 2010) demonstrated in the autoimmune-regulator (AIRE) deficient mice, that IL-1 is capable to modulate the pathogenesis of squamous metaplasia (from nonkeratinized to a nonsecretory keratinized epithelium) in conjunctival epithelium, indicating that targeting IL-1 may be beneficial for treating ocular surface disease associated with pSS. However, preliminary results of IL-1 inhibition did not show effect in clinical characteristics of pSS (fatigue).

2.3.7.7. Chemokines and chemokine receptors

Interactions between chemokines and chemokine receptors cause selective local infiltration of specific cells into inflamed areas. In addition, chemokines are involved in the Th1/Th2 balance and immune responses. Ogawa et al. (Ogawa et al., 2002) reported that Th1 chemokines, such as IFN- γ -inducible 10kd protein (IP-10) (a chemokine that binds CXCR3 and it is chemotactic for monocytes and Th1 cells) and monokine induced by IFN- γ (Mig) are involved in the accumulation of T-cell infiltrates in the SGs of pSS patients. Recently, Moriyama et al. (Moriyama et al., 2012) showed that in addition to IP-10, CXCR3, MIP-1 α , RANTES, TARC (produced by vascular endothelial cells and DCs), MDC (produced by APC) and CCR4 are highly expressed in SGs of patients. TARC, MDC and CCR4 were higher in MSGs with strong lymphocytic infiltration in comparison to those with weak lymphocytic infiltration. TARC and MDC were detected by immunohistochemical in/around the ductal ECs (which may

act as APC in disease state) in MSGs with strong lymphocytic infiltrates, while CCR4 was detected in infiltrating lymphocytes. These findings were accompanied by high salivary concentration of IL-8 and MDC. The interaction of CCR4 with MDC and TARC is suggested to play a critical role in the accumulation of Th2 cells and consequently the pSS progression.

The chemokines and chemokines receptor implicated in B-cell migration and GC formation are discussed in B-cell section.

2.4. Conclusion: current model of the pathophysiology of primary Sjögren's syndrome

SS appears to be triggered by environmental factors such as viral infection and/or hormonal factors. In patients carrying susceptibility genes predisposing to enhanced reaction of the innate immune system via IFN- pathway proteins (IRF5, STAT4), ECs become activated and may also produce IFN- and other cytokines. Subsequently, auto-antigen presentation by the ECs and BAFF overproduction induced by IFN- stimulates B- and T-cell activation. Adaptive immune responses, mediated by B- and T-cell activation, perpetuate the inflammatory process in exocrine glands and ultimately lead to tissue dysfunction and destruction. Neuro-exocrine mechanisms also contribute to decreased glandular secretion.

3. Treatment of pSS

The current treatment of pSS consists in supportive and symptomatic measures aimed at relieving the sicca symptoms. The key issues for the routine management are systemic treatment for specific extra-glandular manifestations and regular assessment by rheumatologists, dentists and ophthalmologists. The good health of mucosal tissues rests upon maintaining a sustained level of dampness in both the eye and the mouth. In the eyes a variety of moisture protection devices, artificial tears, and the use of topical agents including methylprednisolone and cyclosporin may be of great value. A variety of punctual plugs are also available. Dryness of the mouth may be helped by frequent sips of water, artificial saliva and the use of pilocarpine or cevimeline that stimulate the muscarinic receptors. All the available drugs may transiently improve the dryness, but fail to affect the course of the disease. Ultimately, there is a critical need

for disease-modifying drugs to treat patients who have severe organ involvement and factors indicating a high risk of lymphoma. Immunotherapy in pSS has been unsuccessfully launched with TNF- α inhibitors, IFN- α and currently being tested with B-cell-targeted therapies. By far, treatments that cause B-cell depletion have yielded the best results as disease-modifying drugs for pSS. All emerging therapies for pSS, are reviewed in the **appendix 5 (Emerging therapies for Sjögren’s syndrome. *Expert Opin Emerging Drugs* 2010;15:269-282)**. Previous and on-going studies with special emphasis in B-cell targeted therapies are summarized in the **appendix 6 (B Cell-targeted therapies in Sjögren’s syndrome. *Autoimmun Rev* 2010;9:224-228)**.

Inhibition of TNF- α with both infliximab and etanercept have not shown efficacy in clinical and biologic variables (Steinfeld et al., 2001; 2002; Mariette et al., 2004; Zandbelt et al., 2004; Sankar et al., 2004). Concerning B-cell depletion therapies several molecules can be targeted (**Table 10**).

Table 10. Potential B-cell targets, monoclonal antibodies (mAbs) and other treatments used for B-cell depletion in patients with Sjögren’s syndrome

| |
|--|
| Direct targeting of B cells |
| CD-20 antigen |
| • RTX (chimeric mAb) |
| • Ocrelizumab (humanized mAb) |
| • Ofatumumab (human mAb) |
| • Veltuzumab (humanized mAb) |
| • TRU-015 (engineered protein) |
| CD-22 antigen |
| • Epratuzumab (humanized mAb) |
| Indirect targeting of B cells |
| BAFF |
| • Belimumab (LymphoStat B: fully human mAb against BAFF) |
| BAFF receptors |
| • Anti-BR3 |
| • Atacicept (IgG Fc fused to the extracellular TACI receptor domain) |
| • Briobacept/BR3-Fc (IgG Fc fused to the extracellular BAFF receptor, BR3) domain) |

BAFF: B-lymphocyte Activator Factor belonging to the TNF family; **TACI:** Transmembrane activator and calcium modulator and cyclophilin ligand interactor.

The most widely studied target for achieving B-cell depletion in pSS is the CD20 antigen (human B-cell-restricted differentiation Antigen), a hydrophobic transmembrane

protein with a molecular weight of approximately 35 kDa found on pre-B and mature B cells (Einfeld et al., 1988; Valentine et al., 1989), as well as in over 90% of B-cell in NHL (Anderson et al., 1984). RTX, an anti-CD20 chimeric mAb, it is the only anti-CD20 compound thus far tested in pSS. In retrospective (Gottenberg et al., 2005; Galarza et al., 2008), open-label studies (Pijpe et al., 2005; Seror et al., 2007; Devauchelle-Pensec et al., 2007) and randomized trials (Meijer et al., 2010; Dass S et al., 2008), there is a trend for efficacy of RTX for at least six to nine months in pSS patients with active disease, improving both the subjective and the objective complaints. The sicca symptoms abated in some patients, and the objective measures of salivary function improved in those with early disease. Extraglandular clinical manifestations of pSS responded to RTX. Re-treatment with RTX resulted in a similar good clinical response. Laboratory abnormalities associated with B-cell over-activity also improved. To confirm these promising results, additional randomized placebo-controlled trials are presently in progress (TEARS study in France and TRACTISS in the United Kingdom). **Table 11** summarizes the main previous and ongoing (from clinicaltrials.gov) trials with immunotherapy in pSS patients.

Table 11. Immunotherapy in primary Sjögren’s syndrome

| Study | Target | Immunotherapy | Results in clinical trials |
|---|---------------|--------------------------------------|---|
| Published studies | | | |
| Mariette et al., 2004 | TNF- α | Infliximab | No effect in reduction of sicca symptoms. |
| Sankar et al., 2004 | TNF- α | Etanercept | No effect in reduction of sicca symptoms. |
| Shiozawa et al. 1998 Ship et al., 1999. Khurshudian, 2003. Cummins et al., 2003. | IFN- α | Oral administration of IFN- α | Improved in saliva secretion and xerostomia and xerophthalmia. Improvements only in unstimulated salivary flow in Cummins et al. series. |
| Pijpe et al., 2005 Seror et al., 2007 Devauchelle-Pensec et al., 2007 Dass et al., 2008 Meijer et al., 2010 | CD20 | RTX | Efficacy of RTX for at least six to nine months in pSS patients with active disease, improving both the subjective and the objective complaints. Improvement in fatigue and extraglandular manifestations. |
| Steinfeld et al., 2006 | CD22 | Epratuzumab | Improvement in Schirmer test, unstimulated whole salivary flow, and the VAS-fatigue. |
| Ongoing studies | | | |
| NCT01160666 | BAFF | Belimumab | |
| NCT01008982 | BAFF | Belimumab | |
| NCT00740948 (Tears study) | CD20 | RTX | |
| NCT00363350 | CD20 | RTX | |
| NCT00426543 | CD20 | RTX | |

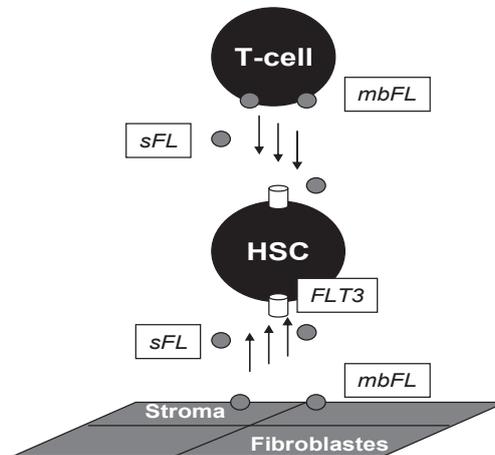
Recently, the effect of IL-1 inhibition with the IL-1 receptor antagonist (anakinra) on fatigue improvement in pSS patients has been described (NCT00683345) (Norheim et al., 2012). In this study, 26 pSS patients were randomized to receive either anakinra or placebo for four weeks. There was no significant difference between the two groups in fatigue scores. The effects of IL-1 inhibition on other symptoms were not reported. Although in animal models IL-1 levels may explain fatigue, the inhibition in this preliminary study did not show any effect. Other therapeutic perspectives that have shown efficacy in other AIDs such as RA may be tested in pSS. One interesting molecule tested in NOD mice models is baminercept (lymphotoxin-beta receptor-Ig fusion protein), that inhibes lymphotoxin-beta, which is a cytokine that governs the GC-like structures in exocrine glands. In this study, baminercept showed reduction of B-cell infiltrates in partial restoration of saliva production (Gatumu et al., 2009). Thus, this strategy may be tested in future clinical trials.

4. FLT3-Ligand

4.1. Introduction

Fms-like tyrosine kinase 3-Ligand (FLT3-L) is an essential cytokine for the development of lymphoid precursors and DC, produced by stromal cells in the BM and many other hematopoietic cells (including T lymphocytes) in both membrane and soluble forms (**Figure 8**) (Lisovsky et al., 1996 ; Antonysamy et Thomson, 2000 ; Bertrand et al., 2000 ; Drexler et Quentmeier, 2004). The administration of FLT3-L mobilizes hematopoietic cells from the BM into the blood, lymphoid organs, and parenchymal tissues. In addition to its effects on hematopoietic stem cells and progenitor cells, FLT3-L increases the number of DCs in the circulatory system (McKenna et al., 2000). Treatment of mice with FLT3-L results in significant stimulation of hematopoiesis, leading to BM hyperplasia, splenomegaly, and enlargement of the lymph nodes and liver. In addition, FLT3-L is abnormally increased in the majority of leukemias (Stirewalt et al., 2003), and FLT3 receptor mutations are present in 30% of patients with acute myeloid leukemia (AML) (Nakao et al., 1996). Although FLT3-L has been basically studied in hematological malignancies, their role in AIDs remains largely unknown. In next sections, their functions and physiological role, intracellular signaling, and pathological findings will be discussed.

Figure 8. FLT3-Ligand (FL)-mediated interactions in the bone marrow microenvironment. HSC: Hematopoietic stem cell; mbFL: membrane-bound FL; sFL: soluble FL.



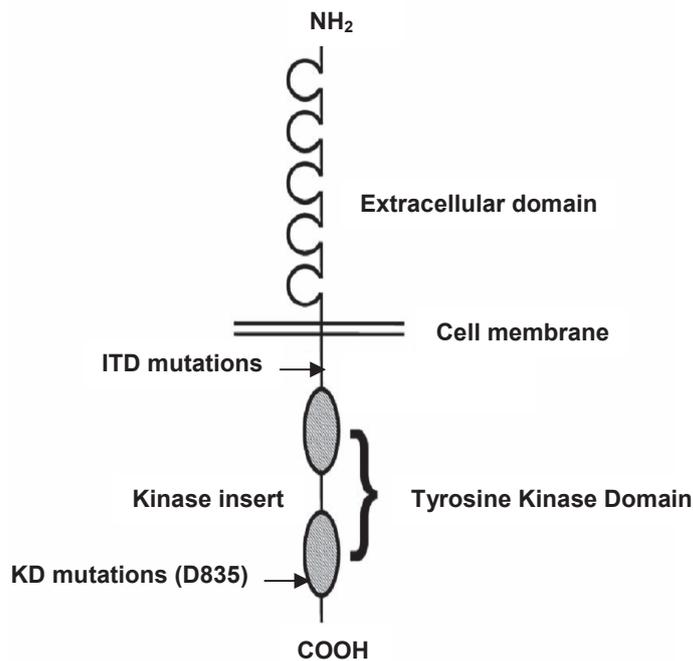
4.2. FLT3-L

FLT3-L was cloned in 1993–1994 by 2 groups of investigators (Lyman et al., 1993; Hannum et al., 1994). It is a cell surface transmembrane type I protein that can be proteolytically processed and released as a soluble homodimeric protein (Lyman et al., 1995; McClanahan et al., 1996). Both the membrane-bound and the cell-free forms can activate FLT3 and stimulate the growth of progenitor cells in the BM and blood. FLT3-L strongly synergizes with other hematopoietic growth factors and ILs (Sitnicka et al., 2003; Vosshenrich et al., 2003; Jensen et al., 2008). In contrast to the restricted distribution of FLT3 receptor, FLT3-L mRNA is ubiquitously expressed in hematopoietic and nonhematopoietic tissues. Despite the widespread expression of mRNA, the protein is found mainly in stromal fibroblasts in the BM microenvironment and T lymphocytes (Hannum et al., 1994; Brasel et al., 1995) (**Figure 8**). Mice lacking FLT3-L are viable but have more severe defects than FLT3 receptor knockouts, including reduced numbers of myeloid and lymphoid progenitors in the BM, and a marked deficiency of NK and DCs in lymph nodes, spleen and thymus (McKenna et al., 2000).

4.3. FLT3 and intracellular signaling

FLT3 receptor was isolated independently by two groups and termed fms-like tyrosine kinase 3 (FLT3) or fetal liver kinase-2 (flk2) (Matthews et al., 1991; Rosnet et al., 1991). It is a type III tyrosine kinase receptor, structurally related to macrophage colony-stimulating factor (M-CSF) and stem cell factor (SCF) receptors *c-fms* and *c-kit*, respectively (**Figure 9**).

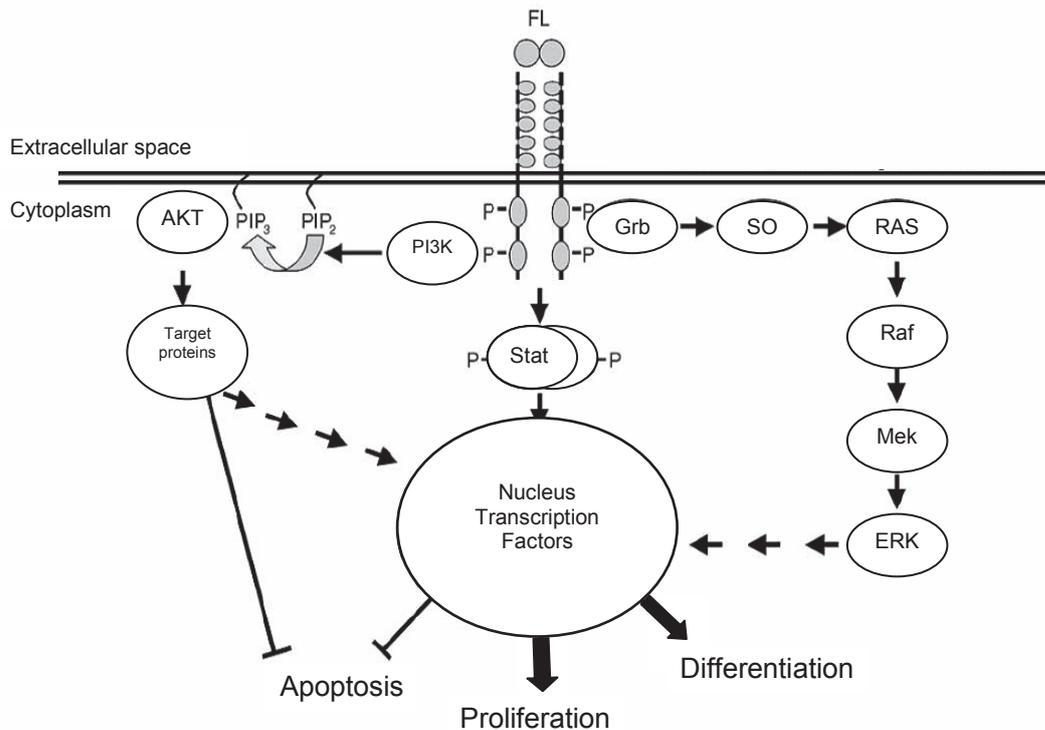
Figure 9. FLT3 structure. Shown in schematic fashion are the 5 immunoglobulin-like folds that make up the ligand-binding extracellular domain, single transmembrane domain, and cytoplasmic domain made up of a kinase domain interrupted by a kinase insert. The juxtamembrane domain where internal tandem duplications occur and aspartic acid 835 where most kinase domain mutations occur are indicated. The human *FLT3* gene is located on chromosome 13q12 with 24 exons, and shares 86% of homology with murine FLT3 (Rosnet et al., 1993; Small et al., 1994). The FLT3 protein contains 993 amino acids in length, with 10 N-glycosylation potential sites (Lyman and Jacobsen, 1998).



Mice with targeted disruption of FLT3 develop into adults but possess specific deficiencies in B-cell development and the multilineage repopulating function of earliest progenitors (Mackarehtschian et al., 1995). Crossing FLT3 knockout mice to mice with spontaneous mutations of *c-kit* resulted in a further decrease in hematopoietic cells (in particular lymphoid cells), indicating that FLT3 and *c-kit* signaling have additive function

in hematopoietic development. FLT3-L mediated triggering of FLT3 induces receptor dimerization and receptor autophosphorylation at tyrosine residues, creating the sites for the signal-transducing effector molecules. The downstream cascade involves the tyrosine phosphorylation and activation of multiple cytoplasmic molecules, either directly or indirectly, including, GAB1, GAB2, GTPase, RAS/mitogen-activated protein kinase (MAPK), phospholipase C- γ , extracellular-signal regulated kinase 1 and 2 (ERK1/2), Src-homology 2 containing protein tyrosine phosphate (SHP2), Src-homology 2 containing inositol phosphatase (SHIP) and the p85 subunit of phosphatidylinositol 3'-kinase (PI3K) (Lyman et al., 1994 ; Rosnet et al., 1996 ; Lavagna-Sevenier et al., 1998 ; Zhang et al., 1999 ; Zhang et al., 1999 ; Zhang and Broxmeyer, 2000).

Figure 10. Intracellular signaling of FLT3. FLT3-L (FL) binding to its receptor induces receptor dimerization and receptor autophosphorylation at tyrosine residues, creating the sites for the signal-transducing effector molecules. Several proteins are active after FLT3 stimulation by FL. STAT 5: Signal transducer and activator of transcription 5, PI3K: phosphatidylinositol-3-kinase, PLC- γ : phospholipase C-gamma, ERK: extracellular-signal regulated kinase.



FLT3-L stimulation selectively activates STAT5 through a Janus kinase-independent mechanism. In addition, STAT3 is indispensable for FLT3-L dependent DCs differentiation (Laouar et al., 2003). In human leukemic myeloid cells, FLT3-L stimulation of wild-type FLT3 increases phosphorylation of Src kinase Lyn (Robinson et al., 2005). These pathways induce proliferation, differentiation and survival effects (**Figure 10**). Protein analyses show that the major signal transduction pathways leading from FLT3 include PI3K, RAS/MAPK and STAT5.

In the other hand, little is known about the protein-tyrosine phosphatases (PTP) to inhibit the FLT3 activation. Arora et al (Arora et al., 2011) using myeloid cells expressing wild type FLT3 and infected with a panel of lentiviral pseudotypes carrying shRNA expression cassettes targeting different PTP, show that DEP-1 is a negative regulator of FLT3 phosphorylation and signaling.

4.4. Distribution of FLT3

In humans, FLT3 expression has been reported in BM, liver, spleen, thymus and the placenta (Rosnet et al., 1993). In the BM, FLT3 is expressed predominantly on primitive hematopoietic precursors, restricted mainly to CD34+ cells lacking the lineage-specific markers. Within the stem cell pool, expression of FLT3 is heterogeneous, found on cells with long- and short-term marrow-reconstitution activity. Expression of FLT3 is lost as hematopoietic cells differentiate. Although this expression is usually lost, DCs are an exception, as mature DCs display persistent FLT3 expression.

In B lymphocytes, Flt-3 expression has been described in pre-pro-B cells and pro-B cells. In our laboratory, we described the expression of FLT3 by some mature B cells (**Article 2**). FLT3 is selectively expressed in some naïve B cells, whereas tonsillar GC B lymphocytes (Bm3/Bm4) do not express FLT3.

Monocytes and granulocytes are weakly positive, and mast cells and erythroid progenitors are negative at both the mRNA and cell surface levels (Gabbianelli et al., 1995).

4.5. Functions of FLT3-L

4.5.1. Functions in hematological precursors

FLT3-L stimulates the growth of hematopoietic progenitors from the BM, peripheral blood and cord blood *in vitro*. Alone, this cytokine is not sufficient, but in synergy with other hematopoietic growth factors and ILs it exerts pleiotropic effects on precursors of myeloid and lymphoid lineages (Hirayama et al., 1995). *In vivo*, the treatment of mice with FLT3-L results in significant stimulation of hematopoiesis, leading to BM hyperplasia, splenomegaly, and enlargement of the lymph nodes and liver. These effects are transient and hematopoietic values return to normal upon cessation of FLT3-L treatment (Brasel et al., 1996). Administration of FLT3-L to mice and rabbits subjected to lethal doses of irradiation protects Hematopoietic Stem Cells (HSC) and allows rapid hematopoietic recovery (Gratwohl et al., 1998). During steady-state hematopoiesis, FLT3-L is expressed constitutively but little of the cytokine is released by cells. Consequently, it is undetectable or very sparse on the cell surface, and FLT3-L levels in circulation are in normal circumstances ≤ 100 pg/mL. Instead, preformed FLT3-L can be detected in the cytoplasm of BM stroma fibroblasts, T cells, B cells and CD34+ progenitors. This mechanism of intracellular retention argues for the existence of a negative regulator controlling the availability of the cytokine during steady-state hematopoiesis. The regulator mechanism remains unknown.

In vivo, FLT3-L release may be triggered by stem cell deficiency in the BM. For example, in the case of blood disorders of the stem cell compartment (Fanconi anemia and acquired aplastic anemia), serum levels of FLT3-L are highly elevated up to 10 ng/mL (Lyman et al., 1995; Wodnar-Filipowicz et al., 1996). Since FLT3-L levels return to normal upon hematopoietic recovery, the serum concentration of this cytokine may serve as a surrogate marker of HSC content in the BM. *In vitro*, IL-2 and IL-7 induces the expression and release of FLT3-L by T cells, whereas CD40 ligand induces a release of FLT3-L from stromal fibroblasts (Chklovskaja et al., 2001).

FLT3 and its ligand regulate proliferation of hematopoietic progenitor cells in an autocrine/paracrine manner. Weisel et al (Weisel et al., 2009) evaluated the inhibition by CEP-701 (lestaurtinib, a specific inhibitor of FLT3) and imatinib (a tyrosin kinase inhibitor, non specific for FLT3). In this study, FLT3 inhibition impaired cell and progenitor cell growth and increased the apoptosis rate. This inhibition resulted in markedly increased of FLT3-L by CD34+ cells as well as upregulation of FLT3 expression, suggesting the autocrine/paracrine regulatory mechanism.

In mice, double deficient model in IL-7R and FLT3-L completely lack lymph nodes, conventional IgM B cells, IgA plasma cells, and B1 cells, and consequently produce no Igs. All stages of committed B-cell progenitors are undetectable in IL-7R α $-/-$ and FLT3 $-/-$ BM that also lacks expression of the B cell commitment factor *Pax5* and their target genes. Thus, these results show that signaling through FLT3 and IL-7R α are indispensable for fetal and adult B-cell development (Sitnicka et al., 2003; Jensen et al., 2008). The effects of both cytokines may converge in the PI3K/AKT pathways (Zhang and Broxmeyer, 2000). The synergic role of both cytokines has been also described in thymic progenitor development, demonstrating an indispensable role of FLT3-L in fetal and adult T lymphopoiesis (Sitnicka et al., 2007). In addition, threshold levels of FLT3-L are required for the generation and survival of lymphoid and B-cell progenitors. In a mice model, haplo-insufficiency of FLT3-L (+/-) reduced the numbers of lymphohematopoietic precursors, lymphoid progenitors and pro-B cells, in a level not as marked as FLT3-L ($-/-$), suggesting that FLT3-L levels set a threshold for B cell lymphopoiesis (Dolence et al., 2011).

In human B lymphopoiesis, FLT3-L acts as a strong synergistic factor in combination with IL-7 (Hunte et al, 1995; Namikawa et al., 1996), stimulating the growth of B lineage cells from the primitive progenitor stage through the pro-B cell stage, showing that FLT3-L has a wide range of activities in early hematopoiesis and B-cell lymphopoiesis (Hirayama et al., 1995).

4.5.2. Functions in dendritic cells

The most remarkable in vivo effects of FLT3-L have been observed within the DCs compartment. These cells express FLT3, and the effect of FLT3-L is extremely important in the differentiation of pre-DCs to mature DCs (McKenna et al., 2000; O'Keeffe et al., 2002). In healthy human volunteers receiving FLT3-L for 10 to 14 days, there is a mobilization of both myeloid- and lymphoid-derived DC1 and DC2 precursors by 48- and 13-fold, respectively (Maraskovsky et al., 2000). These results suggest that FLT3-L is a key regulator of the development of BM-derived DCs in vivo. Moreover, some mice experimental tumors have been successfully treated by FLT3-L injections that induce DCs expansion with antitumor T-cell responses and activation of cytolytic activity of NK cells (Lynch et al., 1997). Ablation of STAT3 in hematopoietic-derived cells shows a contracted DCS compartment and abolishes the effects of FLT3-L on DCs generation (Lanouar et al., 2003). This pathway is stage specific and is restricted

to the transitions from common lymphoid and myeloid precursors to common DCs precursors.

Interestingly, FLT3-L is implicated also in the development of pDCs, which are the main sources of type I IFN-. In vivo pharmacologic inhibition of FLT3-L signaling leads to disruption of pDCs (Onai et al., 2007). In AIDs characterized by “type I IFN- signature” such as pSS and SLE, increased levels of FLT3-L may explain the high synthesis of type I IFN- by pDCs. Chen et al (Chen et al., 2004) have shown that thrombopoietin cooperates with FLT3-L in the generation of pDCs from human hematopoietic precursors. An additional cell type, the Langenhars cells, is derived mainly from myeloid progenitors dependent on FLT3-L stimulation for their development (Mende et al., 2006).

4.6. FLT3 and FLT3-L in pathology

4.6.1. Hematological malignancies

There are no known spontaneous mutations associated with the FLT3-L locus. However, mutations in FLT3 receptor have been detected in approximately 30% of patients with AML. The most frequent mutations are the internal tandem duplications (ITDs) in the juxtamembrane domain of FLT3 (Nakao et coll., 1996). The second type is point mutations occurring in the so-called activation loop of the intracellular tyrosine kinase domain (KD) (**Figure 9**). Both ITDs and point mutations result in constitutive activation of the receptor, presumably due to unfolding of the active tyrosine kinase site allowing the access of ATP and substrates. Clinical data revealed that FLT3 mutations confer a poor prognosis in AML. AML patients who do not have FLT3 mutations at diagnosis have been found to occasionally acquire them at the time of relapse (Shih et al., 2002). Although both ITDs and point mutations appear to activate FLT3 in a FLT3-L-independent manner, mainly in homozygous mutations (Kharazi et al., 2011), new evidence suggest that FLT3-L leads to further activation of FLT3 mutants and is especially important in light of recent findings of FLT3-L increased levels in AML patients in response to chemotherapy (Zheng et al., 2011). This was elegantly demonstrated in a model of FLT3-L deficient murine embryo fibroblast, expressing FLT3/ITD and FLT3/TKD (point mutations). In the FLT3-L deficient cells, an increased of FLT3 autophosphorylation was induced by exogenous FLT3-L, leading to enhanced survival and/or decreased apoptosis. In addition, FLT3 expression is increased in samples of AML, chronic myeloid leukemia (CML) in blast crisis, and B cell from acute

lymphoblastic leukemia (ALL) (Stirewalt and Radich, 2003). FLT3-L alone or in combination with other growth factors induces a proliferative response of leukemic blasts, suggesting that in some cases autocrine stimulation may play a role in the outgrowth of malignant clones.

In the clinical aspects, higher FLT3-L levels are associated with advanced disease before treatment and residual disease after treatment. Retzlaff et al (Retzlaff et al., 2002) showed that before treatment, serum levels of FLT3-L correlate with prognostic variables and high grade lymphoma subtype in patients with primary gastro-intestinal NHL. The crucial role of FLT3-L in sustaining conventional B lymphopoiesis, may be useful to promote immune reconstitution after myeloablation and BM transplantation (Buza-Vidas et al., 2007). Together, these results provide a valid argument that inhibition of FLT3 signaling may be an effective approach to treat hematological malignancies. Currently, many small-molecule FLT3 inhibitors have been developed, but clinical trials have resulted in limited anti-leukemia effects because of off-target toxicities and drug resistance (Weisberg et al., 2009), targeting mainly the mutated FLT3. Clinical trials are ongoing to improve their clinical efficacy. One of the explanations to low clinical effect of FLT3 inhibition by different tyrosine kinase inhibitors in AML patients, is the increase of FLT3-L after chemotherapy treatment (Sato et al., 2011), mainly in patients with relapsing disease. The development of anti-FLT3 Abs might overcome these difficulties and enhance the anti-leukemia efficacy of FLT3 inhibitors. Recently, Yamamoto et al (Yamamoto et al., 2011) developed an antagonist Ab, designated A2, capable to continuously inhibit FLT3-L-induced phosphorylation of FLT3 and MAPK. Thus, this strategy can be a new approach of targeting FLT3 and FLT3-L therapies.

4.6.2. Autoimmune diseases

Several studies have shown the implication of FLT3-L in its receptor in hematological malignancies. In contrast, studies of FLT3-L in AIDs are scarce. Dehlin et al. (Dehlin et al., 2008) have showed that synovial membrane of patients with RA express high levels of FLT3-L and synovial fluid levels of FLT3-L were higher in RA patients compared to those in osteoarthritis patients. However, serum FLT3-L levels were similar in RA patients and normal controls. In addition, intraarticular administration of a B cell line that had been transfected with the FLT3-L gene resulted in highly erosive

arthritis in a mouse model. This highlighted the effect of FLT3-L in RA.

In pSS, we found that serum levels of FLT3-L were higher in patients compared to controls. In addition, high levels were correlated with abnormal Bm2+Bm2'/eBm5+Bm5 B-cell distribution (**Figure 5**). B cell culture experiments showed that FLT3-L potentiated the proliferative effect of anti-IgM stimulation. In SGs, we found that infiltrating B cells expressed FLT3 and epithelial cells produced FLT3-L. Finally, FLT3-L levels were associated with high disease activity scores and two patients with pSS and lymphoma had high levels of FLT3-L showing that this cytokine may be a marker of lymphoproliferation (**Results: article 2: The FMS-like tyrosine kinase3-ligand, a mediator for B cell survival is also a marker for lymphoma in primary Sjögren's syndrome. *Arthritis Rheum* 2010;62:3447-3456**). The role of FLT3-L as a marker of lymphoma in pSS has been confirmed in a large cohort of pSS (**Results: article 3: The FMS-like tyrosine kinase3-ligand is an important immunological marker of lymphoma in primary Sjögren's syndrome. *Ann Rheum Dis* 2012; submitted**).

Additionally, targeting FLT3 with lestaurtinib may be effective in the treatment of AIDs. Whartenby et al. (Whartenby et al., 2005) showed clinical improvement in the experimental autoimmune encephalomyelitis model due to the inhibitory effect in DCs. Thus, targeting FLT3-L may open new therapeutic options in AIDs. In the other hand, some authors propose that expansion of DCs induced by FLT3-L may delay progression of AIDs. In the mice model for Type 1 Diabetes (NOD), treatment with FLT3-L during early stages was shown to delay diabetes onset, inducing the expansion of myeloid DCs and CD4+CD25+ Treg cells (Chilton et al., 2004; O'Keeffe et al., 2005). However, another study showed that FLT3-L stimulation actually accelerates autoimmunity when autoreactive CD8 T cells were detectable in blood before treatment in the NOD model (Van Belle et al., 2009). In this case, expansion of DCs and induction of Treg cells by FLT3-L remained intact, but both the number and the functional response of islet-specific CD8 cells were boosted. These inverse results show that FLT3-L may play an important role in the immune tolerance according to the different phases of development of AIDs. The same hypothesis was evoked in the mice TNF driven model of ileitis (TNF Δ ARE) that recapitulates many features of Crohn's disease. In this study, FLT3-L administration resulted in a preferential expansion of CD103+DC that attenuated the severity of ileitis in 20-week-old mice, mediated by increased CD4+CD25+FoxP3 Treg cells (Collins et al., 2011).

Recently, the effects of FLT3-L inhibition with sunitinib (a multi-tyrosine kinase inhibitor) in a mouse model of induced-arthritis have been described (Dehlin et al., 2011). Sunitinib treatment reduced the intensity of synovitis and the incidence of bone destruction. The reduction in bone destruction was seen when the treatment was started at the time of immunization or at the time of arthritis induction. The antiarthritic effect was achieved by inhibition of DCs, reduction of antibody production, and bone metabolism. This study shows that inhibition of FLT3 is a potent anti-arthritic mechanism reducing antigen presentation, synovial inflammation, and bone resorption. Specific FLT3 inhibitors must be tested in the same pathological context.

5. BAFF

5.1. Introduction

During the establishment of tolerance, low-affinity self-reactive B cells can be rescued from apoptosis by the potent B cell survival factor **BAFF** “**B cell activating factor belonging to the tumor necrosis factor family**”. BAFF, also known as **BLyS** for B-Lymphocyte Stimulator (or TALL-1, THANK, TNFSF13B and zTNF4), is a cytokine that prevents apoptosis of auto-reactive B cells (Mackay and Browning, 2002). BAFF has emerged since 1999 (Schneider et al., 1999) as one of the critical factors controlling B-cell maturation, tolerance and malignancy. BAFF exists both as a type II membrane-bound protein (mBAFF) and as a soluble protein (sBAFF) (Moore et al., 1999; Ng et al., 2005).

This cytokine influences B-cell differentiation, survival, and activation. BAFF drives B-cell maturation mainly at the early transitional stages, and discontinues humoral tolerance by rescuing autoreactive B cells from apoptosis. In addition to BAFF, this TNF family ligands comprises **a proliferation-inducing ligand (APRIL)** (Hahne et al., 1998) and the **TNF-like weak inducer of apoptosis (TWEAK)**; and possess four membrane receptors: BCMA for B-Cell Maturation Antigen; TACI for transmembrane activator and calcium modulator and cyclophilin ligand interactor; BAFF specific BAFF-R (BR3) and APRIL specific proteoglycans (Gross et al., 2000; Thompson et al., 2001).

In the next sections, functions, receptors, physiological and pathological roles of BAFF will be discussed.

5.2. BAFF generalities

Since its description by Schneider et al., BAFF has emerged as one of the critical factors controlling B-cell maturation, tolerance and malignancy. Many of these informations are derived from BAFF-deficient or BAFF-transgenic mice models.

5.2.1. Mice models

5.2.1.1. BAFF transgenic mice

Murine BAFF overexpression was induced by transgenesis with the liver-specific α 1 anti-trypsin promoter. Initially, mice transgenic for BAFF develop features of SLE (Mackay et al., 1999) and with age a clinical presentation of pSS with lymphocytic infiltration of SGs, destruction of submandibular glands and decreased saliva production (Groom et al., 2002). Additionally, this model has increased numbers of peripheral B cells and elevated serum Ig levels (Mackay et al., 1999; Dorner et al., 2001). Although this model does not present the classic auto-Abs of pSS, MZ B-cells proliferate in the spleen and latter infiltrate the SGs. In the SGs of BAFF transgenic mice, the expanded MZ B-cell compartment comprises self-reactive B cells (Groom et al., 2002). This last characteristic is useful to study lymphoproliferation in pSS. Disruption of *Tnf α* in BAFF transgenic mice revealed a critical role of the anti-tumor activity of TNF α in this strain, characterized by a high incidence of B-cell lymphomas (>35%) (Batten et al., 2004). Interestingly, BAFF-transgenic mice develop the same manifestations of pSS when T cells are removed, but in this instance, BAFF exacerbates B cell-TLR activation (Groom et al., 2007). Conversely, BAFF-deficient mice are viable at birth but display severely impaired B-cell maturation beyond the immature, transitional stage with significant defects in peripheral B-cell numbers and Ab responses (Gross et al., 2001; Schiemann et al., 2001; Rahman et al., 2003).

BR3- (the dominant receptor for BAFF) deficient mice have a phenotype identical to that of BAFF-deficient mice and are characterized by the absence of most peripheral B cells.

5.2.1.2. Adaptor molecule Act-1 deficient mice

As a negative regulator of BAFF and CD40, the adaptor molecule Act1 (Act1) crucially modulates the B-cell survival (Li, 2008). In Act1-deficient mice, MZ-like B-cell infiltrate dominates in the exocrine glands. Similar to BAFF transgenic model, Act1-deficient mice develop SLE specific auto-Abs and glomerulonephritis. However, deficiency in

Act1 causes anti-Ro and anti-La Ab production (Qian et al., 2008). This production depends on functional CD40.

5.2.2. BAFF producing cells

A large number of cell types has the capacity to express BAFF, including monocytes, macrophages, and DCs, but also hematopoietic tumor cell lines and malignant B cells. Activated T cells, neutrophils, synoviocytes, astrocytes, ECs and B cells infiltrating the SGs of pSS (Daridon et al., 2007) patients are also able to produce BAFF.

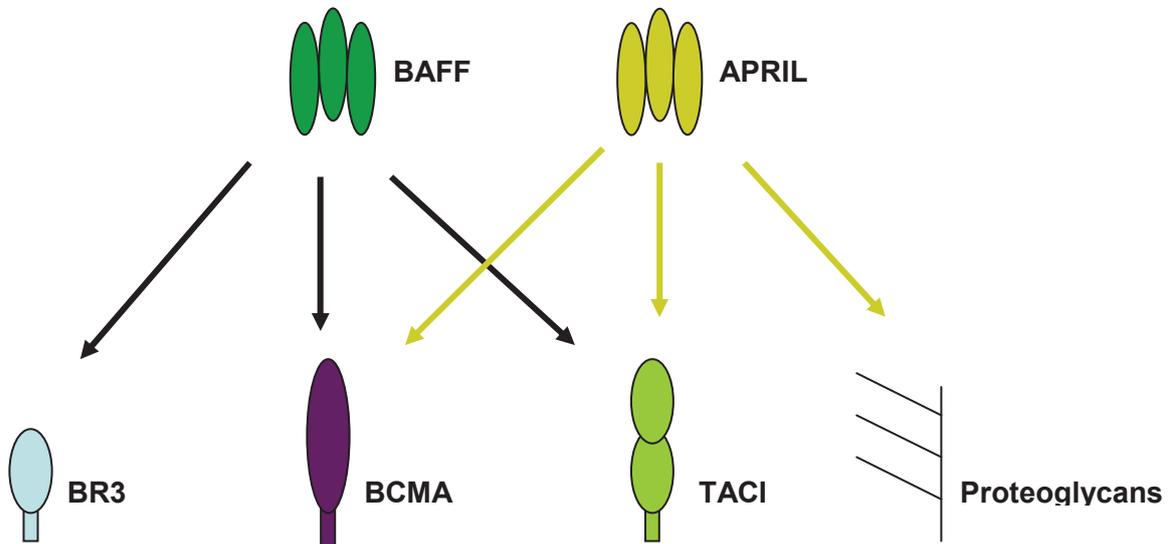
5.2.3. BAFF-from gene to protein-

BAFF gene is located on human chromosome 13q14 and contains six exons and five introns (Schneider et al., 1999). The transcript has 1204 bp and 858 in the the open reading frame. It is a 285-amino-acid glycoprotein (31.2 kDa) originally expressed as a type II membrane-bound protein and then cleaved at a putative furin consensus site and release as a soluble protein of 152 amino acids (17-kDa) (Mackay et al., 2003; Batten et al., 2000). To date, only one separate promoter has been identified for the human BAFF gene, and the nucleotide sequences of this promoter have been reported (Kawasaki et al., 2002). At the protein level, BAFF exists as a membrane associated molecule, or a soluble protein, whereas APRIL is cleaved in the vesicles before release and only exists as a secreted soluble form (Lopez-Fraga et al., 2001).

5.3. BAFF receptors

Interactions between ligands and receptors of this family vary. Thus, BAFF interacts chiefly with BR3 (also known as TNFRSF13c) but also with TACI (also known as TNFRSF13b) and BCMA (also known as TNFRSF17), whereas APRIL interacts with TACI and BCMA, but not with BR3 (Thompson et al., 2001; Hymowitz et al., 2005) (**Figure 11**). In addition, APRIL binds to proteoglycans structures expressed on lymphoid and non-lymphoid cells.

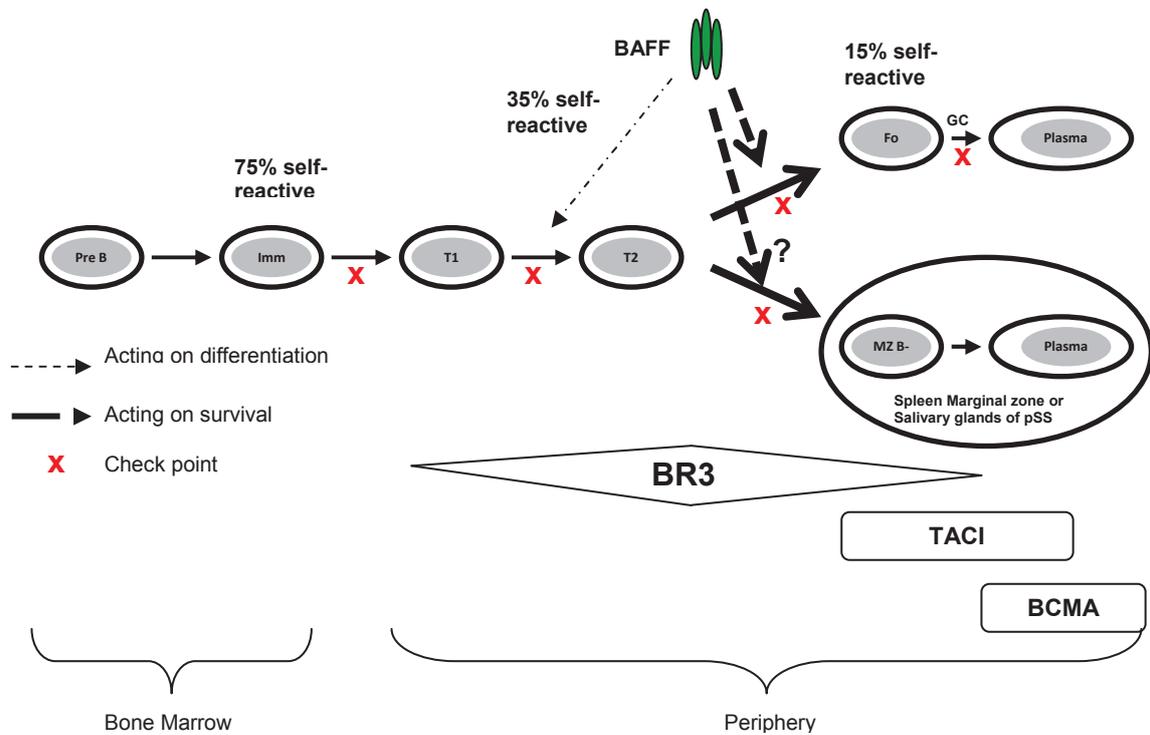
Figure 11. BAFF and APRIL receptors. BAFF binds chiefly BAFF-R (BR3), but also BCMA and TACI. On the other hand APRIL interacts with TACI and BCMA, but not with BR3. In addition, APRIL binds to proteoglycans expressed in membranes of lymphoid and non-lymphoid cells.



BAFF binding to the three receptors activates the NF- κ B pathway to increase the expression of anti-apoptotic molecules such as Bcl-2 and promotes cell survival and cell-cycle progression (Rolink et al., 2002; Sasaki et al., 2008). BAFF receptors are mainly expressed on B cells, but, for each receptor, cell membrane density varies from BT1 to plasma cells (**Figure 12**). In human B-cell, BR3 is present in BT1 cells to memory B cells, but not in plasma cells. TACI expression is low in BT1 but increases in T2 and MZ B and finally predominates on short-lived plasma cells; whereas BCMA is expressed on long-lived plasma cells, plasmablasts and tonsillar GC B cells (Avery et al., 2003).

BR3 is the only BAFF receptor that activates the alternative NF- κ B pathway. BR3 stimulation increases not only the expression of anti-apoptotic molecules by alternative NF- κ B2 pathway, but also results in signals through Akt and Pim 2 that increase metabolic fitness by inducing a metabolic bias toward glycolysis (Woodland et al., 2008). In contrast, TACI and BCMA signal pass through the classic NF- κ B pathway and Mek (mitogen-activated protein extracellular signal-related kinase) pathway to up-regulate anti-apoptotic molecules and down-regulate pro-apoptotic pathways, and through JNK/p38 (c-Jun N-terminal kinase) to drive class-switching.

Figure 12. BAFF receptors cell surface expression and self-tolerance during B cell ontogenesis. Data indicate the proportion of self-reactive B cells at specific B-cell stages before or after check points as determined in the anti-HEL/HEL transgenic models. Fo: follicular, GC: germinal center; Imm: immature; MZ: marginal zone; Pre: precursor; SS: Sjögren's syndrome; T1 or 2: Transitional type 1 or 2; Plasma: Plasmatic cell.



BR3 is the key receptor that triggers BAFF-mediated survival, as mice deficient for this receptor display a phenotype similar to that of BAFF-null mice. BCMA-deficient mice are born with no major immune defect except an impaired survival of some plasma cells in the bone marrow. TACI, by contrast, emerged as a negative regulator of B-cell activation and expansion, as number of B cells are increased in TACI-deficient mice, B cell are hyper-responsive and animals eventually develop SLE-like disease and lymphoid cancers (Seshasayee et al., 2003). Moreover, T-cell independent type II antibody responses are impaired in these mice (von Bulow et al., 2001). Intracellular TACI is present in human macrophages and migrates to the cell surface upon activation (Chang et al., 2006).

5.4. BAFF functions

5.4.1. The effects of BAFF on B lymphocytes

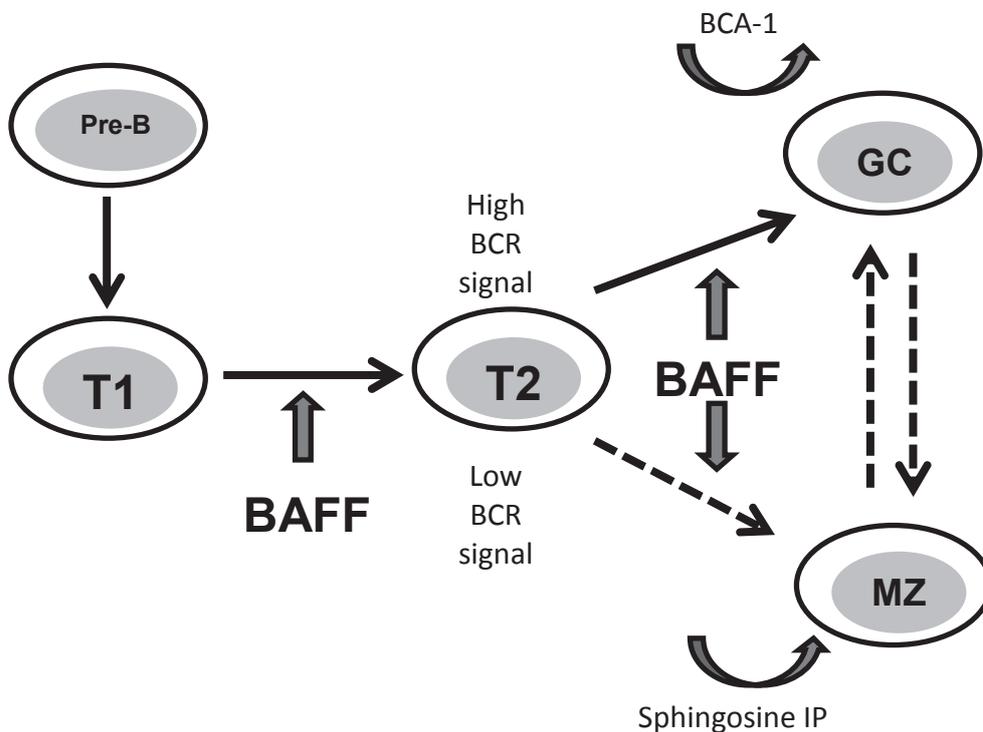
The soluble form of BAFF is a critical survival/maturation factor for peripheral immature B cells (Batten et al., 2000) that may evolve into MZ B cells (Schneider et al., 2001). The biologically active form of BAFF is trimeric and activity is mediated through its three receptors (Novak et al., 2004; Binard et al., 2007). Given the reduced competitiveness of autoantigen-engaged B cells, due to increased dependence on BAFF (Lesley et al., 2004), its pathological excess rescues autoreactive B cells from peripheral deletion and allows them to enter forbidden follicular and MZ niches (Thien et al., 2004). BAFF is particularly important in this step because MZ B cells are prone to activation (**Figure 13**). Normally, few immature B cells successfully pass to the T2 stage. Irrespective of the level of receptor expression, BAFF is the dominant agent in the resistance of BT2 cells to apoptosis. In its absence, B-cell maturation is arrested at the T1 cell stage, while BAFF transgenic mice manifest T2 cell hyperplasia in their exocrine glands, which is reminiscent of the B-cell aggregates in the SGs of pSS patients. The explanation is that excess of BAFF protects self-reactive B cells from deletion and allow their location in forbidden follicle or MZ niches (Thien et al., 2004).

BAFF activates B cells via the Pax-5 transcription factor (Le Pottier et al., 2009) and substitutes for CD40 ligand in the class switching. Thus, BAFF stimulates the GC formation, Ig production, and class-switching recombination (Groom et al., 2002; Mackay and Ambrose, 2003). Activation-induced deaminase is expressed during class-switching recombination, and it has been demonstrated that BAFF induces activation-induced deaminase expression (Litinskiy et al., 2002; Castigli et al., 2005; Yamada et al., 2005).

BAFF plays an important role in humoral immunity. A T cell-independent type II response requires the interaction of BAFF 60-mer or membrane BAFF with TACI (von Bulow et al., 2001; Shulga-Morskaya et al., 2004). This interaction is also vital for T-cell dependent IgM responses, whereas IgG responses are less BAFF-dependent. BAFF-deficient mice fail to develop a proper Follicular Dendritic Cell (FDC) network leading to smaller and unstable GC in which class-switching and somatic hypermutation still occur, but with diminished IgG and secondary responses (Rahman et al., 2003). In contrast, IgA appears to be dependent upon the interaction of APRIL, multimerized by proteoglycans with TACI (Sakurai et al., 2007; Huard et al., 2008). A deficiency of IgA is the major phenotypic abnormality of APRIL-deficient mice, suggesting the role of APRIL in the maintenance of mucosal immunity. Finally, BAFF encourages infiltration

of lymphoid tissues along with T-lymphocyte-derived TNF. This help has long been regarded as necessary.

Figure 13. In secondary lymphoid organs and salivary glands of primary Sjögren's syndrome, immature B cells settle down before further ontogenesis. Transitional type 1 B cells (BT1) evolve to BT2 cells, depending on the affinity of antigen for the B-cell antigen receptor (BCR) and the amount of BAFF. Should the BCR signal be low, they move to the marginal zone (MZB); should it be high, they generate germinal centers within the follicle (FO).



5.4.1.1. BAFF-signaling mediated B-cell survival

In mature B cells, pro-survival signaling is mediated by BR3 stimulation and then NF- κ B signaling pathway activation. The activation of the alternative NF- κ B pathway (processing of NF- κ B and the nuclear translocation of p52/Rel B heterodimers) is a major outcome of BR3 stimulation (Kayagaki et al., 2002; Claudio et al., 2002). BR3 triggers this pathway by preventing the constitutive proteasome-mediated degradation of the serine/threonine kinase NIK (NF- κ B-inducing kinase)-a process that depends on the binding of NIK to TRAF3 (TNF receptor associated factor 3) (Liao et al., 2004). Activated BR3 recruits TRAF3 and triggers its degradation, thus allowing NIK protein to accumulate and increase NF- κ B2 processing. Confirming this model, TRAF3-lacking B-cell lines show elevated levels of NIK and NF- κ B2 processing (He et al., 2006). The

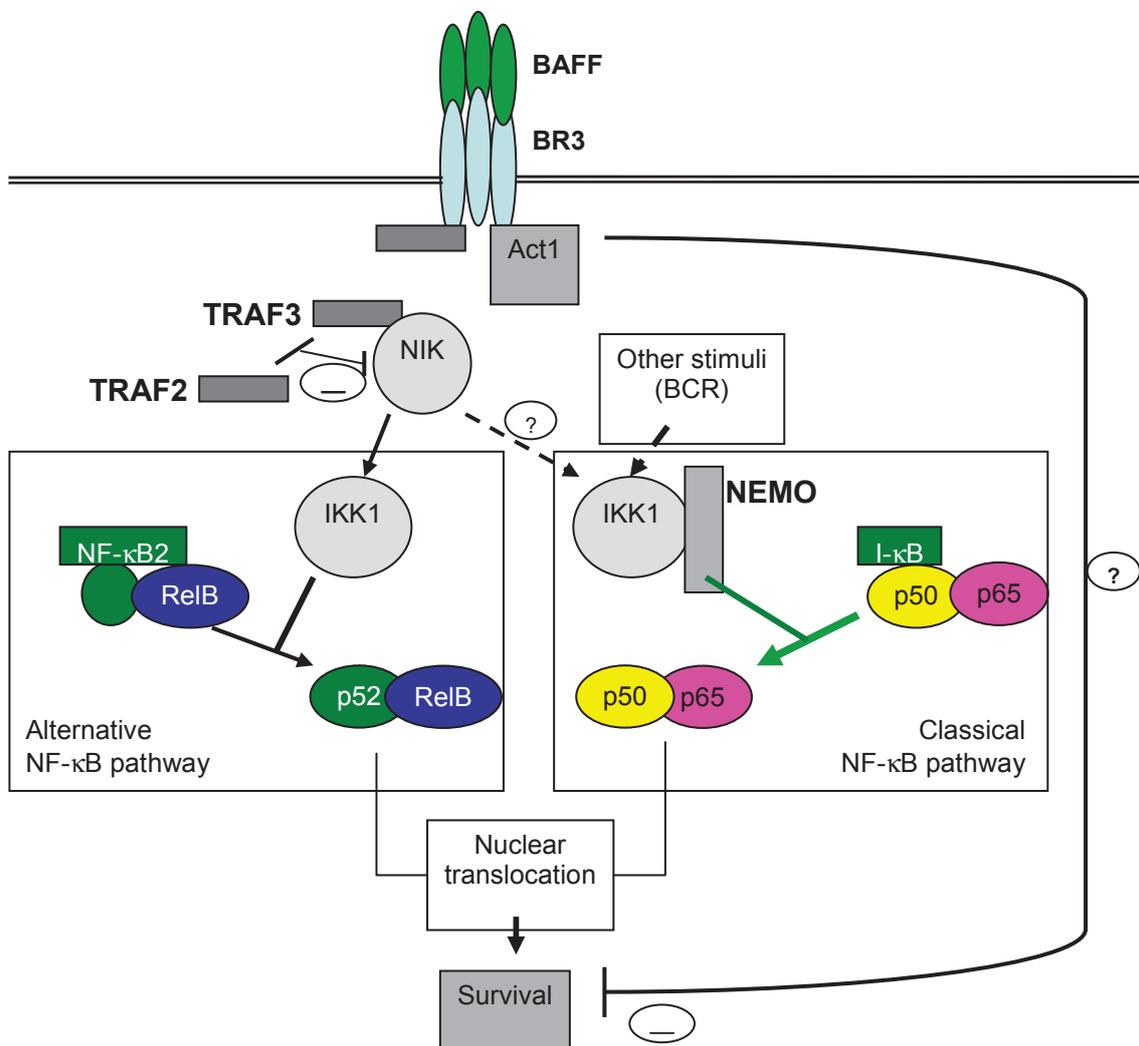
demonstration that mature NF- κ B2-deficient B cells fail to survive in mixed BM chimeras confirms that activation of the alternative NF- κ B pathway through BR3 is essential for the survival of B cells (Miosge et al., 2002).

Conversely, BR3 is a weak activator of the classical NF- κ B pathway in B cells. Although most of the nuclear translocation of p50/RelA DNA-binding activity triggered by BAFF requires TACI, evidence does exist for low level activation of the classical pathway via BR3 (Enzel et al., 2006; Sasaki et al., 2006). The importance of classical NF- κ B activation for mature B-cell survival is indicated by the demonstration that B-lineage-specific inactivation of NEMO (NF- κ B essential modulator), a component of the I- κ B kinase complex essential for activation of the classical NF- κ B pathway, results in the specific depletion of mature B cells (Sasaki et al., 2006). However, the extent to which BR3 mediated activation of this pathway is required is difficult to define, because the BCR is essential for mature-B cell survival, activating the NF- κ B classical pathway **(Figure 14)**.

B-cell survival by BAFF is dependent on NF- κ B-mediated upregulation of anti-apoptotic Bcl-2 family proteins. In addition, inhibition of the nuclear translocation of the proapoptotic protein kinase C δ (PKC δ), appears to be an important component of BAFF-mediated survival signaling in B cells (Mecklenbrauker et al., 2004). In addition, the serine/threonine kinase Akt is another potential partner of BAFF-mediated survival signals. Akt activity counteracts apoptosis at least in part by maintaining a positive balance through the promotion of glucose uptake and glycolysis. Although PKC β and PI3K are required for BAFF-mediated Akt activation, the biochemical link is unknown.

Apart from TRAF3, Act1 is the only signaling molecule known to be recruited to BR3 (Qian et al., 2004), and acts as a negative regulator of BAFF-mediated B-cell survival. However, its mechanism of action remains unclear **(Figure 14)**.

Figure 14. Signaling pathways implicated in BR3 mediated survival in B cells. A clear link between BR3 and the various signaling pathways has only been established in the case of the alternative NF- κ B pathway.



5.4.1.2. BR3 and BCR signaling

The BCR is the main activator of the classical NF- κ B pathway while BR3 primarily activates the alternative NF- κ B2 pathway. As both pathways are required for B-cell development and survival, crosstalk between these two pathways is suggested. Supporting this hypothesis, a study demonstrated that p100, a substrate for NF- κ B2, requires NF- κ B1 activation for its expression in B cells (Stadanlick et al., 2008). In the absence of NF- κ B1 activation, BR3 signaling quickly depletes available p100 in the cell and is unable to sustain permanent NF- κ B2 activation and the expression of downstream survival factors. However, as BR3 also weakly activates the NF- κ B1

pathway, it is possible that BAFF signaling can allow some p100 expression to feed the NF- κ B2 pathway in the absence of BCR-mediated NF- κ B1 activation. In addition, BR3 expression is up-regulated by BCR ligation on mature B cells and it is the predominant receptor expressed on resting memory B cells (Avery et al., 2003).

5.4.2. The effects of BAFF on T lymphocytes

BR3 is also expressed on activated T cells and Treg cells (Mackay and Leung, 2006). Surprisingly, aberrant expression of BAFF in T cells of patients with SLE (Yoshimoto et al., 2006) or RA (Lai Kwan Lam et al., 2008) may engender some T helper-1 responses (Sutherland et al., 2005) and an anti-inflammatory Treg cell expansion (Walters et al., 2009). These observations show that BAFF has a dichotomous role in immune responses.

5.4.3. The effects of BAFF on innate immune response

BAFF is also an essential component of the innate immune response. BAFF synthesis is induced in myeloid DC by type I IFNs (Boule et al., 2004). BAFF up-regulates TLR expression, and together with IL-6 promotes Ig class-switching and plasma cell differentiation (Tremblé et al., 2007; Katsenelson et al., 2007). Activation of intracellular TLRs in B cells by immune complexes containing nucleic acids up-regulates expression of BAFF receptors, particularly TACI, and increases BCR-mediated signaling. The triad of TLRs, type I IFNs and BAFF creates an amplification loop that propagates production of IgG auto-Abs to nucleic acids without any T-cell help (Groom et al., 2007). This mechanism may link the “type I IFN signature”, BAFF excess and autoimmunity in pSS and SLE.

DCs express BAFF receptors (mainly TACI), and when transduced with a siRNA that silences BAFF, remain in an immature state and fail to produce the IL-6 required for the differentiation of Th17 cells (Lai Kwan Lam et al., 2008). Human myeloid DCs stimulated with BAFF up-regulate co-stimulatory molecules, lose their phagocytic ability and produce inflammatory cytokines and chemokines including IL-1, IL-6, CCL2 and CCL5 inducing Th1 responses in vitro (Chang et al., 2008). These studies suggest that BAFF acts on DCs to help them in recruiting immune cells to inflammatory sites and to enhance the proinflammatory activity of T cells.

5.4.4. BAFF functions in immune tolerance

Fifty to 75% of the newly generated B cells in the BM have a self-reactive BCR. Thus, to avoid the generation of pathogenic auto-Abs, self-reactive B cells have to be deleted or anergised during successive checkpoints during B-cell development and maturation. Based on its receptor expression profile, BAFF has no effect on B-cell tolerance in the BM, but does act in the periphery (**Figure 12**). BAFF is needed for survival of T2 cells, which express high levels of BR3. Actually, BAFF is necessary to prevent T2 apoptosis, as has been demonstrated in BAFF or BR3 lacking models, where the B-cell ontogenesis is stopped at the T1 stage (Schiemann et al., 2001).

In BAFF and Δ BAFF (negative regulator of BAFF) transgenic mice models, interbred with transgenic mice for the site-directed 3H9 Ig H chain (whose specificity is derived from a DNA and chromatin-specific hybridoma obtained from a MRL/*lpr* mouse), Ota et al., (Ota et al., 2010) analyzed how BAFF levels alter B-cell tolerance by characterizing changes in the B-cell repertoire that occur with over or under expression of BAFF. BAFF/3H9 mice model presented elevated number of B cells, including L chains generation high-affinity reactivity and auto-Ab production, demonstrating that BAFF signaling leads to tolerance escape and positive selection of autoreactive cells.

5.4.5. APRIL functions

The role of APRIL is more elusive than BAFF functions. Conflicting results emerged from two independently generated APRIL^{-/-} mouse models: one showed no obvious phenotype (Varfolomeev et al., 2004) whereas the other showed impaired switching to IgA, bigger GCs and increased numbers of effector T cells (Castigli et al., 2004).

5.5. BAFF heterogeneity

BAFF offers a lot of forms: mBAFF or sBAFF, monomer or trimers, homotrimers or heterotrimers, heterotrimer formation with APRIL, TWEAK or Δ BAFF, or even virus-like aggregates of 60 monomers (Daridon et al., 2008). The heterogeneity of BAFF may complicate the assessment of this cytokine. This question arises from the observation of normal or low levels of BAFF in patients with AIDs. Also intriguing is that raised serum concentrations of BAFF have been associated with auto-Abs production by some (Mariette et al., 2003) but not all investigators (Collins et al., 2006). Thus, receptor

occupancy B cells by BAFF (Carter et al., 2005), its urinary excretion in case of renal failure (Collins et al., 2006) and sequestration in immune complexes (Gao et al., 2007) all hinder assessment of BAFF. In this respect, it is interesting that intravenous Igs contains natural anti-BAFF Abs (Le Pottier et al., 2007). Furthermore, on the basis that flaws in an ELISA might explain such variations, in our laboratory, we have set an in-house ELISA, as a reliable tool to detect BAFF in these pathologies (Le Pottier et al., 2009).

5.5.1. Alternative splice isoforms

Four different mRNA from BAFF gene have been characterized in humans: the full-length BAFF, the longer variant called ψ BAFF, an exon-3 lacking isoform (Δ BAFF or Δ 3BAFF) and an exon-4 lacking isoform (Δ 4BAFF). The larger transcript ψ BAFF was identified in the human cells lines HL-60 and U937, but sequencing proved this transcript to be non-functional because of incomplete splicing of intronic sequencing leading to premature termination codons (PTCs). An exon-3 (57 bp) lacking isoform of BAFF, referred to as Δ BAFF, has been identified in many mouse and human myeloid cells (Gavin et al., 2003). Based on its ability to multimerize with full-length BAFF, this truncated variant might restrain the effects of the full-length-cytokine, and thereby regulate B-cell homeostasis (Gavin et al., 2005). Currently, Δ BAFF protein has never been characterized. Recently, in our group, we detected an additional transcript (**Results, Article 4: A new BAFF variant acting as a transcription factor in the regulation of immune response. *Nat Genetics* 2012; submitted**) which was devoid of the 113 base pair segment encoded by the exon 4, and we called this new variant Δ 4BAFF. The chromatin immunoprecipitation (ChIP) unveiled that the protein was associated with DNA, and, to our surprise, acted as a transcription factor for the full-length form of BAFF. The discovery of this new variant and their role as a transcription factor are described in the **article 4**.

5.6. BAFF in pathology

5.6.1. Hematological malignancies

In mouse BAFF transgenic models, an increased incidence of B-cell lymphomas is observed (Batten et al., 2004). In humans, overexpression of BAFF has been linked to B-cell neoplasias (Collins et al., 2006; Tai et al., 2006). BAFF levels are elevated in

the serum of NHL patients and these high levels are associated with disease activity progression and worse prognosis (Novak et al., 2004).

Several studies have shown that malignant B cells from NHL-B, Hodgkin's lymphoma and multiple myeloma patients also express abnormal levels of BAFF protein, which protects malignant B cells from spontaneous apoptosis and myeloma cells from apoptosis induced by IL-6 deprivation or dexamethasone (Novak et al., 2002; He et al., 2004; Moreaux J et al., 2004; Novak et al., 2004). Other cells that inhabit or infiltrate tumor microenvironments can also contribute to excessive levels of BAFF. In the other hand, treatment with BAFF antagonists prevented growth and increased apoptosis of tumor cells, showing great interest for the treatment of B-cell malignancies (Novak et al., 2002; Novak et al., 2004).

5.6.2. Autoimmune diseases

Overexpression of BAFF is also described in AIDs. For example, excessive quantities of BAFF have been found in cerebrospinal fluids of SLE patients (George-Chandy et al., 2008), sera and SGs of pSS patients (Groom et al., 2002) and sera and synovial fluids of RA patients (Tan et al., 2003; Bosello et al., 2008). Evidence also suggests that BAFF is involved in auto-Ab production and its upregulation by B-cell depletion in patients with RA (Cambridge et al., 2006), and the inverse correlation between serum concentrations of BAFF before depletion of B cells and the length of time before their return in the blood of pSS patients (Pers et al., 2007). In addition, astrocytes produced BAFF (Krumbholz et al., 2005) levels are elevated in myasthenia gravis (Ragheb et al., 2008), multiple sclerosis (Krumbholz et al., 2008) and neuroBehçet (Hamzaoui et al., 2008). The pathogenic contribution of BAFF to AIDs is also evident as demonstrated by the therapeutic benefit obtained by its neutralization. In animal models, SLE-prone mice treated with TACI-Ig or BR3-Ig fusion proteins developed less and/or delayed proteinuria, resulting in prolonged survival (Ramanujam et al., 2006). These two treatments are equally effective at depleting mature follicular and MZ B cells, but not affect numbers of immature B-1 B cells. The mechanisms of protection have been suggested as auto-Abs independent. The beneficial effects of BAFF inhibition by Belimumab (anti-BAFF Ab) have been confirmed in patients with SLE (Furie et al., 2011).

5.6.3. BAFF in primary Sjögren's syndrome

B-cell hyperactivity characteristic of pSS may be explained, at least in part by the dysregulation of BAFF found in sera, SGs and mice models of pSS.

5.6.3.1. BAFF overexpression in saliva, sera and implications in circulating cells in pSS

High serum levels of BAFF are found in saliva (Pers et al., 2005), and sera of patients with pSS; and they are correlated with auto-Ab titers (Mariette et al., 2003) and their presence in the SGs (d'Arbonneau et al., 2006). Using our in-house assay (Le Pottier et al., 2009) to detect BAFF, serum levels of this cytokine are elevated in most of pSS patients. Estimates of BAFF fluctuate with changes in inflammatory activity and extent of the damages. In addition, the disease activity probably correlates better with leukocyte BAFF mRNA amounts than with plasma BAFF titers (Collins et al., 2006).

In addition, the important finding that BAFF increases expression of CD19 is now extended by the finding that naïve Bm2/Bm2' cells, abnormally increased in peripheral blood of pSS patients, carry more CD19 molecules in patients than in healthy controls (d'Arbonneau et al., 2006). The CD19-induced persistence of the BCRs in lipid rafts may thus be ascribed to BAFF.

5.6.3.2. BAFF in the salivary glands from pSS

5.6.3.2.1. Aberrant expression of BAFF in the SGs impacts B cells exclusively

BAFF is detected in the SGs of pSS patients (d'Arbonneau et al., 2006). Local production of BAFF contributes to deleterious effects of activated B cells by raising their expression of CD19 molecules, and ensuring survival of B-cell aggregates, and auto-antibody isotype switching outside and inside GCs (Le Pottier et al., 2009). ECs and activated T cells, but also B-single cells isolated from SGs have shown aberrant expression of BAFF in the SGs from pSS patients (Daridon et al., 2007). This locally produced BAFF is thought to rescue autoreactive B lymphocytes in an autocrine manner. BR3 is expressed by B cells but not T lymphocytes. In addition, some subgroups of B cells express TACI, indicating that the membrane-bound form of BAFF, but not its soluble form, impacted B cells but not T cells (Daridon et al., 2007).

5.6.3.2.2. Effect of BAFF on MZ-like B cells infiltrating the SGs

T2 B cells and MZ B cells are observed in pSS patients and only a limited proportion of B cell clusters fulfill the criteria for real ectopic GC (Le Pottier et al., 2009). This is perfectly in line with antigen-driven clonal B-cell proliferation and hypermutation in the exocrine tissue (Loder et al., 1999). Engagement of antigen receptor in T2 B cells may induce their apoptosis unless BAFF or T cells signals help them to escape apoptosis. These self-reactive MZ B cells encounter local antigen, and differentiate in memory B cells. The competitiveness of autoantigen-engaged B cells is reduced in the periphery of GCs, due to their increased dependence on BAFF (Lesley et al., 2004). The strongest difference distinguishing pSS patients with GCs from those without GCs, is that the presence of IFN- γ and BAFF in real GCs (Szodoray et al., 2005). Additional interesting information about B-cell infiltration in SGs is derived from sequential biopsies done before and after RTX treatment. Twenty-four months after treatment, B cells aggregating in SGs are T1 B cells. Since transfer experiments have established that T1 B cells give rise to T2 B cells, these T2 B cells present in SGs at baseline should originate from T1 B cells (Pers et al., 2007).

5.6.3.2.3. Autoreactive B cells and ectopic GCs

Although BAFF is not required for the initiation of GC, it plays a critical role in sustaining GC reaction. In pSS, most of the SGs samples contain MZ-like areas, while few contain real ectopic GCs. Clues about the integrity of GC tolerance mechanism have been provided by tracing the emergence of B cell expressing the idiotype targeted by the 9G4 mAb (Le Pottier et al., 2009). These data indicate that the potential tolerance checkpoints that have evolved to restrict autoreactive B cells entering GCs could be defective in pSS. Although rare, these ectopic GCs act as a conduit to recruit and expand autoreactive B cells. Subsequent to these initial processes, self-antigen presentation by autoreactive B cells, or professional APC and ECs may be crucial in sustaining the immune activation and autoimmune response in pSS.

5.6.3.2.4. Effect of BAFF on memory B cells infiltrating the SGs

A minority of TACI- and BCMA-expressing B cells are found in pSS (Daridon et al., 2006). These receptors might expand the population of SGs plasma cells, resulting in local production of auto-Abs.

5.6.3.2.5. BAFF modulates repopulation of B cells in the blood and SGs of pSS patients treated with RTX

Baseline serum levels of BAFF correlated inversely with the duration of B cell depletion (Pers et al., 2007). Thus, repopulation of B cells might be modulated by BAFF. Relevant to this issue is the suggestion that the beneficial effects of RTX-based B cell-depletion may be offset by a BAFF-mediated antiapoptotic effect on reemerging B cells. Advance knowledge of the BAFF levels may help in tailoring the doses and the frequency of RTX administration.

In conclusion, aberrant expression of BAFF in the SGs impact B cells exclusively, favors expansion of T2 and MZ-like B cells and plays a major role in B-cell repopulation after RTX treatment in pSS.

5.6.3.3. BAFF and genetic susceptibility to pSS

Disease susceptibility for anti-Ro/SSA and anti-La/SSB positive pSS has been associated with the CTAT haplotype of 4 SNPs located in the 5' regulatory region of the *BAFF* gene, while the TTTT haplotype has been associated with elevated BAFF levels in pSS (Nossent et al., 2008). No other genetic polymorphism has been implicated in pSS. Thus, dysregulated expression of BAFF in autoimmune-prone individuals is likely to be determined by other genetic or environmental factors that regulate the responsiveness of BAFF-producing cells.

5.7. BAFF targeted therapy

The BAFF targeted therapy is summarized in Table 10 (Chapter 3). This inhibition can be done by anti-BAFF or anti-BR3 monoclonal Abs, as well as BR3 or TACI decoy fusion proteins. Selective BAFF blockers prevent BAFF from interacting with its receptors, leaving APRIL available to interact with TACI and BCMA. Drugs in this class include anti-BAFF Ab (Belimumab or Lymphostat B) and a fusion protein consisting of human Ig Fc and of the extracellular BR3 domain (Briobacept, for BAFF-R-Ig). Non-selective BAFF blockers abolish the interactions of both BAFF and APRIL with all their receptors. To date, there is a single drug in this class (Seshasayee et al., 2003), which is human Ig Fc fused to the extracellular TACI domain (Atacicept, TACI-Ig). Differences in the distribution of the forms of BAFF could denote the potential of SLE patients to respond or to resist to BAFF antagonist therapy. Treatment of B cells with TACI agonist

Ab inhibits proliferation *in vitro* and activation of a chimeric receptor containing TACI intracellular domain induces apoptosis. These results demonstrate also the critical requirement for TACI in regulating B cell homeostasis (Seshasayee et al., 2003).

One issue for monitoring treatment with BAFF antagonists is the assay for BAFF. Given the conflicting results, we have developed an in-house-enzyme-linked immunosorbent assay for BAFF (Le Pottier et al., 2009).

Different forms of BAFF are present in the human serum due to translational modifications. BAFF can indeed been glycosylated or not. The major problem is that glycosylation seems to alter epitope recognition by anti-BAFF Abs. This could also explain why serum concentrations of BAFF vary between studies for a given population of patients. Two on-going studies are studying the therapeutic effects of BAFF inhibition by Belimumab in pSS.

5.8. Induction of BAFF

5.8.1. Induction of BAFF by cytokines

As BAFF is involved in AIDs and B-cell malignancies (Cancro, 2006; Sutherland et al., 2006), several studies have elucidated the possible mechanisms of BAFF induction under different stimulations. CD40L and anti-IgM stimulation induces BAFF expression in normal human B cells by activating both NF- κ B and nuclear factor of activated T cells (NFAT) binding to the BAFF promoter (Fu et al., 2006). Other preliminary reports showed that BAFF was produced by monocytes upon stimulation with IL-10 and IFN- γ . Additionally, other cells types such as macrophages, DCs and neutrophils synthesize BAFF after IFN- γ , IFN- α and lipopolysaccharide (LPS) (via the production of reactive oxygens) stimulation (Moon et al., 2006). On the other hand IL-4 inhibited the expression of BAFF in monocytes (Nardelli et al., 2000; Scapini et al., 2003). IFN- γ and IL-10 are able to increase BAFF mRNA expression in monocytes (Litinskiy et al., Moon et al., 2006; Fu et al., 2006). The mechanisms of BAFF regulation by these cytokines were not elucidated in these previous studies. More recently, Zhou et al (Zhou et al., 2009) evaluated the effects of several inflammatory cytokines on BAFF expression in cultured human promyelocytic leukemia (HL60) cells both at the transcriptional and posttranscriptional level. They confirmed that IL-10 and IFN- γ elevated the expression of membrane-bound and soluble forms of BAFF after a 48 h stimulation and that IL-4 markedly diminished BAFF expression. Transcriptional regulation was evaluated in HL60 transfected cells with a human BAFF promoter/reporter gene construct. Both

cytokines enhanced the BAFF promoter activity, showing that they regulate BAFF expression and synthesis. Another cytokine able to up-regulate BAFF expression in macrophages is the TGF- β (both membrane and soluble forms) (Kim et al., 2008). Using mouse macrophages as model, the authors' report that TGF- β induced BAFF expression in a typical TGF- β signaling pathway (Smad3 and Smad4 promoted BAFF promoter activity). In the same study, IFN- γ stimulation further increased TGF- β -induced BAFF expression via phosphorylation of CREB, implicated the PKA/CREB pathway in the IFN- γ -induced BAFF expression (Kim et al., 2008). Neither Stat1 α (a well-known transducing molecule of IFN- γ) nor JAK affected BAFF expression in response to IFN- γ .

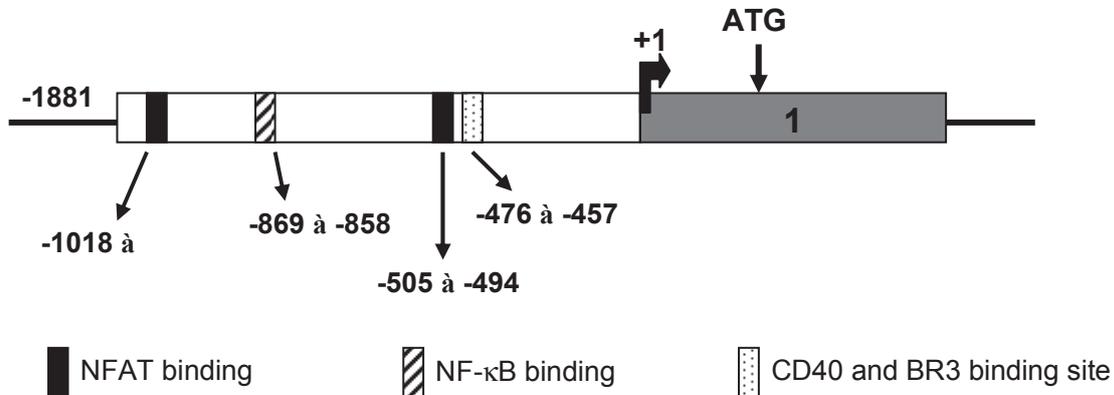
In addition, BAFF and IFN- γ establish an inflammatory loop between myeloid cells and T cells that exacerbates autoimmunity in the lupus-like autoimmunity model Lyn-deficient mice (Scapini et al., 2010). In this study, overproduction of BAFF by hyperactive myeloid cells contributed to inflammation and autoimmunity in part by acting directly on T cells to induce the release of IFN- γ . The increased production of IFN- γ mice feeds back on the myeloid cells to further stimulate BAFF release. Expression of BR3 on T cells was required for their full activation and IFN- γ release.

IFN- γ is also able to regulate the expression of BR3 (Shen et al., 2011). In cultured human multiple myeloma cells (KM3), IFN- γ stimulation induced BR3 expression both at the transcriptional and posttranscriptional levels. This stimulation induced marked enhancement of the human BR3 promoter activity and NF- κ B DNA binding activity. In conclusion, different cytokines especially IFN- γ have shown regulatory effects on BAFF and BR3 expression in human B cells.

5.8.2. The transcription factors for BAFF

In B-cell lymphomas, NF- κ B and NFAT transcription factor were described as regulators of BAFF expression through at least one NF- κ B and 2 NFAT binding sites in the BAFF promoter (Fu et al., 2006). In addition, CD40 behaves as a transcription factor for BAFF in stimulated B-cell (Lin-Lee et al., 2006) (**Figure 15**).

Figure 15. Transcription factors NF- κ B, NFAT, CD40 and BR3 bind to the BAFF promoter. BAFF gene promoter diagram (GenBank accession no. AY129225) showing the putative binding sites for NF- κ B (black lines), NFAT (black) and CD40/BR3 (black points).



5.8.2.1. Nuclear factor- κ B

NF- κ B is a family of important transcription factors that regulate B-cell development, maintenance, and stimulation (Ghosh et al., 1998; Li et al., 2002). BCR signaling and some TNF cytokine signaling induce NF- κ B activation through the NF- κ B1 pathway, where phosphorylation of the NF- κ B inhibitor by I κ B by the I κ B kinase (IKK) complex allows the cytoplasmic NF- κ B proteins p65 and p50 to translocate to the nucleus when their nuclear localization sequence (NLS) is exposed. NF- κ B proteins bind to a specific consensus sequence in the DNA and subsequently activate NF- κ B-regulated genes. Alternatively, NF- κ B can also be activated through the NF- κ B2 pathway, in which the NF- κ B precursor p100 is proteolytically cleaved to activate p52 through the NF- κ B-induced kinase (NIK) and IKK α (Senftleben et al., 2001; Claudio et al., 2002; Kayagaki et al., 2002). Both mechanisms of NF- κ B activation play important roles in B-cell development and survival. BAFF can activate NF- κ B2 through the ubiquitin-mediated proteolysis of p100 and the NF- κ B1 pathway through the proteolysis of I κ B α in vitro (Hatada et al., 2003). BAFF signaling and NF- κ B expression form a positive feedback loop that contributes to prolonged or continuous malignant B-cell survival in NHL.

5.8.2.2. Nuclear factor of activated T cells (NFAT)

NFAT is an important family of transcription factors that play key roles in the transcriptional activation of T-cell cytokines such as IL-2, IL-4, CD40L and Fas ligand. In resting T lymphocytes, these transcription factors are maintained in the cell

cytoplasm in a phosphorylated state. In response to increasing intracellular concentrations of calcium, the N-terminus of the NFAT protein is dephosphorylated by a calcium-sensitive protein phosphatase (calcineurin) that induces exposure of the NFAT protein NLS and leads to NFAT translocation from the cytoplasm to the nucleus (Shibasaki et al., 1996; Beals et al., 1997).

5.8.2.3. CD40

The CD40 receptor is a 45-50 kDa cell surface-associated phosphorylated glycoprotein that is a member of the TNF receptor (TNFRSF-5) superfamily of cytokine receptors. CD40 has a typical type 1 extracellular binding motif, a transmembrane domain and other structural homologies to TNF members that anchor and initiate important signaling pathways in B lymphocytes (Noelle et al., 1992; Nonoyama et al., 1993), DCs, monocytes and macrophages. The binding of CD40 to its cognate ligand (CD154/CD40L) is required for initiating thymus-dependent humoral responses in the immune system. In B cells, CD40 is responsible for B-cell activation, Ig isotype switching, development of GCs and humoral immune memory. Whereas CD40 has been shown to be involved in most crucial B-cell activities after binding its ligand CD40L, Lin-Lee et al. (Lin-Lee et al., 2006) reported that CD40 is also present in the nucleus of normal and neoplastic human B cells, and this nuclear CD40 is able to bind the BAFF promoter, and thus, CD40 appears to be a transcriptional regulator for growth and survival pathways in B lymphocytes. Interestingly, CD40 interacts with the NF- κ B protein, c-Rel but not with p65, and forms complexes on the promoters of CD40L and BAFF (Zhou et al., 2007). This cooperation between nuclear CD40 and c-Rel appears to be important in regulating cell growth and survival genes involved in lymphoma cell proliferation and survival mechanisms.

5.8.2.4. BR3

BR3 contains a NLS, and can be present in the nucleus of both normal and neoplastic B cells (Fu et al., 2009). Nuclear BR3 bridges and enhances the phosphorylation of histone H3 by IKK β and also associates with c-Rel, for subsequent recruitment to NF- κ B target gene promoters for initiation of transcription. Through its novel nuclear function in remodeling chromatin structure and modulating NF- κ B transcriptional activity, BR3 promotes the survival and proliferation of both normal B cells and B-cell

lymphoma. Interestingly, the c-Rel subunit benefits from both CD40 and BR3 association to facilitate the transcription of NF- κ B targeted genes.

5.8.2.5. Δ 4BAFF

Due to the effect of the new described Δ 4BAFF variant as a transcription factor of full-length BAFF (**Results: article 4**), and its overexpression in autoimmunity, in the next sections we will describe the different tools for the study of alternative splicing phenomena.

5.9. Alternative splice study

5.9.1. Alternative RNA splicing

The majority of human genes (up to 94% of genes) express multiple mRNAs via alternative splicing (Wang et al., 2008). These mRNAs can encode functionally diverse protein isoforms or can promote the on/off control of gene expression by introducing premature translation stop codons (Lareau et al., 2004). A larger fraction of these alternative splicing events are regulated according to cell-specific pattern or in response to acute stimulation (Black, 2003). The splicing efficiency of individual exons is determined by multiple features involving gene architecture (e.g. the size of the exon, the size of the flanking introns), a variety of *cis*-acting elements within the exons and flanking introns (RNA sequence), and interactions with components of the spliceosome and auxiliary regulatory factors which transiently co-assemble with the spliceosome. Both alternative and constitutive exons are recognized by multiple weak protein:RNA interactions and different exons differ in the interactions which are determinative for exon usage.

5.9.2. Splicing machinery

The basic splicing machinery, known as the spliceosome, recognizes and binds to 5' and 3' splice sites, the branch point and the polypyrimidine tract; and executes a highly complicated task of correctly defining and joining the exons together. The major spliceosome, a multi-megadalton ribonucleoprotein particle, consists of both RNAs (small nuclear RNAs, snRNAs: U1, U2, U4/U6 and U5) and a variety of protein factors

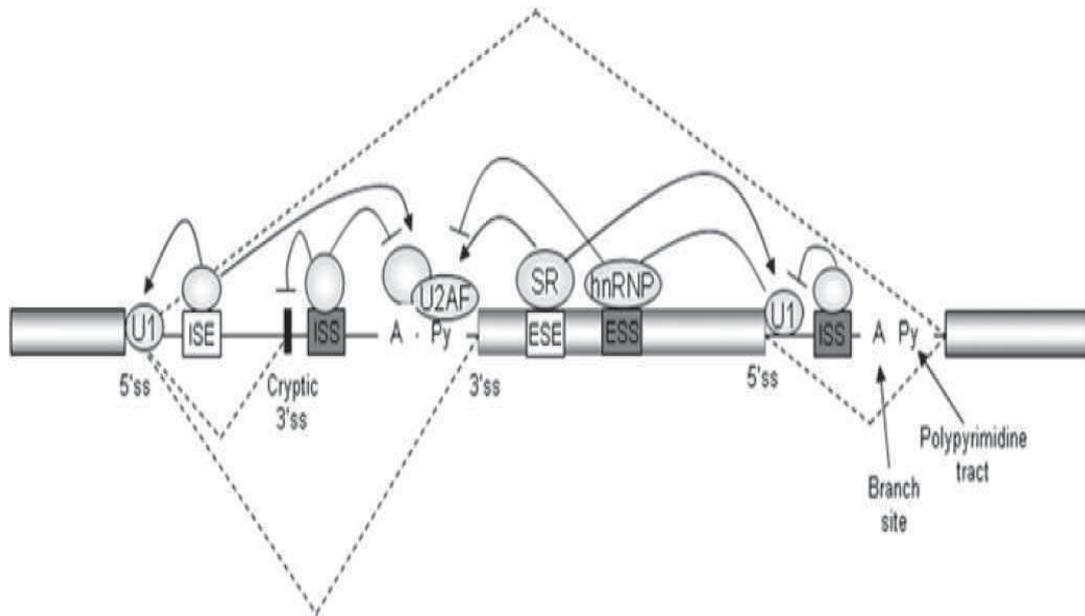
(forming together with the snRNAs five small nuclear ribonucleoprotein complexes, or snRNPs).

The 5' and 3' ss (splice sites) are recognized by spliceosome components U1 snRNP and U2AF (U2 snRNP auxiliary factor) respectively and of the branch point by SF1 (splicing factor 1)/mBBP (mammalian branch-point-binding protein), which results in the formation of the pre-splicing complex or E (early) complex. Subsequently, the E complex recruits the branch-point-binding factor U2 snRNP and is converted into the A complex. Addition of the U4/U5/U6 tri-snRNP (B complex) to the pre-splicing complex (A complex) produces a fully assembled spliceosome, which undergoes conformational changes resulting in the first catalytic step of the splicing reaction (C complex), branch formation. After subsequent conformational changes and proper alignment of the two exons to be spliced, the second catalytic reaction, ligation of the exons, occurs. Spliceosome function is executed through dynamic RNA-RNA, RNA-protein and protein-protein interactions (**Figure 16**).

5.9.3. Splicing regulatory elements

Alternative exons are often regulated according to cell-specific patterns and regulation is mediated by specific sets of *cis*-acting elements and *trans*-acting factors (SR proteins and hnRNPs). Exons contain exonic splicing enhancers (ESEs) and exonic splicing silencers (ESSs) that enhance or repress exon usage, respectively (**Figure 16**). The best characterized ESEs are purine-rich and their enhancer activities are mediated through bindings to members of the SR protein family (Cartegni et al., 2002). A lesser characterized A/C-rich family of ESEs mediate their effects by binding to the cold box protein, YB-1 (Stickeler et al., 2001). ESSs bind to hnRNP proteins and functional analysis using minigenes have demonstrated that this binding of hnRNP proteins mediates repression (Wang et al., 2004). Intronic splicing enhancers (ISEs) and intronic splicing silencer (ISSs) within the upstream and downstream introns also serve to enhance or repress, respectively, the use of associated splice sites (Wagner et al., 2001). Occasionally, the same element is able to activate and suppress splicing of two mutually exclusive exons.

Figure 16. Schematic representation of mRNA splicing. Splicing regulatory motifs include the 3' ss and 5' ss; the branch point and polypyrimidine tract are recognized by the spliceosome components (U1 and U2AF) early in splicing. Splicing efficiency is influenced by a range of *trans*-acting factors as the SR proteins (SR) that bind to the positive regulatory motifs known as ESEs and ISEs or factors such as hnRNPs that bind to negative splicing regulatory motifs known as ESSs and ISSs. From Jensen et al., 2009.



5.9.4. Serine/Arginine-rich protein splicing factors (SR proteins)

The serine/arginine-rich proteins (SR proteins) constitute a family of predominantly nuclear RNA-binding proteins that are present in all organisms. SR proteins function in both constitutive and alternative splicing and can act in the assembly of spliceosome body. Via binding to the exon (predominantly in the ESEs where they influence the splicing of the exon in which they are located), SR proteins are known to increase the binding of U2AF snRNP to an upstream 3' ss or that of U1 snRNP to a downstream 5' ss (**Figure 16**). The first SR protein was identified nearly 20 years ago as an essential pre-mRNA splicing factor that could also influence alternative splicing in human cell extracts (Ge et al, 1991; Krainer et al., 1991). These proteins are characterized by one or two N-terminal RNA-binding domains (RBDs), also known as an RNA recognition motif (RRM) that interacts with the pre-mRNA and a C-terminal region enriched in Arg-Ser dipeptides (RS domain) that promotes protein-protein interactions with the splicing complex (Zahler et al., 1992; Cáceres et al., 1997). The SR proteins have since been extensively studied in several species, and found to play important roles in splicing,

both as general splicing factors and as regulators of alternative splicing. Unexpectedly, studies during the last decade have extended the role of SR proteins to a diverse set of additional cellular processes, including mRNA nuclear export, mRNA stability and quality control, translation, maintenance of genomic stability, and oncogenic transformation (Huang and Steitz, 2005; Long and Cáceres, 2009; Zhong et al., 2009). In different genetic contexts, SR proteins can act as ISSs. SR proteins are highly regulated by the phosphorylation. Several kinases, such as SR-protein kinases 1 and 2 (SRPK1 and SRPK2), the cell cycle-dependent dual specificity kinase (Clk/Sty), Akt/protein kinase B and DNA topoisomerase I (Topo I) could phosphorylate SR proteins and regulate their function (Gui et al., 1994; Colwill et al., 1996; Rossi et al., 1996; Wang et al., 1998).

5.9.4.1. Nomenclature for Serine/arginine-rich protein splicing factors (SR proteins)

A simple nomenclature of SR proteins has been recently published (Manley and Krainer, 2010). SR proteins are defined based entirely on their properties, with one or two N-terminal RBDs, followed by a downstream RS domain of at least 50 aminoacids with >40% RS content, characterized by consecutive RS or SR repeats. This definition yields 12 SR proteins in humans (**Table 12**).

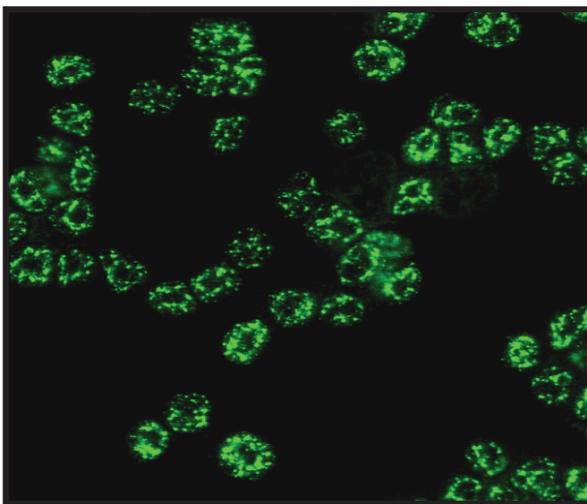
Table 12. SR proteins/gene symbols. The nomenclature is based on the root « SRSF » (SR splicing factor) followed by the numbers 1-12, that reflect the chronological order in which the genes/proteins were discovered. From Manley and Krainer, 2010.

| Previous gene symbol | Aliases | New protein/gene symbol |
|----------------------|---------------------|-------------------------|
| SFRS1 | ASF, SF2, SRp30a | SRSF1 |
| SFRS2 | SC35, PR264, SRp30b | SRSF2 |
| SFRS3 | SRp20 | SRSF3 |
| SFRS4 | SRp75 | SRSF4 |
| SFRS5 | SRp40, HRS | SRSF5 |
| SFRS6 | SRp55, B52 | SRSF6 |
| SFRS7 | 9G8 | SRSF7 |
| SFRS2B | SRp46 (human only) | SRSF8 |
| SFRS9 | SRp30c | SRSF9 |
| SFRS13A | TASR1, SRp38, SRp40 | SRSF10 |
| SFRS11 | SRp54, p54 | SRSF11 |
| SFRS13B | SRp35 | SRSF12 |

5.9.4.2. SR proteins localization

SR proteins are concentrated within nuclear speckles of cells that correspond to interchromatin granule clusters (IGCs) at the electron microscopic level (**Figure 17**) and shuttle to sites of active transcription to initiate their co-transcriptional splicing activity. The activity and subcellular localization of these splicing factors are highly regulated by phosphorylation through SRPKs (SR protein kinases) and Clk/Sty, many of which are located by intracellular signaling. In this way, alternative splicing can be regulated by extracellular signals (Cáceres et al., 1998; Huang et al., 2004; Ding et al., 2006). Overexpression of SRPKs and Clk/Sty causes dispersal of speckles in the nucleoplasm in interphase cells and disrupts the coordination between transcription and splicing (Gui et al., 1994; Colwill et al., 1996; Sacco-Bubulya and Spector 2002). In addition, whereas most of SR proteins are confined to the nucleus, some of them (SF2/ASF, SRp20 and 9G8) shuttle rapidly and continuously between the nucleus and the cytoplasm. Phosphorylation of the RS domain affects the shuttling properties of SR proteins. These findings show that different SR proteins have unique intracellular transport properties and not only a role in nuclear pre-mRNA splicing (Cáceres et al., 1998).

Figure 17. Immunofluorescence staining of SC35 protein in Hep2 cells, showing a nuclear speckled pattern, characteristic of SR distribution.



5.9.4.3. SC35 protein

One of the most important and studied member of the SR family is the splicing factor SC35. SC35 was identified by mAbs directed against purified spliceosomes (Fu et al., 1990). Further analysis of our study about the alternatively spliced Δ 4BAFF variant showed a significant prediction of SC35 to bind 2 ESEs in the exon 4, suggesting that this protein may be implicated in the constitutive splicing of BAFF. Adding to their functions as a splicing factor, SC35 affects transcriptional elongation in a gene-specific manner. This effect has been demonstrated by Lin et al. (Lin et al., 2008). Interestingly, SC35 can regulate its own expression. This is accomplished via a feedback loop that connects the level of SC35 protein to the productive splicing of the SC35 transcript. The SC35 protein binds to its own pre-mRNA uncovering ss downstream of the termination codon. When these alternative ss in the 3'UTR are used, the normal termination codon becomes a PTC based on the 50 nucleotide rule. Nonsense-mediated decay (NMD) then degrades the transcript, down-regulating the expression of SC35. In this way, SC35 appears to auto-regulate its own production (Sureau et al., 2001).

5.9.5. Heterogeneous nuclear ribonucleoproteins (hnRNPs)

The hnRNPs are a diverse family of approximately 20 ubiquitously expressed major proteins including the important members hnRNP A1 and PTB (polypyrimidine tract-binding protein)/hnRNP I. They are designated from hnRNP A1 through hnRNP U. This protein family can be defined by containing an RNA recognition motif and a KH (hnRNP K homology) domain or an arginine-glycine box (He and Smith, 2009). The hnRNP A1 is the best characterized member as splicing factor (Caceres et al., 1994; Hanamura et al., 1998; Pollard et al., 2002). The hnRNPs can bind to nascent pre-mRNA and operate predominantly as negative regulator of splicing, often in direct competition with SR proteins, or other splicing factors for binding sites. Binding of hnRNPs to silencer sequences inhibits SR protein binding, forcing a shift of splicing to a distal splicing site. Like SR proteins, hnRNP activity is regulated in part by intracellular location and by interaction with other proteins (Ji and Tulin, 2009). As well as their role in alternative splicing, hnRNPs have been reported to be involved in mRNA transport, stability and translation initiation.

Finally, shuttling of both SR proteins (Cáceres et al., 1998) and hnRNPs between the nucleus and the cytoplasm (Choi and Dreyfuss, 1984), is an important mechanism for

the regulation and specificity of splicing for these factors. In the nucleus these two antagonistic protein families have their effect on alternative splicing with the ratio of these proteins within the nucleus predetermining their functional roles in splicing. The hnRNP A1, D, E, F/H and K have been demonstrated in both the nucleus and cytoplasm, suggestive of cytoplasmic roles for these hnRNPs (Dreyfuss et al., 2002).

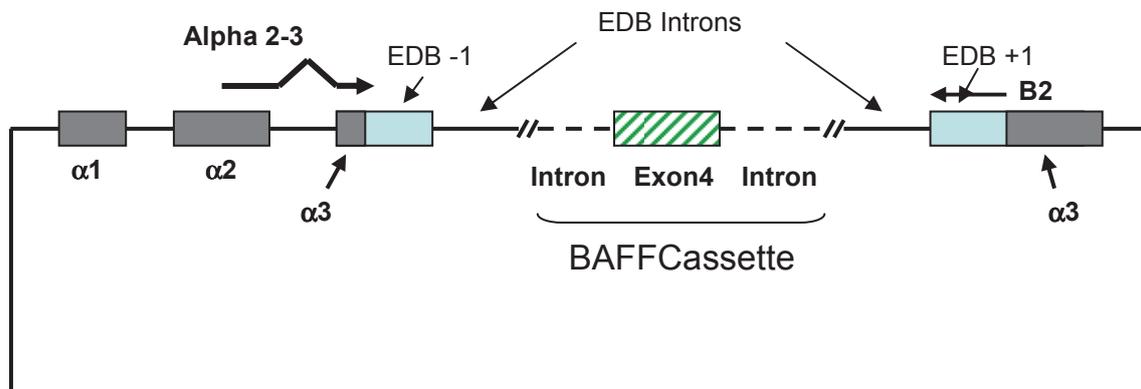
5.9.6. Minigene technique to study alternative splice

Transient expression of minigenes is a commonly used in vivo assay to identify the intrinsic features of a gene that control exon usage, to identify specific *cis*-acting elements that control usage of constitutive and alternative exons, to identify *cis*-acting elements that control cell-specific usage of alternative exons, and once regulatory elements have been observed, to identify the *trans*-acting factors that bind to these elements and modulate splicing. Expression of minigene pre-mRNAs by transient transfection provides a rapid assay for loss-of-function and gain-of-function analysis for *cis*-elements and *trans*-acting factors that affect splicing regulation.

5.9.6.1. Minigene design

Minigenes contain a genomic segment from the gene of interest that includes the alternatively spliced region and flanking genomic regions. The genomic segment is generated by PCR amplification from genomic DNA or cloned genomic DNA fragment as template. To determine if a specific motif is the response element for a putative regulatory factor, the minigene will include the *cis*-element (s) of interest. The primary elements regulating alternative splicing are typically within 200-300 nucleotides upstream and/or downstream of the regulated exon. **Figure 18** shows our minigene construction centered in the exon 4 of BAFF amplified from genomic DNA. The choice of the cells used for transfection depends upon the goals being pursued. In our experiments (**results: article 5**) we have used the B-cell line RAMOS.

Figure 18. Minigene construct centered on exon 4 of BAFF.



5.9.6.2. Identification of exonic and intronic elements that enhance or repress splicing

Minigenes are useful tools to identify and demonstrate the activities of exonic and intronic elements *in vivo*. An analysis of ESEs should begin with the use of biocomputational tools such as ESEFinder (Cartegni et al., 2003), and RESCUE-ESE to identify putative elements (Fairbrother et al., 2004). Biocomputational resources for intronic elements have not progressed as far as for exonic elements. To test whether a motif within an exon functions to enhance or suppress exon inclusion, results from both loss of function and gain of function mutations analyses provides the strongest support.

5.9.7. Alternative splicing of the immune system

Many genes in the immune system have been shown to undergo alternative splicing (e.g. HLA, TCR ζ -chain, cytokines and NF-kB). Nevertheless, the knowledge of the global involvement of alternative splicing in different aspects of immunity is still limited. For instance, alternative splicing events have been described at virtually every level of NF-kB signaling, from the initiating receptors to the transcription factors regulating target gene expression (reviewed by Leeman and Gilmore, 2008). In many cases, the mRNAs encoding these protein isoforms can be induced, and their proteins display altered structures and function. Expression of these variants can enhance or inhibit NF-kB signaling. It is still unclear whether alternative splicing of these genes plays a causative role in disease or is simply a consequence of a stressed cell. Other alternative splicing variants may also play a critical role in the maintenance of peripheral immune tolerance. Peripheral tolerance depends upon the expression of

tissue-specific antigens in secondary lymphoid tissues in a manner that triggers functional deletion of autoreactive cells. The most studied regulator is the AIRE protein. However, a second, independent regulator Deaf1 was recently identified (Yip et al., 2009). A Deaf1 splice variant acts as a dominant inhibitor of the wild-type protein and is upregulated in the pancreatic-draining lymph nodes of pre-diabetic NOD mice and subjects with T1D. Another class of splice variant associated with autoimmunity is that involved in immune recognition and regulation including PD-1 (Nielsen et al., 2005), FAS (Drappa et al., 1996), CD45 (Jacobsen et al., 2002), TCR-associated CD3 ζ chain (Nambiar et al., 2001) and CD200 (Chen et al., 2010).

5.9.7.1. Alternative splicing, immune system and regulatory proteins

In a recent study (Oberdoerffer et al., 2008), the hnRNPL-like (hnRNPLL) was identified as a critical inducible regulator of CD45 alternative splicing, from the larger isoforms known as CD45RA to the shortest isoform, CD45RO express by activated and memory T cells.

Nambiar et al. (Nambiar et al., 2001) have shown that T cells from patients with SLE exhibit increased levels of the alternatively spliced variant of the TCR-associated CD3 ζ chain isoform (344-bp) compared to healthy controls that express predominantly the wild type isoform (906-bp). Because the alternative splicing affects the UTR and not the coding region, the size of this protein generated by these two isoforms is identical. However, the alternatively spliced isoform lacks two regulatory adenosine/uridine-rich elements and a translation regulatory sequence, generating an unstable transcript. This short isoform is associated with lower TCR-associated CD3 ζ chain protein level found in SLE T cells and the aberrant function of this protein. The ASF/SF2 protein (of the SR family) binds specifically to the 3'-UTR of CD3 ζ and regulates the expression of CD3 ζ protein limiting the production of the alternatively spliced isoform, showing the important role of this protein in the regulation of the CD3 ζ protein expression in human T cells (Moulton and Tsokos, 2010). Similar evidence for ASF/SF2 has been described in the alternative splicing of CD200 (Chen et al., 2010). CD200 is a type I membrane glycoprotein, important in the prevention of inflammatory disorders, graft rejection and AIDs (Gorczynski et al., 2004). A truncated form resulting from alternative splicing is characterized as a functional antagonist to full-length CD200. In this study, ASF/SF2 regulates the function of CD200 by controlling CD200 alternative splicing, through

direct binding to an ESE located in exon 2 of CD200. Taken together, these data show that members of SR protein family, may mediate constitutive splicing, limiting the generation of alternative splicing isoforms, which in some cases may have a deleterious role in the normal immune response.

5.9.7.2. Alternative splicing and immune activation of human T and B cells

In a systematic genome wide analysis of activated human T and B lymphocytes using whole exon DNA microarrays integrating alternative splicing and differential gene expression (Affymatrix Human Exon 1.0 ST array), Grigoryev et al. (Grigoryev et al., 2009) detected 3 distinct classes of genes in both T and B cells that changed depending of the function of immune activation: 1) differentially expressed and alternatively spliced; 2) constitutively expressed and alternatively spliced; and 3) differentially expressed without alternative splicing. In T cells, 60% of more of all the alternatively spliced genes were differentially expressed at the mRNA transcript level during activation, and only 20-30% in B cells. However, analysis of constitutively expressed transcripts demonstrated that 50-70% in T cells and about 40% in B cells are alternatively spliced. These results show the significant increase in transcriptional diversity generated by alternative splicing during lymphocyte activation.

5.9.8. Tissue-specific splicing

Differential expression of SR proteins, and their negative regulators, the hnRNPs, will bring about tissue- and cell-type-specific splicing in vivo (Hanamura et al., 1998). For example, artificially altering the ration of the SR protein SF2/ASF (SRSF1) to hnRNP A1 can lead to dramatic changes in alternative splicing (Mayeda et al., 1993; Cáceres et al., 1994; Wang et al., 1995). In addition to tissue-specific splicing that is regulated by constitutive splicing factors, there are tissue-specific *trans*-splicing factors that are expressed only in their target tissues such as Nova (restricted to neurons), Fox-1, CELFs [CUG-BP (CUG-binding protein)], ETR3-like factors, MBNL (muscleblind-like), TIA (T-cell-restricted intracellular antigen) and HuR proteins (Barreau et al., 2006; Pascual et al., 2006; Fukumura et al., 2007).

5.9.9. Consequences of aberrant spliced transcripts

Changes in splicing regulatory elements can lead to exon skipping, intron retention, and the creation of ectopic splice sites or the activation of cryptic sites. Aberrantly spliced transcripts may lead to protein isoforms with altered properties or included exons with premature termination codons (PTCs), a signal that activated NMD (a mechanism in which transcripts with PTCs are identified and efficiently degraded) with the effect of preventing translation of truncated proteins. Alternative splicing of the untranslated regions of transcripts may lead to changes in mRNA stability, localization or control of translation.

5.9.10. Alternative splicing induced by IFN- γ

The induction of BAFF by IFN- γ prompted us to study if IFN- γ might be implicated in alternative splicing, and eventually may induce Δ 4BAFF expression. Some authors have shown that IFN- γ is able to regulate isoforms of human tryptophanyl-tRNA synthetase (TrpRS) *in vivo* through alternative mRNA splicing. The miniTrpRS (produced by alternative splicing) has anti-proliferative and anti-angiogenic activity, but these effects are not seen with the full-length protein. An IFN- γ -sensitive promoter was demonstrated to drive the production of miniTrpRS and two new splice variants of TrpRS, showing that this cytokine may explain alternative splicing variants with different effects (Liu et al., 2004).

Recent studies confirm that different cytokines including IFN- γ may modify the global expression pattern of genes, including spliced variants. Ortis et al. (Ortis et al., 2010) in purified rat pancreatic β -cells showed that IFN- γ in association with other cytokines (IL-1 β and TNF- α) modified the expression of more than 20 genes involved in RNA splicing, and changes in alternative splicing of >50% of the cytokine-mediated genes (by exon-array analysis), showing that these cytokines may affect the alternative splicing in a tissue specific manner. These results were confirmed by RT-PCR in three modified genes, including NF-kB2 (Δ exon 22). This information is of capital importance for our projet, because preliminary results showed that different cytokines stimulation [IL-10, CD40L and mainly IFN- γ (**article 5: Alternative splicing in exon 4 of BAFF is regulated by interferon-gamma and SC35 protein**)] induced Δ 4BAFF transcript expression in normal tonsillar B cells.

OBJECTIVES

In the first section, continuing with the main thematic of the laboratory on the study of the clinical and immunological characteristics of B cells in AIDs, we evaluated the contribution of skin biopsy in the diagnosis of pSS. Then, **in the second section**, prompted by the reports on the effects of FLT3-L on lymphocyte development, lymphoid malignancies, B-lymphocyte alterations, and the risk of developing lymphoma, we evaluated the role of FLT3L in pSS. First, serum levels of FLT3-L in patients with pSS were analyzed and compared with those in normal control subjects. FLT3-L levels were then correlated with abnormalities in B lymphocyte distribution. Second, the expression of FLT3 on B-cell subsets in the blood and SGs was determined. Third, the role of FLT3-L in the differentiation of B lymphocytes in primary SS patients was evaluated functionally, and finally, FLT3-L expression was associated with disease activity scores. Our initial interesting results showing that FLT3-L may be a marker of lymphoma in pSS, prompted us to evaluate FLT3-L in a larger cohort of patients (the French cohort of pSS-ASSESS-). **In the third and last section** of my thesis, we wanted to study the role of the new variant of BAFF, called Δ 4BAFF (due to the alternative splicing of exon 4) which has been recently described in our laboratory. Interestingly, this new variant is mainly detected in AIDs (including PBMC and ECs from pSS patients). Thus, our objective was to understand the role of this new variant and the study of the alternative splicing of the exon 4. Our starting hypothesis was that Δ 4BAFF is a transcription factor of its own gene, due to increased levels of full-length BAFF after Δ 4BAFF transfection. To confirm the role of Δ 4BAFF we used Electrophoresis Mobility Shift Assay (EMSA) and reporter gene assays. Our initial results confirmed this role of Δ 4BAFF as a transcription factor, and the need of a co-factor of the NF- κ B family for its entry into the nucleus. To confirm which member of NF- κ B was associated with Δ 4BAFF, we performed EMSA and co-immunoprecipitation. Additionally, a microarray analysis of a stable Δ 4BAFF-transfected-cell line showed that this variant was actually a transcription factor for hundreds of genes, mainly implicated in immune response. At the end, due to the increased expression of Δ 4BAFF in pSS, and the important role of this variant, we analyzed the mechanisms of induction and regulation of Δ 4BAFF. We examined the effects of IFN- γ (a cytokine known as a positive regulator of BAFF) on Δ 4BAFF expression in different cell lines. Finally, to study the mechanisms implicated in the alternative splicing of exon 4, we transfected in RAMOS B cells a minigene construct, centered on exon 4, and this minigene was then co-transfected with coding plasmids for splicing proteins (SR and hnRNP proteins).

Collectively, these results are of clinical and fundamental basic interest in pSS. Of **clinical** interest for all these three points: 1) Memory B cell infiltrates are present in

pSS and may be used as a diagnostic and follow-up tool; 2) Levels of FLT3-L are high in pSS and may favor the development of lymphoma. Thus this cytokine can be use as marker of activity and severity in pSS; 3) the important regulatory role of Δ 4BAFF in the overexpression of BAFF provide an expanded conceptual view of BAFF gene regulation in AIDs, and may open new therapeutic options targeting BAFF in pSS. Of **basic and fundamental research**: 1) the accumulation of memory B cells in the skin of pSS highlights the importance of these cells in pSS; 2) FLT3-L proliferation effect may explain survival of infiltrating B cells in exocrine glands of pSS patients. In addition, FLT3 expression by naïve B cells may explain, in part, the distribution abnormalities seen in peripheral blood of patients; 3) the demonstration that IFN- γ induces Δ 4BAFF, leading to an increase of BAFF provides an expanded conceptual view of *BAFF* gene regulation, and contributes to a better understanding of the mechanisms involved in BAFF up-regulation in autoimmunity.

RESULTS

Skin biopsy in primary Sjögren's Syndrome

Article 1

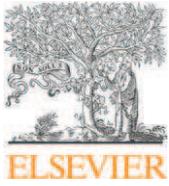
Memory B-cell aggregates in skin biopsy are diagnostic for primary Sjögren's syndrome.

**Roguedas AM, Pers JO, Lemasson G, Devauchelle V, Tobón GJ,
Saraux A, Misery L, Youinou P.**

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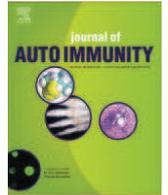
Memory B-cell aggregates in skin biopsy are diagnostic for primary Sjögren's syndrome

Faced with the ever-growing complexity of criteria for classification of pSS patients, new diagnostic test for the pathology are needed. In particular, the skin may function as a diagnostic window of the internal organs and other exocrine tissues involvement in pSS. **In this first article**, our objective was to evaluate the frequency of xerosis in patients with pSS, and compare histopathology of cutaneous sweat glands with MSGs biopsies, and to analyze the contribution of the skin biopsy to pSS diagnosis. In pSS patients, the skin was dryer, and xerosis more frequent compared to controls. In addition, lymphocytic infiltration was seen in the skin of 8 of the 12 patients tested. Two of them had normal MSGs. Most interestingly, B cell infiltrates (memory CD10-/CD20+/CD27+/IgD- B lymphocytes and immature CD20+/CD24+) were identified in patients' skin infiltrates, so that their presence might be a clue to the diagnosis of pSS. As concluding remarks of this article, skin biopsy may serve as an additional tool for the diagnosis of pSS, with supplemental importance in patients with repeatedly negative MSG biopsies. In clinical practice, the skin of patients suspected of pSS must be carefully examined and, one step further, the epidermis should be evaluated to search for B cell infiltrates. A diagnostic flow chart including skin evaluation in pSS suspicion is proposed in **appendix 3**.



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Memory B-cell aggregates in skin biopsy are diagnostic for primary Sjögren's syndrome

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A B S T R A C T

Keywords:

B lymphocyte
Sjögren's syndrome
Skin biopsy
Memory B lymphocyte

There is a crucial need for reliable diagnostic criteria for SS. Our objective was to evaluate the frequency of xerosis in patients with primary Sjögren's syndrome (SS), and compare histopathology of cutaneous sweat glands and labial salivary glands (LSGs), with respect to their contribution to the diagnosis. Twenty-two patients with primary SS and 22 matched normal volunteers were invited to rate their skin dryness on a visual analog scale. The skin was dryer (58.3 ± 10.1 versus 38.9 ± 7.6 , $P < 0.01$), and xerosis more frequent (9 of 22 versus 2 of 22, $P < 0.02$) in the patients than in the controls. The axilla skin was chosen for a 6-mm punch biopsy. Lymphocytic infiltration was seen in the skin of 8 of the 12 patients tested. Two of them had normal LSGs. Most interestingly, B cell infiltrates were identified in patients' skin infiltrates, so that their presence might be a clue to the diagnosis of primary SS. These cell aggregates associated memory CD10⁺/CD20⁺/CD27⁺/IgD⁺ B lymphocytes and immature CD20⁺/CD24⁺ lymphocytes. These latter findings strongly suggest that skin biopsies warrant inclusion into the routine clinical care of patients suspected of suffering from primary SS.

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1. Introduction

A dermatologist was the pioneer who linked dry eyes, dry mouth and dry skin to a single entity, but Henrik Sjögren was the first to extend the sites of involvement throughout the body, so that "Sjögren's syndrome" (SS) came to bear his name. However, Moutsopoulos was the one who perceived the disruption of epithelial cells with lymphoplasmocytic infiltration of exocrine tissues, and coined the terms "autoimmune epithelitis" for the disease [1], stressed the link between innate and acquired responses in this disease [2], and recently described the characteristics of the labial salivary gland (LSG) infiltrates [3]. It can occur alone as primary SS, or against a background of connective tissue disease, as secondary SS. Dryness of the eyes is referred to as xerophthalmia, and dryness of the

mouth as xerostomia. It happens that these two symptoms are not reliable enough to allow therapeutic decisions to be made [4]. Given that new insights have been gained into the pathophysiology of SS from animal models [5], gene analyses [6], and the advent of molecular biology [7], treatments are now endowed with significance by the emergence of potentially active biotherapies.

In everyday practice, the diagnostic path remains a matter of personal choice for SS and is faced with the ever-growing complexity of classification criteria [8]. Given the multiorgan spread of the autoimmune process, sicca symptoms other than xerophthalmia and xerostomia may arise. Astute clinicians look for the appearance of such symptoms, and the ideal organ to investigate could well be the skin, which functions as an easily reachable window to internal diseases. However, the number of patients with SS presenting with cutaneous manifestations is greater than that of the SS patients reporting a history of xerosis at the clinician's request [9]. Such neglect of skin complaints may be simply ascribed to eye and mouth dryness that overshadow the less intrusive symptoms. Furthermore, doubt has been cast on the efficacy of LSG biopsy to support the diagnosis of SS. Initial interpretation of the histopathology has indeed proven to be erroneous in half of the

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biopsies on a second evaluation [10]. In extreme cases, sialadenitis may be absent in new patients, whilst present in healthy individuals [11]. Due to variations over time [12], original lesions, like insertion of B lymphocytes between epithelial cells [13], might be more characteristic of SS than the focal lymphocytic infiltrates. Other sites, such as sublingual [14] or parotid glands [15], are also appropriate for assessment of the oral component of the disease.

The view that any exocrine glands are at risk of undergoing the autoimmune assault connotes that examination of sweat glands could contribute to the histopathological diagnosis. Given the evidence for disturbed selection and differentiation of B lymphocytes [16] in SS, and the imprint of T lymphocytes to migrate to the epidermis [17], the detection of the former cells in the skin would constitute a conclusive clue to the diagnosis of SS. To explore this possibility, we set out to address three issues. 1)- Is xerosis more frequent in SS patients compared with normal controls? 2)- Should sweat gland biopsy be substituted for LSG biopsy in the early diagnosis of SS? 3)- Do the dominance of B lymphocyte in the pathogenesis of SS [18] raise the question as to whether these cells participate in involving the skin? It appeared that, contrary to expectations, memory B lymphocytes, were seen to infiltrate the epidermis of these patients.

2. Patients and methods

2.1. Study population

Twenty-two consecutive patients fulfilling the criteria of the American-European Consensus Group for primary SS [8] and 22 healthy volunteers from the staff or from a home for the elderly, participated in the study. Five patients had disease limited to exocrine glands, and the remaining 7 displayed at least one extraglandular complication. The patients did not have associated lymphoma, nor were they taking corticosteroids or immunosuppressive drugs. Ro/SSA and La/SSB antibodies (Abs) were detected using-commercial enzyme-linked immunosorbent assays (BMD, Marnes-La-Vallée, France). Patients and controls were matched for age, sex and ethnicity. We excluded those who had other causes of sicca symptoms, including viral infection and drugs. All participants gave informed consent, and the experimental procedures were approved by the Ethics Committee at the Brest University Medical School Hospital. In addition to the symptoms that they related spontaneously, patients and controls were further enquired, and invited to rate their skin dryness on a 100-mm visual analog scale (VAS).

2.2. Tissue samples

All the patients had an LSG biopsy. This tissue was embedded in paraffin, processed routinely, and assigned the focus score advocated by Daniels and Whitcher [18]. Twelve of the 22 patients with otherwise-proven primary SS underwent also a skin biopsy in the same session (12 women aged 55.0 ± 10.7 years), as well as 10 of the 22 healthy volunteers (10 women aged 49.5 ± 8.8 years). Axillae were ideal for the 6-mm punch biopsies of non-lesional skin, given the concomitance of eccrine, apocrine and apoecrine sweat glands [19]. One piece of the sample was placed into formalin fixative for hematoxylin-eosin and toluidine blue-staining, and subsequently for immunohistochemical study. A second piece was OCT-embedded (Miles, Naperville, IL) and snap-frozen in isopentane for immunofluorescence (IF) staining.

2.3. Skin lymphocyte staining

Anti-T cell and anti-B cell Abs were used in this pilot examination of the skin. *Immunohistochemical analysis* of 6 μm -thick

microtome sections was based on the use of unconjugated anti-CD3 and anti-CD20 monoclonal-Abs (mAbs), with the binding being revealed by horseradish peroxidase-conjugated goat anti-mouse IgG polyclonal Ab (pAb) and developed with diaminobenzidine.

IF staining was performed on sequential 4 μm -thick cryostat sections mounted onto poly-L lysine-coated slides. Some slides were incubated for 40 min at room temperature with unconjugated anti-CD3, anti-CD4 or anti-CD8 mAb alone, and others with goat anti-CD20 pAb (Neomarker, Fremont, CA) plus unconjugated anti-CD5, anti-CD10, anti-CD24, anti-CD27 or anti-IgD mAbs (all from Beckman-Coulter, Villepinte, France). After three washes in phosphate-buffered saline (PBS), they were all left for another 40 min with fluorescein isothiocyanate (FITC)-conjugated donkey anti-mouse pAb, together with tetramethyl rhodamine isothiocyanate (TRITC)-conjugated donkey anti-goat pAb (both from Jackson, West Grove, PA) in PBS supplemented with 2% donkey serum (Sigma, St Louis, MO). After 5 rinses, the sections were fixed with 4% cold paraformaldehyde and analyzed with a TCS-NT Leica confocal imaging system (Leica Microsystems, Westlar, Germany). The overlay of FITC-generated green-stain and TRITC-generated red-stain was seen yellow. Neither control mouse anti-CD16 mAb, nor control goat anti-hepatitis B surface antigen A pAb (Cytoshop, Rehovot, Israel) showed any background IF.

2.4. Statistics

The results were expressed as arithmetic means with standard deviations. Comparisons were made using the Mann–Whitney *U* test for unpaired data, and Chi-squared test with Yates' correction when required.

3. Results

3.1. Clinical assessment of cutaneous dryness

The 22 primary SS patients scored their skin dryness as being worse than the 22 healthy volunteers on the VAS, *i.e.*, 58.3 ± 10.1 versus 38.9 ± 7.6 ($P < 0.01$). In addition, although not reported spontaneously, xerosis was more frequently experienced by 9 of the 22 patients than by 2 of the 22 controls ($P < 0.02$). Serological and clinical parameters were reviewed to determine whether there were any correlations with cutaneous dryness. There was a trend for more frequent extraglandular complications in the patients with than in those without this symptom. In particular antinuclear Abs, including anti-SSA and anti-SSB Abs were not associated with cutaneous dryness. Whether these findings are significant requires a larger series of patients.

3.2. The diagnostic value of LSG and skin biopsies

All of the 22 LSG specimens, but two, contained at least two foci of inflammatory cells which are diagnostic for SS (Table 1). Of the 12 patients, whose skin biopsy was available, including those two with normal LSG biopsy, lymphocytic infiltration of the skin was identified in 8 (underlined in Table 2) including the 2 patients with non-contributive LSG biopsy (Fig. 1A, and B left). This discrepancy between the LSGs and the skin in primary SS patients is not that surprising, since the extent of infiltration varies between the fields in one LSG specimen, between the LSGs in one biopsy, and between the LSG samples in one patient [13,14,16], and over time in a given patient [17]. In contrast, the 10 skin specimens from matched healthy volunteers were absolutely devoid of lymphocytes (Fig. 1Bright).

Table 1

Antinuclear antibody, clinical complications, labial salivary score (LSG) and skin biopsy in 12 women with primary Sjögren's syndrome.

| Age (years) | Antinuclear antibody | | Extraglandular complication | LSG Focus score | Skin Biopsy |
|-------------|----------------------|----------|-----------------------------|-----------------|-------------|
| | Anti-SSA | Anti-SSB | | | |
| 53 | Positive | Negative | None | 3 | Negative |
| 50 | Positive | Positive | Renal disease | 3 | Positive |
| 39 | Negative | Negative | None | 4 | Negative |
| 68 | Positive | Positive | Nonerosive arthritis | 1 | Positive |
| 67 | Positive | Negative | Raynaud's | 2 | Positive |
| 46 | Positive | Positive | None | 2 | Positive |
| 71 | Negative | Negative | None | 3 | Negative |
| 64 | Negative | Negative | Raynaud's | 1 | Positive |
| 41 | Negative | Negative | None | 2 | Positive |
| 48 | Negative | Positive | Nonerosive arthritis | 3 | Negative |
| 54 | Negative | Negative | Nonerosive arthritis | 4 | Positive |
| 66 | Positive | Positive | Lung involvement | 5 | Positive |

3.3. Phenotyping of cutaneous lymphocytes

In addition to T lymphocytes (CD3 arrowed in Fig. 1C left), numerous B lymphocytes were found (CD20 arrowed in Fig. 1C right) within the cutaneous infiltrates. As expected [3], most of the T lymphocytes, as defined by the membrane expression of CD3, were positive for CD4 and negative for CD8 (not shown).

Aggregates of B cells were detected in the skin by CD20 staining. The view that B cells underpin autoimmunity prompted us to analyze these B cell aggregate further in depth. Two-color IF confirmed their presence, with as few as 1% expressing CD5 which is a marker for polyspecific autoAb-producing B lymphocytes (CD20 arrowed in green, and CD5 arrowed in red in Fig. 2A) from a separate lineage, or the hallmark of anergy in autoreactive B cells. Unexpectedly, there were CD20+/CD27+/IgD- (the overlay of CD20 and CD27 is arrowed in yellow in Fig. 2B, while CD20 is pointed green and IgD pointed red in Fig. 2C) memory B cells in the skin of these patients. They were associated with CD20+/CD24 + immature B cells resembling some marginal-zone B cells (Fig. 2D).

4. Discussion

SS presents as a multifaceted condition with a broad variety of clinical manifestations and biological abnormalities [5]. This polymorphism accounts for the delay in the diagnosis, associated with underestimation of the patients' concerns made by the physician, which consequently discourages patients to take medical advice as symptoms arise. As a consequence, there is every likelihood that the prevalence of the disease is far higher than previously estimated [20].

These disadvantages are being overridden by the emergence of new and potentially active treatments, most notably anti-B lymphocyte antibodies (Abs). In the setting of autoimmune diseases, the latter have proven effective in rheumatoid arthritis

Table 2

Histopathology of exocrine glands in 12 patients with primary Sjögren's syndrome patients and 10 healthy volunteers.

| Final interpretation | | No of patients With pSS | No of healthy volunteers |
|----------------------|--------------|-------------------------|--------------------------|
| Salivary glands | Sweat glands | | |
| + | + | 6 | NA |
| + | - | 4 | NA |
| - | + | 2 | 0 |
| - | - | 0 | 10 |

NA: not applicable. The controls were matched for age, sex and ethnicity to the patients.

and systemic lupus erythematosus, and are currently being tested in primary SS [21,22]. Thereby, the need for an early diagnosis of pSS has become a priority. To this end, criteria for classification of the disease have been defined [23] by the European Community Study Group on diagnostic criteria for SS, and amended [20] by the American-European Consensus Group (AECG). At this time, special emphasis has been placed on laboratory tests. Yet, the question is not settled and reliable diagnostic criteria are needed.

In this respect, concomitant evidence has sparked a great deal of interest in the possibility that B cells play a leading role in the pathogenesis of pSS [24–26]. Among major breakthroughs in this interpretation is the dissection of B-lymphocyte subsets. In particular, the respective membrane expression of IgD and CD38 distributes mature B cells (Bm) into sequential stages from Bm1 through Bm5 cells [27]. Once activated in secondary lymphoid organs, naïve Bm1 (IgD+/CD38-) become Bm2 (IgD+, CD38+), and progress to germinal center (GC) founder Bm2' cells (IgD+/CD38++). There, they evolve to Bm3 centroblasts and Bm4 centrocytes (IgD-/CD38+), which differentiate into either plasma cells, or early (eBm5) and late memory Bm5 (IgD-/CD38+, and IgD-/CD38-, respectively). Inside the GCs, a few cells of each subset escape into the circulation. For unknown reasons, patients with pSS exhibit a high ratio of increased percentages of blood Bm2-plus-Bm2' cells to decreased percentages of eBm5-plus-Bm5 cells in their blood, [28,29] and accumulate these latter memory B cells within their exocrine glands [28,30]. It appeared recently that this ratio, more readily available to the community physician than the LSG biopsy, could provide a new additional criterion for SS [31]. However, an additional asset for the diagnosis of SS in a multicenter prospective study is needed.

In this study, we report, for the first time, that B-cell and T-cell infiltrates in the skin biopsies could, as B cell disturbances in the blood, be considered as new criterion for the diagnosis and warrant inclusion in the routine care of patients suspected of suffering from primary SS. Patients with primary SS are indeed so prone to skin dryness that the low frequency of their cutaneous manifestations becomes inconsistent (reviewed in [9]). Although reserved for patients with dubious oral and ocular tests, the LSG biopsy remains the benchmark diagnostic test for primary SS. It is intriguing that subjects with symptoms suggestive of dry eyes and dry mouth do not display a higher probability of presenting with abnormalities in the ocular and oral tests than those without such complaints [4]. Therefore, xerotic skin may serve as an additional factor for the diagnosis of primary SS. Obviously, this examination gains supplemental importance for the diagnosis in primary SS patients who have repeatedly negative LSG biopsies. There is no doubt that the incidence of xerosis increases in the elderly, but still the diagnosis of primary SS warrants consideration in these individuals.

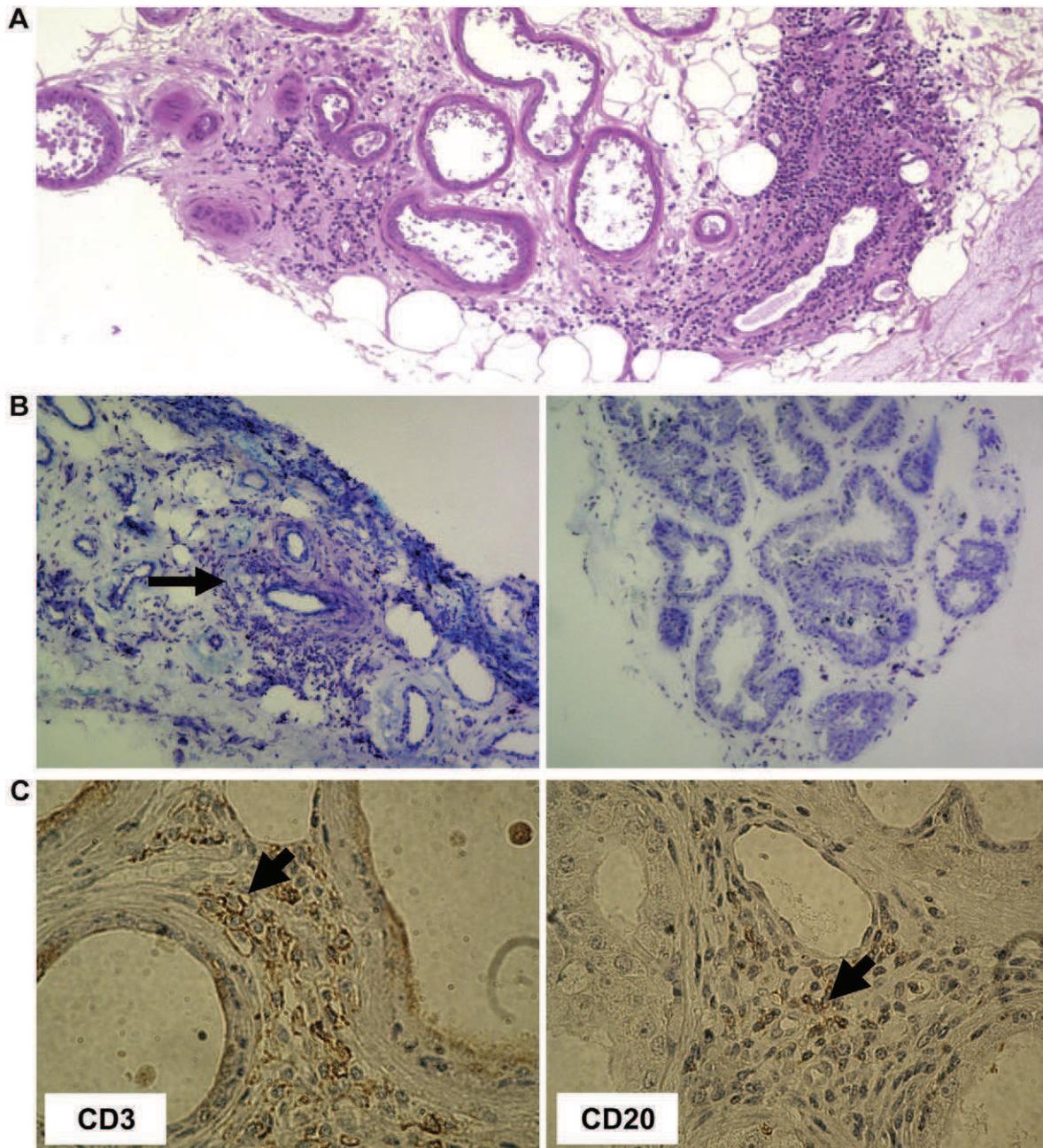


Fig. 1. Pathological changes in the cutaneous tissue of patients with primary Sjögren's syndrome (SS). A. Epidermis infiltrated with mononuclear cells (MNCs) stained with hematoxylin-and-eosin. B. Toluidine blue-staining reveals MNCs (arrow) in the sweat glands of the skin biopsy of a patient with primary SS on the left, but not in those of the skin biopsy from a healthy volunteer on the right (x10). C. Immunohistochemical analysis of a skin specimen from an individual suspected of primary SS shows CD3-positive T lymphocytes on the left, and CD20-positive B lymphocytes on the right (x40).

Several investigators hold strong reservations about the biopsy of LSGs, arguing that this site is non-specific for primary SS, and that some patients may be left with distressing sensory neuropathy in a portion of the chin dermatome. In contrast, the morbidity of skin biopsy is negligible. Additionally, some studies indicate that there is no reduction in cutaneous glandular function, despite the inflammatory response around the eccrine sweat glands [9]. If this is the case, anti-muscarinic autoAbs could underlie the mechanism responsible for the exocrine gland hypofunction [32].

Assuming that homing receptors for the epidermis are specific for T lymphocytes [17], one may consider the migration of B lymphocytes to the skin as unlikely. The simplest explanation for their detection among the cutaneous lymphocytes is that one or

several of the B-lymphocyte chemotactic factors described in the LSGs [33] are shared by all exocrine tissues. Consequently, these include also cutaneous sweat glands [34].

Furthermore, one is struck by our finding that memory B lymphocytes infiltrate not only the LSGs [35], but also the skin. The local production of B cell activating factor of the tumor-necrosis factor family (BAFF) deserves further investigation. Retention of memory B cells in the exocrine tissues [30] accounts, at least in part, for their decrease of the peripheral blood of patients with primary SS. Classically, CD5-expressing B lymphocytes are taught not to generate memory [36], which is in line with the absence of these cells in the cutaneous B cell aggregates. Nonetheless, their relevance is still being questioned.

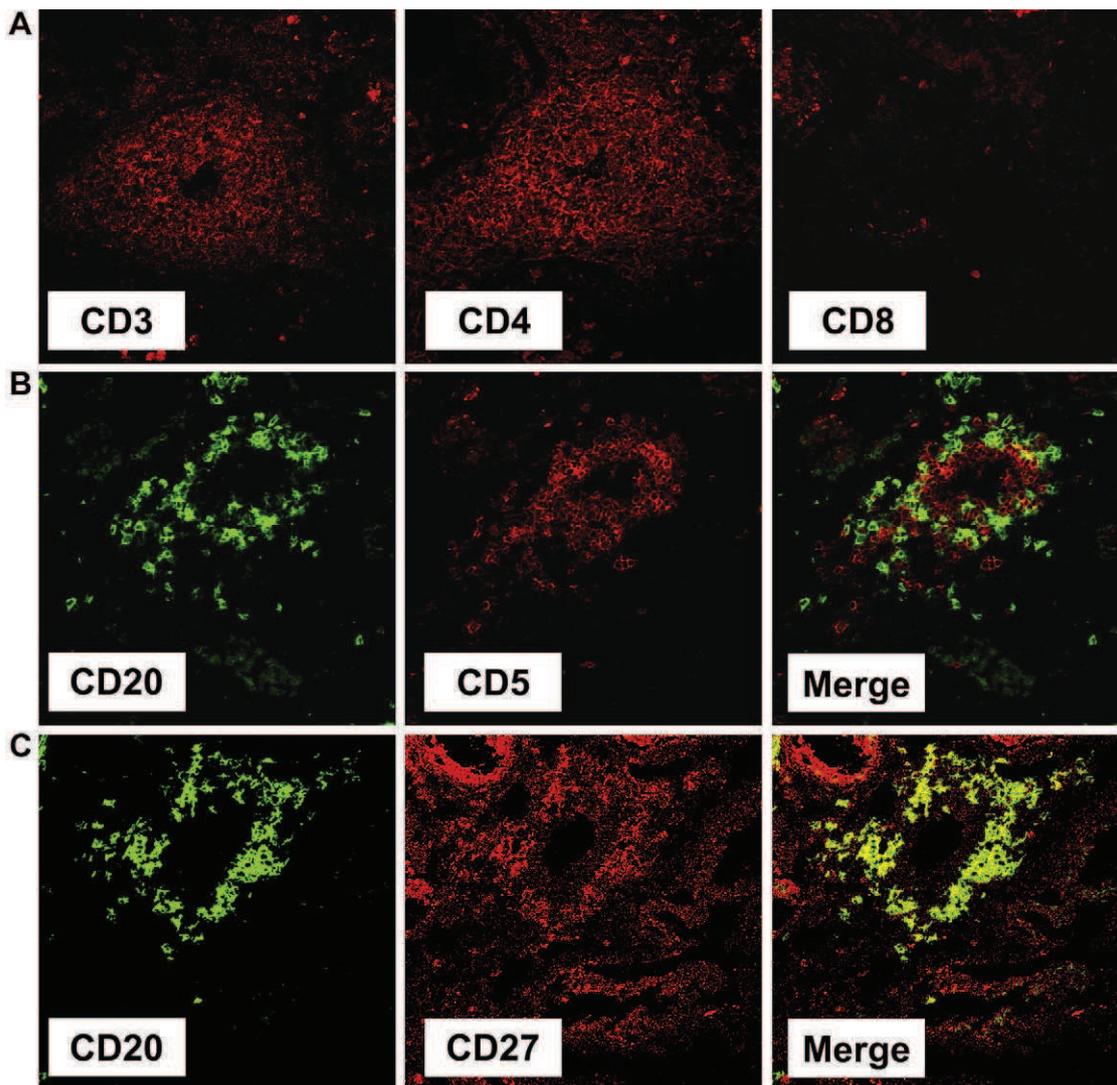


Fig. 2. Phenotype analysis of cutaneous B lymphocytes from patients with primary Sjögren's syndrome. B cells, as defined by the expression of CD20 (left panels) stained green with fluorescein isothiocyanate-conjugated anti-CD20 antibody (Ab). The overlay of this green with tetramethyl rhodamine isothiocyanate-generated red-stain anti-CD5, anti-CD27, anti-IgD and anti-CD24 Abs (is seen yellow). A. B lymphocytes, defined as CD20-positive (left panel), are negative for CD5 (middle panel), as confirmed by the absence of overlay in the left panel (one green arrow points to B cells, and another red to CD5). B. These CD20-positive B cells are positive for CD27. The overlay is seen yellow (arrowed in yellow in the right panel). C. The B cells are negative for IgD. D. Some of them are positive for CD24.

Among memory B cells, some B cells harbor the CD20+/CD24+ profile, which is closer to that of transitional type 2 (T2) B cells than to that of marginal-zone B cells, although marginal-zone-like B cells have also been found in the salivary glands of BAFF-transgenic mice [37]. It is interesting that in this murine model, T2 B cells can differentiate into marginal-zone B cells, given their normal numbers and the expansion of the T2 B cells. This expansion of T2 B cells and marginal-zone B cells in the salivary glands [38] and the skin of patients with primary SS is perfectly in line with antigen-driven clonal B cell proliferation and hypermutation in the exocrine tissue. Transitional B cells are indeed the target of negative selection [39]. Engagement of their antigen receptor may induce those T2 B cells into apoptosis, unless T-cell signals help them escape apoptosis [40]. Inevitably, autoreactive marginal-zone B cells encounter local autoantigen in BAFF-transgenic mice, so that they differentiate into memory B cells. The alternative explanation is that marginal-zone B cells derive from GCs in which hypermutation may be taking place. However, we have not observed ectopic GCs within the cutaneous infiltrates. This might be in line with the fact that, in LSGs, a minority of B-cell clusters represent genuine GC

cells [41]. One possibility exists that BT2 cells found in the LSG and the skin biopsies would thus be at the crossroads of B-cell development into marginal-zone-like B cells forming marginal-zone equivalents or GC-forming B cells. In either case, the fate of B cells during their development is the result of a competition for BAFF, and the overexpression of BAFF in BAFF-transgenic mice or in patients with primary SS augments survival signals to self-reactive T2 B cells, allowing them to resist death signals. Finally, these cells constitute the first B lymphocytes to repopulate the LSG after B-cell depletion [33]. As pinpointed by Dörner et al. [42], they may indeed accumulate in inflamed tissues [41]. The landscape on Sjögren's syndrome has changed dramatically not only with respect to diagnosis but, more importantly, with our general understanding of the genetics, use of animal models and especially the effector mechanisms. We will not review this literature but highlight a number of recent contributions, especially papers from the most recent international Sjögren's meeting held in Brest in Fall 2009 [5–7,43–75].

In practice, the skin of patients suspected of primary SS must be carefully examined, and, one step further, the epidermis warrants to be

biopsied to search for B cell infiltrates. Our group has undertaken confirmation of these peculiarities in larger numbers of patients including disease controls. Furthermore, our findings indicates that T2, marginal-zone-like and memory-type B lymphocytes accumulate in the skin of these patients as previously described in the salivary glands.

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FLT3-L in primary Sjögren's Syndrome

Article 2

The FMS-like tyrosine kinase3-ligand, a mediator for B cell survival is also a marker for lymphoma in primary Sjögren's syndrome.

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The FMS-like tyrosine kinase3-ligand, a mediator for B cell survival is also a marker for lymphoma in primary Sjögren's syndrome.

B-cell biological abnormalities, such as hypergammaglobulinemia, presence of auto-Abs, and abnormal distribution of mature B lymphocytes in peripheral blood are characteristic of pSS. In addition, the most severe complication in pSS patients is the increased risk of NHL, found in up to 5% of cases. Thus, it is important to identify which patients present high risk of lymphoproliferation, determined by predictive markers.

In this second article, prompted by the reports on the effects of FLT3-L on lymphocyte development, lymphoid malignancies, B-lymphocyte alterations, and the risk of developing lymphoma, we evaluated the role of FLT3-L in pSS, to determine if this cytokine might be responsible for the increased numbers of Bm2+Bm2' cell subsets and risk of lymphoma in pSS. In this study, we found that serum levels of FLT3-L were higher in pSS patients compared to controls, and these levels were correlated with abnormal B cell distribution. In addition, FLT3 was mainly expressed by Bm2+Bm2' cells, which are the cell type increased in peripheral blood of patients. B-cell culture experiments showed that this cytokine potentiated the proliferation of B cells in addition to IgM stimulation. In SGs from pSS patients, FLT3 and FLT3-L were expressed by infiltrating B cells and ECs, respectively. Finally, FLT3-L levels were higher in two patients with pSS and NHL, and also associated with high disease activity score (using the ESSDAI score), showing that this cytokine may be implicated in B-cell distribution abnormalities, proliferation of infiltrating B cells in SGs, higher disease activity scores and risk to lymphoproliferation. The results of this article may open the possibility to target FLT3-L in pSS.

The Fms-like Tyrosine Kinase 3 Ligand, a Mediator of B Cell Survival, Is Also a Marker of Lymphoma in Primary Sjögren's Syndrome

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Objective. To determine if the Fms-like tyrosine kinase 3 ligand (Flt-3L), a cytokine implicated in B cell ontogenesis and proliferation in hematologic malignancies, might be responsible for the increased numbers of circulating Bm2 and Bm2' B cell subsets in patients with primary Sjögren's syndrome (SS).

Methods. Serum levels of Flt-3L were measured in 64 patients with primary SS and in 20 healthy controls matched for age and sex. Flt-3L and its receptor Flt-3 were quantified in circulating B cells and in salivary gland (SG) biopsy tissues by immunofluorescence analysis. The effect of Flt-3L on circulating B lymphocytes was then determined by coculture with cells of a human SG (HSG) epithelial cell line.

Results. Serum levels of Flt-3L were increased in patients with primary SS as compared with controls (mean \pm SD 135.8 \pm 5.5 versus 64.4 \pm 4.5 pg/ml; $P < 0.001$). Serum levels of Flt-3L in primary SS patients correlated with the numbers of Bm2 and Bm2' cells ($r = 0.46$, $P < 0.0006$), and Flt-3 was selectively expressed in Bm2 and Bm2' cells. B cell culture experiments showed that Flt-3L potentiated the proliferative effect of anti-IgM stimulation. In SGs, we found that infiltrating B cells expressed Flt-3 and epithelial cells produced Flt-

3L. Finally, Flt-3L levels were associated with high disease activity scores and increased risk of developing lymphoma.

Conclusion. Serum levels of Flt-3L are elevated in patients with primary SS and correlate with abnormal B cell distribution. Flt-3 is mainly expressed by Bm2 and Bm2' cells. Serum levels of Flt-3L might explain the clinical evolution of primary SS to B cell lymphoma that is observed in some patients, thus opening the possibility of new avenues for therapy.

Autoimmune epitheliitis (1), which is also known as Sjögren's syndrome (SS), occurs either alone, as primary SS, or against a background of connective tissue diseases, as secondary SS. Although keratoconjunctivitis sicca and xerostomia, which result from involvement of the lacrimal glands and the salivary glands (SGs), respectively, are usually prominent, SS may also present as a multifaceted condition with a wide variety of clinical manifestations and extraglandular complications (2). This variable presentation accounts for the delay in diagnosis, and coupled with an underestimation of the patient's concerns on the part of the physician, patients become discouraged and seek medical advice only as further symptoms arise (3). As a consequence, there is every likelihood that the prevalence of the disease is far higher than is currently estimated (4).

Biologic abnormalities associated with B lymphocytes in SS are manifested as rheumatoid factor (RF), hypergammaglobulinemia, and anti-SSA and anti-SSB antibodies, together with an increased risk of non-Hodgkin's lymphoma (NHL) (5,6). The development of NHL in SS patients can be predicted by the presence of neutropenia, cryoglobulinemia, splenomegaly, lymphadenopathy, and low levels of C4 (7). Indeed, patients with any of these characteristics have a >5-fold increased risk of developing NHL.

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Although classification criteria for SS have been defined by the American–European Consensus Group (2), the diagnosis in some patients may be difficult to make and may therefore be delayed. We recently demonstrated that the distribution of mature B cells (from Bm1 through Bm5) in the peripheral blood (PB) of patients with primary SS is different from that in patients with other autoimmune diseases and in normal control subjects and that this might provide a useful diagnostic tool (8). This Bm1–Bm5 distribution is based on the relative membrane expression of IgD and CD38 (9). Once activated in secondary lymphoid organs, naive Bm1 cells (IgD+CD38–) become Bm2 cells (IgD+CD38+) and progress to become germinal center founder Bm2' cells (IgD+CD38++). There, they evolve into Bm3 centroblasts and Bm4 centrocytes (IgD–CD38++), which differentiate into either plasma cells or early (eBm5) and late memory Bm5 cells (IgD–CD38+ and IgD–CD38–, respectively). A few cells of each subset escape into the circulation from the germinal centers. For unknown reasons, patients with primary SS have an abnormal distribution of the blood B cell subset and accumulate memory B cells in their exocrine glands (10,11). These findings are not related to serum levels of cytokines of the tumor necrosis factor (TNF) family, such as BAFF or TNF α .

Precursor lymphocytes are dependent on Fms-like tyrosine kinase 3 (Flt-3) for their development. Thus, administration of Flt-3 ligand (Flt-3L) mobilizes hematopoietic cells from the bone marrow into the blood, lymphoid organs, and parenchymal tissues. In addition to its effects on hematopoietic stem cells and progenitor cells, Flt-3L increases the number of dendritic cells in the circulatory system (12–15).

Flt-3L was cloned in 1993–1994 by 2 groups of investigators (16,17). It is a type I transmembrane protein that can be released as a soluble homodimeric protein (12–14). Both the membrane-bound and the cell-free forms can activate Flt-3 and stimulate the growth of progenitor cells in the bone marrow and blood. Flt-3L strongly synergizes with other hematopoietic growth factors and interleukins (18–20). Treatment of mice with Flt-3L results in significant stimulation of hematopoiesis, leading to bone marrow hyperplasia, splenomegaly, and enlargement of the lymph nodes and liver (12–14). In addition, Flt-3L is abnormally increased in the majority of leukemias (21), and Flt-3 mutations are present in 30% of patients with acute myeloid leukemia (22).

Prompted by the reports of the effects of Flt-3L

on lymphocyte development, lymphoid malignancies, B lymphocyte alterations, and the risk of developing lymphoma, we evaluated the role of Flt-3L in SS. First, serum levels of Flt-3L in patients with primary SS were analyzed and compared with those in normal control subjects. Flt-3L levels were then correlated with abnormalities in B lymphocyte distribution. Second, the expression of Flt-3 on B cell subsets in the blood and SGs was determined. Third, the role of Flt-3L in the differentiation of B lymphocytes in primary SS patients was evaluated functionally, and finally, Flt-3L expression was associated with disease activity scores.

PATIENTS AND METHODS

Patients and controls. Serum levels of Flt-3L were determined in 64 patients with primary SS and in 20 healthy controls matched for age and sex. All patients with primary SS fulfilled the criteria amended by the American–European Consensus Group (2). An aliquot of serum was retained for measurement of Flt-3L at the time blood samples were drawn for routine serologic testing for connective tissue diseases in patients attending the Rheumatology Ward at Brest University Medical School Hospital. All patients and normal controls gave their informed consent for study, and the study was approved by the Ethics Committee at Brest University Medical School Hospital.

Enzyme-linked immunosorbent assay (ELISA) for Flt-3 ligand. The levels of Flt-3L were measured with a commercial kit that uses a 2-step sandwich ELISA (R&D Systems). Briefly, a monoclonal antibody (mAb) specific for Flt-3L was precoated onto the wells of a microtiter plate, followed by the addition of 100 μ l of assay diluents with 50 μ l of the standards and samples. After a 2-hour incubation, serum Flt-3L was caught by the immobilized antibody. After washing each well 5 times, 200 μ l of a peroxidase-conjugated anti-Flt-3L antibody was added to form an antigen–antibody complex in each well, and the wells were incubated for 2 hours. After 5 further washes, 200 μ l of substrate was added to the wells and incubated for 30 minutes. The resulting color development was proportional to the amount of Flt-3L in the sample; color intensity was measured at 450 nm. The concentration of Flt-3L was read from a calibration curve based on reference standards. The sensitivity of this assay was \sim 7 pg/ml.

Purification of B lymphocytes. Samples of PB obtained from SS patients and normal controls was layered onto Ficoll-Hypaque and centrifuged for 30 minutes at 450g. Mononuclear cells were incubated with neuraminidase-treated sheep red blood cells for 1 hour at 4°C, and T cells were depleted by a second 25-minute round of centrifugation to achieve a B cell purity of \sim 90%.

All mAb were purchased from Beckman Coulter, except where indicated otherwise. Purified B cell preparations were stained with phycoerythrin (PE)–conjugated anti-CD3 mAb and fluorescein isothiocyanate (FITC)–conjugated anti-CD19 mAb. CD3– and CD19+ B cells were sorted with an Epics Elite fluorescence-activated cell sorter (FACS; Beckman

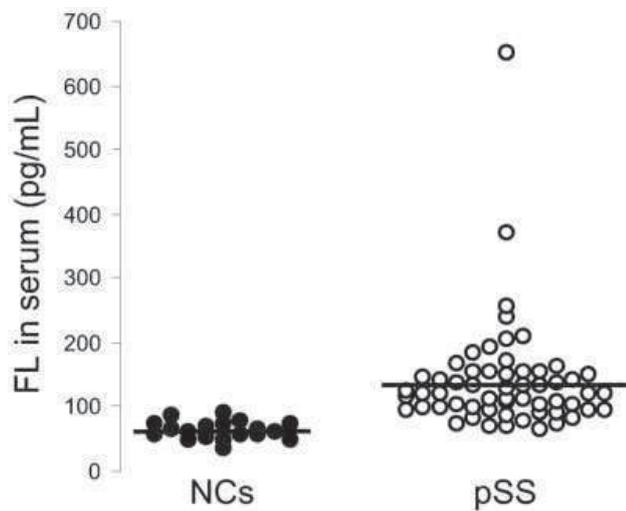


Figure 1. Fms-like tyrosine kinase 3 ligand (Flt-3L) levels in the sera of 64 patients with primary Sjögren's syndrome (pSS) and in 20 normal controls (NCs). Each data point represents a single subject; horizontal lines show the median. The median levels of Flt-3L (FL) were 135.83 pg/ml (range 67.39–650.59) in primary SS patient sera and 64.36 pg/ml (range 33.83–122.87) in normal control sera.

Coulter). All preparations were >98% pure CD19+ cell subset, as determined by FACS.

Cells from a human salivary gland (HSG) epithelial cell line were cocultured with B lymphocytes (23).

Quantitative reverse transcription–polymerase chain reaction (RT-PCR). The relative levels of gene expression of Flt-3, Flt-3L, and GAPDH were quantified by RT-PCR using SYBR Green Master Mix reagent in an ABI Prism 7000 sequence detection system (Applied Biosystems) according to the manufacturer's instructions. The number of threshold cycles (C_t) was counted using the $2^{-\Delta C_t}$ method, with GAPDH transcripts as an internal control. The following primers were used: for Flt-3, 5'-CACGGGAAAGTGGTGAAGAT-3' and 5'-GGAATGCCAGGGTAAGGATT-3'; for Flt-3L, 5'-TTGGGCCCTCTCATTC-3' and 5'-GGGCTTTGTACAGAGTTGGGTAA-3'; and for GAPDH, 5'-CTTAGCACCCCTGGCCAAGG-3' and 5'-CTTACTCCTTGGAGGCCATG-3'. DNA fragments of 228 bp for Flt-3, 63 bp for Flt-3L, and 522 bp for GAPDH were obtained.

Flow cytometric analysis of Flt-3 and Flt-3L expression. PE-Cy7 (PC7)-labeled anti-CD19 mAb (J4:119) was used to tag B cells, and FITC-labeled anti-IgD mAb (IA6-2) plus PC5-labeled anti-CD38 (LS198) was used to identify Bm1–Bm5 subsets. PE-labeled anti-Flt-3 mAb (Santa Cruz Biotechnology) was used to determine the expression of Flt-3 in B cells. To determine the expression of Flt-3L in B cells, fixed/permeabilized cells were analyzed using a Cytostain kit containing Cytofix/Cytoperm Plus (PharMingen). Briefly, 10^6 cells were stained for 30 minutes at 4°C with the fluorochrome labeled anti-CD19, anti-CD38, and anti-IgD antibodies described above. After washing twice with phosphate buffered

saline (PBS), 250 μ l of Cytofix/Cytoperm solution was added for 20 minutes at 4°C. After 2 washes with 1 \times Perm/Wash solution, rabbit anti-Flt-3L antibody (Abcam) was added for 30 minutes at 4°C. After 2 washes, this anti-Flt-3L antibody was revealed with the use of a PE-conjugated anti-rabbit antibody to determine the expression of Flt-3L in B cells.

Immunofluorescence staining of SGs and tonsils. Biopsy specimens of SGs from 6 patients with primary SS were embedded in OCT (Miles), snap-frozen in isopentane, and stored at -80°C until examined. Serial sections measuring 6 μ m in thickness were prepared and mounted onto poly-L-lysine-coated slides. A 2-color staining procedure was performed on 2 serial slides. In the first step, rabbit anti-human CD20 antibody (Interchim) or FITC-conjugated anti-CD20 was used in conjunction with PE-coupled anti-human Flt-3 mAb (Santa Cruz Biotechnology) or rabbit anti-human Flt-3L mAb (Abcam), respectively. After a 40-minute incubation and 3 washes in PBS, the second staining step consisted of incubating the first slide for a further 40 minutes with FITC-conjugated donkey anti-rabbit antibody along with tetramethylrhodamine isothiocyanate (TRITC)-conjugated donkey anti-mouse antibody (both from Jackson ImmunoResearch), whereas the second slide was incubated with TRITC-conjugated donkey anti-rabbit antibody. After 3 washes in PBS, the sections were fixed with 4% cold paraformaldehyde, a coverslip was added (Vector), and the tissue was analyzed using a TCS-NT Leica confocal imaging system (Wetzlar). Neither control rabbit IgG plus FITC-conjugated donkey anti-rabbit antibody nor control mouse IgG plus TRITC-conjugated donkey anti-mouse antibody showed any background fluorescence.

Cell culture and coculture experiments. B lymphocytes isolated from the PB of 6 patients with primary SS and 6 normal controls were cultured in RPMI 1640 medium (Invitrogen Life Technologies) supplemented with 10% heat-inactivated fetal calf serum, 2 mM L-glutamine (Invitrogen Life Technologies), 200 units/ml of penicillin, and 100 μ g/ml of streptomycin. B lymphocytes were seeded at 2×10^5 cells/well and were cultured with or without recombinant Flt-3L (PeproTech) at 100 ng/ml. In some experiments, cells were also stimulated with 10 μ g/ml of anti-IgM antibody-coated beads. To determine the proportion of apoptotic cells in the cell culture, FITC-coupled annexin V and propidium iodide (PI) (Immunotech) from day 5 of culture was classified by FACS, according to the manufacturers' instructions. B cells were labeled with carboxyfluorescein succinimidyl ester (CFSE; 5 μ M) on day 2 to measure cell proliferation.

B lymphocytes from 6 patients with primary SS and 6 normal controls were also analyzed in coculture with cells of the HSG epithelial cell line, and the inhibitory effects of anti-Flt-3L and anti-Flt-3 antibodies were evaluated. HSG cells (5×10^5) were seeded in 96-well plates and were incubated for 8 hours. Supernatants were removed, and B lymphocytes were cultured with HSG cells at a 1:1 ratio. In addition, B lymphocytes were cultured in the presence of the HSG cell supernatant. Anti-Flt-3L and anti-Flt-3 antibodies were added to the culture wells (10 μ g/ml), and the effects were analyzed at 48 and 72 hours. Rabbit and mouse IgG polyclonal antibodies (10 μ g/ml) were used as controls. These

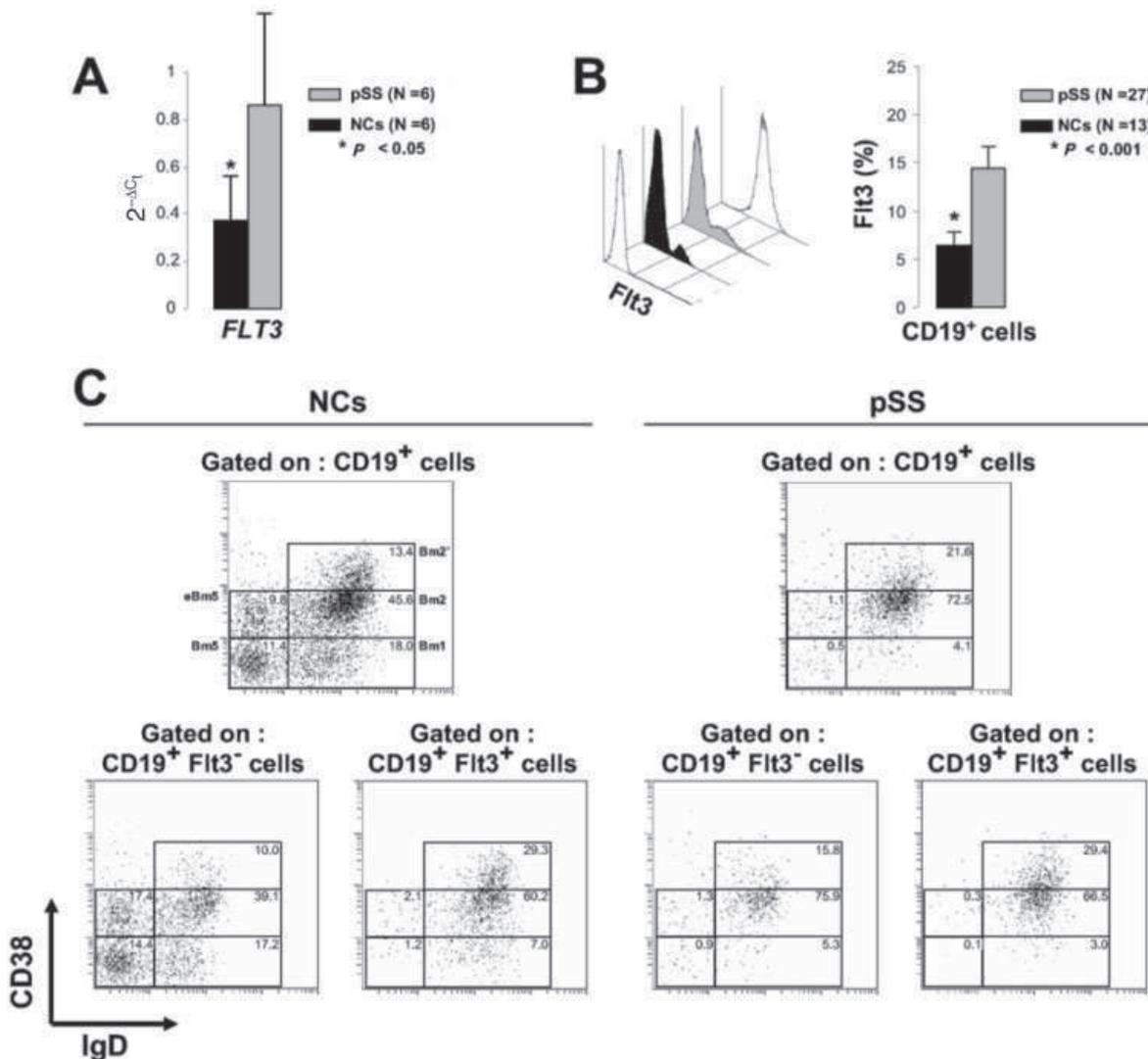


Figure 2. Fms-like tyrosine kinase 3 (Flt-3) expression in B cells from the peripheral blood (PB) of patients with primary Sjögren's syndrome (pSS) and normal controls (NCs). **A**, Levels of mRNA for Flt-3 were determined by quantitative reverse transcription-polymerase chain reaction in B cells from 6 primary SS patients and 6 normal controls and were normalized relative to GAPDH. Values are the mean and SD. **B**, Representative fluorescence-activated cell sorter profiles of Flt-3 expression in PB CD19⁺ B cells from normal controls (solid histogram) and primary SS patients (shaded histogram), and in CD34⁺ B cells from control bone marrow (open histogram) are shown at the left. A representative isotype control histogram profile (broken line) is also presented for comparison. CD19⁺ cells in 13 normal controls and 27 primary SS patients (mean and SD) are shown at the right. **C**, Distribution of CD19⁺ B cells in primary SS patients and normal controls is shown in the top 2 histograms, with the typical abnormal distribution of B cells (increase in Bm2 and Bm2' cells, but not early Bm5 [eBm5] or Bm5 cells) in primary SS patients and the typical normal distribution of B cells in normal controls. The 4 histograms at the bottom represent the gating of CD19⁺Flt3⁻ and CD19⁺Flt3⁺ B cell compartments and their analysis according to IgD and CD38 expression in primary SS patients and controls. Numbers in each compartment represent the percentages of cells.

cells were also stained with FITC-conjugated annexin V and PI and analyzed by FACS at 36 hours.

Assessment of disease activity. Disease activity was evaluated in 34 primary SS patients according to the EULAR

SS Disease Activity Index (ESSDAI) (24). This recently proposed index takes into account 12 organ-specific domains that contribute to disease activity (constitutional, lymphadenopathy, glandular, articular, cutaneous, pulmonary, renal, mus-

cular, peripheral and central nervous system, hematologic, and biologic domains). Features of disease activity in each domain were classified into 3 or 4 levels according to their severity (0 = no activity, 1 = low activity, 2 = moderate activity, and 3 = high activity). Domain weights ranged from 1 to 6. The score for each domain is the product of the weight of the domain multiplied by the level of activity (e.g., the weight of the articular domain is 2 and that of the renal domain is 5). The total score is the sum of the scores for all domains. The activity index was evaluated retrospectively based in the clinical charts at time of blood sampling for the measurement of Flt-3L.

Statistical analysis. The results are expressed as the mean \pm SD. Comparisons between means were performed by the Mann-Whitney U test. Differences between groups were calculated by Fisher's exact test. Correlations were evaluated with Pearson's correlation test.

RESULTS

Expression of Flt-3 mainly in Bm2 and Bm2' B cell subsets. Serum levels of Flt-3L were significantly higher in the 64 primary SS patients as compared with the 20 normal control subjects (mean \pm SD 135.8 \pm 5.5 pg/ml versus 64.4 \pm 4.5 pg/ml; $P < 0.001$) (Figure 1).

Although Flt-3 has been shown to be expressed predominantly on primitive hematopoietic progenitors (CD34+ cells) and dendritic cell precursors, it has not previously been described in mature B cells. We therefore examined B cells derived from the PB of patients with primary SS for Flt-3 using a quantitative RT-PCR analysis. Flt-3 transcripts were expressed in B cells from patients with primary SS, but fewer were detected in B cells from the controls (mean \pm SD 0.87 \pm 0.41 versus 0.37 \pm 0.19, $P < 0.05$) (Figure 2A). In addition, FACS analysis showed that significantly more B cells from primary SS patients than from healthy controls expressed Flt-3 (mean \pm SD 14.5 \pm 4.2 versus 6.5 \pm 2.5%; $P < 0.001$) (Figure 2B). Finally, we found that Flt-3 expression was mainly expressed by some, but not all, Bm2 and Bm2' lymphocytes (Figure 2C).

Flt-3L induction of B cell proliferation in association with anti-IgM stimulation. B lymphocytes from the PB of patients with primary SS were incubated in the presence or absence of Flt-3L, which itself alone, does not protect the cells from spontaneous apoptosis or alter the percentages of Flt-3 expression in B cells. However, in the presence of anti-IgM antibody-coated beads, Flt-3L enhanced the survival effect of anti-IgM stimulation measured on day 5 ($P < 0.05$) (Figure 3A). This effect was not observed with B cells from normal control subjects.

B cell proliferation evaluated by CFSE staining on day 2 showed higher percentages of dividing cells in

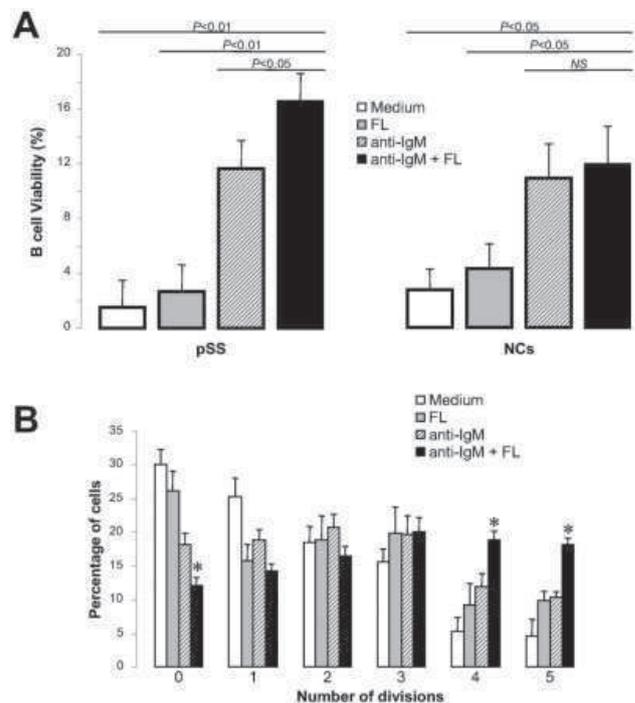


Figure 3. In vitro effects of Fms-like tyrosine kinase 3 ligand (Flt-3L) in cells from patients with primary Sjögren's syndrome (pSS) and from normal controls (NCs). **A**, B cells from the peripheral blood (PB) of 6 patients with primary SS and 6 normal controls were purified, and the effects of Flt-3L were determined. B cells were cultured for 5 days in medium alone or in the presence of 100 ng/ml of recombinant Flt-3L (FL). In some experiments, cells were also stimulated with 10 μ g/ml of anti-IgM antibody-coated beads in the presence or absence of recombinant Flt-3L. Survival of B cells was defined as annexin V and propidium iodide negativity. Values are the mean and SD of 6 different experiments. NS = not significant. **B**, For measurement of cell proliferation, B cells were labeled with carboxyfluorescein succinimidyl ester (CFSE; 5 μ M) and cultured in medium, Flt-3L (100 ng/ml), anti-IgM antibody-coated beads (10 μ g/ml), or Flt-3L plus anti-IgM antibody-coated beads. Proliferation was defined as a reduction in the intensity of CFSE fluorescence on day 2, as determined by fluorescence-activated cell sorting. Data are presented as the percentage of cells in each number of divisions, from 0 to 5 divisions. Values are the mean and SD of 6 different experiments. * = $P < 0.05$ versus medium alone.

the fourth and the fifth divisions in cultures stimulated with Flt-3L plus anti-IgM ($P < 0.05$) (Figure 3B). In addition, there was a reduced percentage of nondividing cells in cultures stimulated with Flt-3L plus anti-IgM ($P < 0.05$) (Figure 3B).

Expression of Flt-3L by B cells. In contrast to the restricted distribution of Flt-3, Flt-3L messenger RNA is ubiquitously expressed in hematopoietic and nonhematopoietic tissues. By FACS analysis, after membrane

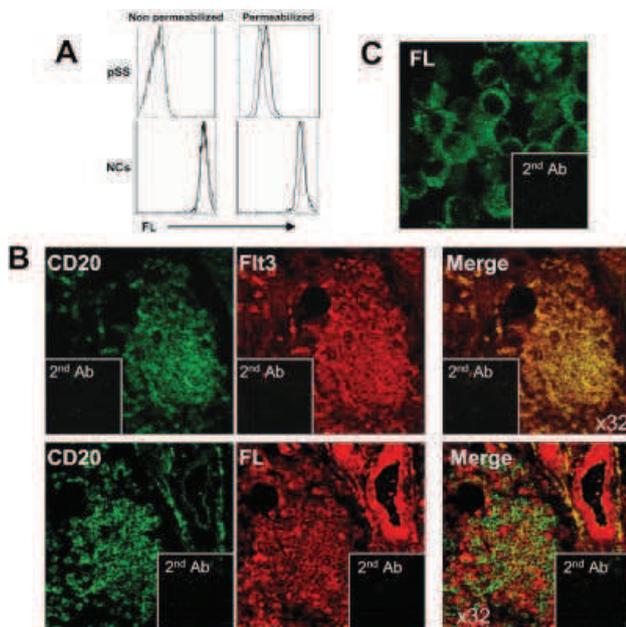


Figure 4. Fms-like tyrosine kinase 3 (Flt-3) and Flt-3 ligand (Flt-3L) expression in salivary glands (SGs) from patients with primary Sjögren's syndrome (pSS). **A**, Flt-3L (FL) was detected in peripheral blood from patients with primary SS and normal controls (NCs). Nonpermeabilized (left) or permeabilized (right) B cells from patients and controls were stained with fluorescein isothiocyanate (FITC)-conjugated anti-Flt-3L mouse monoclonal antibody (solid line) or with FITC-conjugated IgG1 isotype control (broken line). **B**, SG biopsy sections were stained with rabbit anti-human CD20 antibody and revealed with FITC-conjugated donkey anti-rabbit monoclonal antibody (mAb) or were stained with FITC-conjugated anti-CD20 mAb. CD20 staining was associated with anti-Flt-3 mAb, which was revealed with tetramethylrhodamine isothiocyanate (TRITC)-conjugated donkey anti-mouse antibody, or (in the bottom panel) was associated with rabbit anti-Flt-3L antibody, which was revealed with TRITC-conjugated donkey anti-rabbit antibody. The overlay of green-stained B cells with red-stained Flt-3 or Flt-3L cells is seen as yellow. Images are representative of SG biopsy tissues from 6 patients with primary SS. **C**, Flt-3L was detected in the cytoplasm of cells from a human SG epithelial cell line. **Insets** in **B** and **C** show staining with the secondary antibody (2nd Ab) but without primary reagents. (Original magnification $\times 32$.)

permeabilization, the presence of Flt-3L was observed in $32.7 \pm 5.2\%$ (mean \pm SD) of B cells from the PB of patients with primary SS and in $29.0 \pm 9.1\%$ of PB B cells from normal controls (Figure 4A). Flt-3L was also shown to be present in Bm2 and Bm2' cells. These results suggest that Flt-3L, which is secreted by PB B cells, could have an autocrine effect.

Expression of Flt-3 and Flt-3L by salivary gland B cells. Flt-3 expression was found in infiltrating B cells in SGs from primary SS patients (Figure 4B). In addition,

with permeabilization, Flt-3L was detected in epithelial cells from the SG biopsy tissues as well as in cells of the HSG epithelial cell line (Figure 4C). Control rabbit IgG plus FITC-conjugated donkey anti-rabbit antibody and control mouse IgG plus TRITC-conjugated donkey anti-mouse antibody showed no background fluorescence.

Abrogation of the survival of B cells cocultured with HSG cells by treatment with anti-Flt-3L or anti-Flt-3 antibodies. Next, the role of Flt-3L+ epithelial cells on Flt-3+ infiltrating B cells was evaluated. B cells from primary SS patients and normal controls were isolated, and each of the resulting suspensions was distributed into 11 aliquots. The first aliquot was cultured alone, the second with HSG cells, the third and the fifth in the same manner as the second, but with $10 \mu\text{g/ml}$ of anti-Flt-3L antibody or with $10 \mu\text{g/ml}$ of anti-Flt-3 mAb, respectively, and the fourth and the sixth were cultured with the isotype controls of these 2 antibodies (Figure 5). The same experiments were performed with aliquots 7–11 in culture with addition of supernatant from cultured HSG cells. After incubation for 48 hours, the percentages of annexin V-/PI-cells were determined.

After 48 hours, the percentages of viable B cells from primary SS patients were found to be increased in cultures in which HSG cells were added as compared with cultures in medium alone (mean \pm SD $48.1 \pm 8.7\%$ live cells versus $18.5 \pm 4.1\%$ in medium alone; $P < 0.05$). This increase was also significant when HSG cell culture supernatant was added ($43.1 \pm 3.8\%$; $P < 0.05$ versus medium alone), suggesting that cell-free Flt-3L was sufficient for increased survival of B cells. This effect was abrogated by anti-Flt-3L antibody and by anti-Flt-3 mAb (7.8 ± 2.5 and $8.1 \pm 3.9\%$, respectively; $P < 0.05$) in the presence of HSG cells or in the presence of HSG cell supernatant ($7.4 \pm 3.3\%$ and $8.1 \pm 3.0\%$, respectively; $P < 0.05$). Control rabbit anti-IgG and mouse anti-IgG antibodies did not alter the B cell viability.

This survival effect, although less pronounced, was also observed with normal control B cells in the presence of HSG cells or supernatant from HSG cells (Figure 5). This could be explained by a more limited number of Flt-3 molecules on the B cells of normal controls (Figure 2B).

Collectively, these results showed that Flt-3L produced by B cells favor survival in an autocrine manner and that SG epithelial cells could also help the survival of adjacent B cells by virtue of cell-free Flt-3L and Flt-3 interactions in B lymphocyte-infiltrating SGs.

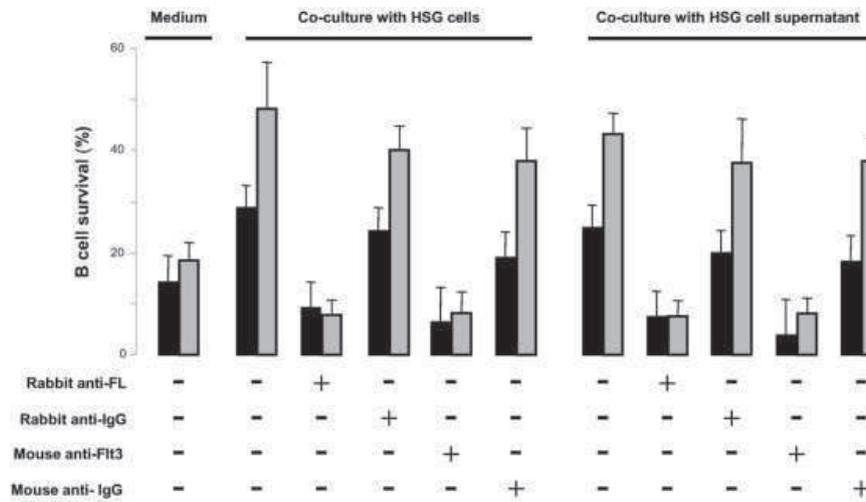


Figure 5. Effect of Fms-like tyrosine kinase 3 (Flt-3) and Flt-3 ligand (Flt-3L) from a human salivary gland (HSG) epithelial cell line on B lymphocytes from patients with primary Sjögren's syndrome (SS). Peripheral blood B cells from 6 normal controls (solid bars) and 6 patients with primary SS (shaded bars) were cocultured with HSG cells. B cells were cultured in medium or were cocultured with HSG cells or with supernatant from cultured HSG cells plus HSG cells. Anti-Flt-3L (anti-FL), anti-Flt-3, or isotype control (anti-IgG) antibodies were added to the culture. Survival of B cells was defined as annexin V and propidium iodide negativity. Values are the mean and SD of 6 different experiments.

Relationships between Flt-3L and serologic and clinical parameters. We have recently shown that the percentages of the Bm2 and Bm2' B cell subsets are increased and the eBm5 and Bm5 cell subsets are decreased in the blood of patients with primary SS (8). In the present study, we further showed that serum levels of Flt-3L correlated positively with the elevated ratio of Bm2 plus Bm2' cells to eBm5 plus Bm5 cells in 54 patients with primary SS ($r = 0.46, P < 0.0006$) (Figure 6A). The higher expression of Flt-3L in the serum of patients with primary SS was associated with an increase in the percentages of Bm2 and Bm2' B cells and, consequently, with an alteration of B cell homeostasis.

Relationships between the ESSDAI score and serum levels of Flt-3L were evaluated in 34 primary SS patients (Figure 6B). The higher the levels of Flt-3L, the higher the ESSDAI score ($r = 0.77, P < 0.0001$). This correlation held true when the 2 patients with lymphoma were excluded from analysis ($r = 0.41, P < 0.01$). Consequently, patients who had an ESSDAI score of ≥ 12 had elevated levels of Flt-3L as compared with those who had an ESSDAI score of < 12 (mean \pm SD 241.5 ± 41.6 versus 140.0 ± 5.1 pg/ml; $P < 0.03$).

The study patients were then divided into 2 groups according to an Flt-3L level higher or lower than

160 pg/ml, which is 4 SD above the mean in normal controls. Patients who had higher Flt-3L levels had higher ESSDAI scores as compared with the patients who had lower levels (mean \pm SD 12.2 ± 2.7 versus 4.7 ± 0.7 ; $P < 0.01$). Patients with the higher Flt-3L levels also had higher levels of anti-SSA or anti-SSB antibodies ($P < 0.03$), but there were no differences in the levels of RF, serum IgM, IgG, or IgA, or β_2 -microglobulin.

Interestingly, the 2 patients who presented with lymphoma during the study had high serum levels of Flt-3L (650.0 pg/ml and 182.4 pg/ml, respectively). As a matter of fact, the highest level of Flt-3L in our cohort was seen in association with lymphoma. However, patients with at least 1 clinical or biologic manifestation associated with the risk of developing lymphoma in association with the primary SS (i.e., purpura, low C4 levels, enlargement of the parotid glands, monoclonal proteins, cryoglobulinemia) did not have significantly increased levels of Flt-3L.

DISCUSSION

Although the pathogenesis of primary SS remains unclear, the disease has traditionally been ascribed to T cells. Recent evidence, however, indicates a major con-

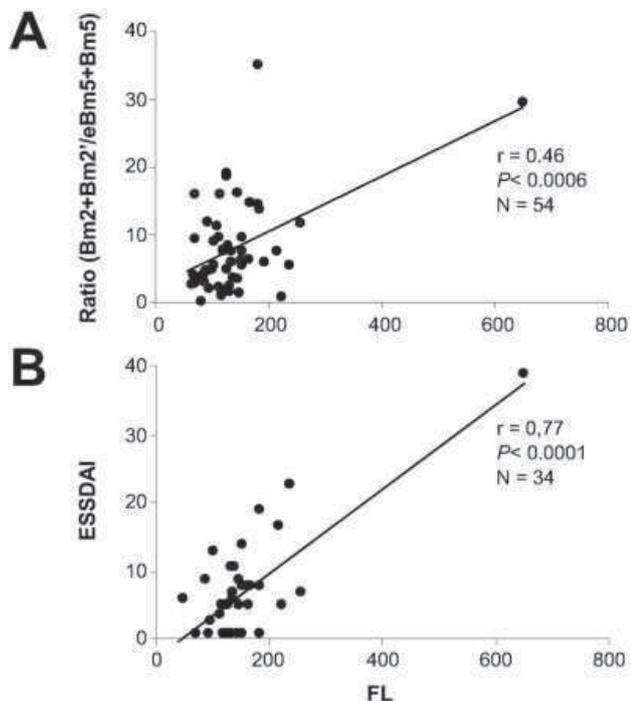


Figure 6. Correlation of serum levels of Fms-like tyrosine kinase 3 ligand (Flt-3L) with abnormal B cell distribution and with the EULAR Sjögren's syndrome Disease Activity Index (ESSDAI) scores in primary Sjögren's syndrome (SS) patients. **A**, Levels of Flt-3L (FL) were compared with the ratio of Bm2 plus Bm2' cells to early Bm5 (eBm5) plus Bm5 cells. The distribution of mature B lymphocytes was classified according to the relative membrane expression of IgD and CD38. There was a positive correlation in the 54 primary SS patients evaluated. **B**, Levels of Flt-3L were compared with the ESSDAI scores in 34 patients for whom all clinical data were available. There was a positive correlation in this group of patients. The higher the levels of Flt-3L, the higher the ESSDAI scores.

tribution by B cells (10,25,26). Moreover, the distribution of mature B cell subsets from Bm1 through Bm5 in the PB of patients with primary SS differs from that in patients with other autoimmune diseases as well as in controls, being characterized by higher percentages of Bm2 and Bm2' cells (8). Thus, B cells are involved in the development of primary SS and therefore hold promise as therapeutic targets. Since Flt-3L and Flt-3 play a role in normal B lymphocyte development and since these cells are altered in hematologic malignancies, we tested this cytokine and its receptor in patients with primary SS.

Levels of Flt-3L have been shown to be increased in patients with hematologic malignancies (21,27), and mutations of Flt-3 have been demonstrated in 30% of patients with acute myeloid leukemia (22). In contrast,

studies of Flt-3L and its receptor in autoimmune diseases are scarce. Our first results from the present study showed that serum Flt-3L is significantly higher in primary SS patients than in normal controls. High synovial membrane levels have recently been found in rheumatoid arthritis (RA) patients, with synovial fluid levels of Flt-3L higher than those in osteoarthritis patients (28). However, serum Flt-3L levels were similar in RA patients and normal controls. In addition, intra-articular administration of a B cell line that had been transfected with the Flt-3L gene resulted in highly erosive arthritis in a mouse model. This highlighted the effect of Flt-3L in RA.

This study is the first to show that Flt-3L levels are increased in patients with primary SS. Even more interesting, these higher levels of Flt-3L correlated with the abnormalities of B cell subset distribution. Our hypothesis was that Bm2 and Bm2' subsets, which are abnormally increased in the PB of primary SS patients, expressed Flt-3. In B lymphocytes, Flt-3 expression has been described in prepro-B cells and pro-B cells, but not in mature B cells (29). The present study confirmed by several techniques that Flt-3 is selectively expressed in some Bm2 and Bm2' subsets. In contrast, tonsillar germinal center B lymphocytes (Bm3/Bm4) did not express Flt-3 (Tobón GJ, et al: unpublished observations). Flt-3 expression, even weak expression, may be implicated in B lymphocyte survival in primary SS.

We have also observed that Flt-3L alone has no effect on B cell proliferation but may potentiate the proliferation effect of anti-IgM stimulation. This result is not surprising, since Flt-3L alone has no effect on normal hematopoiesis (18–20). In primary SS, this synergy may be due to certain cytokines, such as BAFF, which is also altered in this disease (11). In addition, we have found that mature B cells also express Flt-3L, thus suggesting the possibility of an autocrine effect.

SG biopsy documented that infiltrating B lymphocytes also expressed Flt-3, whereas epithelial cells produced Flt-3L, suggesting that both molecules are produced in situ. Flt-3L from epithelial cells would therefore be implicated in the survival of B lymphocytes in the local infiltrates. To confirm this interaction, we developed a coculture system, and we found that anti-Flt-3L and anti-Flt-3 antibodies blocked B cell proliferation. These results showed indirectly that B cell survival in SGs is associated with Flt-3L. Since stromal cells are the major producers of Flt-3L in the bone marrow, further analysis needs to be performed to determine the levels of Flt-3L in bone marrow from primary SS patients. The antiproliferative effects of anti-Flt-3L and

anti-Flt-3 antibodies suggest a possible rationale for therapy in primary SS.

Several Flt-3 inhibitors have been developed for the treatment of hematologic malignancies (30), but these drugs target the effects of the mutated Flt-3. Clinical trials are ongoing to improve their clinical efficacy. Previous studies by other investigators have shown that targeting Flt-3 may be effective in the treatment of autoimmune diseases, in this case, experimental autoimmune encephalomyelitis (31). Here, they showed that clinical improvement was due to the inhibitory effect in dendritic cells. These cells express Flt-3, and the effect of Flt-3L is extremely important in the differentiation of pre-dendritic cells to mature into dendritic cells (15,32).

A key characteristic of primary SS is the high risk of developing B cell lymphomas (6). Several markers have been proposed for the determination of this risk in a study population. These include immunoglobulin free light chains (33), low complement levels (34), and serum levels of β_2 -microglobulin (35). From our results, serum levels of Flt-3L would be predictive of the risk of lymphoma in primary SS. However, a prospective study concerning this issue needs to be undertaken.

In conclusion, Flt-3L levels were found to be elevated in patients with primary SS and were correlated with an abnormal B cell profile in the PB. Flt-3 is expressed by Bm2 and Bm2' cells (subsets of B cells altered during the disease). The role of Flt-3L in B cell proliferation may explain the clinical evolution to B cell lymphoma in some patients, and targeting Flt-3L may open a new chapter in primary SS patient therapy.

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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 published. Dr. Youinou had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study conception and design. Tobón, Pers.

Acquisition of data. Tobón, Renaudineau, Cornec, Pers.

Analysis and interpretation of data. Renaudineau, Hillion, Devauchelle-Pensec, Youinou, Pers.

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Article 3

The FMS-like tyrosine kinase3-ligand is an important immunological marker of lymphoma in primary Sjögren's syndrome.

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The FMS-like tyrosine kinase3-ligand (FLT3-L) is an important immunological marker of lymphoma in primary Sjögren's syndrome.

The development of NHL in pSS can be predicted by the presence of persistent enlargement of parotid glands, lymphadenopathy, splenomegaly, palpable purpura, cryoglobulinemia, low levels of C4, neutropenia and lymphopenia. In the previous article, we have shown that FLT3-L levels were associated with B-cell abnormal distribution in peripheral blood and also that these levels were high in two patients with pSS and NHL. **In this third article**, our objective was to confirm whether FLT3-L might be associated with history of lymphoma in pSS in a large cohort of pSS patients. In this article, history of lymphoma was significantly associated with higher levels of FLT3-L. In addition, risk factors for lymphoma development such as purpura, low C4, lymphocytopenia, low IgM and high levels of β 2-microglobulin were associated with higher levels of FLT3-L whereas enlargement of parotid glands, lymphadenopathy and splenomegaly were not. In conclusion, FLT3-L was correlated with history of lymphoma in pSS and constitutes the best biological marker associated with previous lymphoma in pSS. The role of Flt3-L in B-cell proliferation may explain the clinical evolution to lymphoma in pSS and the predictive value of high levels of FLT3-L will be studied in the prospective analysis of this cohort.

The FMS-like tyrosine kinase 3-ligand is an important biological marker of lymphoma in primary Sjögren's syndrome

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3 **The FMS-like tyrosine kinase 3-ligand is an important biological marker of**
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48 **Key words:** primary Sjögren's Syndrome, autoimmune epithelitis, lymphoma, Flt3-
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Abstract

Objective: Patients with primary Sjögren's Syndrome (pSS) are at greater risk of developing lymphoma. We have shown that the FMS-like tyrosine kinase 3-ligand (Flt3-L), a cytokine implicated in B-cell ontogenesis and malignant proliferation, is associated with B-cell abnormal distribution in pSS. In this study, we wanted to determine whether Flt3-L might be associated with history of lymphoma in pSS.

Methods: Serum levels of Flt3-L were measured in 369 patients enrolled in the French pSS cohort. We analyzed association between increased level of Flt3-L and: 1- lymphoma history; 2- previous criteria related to a high risk of lymphoma; 3- disease activity.

Results: History of lymphoma was significantly associated with higher levels of Flt3-L ($p = 0.0001$). ROC analysis showed that 175 pg/mL was ideal cut-off to detect the association with lymphoma (sensitivity: 44%, specificity: 97.5%). Markers for lymphoma development such as purpura, low C4, lymphocytopenia, low IgM and high levels of β 2-microglobulin were associated with higher levels of Flt3-L whereas enlargement of parotid glands, lymphadenopathy and splenomegaly were not. The sex ratio and subjective symptoms were not associated with levels of Flt3-L. In contrast, the disease activity score was significantly associated with elevated levels of Flt3-L.

Conclusion: Flt3-L is correlated with history of lymphoma in pSS, and constitutes the best biological marker associated with previous lymphoma. The role of Flt3-L in B-cell proliferation may explain the clinical evolution to lymphoma in pSS. The predictive value of high levels of Flt3-L will be studied in the prospective analysis of this cohort.

INTRODUCTION

Autoimmune epithelitis which is also known as Sjögren's Syndrome (SS), occurs either alone, as primary SS (pSS), or associated with other autoimmune diseases, as secondary SS. SS predominantly affects females during the fourth and fifth decades of life.[1] The spectrum of SS extends from an organ-specific autoimmune disorder to a systemic process that may involve the musculoskeletal system, lungs, kidneys and blood vessels.[2] Biological abnormalities associated with B lymphocytes in SS are also a hallmark of the disease and these abnormalities are characterized by the presence of rheumatoid factor (RF), hypergammaglobulinemia, anti-SSA and anti-SSB antibodies, abnormal distribution of mature B lymphocytes in peripheral blood, in addition to an increased risk of non-Hodgkin's lymphoma in 5% of patients (NHL).[3-5] Several clinical and serological markers have been reported to predict the development of NHL in pSS. Among these parameters, patients with pSS and splenomegaly, persistent enlargement of parotid glands, lymphadenopathy,[6-8] palpable purpura,[9] cryoglobulinemia,[10] low levels of C4,[11], neutropenia,[12] or lymphocytopenia [13] have more than 5-fold increased risk of NHL compared to patients without risk factors.

Recently, we have demonstrated that serum levels of the cytokine called Fms-like tyrosine kinase 3 Ligand (Flt3-Ligand), a cytokine implicated in B cell ontogenesis and proliferation in hematological malignancies, were elevated in 64 pSS patients, compared to controls.[14]. These higher levels were correlated with the abnormal B cell distribution in peripheral blood (naïve/memory B cells ratio ≥ 5),[15] and activity of the disease evaluated by the EULAR SS Disease Activity Index (ESSDAI). Additionally, two patients who presented lymphoma during the study had high serum levels of Flt3-L.

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3 Thus, in order to confirm that Flt3-L levels may be a marker of lympho-proliferation in
4 patients with pSS, we evaluated serum levels of Flt3-L in 369 patients enrolled in the
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8 “Assessment of Systemic Signs and Evolution of Sjögren’s Syndrome” (ASSESS)
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10 prospective cohort.
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PATIENTS AND CONTROLS

Patients and controls. Serum levels of Flt3-L were determined in 369 patients of the French cohort of pSS patients (ASSESS) and in 50 healthy controls. All patients fulfilled the criteria amended by the American-European Consensus Group.[16] The ASSESS cohort is a national wide cohort created in 2006 to evaluate the systemic complications and the development of NHL in pSS. Fifteen centers are recruiting patients (Gottenberg JE et al, Submitted). All patients and controls gave their informed written consent for the study, and the study was approved by the Ethics Committee of Hôpital Bichat and the “Commission Nationale Informatique et Libertés” in 2006. An aliquot of serum was frozen at the inclusion visit for measurement of Flt3-L.

Enzyme-linked immunosorbent assay (ELISA) for Flt3-Ligand. The Flt3-L levels were measured with a commercial kit that uses a 2-step sandwich ELISA (R & D Systems, Minneapolis, United States). Briefly, a monoclonal antibody (mAb) specific for Flt3L was precoated onto the wells of a microtiter plate, followed by the addition of 100 μ L of assay diluents with 50 μ l of the standards and samples. After a 2-hour incubation, serum Flt3-L was caught by the immobilized antibody. After washing each well 5 times, 200 μ l of a peroxidase-conjugated anti-Flt3-L antibody was added to form an antigen-antibody complex in each well, and incubated for 2 hours. After 5 further washes, 200 μ l of substrate was added to the wells and incubated for 30 minutes. The resulting color development was proportional to the amount of Flt3-L in the sample; color intensity was measured at 450 nm. The concentration of Flt3-L was read from a calibration curve based on reference standards. The sensitivity of this assay was \sim 7

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3 pg/mL. FL superior to 120 pg/mL was considered as positive (FL+) i.e. 4 SD above the
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6 mean of 50 controls.
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10 **Clinical and serological variables.** The association between Flt3-L positivity (FL+)
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12 and: 1- previous occurrence of lymphoma; 2- criteria linked to a high risk of lymphoma
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14 such as purpura, low levels of C4, enlargement of the parotid glands, cryoglobulinemia;
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17 3- subjective patients' symptoms according to the Eular SS Patient Reported Index
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19 (ESSPRI) [17] and disease activity evaluated by the Eular SS Disease Activity Index
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21 (ESSDAI) were analyzed.[18] Flt3-L determination and all the clinical and serological
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23 characteristics of patients accumulated were made at the inclusion visit and collected
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25 from the clinical charts, using a predetermined protocol form. Additionally, new
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27 clinical and/or serological characteristics were prospectively registered in the form.
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29 Special emphasis was made in lymphoma related risk factors and clinical characteristics
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39 **Statistical analysis.** Sensitivity and specificity for Flt3-L to be associated with
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41 lymphoma were calculated at different cut-off values. To compare data between patients
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43 with FL+ and FL-, we used the chi-square or Fisher exact test for qualitative variables
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45 and the Mann-Whitney test for quantitative variables (Statistical Package for the Social
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47 Sciences). Logistic regression was used to identify independent markers of lymphoma
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49 in pSS patients.
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RESULTS

Flt3-L Levels are increased in pSS patients

Among the 369 patients, 18 had previous lymphoma 7.4 ± 3.38 years before the inclusion in the cohort. Fifteen patients were in complete remission without treatment for 7 ± 2.2 years before the inclusion in the cohort and three patients were still on treatment.

Serum levels of Flt3-L were significantly higher in the 369 pSS patients compared to 50 healthy controls (mean \pm SD: 107.1 ± 30.0 pg/mL vs 64.4 ± 14.5 pg/mL; $p < 0.01$). 103 patients (28%) were considered as positive (FL+) with Flt3-L ≥ 120 pg/mL that corresponds to 4 SD above the mean of the controls. Although no sex ratio association was observed, FL+ patients were older compared to FL- patients ($p < 0.0001$) (mean \pm SD: 62.2 ± 11.3 years vs. 56.2 ± 12.1 years), and had a longer disease duration (Table 1). In healthy controls (mean \pm SD: 55.3 ± 10.4 years) Flt3-L levels were not correlated to age ($p=0.37$). Subjective symptoms of dryness, pain or fatigue evaluated by the ESSPRI questionnaire were not statistically associated with Flt3-L levels. In contrast, ESSDAI was significantly associated with elevated levels of Flt3-L ($p = 0.03$). This correlation persisted if we excluded the 3 patients with active lymphoma at evaluation ($p = 0.05$). Finally, Flt3-L levels were not associated with the presence of RF, anti-SSA or anti-SSB antibodies (Table 1).

Table 1. Main demographic and clinical characteristics of 369 primary Sjögren's syndrome according to Flt3-L levels (Flt3-L levels superior to 120 pg/mL were considered as FL+).

| | <i>FL+</i> (<i>n</i> =103) | <i>FL-</i> (<i>n</i> = 266) | <i>p</i> |
|--------------------------------------|-----------------------------|------------------------------|----------|
| Demographic data | | | |
| Age (years) (mean ± SD) | 62.18 ± 11.3 | 56.25 ± 12.1 | < 0.0001 |
| Sex Male/Female | 5/98 | 19/247 | NS |
| Disease duration (years) (mean ± SD) | 7.53 ± 5.7 | 6.12 ± 5.7 | 0.007 |
| Disease activity | | | |
| ESSPRI (mean ± SD) | 5.3 ± 2.3 | 5.3 ± 2.05 | NS |
| ESSDAI [median (range)] | 4 (0-31) | 2 (0-24) | 0.03 |
| Biological parameters | | | |
| Anti-SSA antibodies (y/n) | 65/38 | 152/114 | NS |
| Anti-SSB antibodies (y/n) | 31/72 | 88/178 | NS |
| Rheumatoid factor (mean ± SD) | 20.18 ± 44 | 23.06 ± 75.92 | NS |

NS: not significant; y/n: yes/no; SD: standard deviation; VAS: visual analog scale.

Flt3-L is associated with previous lymphoproliferative disease in pSS

Several clinical and immunological characteristics were previously described as markers of lymphoproliferative disease in pSS. Among these parameters, patients with pSS and splenomegaly, persistent enlargement of parotid glands, lymphadenopathy, palpable purpura, cryoglobulinemia, low levels of C4, neutropenia or lymphocytopenia have more than 5-fold increased risk of NHL compared to patients with no risk factors. The analysis of clinical parameters showed that lymphoma in our cohort was associated with antecedents of purpura, splenomegaly, and lymphadenopathy. In addition, the analysis of biological parameters showed that lymphocytopenia, low levels of C4, and high levels of Flt3-L were associated with previous occurrence of lymphoma (Table 2). Among these biological markers, Flt3-L was the most significantly associated to previous lymphoma in univariate analysis. ROC analysis showed that 175 pg/mL was the ideal cut-off to detect the association with previous lymphoma with a sensitivity of 44%, and a specificity of 97.5%.

Table 2. History of clinical features and biological findings at inclusion in primary SS patients according to history of lymphoma.

| | <i>Lymphoma + (n =18)</i> | <i>Lymphoma - (n =351)</i> | <i>p</i> |
|---|-------------------------------|--------------------------------|----------------------|
| <i>History of clinical features</i> | | | |
| Enlargement of parotid glands (y/n) | 5/13 | 55/296 | NS |
| Purpura (y/n) | 6/12 | 38/308 | 0.007 |
| Splenomegaly (y/n) | 4/14 | 8/341 | 0.0001 |
| Lymphadenopathy (y/n) | 9/9 | 42/309 | 0.0001 |
| <i>Biological findings at inclusion</i> | | | |
| <i>Flt3-L levels \geq 120 pg/mL (y/n)</i> | <i>13/5</i> | <i>90/261</i> | <i>0.0001</i> |
| Mixed monoclonal cryoglobulinemia (y/n) | 5/13 | 55/281 | NS |
| Lymphocytopenia (y/n) | 6/12 | 15/323 | 0.004 |
| IgM levels \leq 0.5 g/L (y/n) | 4/14 | 43/306 | NS |
| β 2-microglobulin levels \geq 2.3 mg/L (y/n) | 9/9 | 111/240 | NS |

NS: not significant; y/n: yes/no; SD: standard deviation.

Flt3-L levels and associations with clinical and serological markers of NHL in pSS.

Then, we compared Flt3-L levels with every clinical and biological parameter previously described as markers of lymphoproliferation in pSS. Previous splenomegaly, lymphadenopathy, enlargement of parotid glands, and cryoglobulinemia were not associated with higher levels of Flt3-L. In contrast, an antecedent of purpura was significantly associated with elevated levels of Flt3-L. Biological markers such as low levels of C4, lymphocytopenia, low levels of IgM and high levels of β 2-microglobulin were also associated with higher levels of Flt3-L. Table 3 summarizes the main risk factors of NHL in pSS according to Flt3-L levels.

Table 3. History of clinical features and biological findings at inclusion in primary SS patients according to Flt3 Ligand levels.

| | <i>FL+</i> (n =103) | <i>FL-</i> (n =266) | <i>p</i> |
|--|---------------------|---------------------|----------------------|
| <i>History of clinical features</i> | | | |
| Enlargement of parotid glands (y/n) | 16/87 | 44/222 | NS |
| Purpura (y/n) | 20/83 | 24/237 | 0.008 |
| Splenomegaly (y/n) | 4/97 | 7/259 | NS |
| Lymphadenopathy (y/n) | 19/84 | 33/233 | NS |
| <i>Previous lymphoma</i> (y/n) | <i>13/90</i> | <i>5/261</i> | <i>0.0001</i> |
| <i>Biological findings at inclusion</i> | | | |
| Mixed monoclonal cryoglobulinemia (y/n) | 20/78 | 32/224 | NS |
| Lymphocytopenia (y/n) | 16/84 | 8/248 | 0.004 |
| IgM levels \leq 0.5 g/L | 20/82 | 27/238 | 0.024 |
| β 2-microglobulin levels \geq 2.3 mg/L (y/n) | 47/56 | 73/193 | 0.001 |
| Levels of C4 (mean \pm SD) | 0.21 \pm 0.088 | 0.24 \pm 0.16 | 0.05 |

NS: not significant; y/n: yes/no; SD: standard deviation.

Analysis of independent parameters associated with history of lymphoma

When we compared risk factors associated with previous lymphoma by multivariate analysis, we observed that Flt3-L level \geq 120 pg/mL, lymphadenopathy and splenomegaly were independent factors. Thus, we conducted a logistic regression analysis to determine which criterion is the most associated with lymphoma. Only 2 items were selected: splenomegaly (OR=56.39) and Flt3-L \geq 120 pg/mL (OR= 17.27), confirming that Flt3-L is the best biological criterion associated with history of lymphoma.

Flt3-L and relation to NHL in pSS

Eighteen patients presented previous NHL (4.8%). This clinical antecedent was statistically associated with higher levels of Flt3-L ($p < 0.0001$). Among NHL types, 8 patients presented MALT lymphoma, 4 had nodal marginal zone B-cell lymphoma, 3

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3 diffuse large B-cell lymphoma and 3 patients presented other types of
4 lymphoproliferation. All 8 patients with previous MALT lymphoma had FL+ levels
5 (216.5 ± 119.0 pg/mL). These levels were higher in patients with previous MALT
6 compared to patients with previous diffuse large B-cell lymphoma (167.0 ± 59.2 pg/ml)
7 and previous nodal marginal zone B-cell lymphoma (134.0 ± 30.6 pg/mL). Flt3-L levels
8 remained higher in NHL patients even after treatment that might indicate persistent B-
9 cell activation. High levels of Flt3-L were present in patients with relapsing disease and
10 in current treatment at the inclusion (343.0 pg/mL, 211.0 pg/mL, and 157.0 pg/mL).
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22 The clinical characteristics of patients with lymphoma are represented in Table 4.
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Table 4. Clinical characteristics of 18 lymphomas and relation to Flt3-L levels.

Abbreviations: Diffuse large B-cell lymphoma (DLBCL); nodal marginal zone (NMZ); MALT: mucosa-associated lymphoid tissue; EBV: Epstein-Barr Virus

| Case | Sex | Lymphoma type | Lymphoma location | Clinical staging | Main treatments | Relapse/current treatment | Flt3-L (pg/mL) |
|------|-----|-------------------|---------------------------------------|------------------|--|---------------------------|----------------|
| 1 | F | DLBCL | Skin | Ie | Chemotherapy (Interferon alpha) | N | 99.5 |
| 2 | M | MALT | Parotid gland | IV | Surgical | N | 131.0 |
| 3 | F | MALT | Parotid gland | IV | Chemotherapy (Chlorambucil-Rituximab) | Y | 343.9 |
| 4 | F | NMZ | Lymphatic node | IV | Rituximab | Y | 157.0 |
| 5 | F | MALT | Parotid gland | IV | Chlorambucil | Y | 211.5 |
| 6 | F | NMZ | Lymphatic node + bone marrow | IV | Mini-CHOP | N | 92.6 |
| 7 | F | MALT | Submaxilar gland | Ie | Mini-CHOP | N | 178.0 |
| 8 | M | NMZ | Lymphatic node + bone marrow | IV | Chlorambucil + fludarabine | N | 176.9 |
| 9 | F | NMZ | Spleen | I | Surgical | N | 112.0 |
| 10 | F | EBV associated | Lymphatic node + bone marrow + spleen | IV | Chemotherapy (ACVBP) + Rituximab | N | 131.0 |
| 11 | F | MALT | Gastric | Ie | Chlorambucil-Rituximab | N | 120.4 |
| 12 | F | DLBCL | Lymphatic node | III | CHOP-Rituximab | N | 208.0 |
| 13 | F | MALT | Skin | IV | Surgical | N | 121.0 |
| 14 | F | MALT | Lung | Ie | Chlorambucil | N | 451.4 |
| 15 | F | DLBCL | Lymphatic node + bone marrow | IV | Rituximab + autologous stem cell transplantation | N | 195.9 |
| 16 | F | MALT | Parotid gland + lymphatic node | IV | Chlorambucil + Rituximab | N | 185.5 |
| 17 | F | Mycosis fungoides | Skin | IV | Local chlorethamine | N | 130.0 |
| 18 | F | MALT | Parotid gland | Ie | Mini-CHOP | N | 123.9 |

DISCUSSION

Recent evidence indicates a major role for B cells in the pathogenesis of pSS.[16,18] In addition to biological abnormalities of B cells in this disease,[15], 5% of patients may develop a NHL. The clinical association between pSS and NHL has been shown in several studies.[5,6] Thus, it is important to determine which patients are at risk of progression to lymphoma. In this study we established that Flt-3L is elevated and correlated with history of lymphoma in pSS.

Flt3-L plays a role in normal B cells development. Its level is increased in hematologic malignancies and we had previously demonstrated that serum levels of Flt3-L were correlated with the abnormal distribution of B cells in peripheral blood and the activity of the disease in pSS patients.[14] In this larger study, we confirmed that levels of Flt3-L were higher in pSS patients (n=369) compared to healthy controls and were associated with disease activity determined by ESSDAI score. In addition, previous history of lymphoma was significantly associated with higher levels of Flt3-L ($p < 0.0001$). The most specific cut-off value of Flt3-L associated with NHL was 175 pg/mL (Sensitivity: 44%, Specificity: 97.5%). Flt3-L higher expression is also associated with the others lymphoma risk factors classically described such as palpable purpura, low levels of C4, lymphocytopenia, low levels of IgM and high levels of β 2-microglobulin. However no association with persistent enlargement of parotid glands, splenomegaly, cryoglobulinemia, low levels of C3 and neutropenia was seen. Among all the biological markers already described, Flt3-L was the most significantly associated with lymphoma.

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3 In addition, patients previously treated with immunosuppressive drugs
4 (cyclophosphamide, intravenous immunoglobulin or rituximab) presented higher Flt3-L
5 levels (p = 0.002; p = 0.026; p = 0.003, respectively, data not shown). These findings
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11 may be explained by a more severe disease with systemic complications and lymphoma,
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13 requiring immunosuppressive treatment.
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17 More interestingly, levels of Flt3-L remained high in treated patients with lymphoma.
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19 In hematological malignancies higher Flt3-L levels are associated with advanced
20 disease before treatment and residual disease after treatment. Retzlaff et al showed that
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22 before treatment, serum levels of Flt3-L correlate with prognostic variables and high
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24 grade lymphoma subtype in patients with primary gastro-intestinal NHL.[19] In acute
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26 lymphoblastic leukemia, Flt3-L treatment renders cell-lines and primary leukemia cells
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28 resistant to chemotherapy agents,[20] showing that high levels of Flt3-L may contribute,
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30 at least in part, to persistent minimal residual disease in bone marrow. Thus, persistent
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32 high levels of Flt3-L after lymphoma treatment may indicate minimal residual disease,
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34 B-cell activation and probably high relapsing risk. In our study, three patients with
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36 relapsing disease after chemotherapy presented high levels of Flt3-L. This observation
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38 needs to be confirmed in the prospective follow-up of our cohort. Higher levels were
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40 also associated with previous MALT type lymphoma, which is the most characteristic
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42 lymphoproliferative disorder in patients with pSS, compared to other types such as
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44 diffuse large B-cell and marginal zone lymphomas.
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55 An alternative explanation to our results would be that Flt3-L is a marker of activity and
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57 that patients with pSS and lymphoma have a higher activity of the disease which
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59 remains even after cure of lymphoma. In favor of this hypothesis, Flt3-L was correlated
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3 with the ESSDAI, a new clinical activity score recently developed and in process of
4 validation. Moreover, this correlation persisted if we excluded the 3 patients with active
5 lymphoma at evaluation, these 3 latter patients having obviously an important increase
6 of the ESSDAI score due to active lymphoma (addition of 12 points).[18]
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15 In conclusion, routine evaluation of Flt-3L in pSS is useful to evaluate clinical severity
16 and activity of the disease. The role of Flt3-L in B cell proliferation may explain the
17 clinical evolution to B cell lymphoma in some patients and targeting Flt3-L might open
18 new therapeutic options. The follow-up of the ASSESS cohort will allow to assess if
19 the baseline level of Flt3-L will be also predictive of occurrence of incident cases of
20 lymphoma.[21]
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BAFF

Article 4

A new BAFF variant acting as a transcription factor to enhance the production of BAFF

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Nat Genetics 2012; submitted

A new BAFF variant acting as a transcription factor to enhance the production of BAFF

Elevated expression of BAFF, contributes to the expansion of low-affinity self-reactive B cells during the establishment of tolerance. However, mechanisms leading to BAFF over-expression in AIDs such as SLE or pSS and in lymphoproliferation such as CLL are not fully understood. **In this fourth article**, we report the discovery of a new transcriptional variant for BAFF, $\Delta 4$ BAFF in humans (in which exon 4 is excised) or $\Delta 5$ BAFF in mice (in which exon 5 is excised), which acts, in association with p50 from the NF- κ B pathway, as a transcription factor for its own parent gene, and which is mainly detected in autoimmune diseases (including ECs and B lymphocytes from pSS patients) and CLL B cells.

This work introduces an entirely novel concept in biology suggesting that a human cytokine gene can be transcriptionally regulated by the activity of one of its own splice variants.

A new BAFF variant acting as a transcription factor in the regulation of immune response

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ABSTRACT

Elevated expression of 'B cell activating factor' (BAFF), a potent B-cell survival cytokine, contributes to the expansion of low-affinity self-reactive B cells during the establishment of tolerance. Here we report the discovery of a new transcriptional variant for BAFF, $\Delta 4$ BAFF in humans, involved in the regulation of immune response and acting in association with p50 from the NF- κ B pathway, as a transcription factor for its own parent gene. Our work introduces an entirely novel concept in biology suggesting that a human cytokine gene can be transcriptionally regulated by the activity of one of its own splice variants.

INTRODUCTION

Excess of 'B cell activating factor belonging to the tumor necrosis factor family' (BAFF) production and circulation can precipitate autoimmunity not only by increasing the numbers of self-reactive B cells but also by promoting them into microenvironments where their location increases their chances of inappropriate activation.^{1,2}

However, mechanisms leading to BAFF over-expression in autoimmune diseases such as primary Sjögren's syndrome (pSS), rheumatoid arthritis (RA) or systemic lupus erythematosus (SLE)³ and in lymphoproliferative disorders such as chronic lymphocytic leukemia (CLL)⁴ are not fully understood.

BAFF is mainly produced by innate immune cells such as neutrophils, macrophages, monocytes, and dendritic cells but also synoviocytes in RA and epithelial cells (EC) in pSS,⁵ providing local niches to modulate the survival and function of B cells in autoimmune diseases.

The BAFF gene is mapped to human chromosome 13q32-q34 and contains six exons in humans⁶ (chromosome 8 and seven exons in mice). This gene encodes three different mRNAs: the well-characterized full-length *BAFF*, a longer variant called ϕ *BAFF*, and a shorter variant designated Δ *BAFF*.⁷ The larger transcript ϕ *BAFF* was identified in the human cell lines HL-60 and U937, but sequencing proved this transcript to be non-functional because of incomplete splicing of intronic sequences leading to formation of premature stop codon.⁷ The smaller transcript Δ *BAFF*, which lacks exon 3

in humans (exon 4 in mice), appears to negatively regulate BAFF in mice, by forming non-functional heterotrimers with full-length BAFF.⁸

Like most of the ligands of the TNF family, BAFF is a transmembrane type II protein (32 kDa) that can be cleaved by a furin convertase to produce a soluble form (17 kDa).⁶ The biologically active form of BAFF is trimeric but these trimers may associate to form a virus-like 60-mer structure.⁵ BAFF possesses two potential *N*-glycosylation sites but only residue N¹²⁴ is glycosylated.⁶ BAFF expression requires NF- κ B components that act as pivotal transcription factors.⁹ The BAFF gene promoter has an NF- κ B binding site that can be activated through NF- κ B1 or NF- κ B2 pathways. In the NF- κ B1 pathway, phosphorylation of the NF- κ B inhibitor I κ B by the I κ B kinase (IKK) complex allows the cytoplasmic NF- κ B proteins p65 and p50 to translocate to the nucleus. Alternatively, in the NF- κ B2 pathway, precursor p100 is proteolytically cleaved to activate p52 through the NF- κ B-inducing kinase (NIK) and IKK α .¹⁰ Another NF- κ B component, c-Rel, can exert its function on the promoter of BAFF gene by recruiting unusual factors such as cytokine receptors CD40¹¹ and BAFF-receptor.¹² Cytokines such as IL-10, IFN- α and IFN- γ increase BAFF production by monocytes, macrophages and dendritic cells.¹³ IFN- γ is a highly expressed cytokine in autoimmune diseases and CLL and is involved in their pathogenesis. Although IFN- γ is a potent inducer of BAFF expression, the relationship between IFN- γ and the activation of BAFF gene expression are not clear. While IFN- γ responses are mainly mediated via Jak-Stat, this pathway is not thought to be involved in the IFN- γ -induced BAFF expression. Instead, protein kinase A/CREB has been reported as the dominant pathway.¹⁴

Given the fact that estimates of BAFF in humans fluctuate with changes in inflammatory activity, extent of the disease activity,¹⁵ we investigated the presence of other intragenic splice variants.

RESULTS

Demonstration of a new *BAFF* variant, Δ *BAFF*, expressed in cell lines and healthy individuals

RT-PCR analysis of cells from healthy donor peripheral blood cells (monocytes, B cells, T cells and neutrophils) or from cell lines (HeLa, U937 and RAMOS) that produce BAFF revealed different results according to the specific oligonucleotides used (**Fig. 1a**). With commonly used oligonucleotides described by Schneider *et al.* (JT1322 and JT1323, forward exon2 and reverse exon5),⁶ we can observed the 3 mRNA forms already described *i.e.* native full-length *BAFF*, a shorter variant, Δ *BAFF*, and a longer, non-functional variant, ϕ *BAFF*. In contrast, using oligonucleotides LLP2008 and LLP2009 (forward exon 3 and reverse exon 6), we observed the presence of the full-length *BAFF* transcript and, in some cases, ϕ *BAFF* but also another shorter form that did not correspond to Δ *BAFF* (exon 3 excised) because the forward primer annealed within exon 3.

A 3' RACE-PCR was then performed to detect this alternate mRNA splicing configuration. The predominant product (**Fig. 1b**) was 584 bp in length, one longer product was 817 bp and two smaller products were 527 bp and 471 bp in length, respectively. Sequencing analysis revealed that the smaller fragment at 471 bp was identical to the published *BAFF* sequence (NCBI NM_006573) but lacked 113 bp encoding the predicted exon 4 (deletion of nucleotides 749 to 861) (**Fig. 1c**). This result demonstrated the existence of a previously unrecognized mRNA transcript, designated Δ *BAFF*, derived from the same genomic sequence. The last nucleotide (748) in exon 3 is linked to the first base (862) within exon 5. A new in-frame stop codon (TGA) within

exon 5 is generated resulting in an open reading frame of 495 nucleotides (**Fig. 1d**), encoding a predicted truncated 164 amino acid protein (**Fig. 1e and Supplementary Fig. 1a**) (GenBank accession number HM 636064). We tested if $\Delta 4BAFF$ could be degraded by non-sense mediated decay (NMD), an integral quality control mechanism that initiates active degradation of nonsense mRNA in order to maintain high fidelity gene expression when translation terminates more than 50-55 nucleotides upstream the last intron-exon junction.^{16,17} Incubation with cycloheximide, a known indirect inhibitor of NMD showed that $\Delta 4BAFF$ is not increased after treatment, indicating that the transcript is stable and not subjected to NMD degradation (**Supplementary Fig. 2**). However, while both the N-glycosylation site at asparagine residue 124 (N¹²⁴) and the furin cleavage site are conserved, exons 5 and 6 of the transcript are not translated, thus encoding a protein that will be unlikely to form trimers or bind to BAFF receptors.

Expression and characterization of this new variant in mice

Since exon 4 in humans and exon 5 in mice are very similar (90% sequence identity using BLASTn), we then investigated whether a counterpart splice variant was present in mice. PCR amplifying the exon 4 and 6 junction demonstrated that exon 5 was spliced out in splenocytes from Balb/c and Swiss mice (**Supplementary Fig. 3a**). The murine splicing event generates a stop codon further upstream in exon 7 resulting in an open reading frame of 732 nucleotides, predicting a truncated 243 amino acid protein (**Supplementary Fig. 1b**). Interestingly, the $\Delta 5BAFF$ alternative splice variant was not observed in splenocytes from C57Bl/6 mice.

Characterization of Δ 4BAFF protein

To assess the biochemical properties of the new variant of *BAFF* in humans, a Δ 4BAFF construct was associated to EGFP expression via an internal ribosomal entry site (IRES), which allowed detection of the positively transfected cells using FACS (**Supplementary Fig. 4a**) and transfected in RAMOS, which do not express Δ 4BAFF constitutively. RAMOS cells transiently transfected with p Δ 4BAFF-IRES2-EGFP or empty vector alone (control) were sorted and analyzed by Western blot for BAFF expression using 2 different anti-BAFF antibodies (Abs), one mAb and one polyclonal (pAb). Anti-BAFF mAb, clone 137314, was obtained from an *Escherichia coli*-derived BAFF recombinant, while the pAb was a rabbit-derived anti-BAFF characterized by a 13-amino acid peptide located in the extra-membrane region encoded by exon 6. Consequently, Δ 4BAFF protein, which lacks amino acids encoded by exon 6, cannot be recognized by this rabbit anti-BAFF pAb. Anti-BAFF mAb 137314 recognizes the full length BAFF at 32kDa (**Fig. 2a**, upper panel) but also, in the Δ 4BAFF construct, two others forms at 21 and 17 kDa. The fact that the rabbit anti-BAFF pAb only recognizes the 32kDa form further indicated that the two bands at 21 and 17 kDa belong to Δ 4BAFF (**Fig. 2a**, lower panel).

Since they predicted form of Δ 4BAFF should correspond to a molecular weight of 17 kDa, the presence of a primary 21 kDa-form suggests a post-translational modification. Schneider et al. have shown that residue N¹²⁴ was glycosylated,⁶ with N-linked carbohydrates, in the full-length form of BAFF. In order to confirm the N¹²⁴ glycosylated status of Δ 4BAFF, two experiments were conducted. The first consisted of removing the N-linked carbohydrates using PNGase F (**Fig. 2b**) and, the second in transfecting RAMOS cells with a mutated form of Δ 4BAFF at N¹²⁴ (p Δ 4^[N124→D]BAFF-

IRES2-EGFP, **Fig. 2a**). Incubation with PNGase F revealed that $\Delta 4$ BAFF was glycosylated and led to the predicted mobility of $\Delta 4$ BAFF at 17kDa (**Fig. 2b**). Transfection of mutated $\Delta 4^{[N^{124} \rightarrow D]}$ BAFF variant revealed the absence of glycosylated form and confirmed that $\Delta 4$ BAFF was glycosylated on N¹²⁴ (**Fig. 2a**).

We then transfected RAMOS cells with p $\Delta 4$ BAFF-EGFP (**Supplementary Fig. 4b**) to observe the $\Delta 4$ BAFF protein localization using confocal microscopy (**Fig. 2c**). p $\Delta 4$ BAFF-EGFP was observed in the organelles, especially the endoplasmic reticulum and the Golgi apparatus, and also on the nuclear membrane and in the nucleus. However, p $\Delta 4$ BAFF-EGFP was not observed in the cytoplasm or the plasma membrane. The p $\Delta 4^{[N^{124} \rightarrow D]}$ BAFF-EGFP transfection leads to a similar localization pattern albeit with less marked detection within the nucleus.

To confirm these results and to avoid inability of shift due to EGFP molecular weight, RAMOS cells were transfected with p $\Delta 4$ BAFF-IRES2-EGFP and pIRES2-EGFP, selected by cell sorting and subjected to subcellular fractionation. The $\Delta 4$ BAFF variant was observed in the organelles and nuclear fractions (**Fig. 2d**). Consequently, despite the absence of a putative nuclear localization signal (NLS) sequence, $\Delta 4$ BAFF is present in the nucleus and its nuclear location requires its glycosylation on N¹²⁴ (**Fig 2c**).

Gene expression profile of $\Delta 4$ BAFF transfected RAMOS B cells.

We hypothesized that, since $\Delta 4$ BAFF protein is distributed throughout the nucleus, $\Delta 4$ BAFF might function as a transcription regulator. The gene expression profiles of RAMOS B cells stably transfected with pIRES2-EGFP, p $\Delta 4$ BAFF-IRES2-EGFP or p $\Delta 4^{[N^{124} \rightarrow D]}$ BAFF-IRES2-EGFP was then carried out. 6503 genes were found to be

differentially expressed between p Δ BAFF-IRES2-EGFP and pIRES2-EGFP transfected cells (2-fold difference, $p < 0.01$). Interestingly, by applying the same ± 2 -fold cut-off, 6303 genes were also up- and down-regulated between p Δ BAFF-IRES2-EGFP and p Δ ^[N124→D]BAFF-IRES2-EGFP transfected RAMOS B cells ($p < 0.01$). A Venn diagram (**Fig. 3a**) showed that 6155 RAMOS B cell genes have their expression specifically modified by Δ 4BAFF transfection in comparison to Δ 4^[N124→D]BAFF and empty vector (pIRES2-EGFP) transfection. These observations confirm the suspected role of Δ 4BAFF as a transcription factor and the importance of the glycosylation state on N¹²⁴ in the modulation of Δ 4BAFF activity.

Gene-lists were analyzed by functional annotation clustering using DAVID. This enabled the identification of common biological charts (**Supplementary Table 1**). Among the 6155 genes, 2904 genes were up-regulated and 3251 genes were down-regulated by p Δ BAFF-IRES2-EGFP transfection in comparison to pIRES2-EGFP and p Δ ^[N124→D]BAFF-IRES2-EGFP transfection. Up-regulated genes were associated with immune response and especially innate immunity, protein localization processes, RNA processing, translation and regulation of apoptosis. Down-regulated genes were involved predominantly in the regulation of transcription, immune response and cellular homeostasis.

Real-time PCR validated the expression patterns of a subset of genes selected from the microarray patterns which were up-regulated (*TLR2*, *TLR6*, *TLR9*, *TLR10*, *AICDA*, *TBX21*, *miR155*) or down-regulated (*MBD2*) after Δ 4BAFF transfection in RAMOS B cells (**Fig. 3b**).

$\Delta 4$ BAFF induces up-regulation of full-length BAFF and acts as a transcription factor of its own parent gene

Real-time PCR analysis in mice showed that the full-length form of *BAFF* was increased when the corresponding alternative splice variant $\Delta 5$ *Baff* was present (**Supplementary Fig. 3b**).

Interestingly, we also observed that $\Delta 4$ *BAFF* transfection in RAMOS cells (using p $\Delta 4$ *BAFF*-IRES2-EGFP construct) induced an up-regulation of full-length BAFF as shown by the increase in the BAFF (32kDa)/ β -actin ratio (**Fig. 3c**). The mean \pm SD of BAFF (32kDa)/ β -actin ratio in RAMOS p $\Delta 4$ *BAFF*-IRES2-EGFP transfected cells was higher than those transfected with pIRES2-EGFP alone (1.44 ± 0.11 vs 0.87 ± 0.05 respectively, $p < 0.01$, averages of 3 independent experiments). However, this ratio was not increased in RAMOS p $\Delta 4^{[N124 \rightarrow D]}$ *BAFF*-IRES2-EGFP transfected cells (0.82 ± 0.07).

We then examined whether $\Delta 4$ BAFF might function as a transcriptional regulator of the BAFF gene. To test this hypothesis, we performed ChIP analysis within the *BAFF* promoter using anti-BAFF mAb (137314) and primers that amplified different regions in the promoter of *BAFF*. The first primers target a region located at -681 and -377 known for binding CD40¹⁸ and the second primers target a region located at -1040 and -840 chosen because of its capacity to bind NF- κ B components.⁹ $\Delta 4$ BAFF binds to the specific sequence located within the -1040 to -840 region of *BAFF* promoter but did not bind to DNA precipitated with the rabbit anti-BAFF pAb or an IgG control (**Fig. 4a**). PCR analysis using primers upstream of the *BAFF* promoter (located at -1474 to -1261) also did not show $\Delta 4$ BAFF binding, indicating that the $\Delta 4$ BAFF protein binds to a specific

region within the *BAFF* promoter. To confirm whether $\Delta 4$ BAFF exerts transcriptional regulatory functions on its own gene, the NF- κ B component binding region of the *BAFF* gene promoter within -1040 to -840 was cloned into pDsRed-Express1 reporter plasmid (**Supplementary Fig. 4c**), leading to a p*BAFF* promoter-DsRed construct, and analyzed after transient transfection in RAMOS B cells stably transfected with pIRES2-EGFP, p $\Delta 4$ BAFF-IRES2-EGFP or p $\Delta 4^{[N124 \rightarrow D]}$ BAFF-IRES2-EGFP. By FACS, the mean fluorescence intensity of the p*BAFF* promoter-DsRed construct increased greater than two-fold rising from a baseline value of 0.30 ± 0.01 with empty vector to 0.65 ± 0.02 in p $\Delta 4$ BAFF-IRES2-EGFP RAMOS B cells (**Fig. 4b**). Together, these results indicate that glycosylated $\Delta 4$ BAFF acts as a transcription factor of its own parent gene and binds to the NF- κ B component binding region of the *BAFF* promoter.

$\Delta 4$ BAFF forms complexes with the transcription factor p50 from the NF- κ B pathway

The NF- κ B components binding to the *BAFF* promoter in humans included predominantly p50, p52, c-Rel and to a lesser extent p65.⁹ As a putative NLS sequence was absent, we hypothesized that $\Delta 4$ BAFF can translocate to the nucleus in association with one of the NF- κ B family members. We then synthesized digoxigenin-labelled consensus NF- κ B binding oligonucleotides (-1040 to -840) and performed gel shift assays. When nuclear extracts from p $\Delta 4$ BAFF-EGFP-transfected RAMOS B cells were incubated with the NF- κ B binding probe, a protein DNA complex was visualized (**Fig. 4c**) and the specificity of the binding was confirmed by competition with the excess (20 X) unlabeled oligo probe. A supershift was detected with addition of anti-BAFF mAb (clone 137314) but was not observed with the rabbit anti-BAFF pAb, which is unable to

bind $\Delta 4$ BAFF. Remarkably, the combination of anti-p50 and anti-BAFF mAbs further shifted the complex. As expected, a supershift was also observed with anti-cRel, anti-p52, anti-p65 and anti-p300, confirming their ability to bind the BAFF promoter but none of these in combination with the anti-BAFF mAb was able to further shift the complex (**Supplementary Fig. 5**).

Co-immunoprecipitation experiments confirmed that $\Delta 4$ BAFF interacts with p50 since these two molecules co-immunoprecipitated each other in nuclear lysates from p $\Delta 4$ BAFF-EGFP-transfected RAMOS B cells (**Fig. 4d**). Furthermore, only a weak association between $\Delta 4$ BAFF and p65 exists since $\Delta 4$ BAFF coimmunoprecipitate with p65 whereas p65 did not coimmunoprecipitate with $\Delta 4$ BAFF. Collectively these data suggest that $\Delta 4$ BAFF and p50 bind together at the NF- κ B consensus-binding site of the BAFF promoter providing $\Delta 4$ BAFF with a mechanism to translocate to the nucleus where it can act as a transcription factor for its own parent gene.

$\Delta 4$ BAFF is required for BAFF secretion by IFN- γ stimulated monocytes

In order to clarify the physiological context and impact of $\Delta 4$ BAFF isoform expression, we investigated the regulation of BAFF levels in monocytic cells (U937 cell line) in response to stimulation. IFN- γ is indeed known to stimulate BAFF synthesis by monocytes^{14,19,20} and to enhance the release of soluble BAFF that is active in B cell proliferation assays.¹⁹ We evaluated the consequences of specific $\Delta 4$ BAFF suppression on soluble BAFF release in the supernatant of U937 cells stimulated by IFN- γ . In order to generate cells deficient in $\Delta 4$ BAFF, U937 cells were transfected with $\Delta 4$ BAFF-specific siRNA (si $\Delta 4$ BAFF). As shown in **Fig. 5a** (upper panels), IFN- γ stimulation induced

Δ4BAFF expression in U937 cells and *Δ4BAFF* mRNA expression levels were not detectable in cells transfected with *siΔ4BAFF* while the expression levels of *BAFF* mRNA were not affected. Stimulation of U937 cells with IFN- γ induces BAFF release except in cells transfected with *siBAFF* and *siΔ4BAFF* (**Fig. 5a**, lower panels). These results clearly demonstrate that IFN- γ -induced BAFF release require the presence of the transcription factor $\Delta 4$ BAFF.

$\Delta 4$ BAFF expression is increased in patients with autoimmune diseases and chronic lymphocytic leukemia

There are several lines of evidence that support a role for IFN- γ in promoting the survival of B cells in CLL and the production of autoantibodies through BAFF activation in autoimmune diseases.^{21,22}

We then checked whether this new variant could explain the high levels of BAFF observed in autoimmune diseases and CLL. Excessive BAFF production has been previously reported to support the survival of low-affinity self-reactive B cells in autoimmune diseases.^{1,2} Previously, we observed the production of BAFF by synoviocytes from some patients with RA,²³ and also by EC and B cells from patients with pSS.²⁴ RT-PCR analysis of these cells that produce BAFF revealed that *Δ4BAFF* is overexpressed in cells from patients with autoimmune disease (**Fig. 5b**). However, elevated expression of *Δ4BAFF* transcript was not associated with an elevated expression of the classical *BAFF* transcript, although the detection of BAFF, at the protein level, was high in these cells (data not shown).

B-CLL cells can be rescued from apoptosis through an autocrine process involving BAFF⁴ and the basal level of NF- κ B activation in these cells is already high, contributing to apoptotic resistance.²⁵ To further understand this resistance, we tested the presence of Δ 4BAFF in lysates of B cells from CLL patients (**Fig. 6a**). Western blotting with specific mouse anti-BAFF mAb (clone 137314) confirmed the presence of Δ 4BAFF protein in some CLL B cells. Δ 4BAFF/ β -actin ratio was higher in B cells from some CLL patients than in those from controls and was associated with increased BAFF (32 kDa)/ β -actin ratio.

Δ 4BAFF is required for BAFF secretion by IFN- γ -stimulated monocytes and for B-chronic lymphocytic leukemia cell survival

B-CLL cells can be rescued from apoptosis through an autocrine process involving BAFF.^{4,26} We then generate B-CLL and healthy donor B cells deficient in Δ 4BAFF by transfecting these cells with si Δ 4BAFF and tested spontaneous cell death after 24 hours of culture in medium (**Fig. 6b**). Although no increase of spontaneous apoptosis was observed in control B cells after BAFF or Δ 4BAFF suppression in a short time culture, Δ 4BAFF-deficient B-CLL cells were more sensitive to cell death than those transfected with control siRNA. B cell survival was $20.6 \pm 7.2\%$ vs $68.5 \pm 8.4\%$, $P < 0.004$, respectively. B cell survival was also reduced in si Δ 4BAFF-transfected B-CLL cells in comparison to siBAFF-transfected B-CLL cells ($20.6 \pm 7.2\%$ vs $33.5 \pm 6.3\%$, $P < 0.03$ respectively, which suggests that Δ 4BAFF could acts as a transcription factor for other key regulators of B-CLL homeostasis.

DISCUSSION

This study reports the discovery of a previously unidentified transcript for BAFF ($\Delta 4BAFF$) in humans and $\Delta 5BAFF$ in mice. When transfected to RAMOS B cells, $\Delta 4BAFF$, induces the differential expression of many genes involved in immune response. For example, TLRs (-2, -6, -9 and -10) were markedly increased as *AICDA* (AID gene) and miR-155. These genes have critical roles in the establishment and the control of tolerance.²⁷⁻²⁹ Indeed, TLR ligation results in the production of pro-inflammatory cytokines, increased antigen expression, antibody production, proliferation and differentiation in B cells.^{30,31} We also described an entirely novel function of $\Delta 4BAFF$ as a transcription factor that enhances expression of its own parent gene and report its increased expression in autoimmunity (and particularly in synoviocytes from RA patients and in B cells and EC from pSS patients) and in lymphoproliferative disorders (B cells from CLL patients). These findings are of particular interest because BAFF overexpression is a central driver of such pathologies and is also associated with B cell tolerance breakdown and autoantibody production.³² The combination of the neoplastic accumulation of mature B lymphocytes with the presence of autoimmune phenomena is also a characteristic finding in CLL, and BAFF constitutes a crucial survival factor that sustains neoplastic B lymphocytes.²⁶

Elevated expression of $\Delta 4BAFF$ transcript was not systematically associated with an elevated expression of the classical *BAFF* transcript in synoviocytes from RA or EC from pSS patients. This can not be explained by NMD degradation. Thus, a fine-balance must exist between $\Delta 4BAFF$ mRNA levels, the time-frame in which it controls *BAFF*

transcription and the rate of NMD activity. Consequently, mechanisms leading to alternative splicing, for regulating protein expression in a cell-specific manner or in response to precise environmental cues, need to be more extensively studied. Consideration must be given to the differential expression of $\Delta 4$ BAFF in explaining the intriguing issues of serum concentrations of BAFF that remain within, or below the normal range in some autoimmune patients³ and why estimates fluctuate with changes in inflammatory activity, extent of the disease and classification criteria chosen by the investigators for SS, RA or CLL.

The $\Delta 4$ BAFF counterpart splice variant was observed in splenocytes from Balb/c and Swiss mice. BAFF gene in mice possesses seven exons. We demonstrated that exon 5, which shares significant sequence identity with exon 4 in humans, was spliced out. The resulting $\Delta 5$ BAFF protein was also truncated as the splicing event generates a stop codon further upstream in exon 7. However, the $\Delta 5$ BAFF variant was not observed in C57Bl/6 mice. This difference may be due to the mouse strains used. Balb/c mice have been shown to present increased susceptibility to several intracellular pathogens when compared to C57Bl/6 mice.³³ Splenocytes from Balb/c mice showed a constitutive increased production of IL-10 when compared with C57Bl/6 mice.³⁴ Interestingly, splenocytes from C57Bl/6 mice stimulated with IL-10 were induced to produce $\Delta 5$ BAFF (data not shown). Consequently, IL-10, a cytokine strongly associated with inflammatory response could also promote $\Delta 5$ BAFF expression leading to BAFF production in such circumstances.

We observed that N-glycosylation of $\Delta 4$ BAFF was not only required for nuclear entry but also for promoter binding. The importance of the N-glycosylation status for BAFF has been already described, particularly for the Δ BAFF variant,⁷ but also for the full-length form of BAFF in the serum of patients with autoimmune diseases.³⁵ In this respect, a number of conflicting results have cast doubt on the reliability of the enzyme-linked immunosorbent assays (ELISA) presently in use for its quantification, most of them being unable to recognize the non-glycosylated form of BAFF.³⁵ Furthermore, N-glycosylation was recently described to be required for the full activation of the transcription factor cyclic AMP-responsive element-binding (CREB)-H. Unglycosylated or deglycosylated CREB-H was retained in an inactive form in the endoplasmic reticulum and less capable of activating transcription by binding to its promoters.³⁶

Cooperation between $\Delta 4$ BAFF and p50 appears to be important in regulating BAFF expression in normal health, CLL and autoimmune diseases. The interactions of NF- κ B dimers or monomers with heterologous transcription factors through direct binding has been already described and profoundly influences transcriptional responses.³⁷ NF- κ B p50 lacks a transactivation domain and therefore usually form a heterodimer to be transcriptionally active.³⁸ *In vitro* studies have shown that p50 can associate with other transcriptional activators such as Bcl-3³⁹ or p300⁴⁰ to activate transcription. NF- κ B p50 can also form a complex with the transcriptional co-activator CREB to activate IL-10 transcription in macrophages.⁴¹ Interestingly, among the genes up-regulated after $\Delta 4$ BAFF transfection, the promoter of the BIC gene encoding miR-155 contains two putative NF- κ B sites able to bind *in vitro* the NF- κ B proteins p50 and p65 in nuclear extract from MC3 cells.⁴² Furthermore, p50 has a critical role in the induction of the AID

gene expression as AID induction in B cells was impaired in $p50^{-/-}$ mice.⁴³ Finally by blocking ubiquitination of p50, Bcl-3 stabilizes a p50 complex that inhibits TLR gene transcription and limits the strength of the TLR responses.⁴⁴ Consequently the cooperation between $\Delta 4$ BAFF and p50 may be an important regulatory mechanism for the transcription of a large number of genes. Further investigation of the role of $\Delta 4$ BAFF and its interaction with p50 in the context of both autoimmunity and B cell leukemia could provide novel therapies targeted at modulating its function.

We also explored the effects of IFN- γ on BAFF expression by U937 monocytes to see if the transcription factor $\Delta 4$ BAFF regulates this expression. Once inflammation is initiated, IFN- γ acts to induce BAFF production by monocytes,¹⁹ intensifying the inflammation process and autoimmune diseases. The links between IFN- γ and BAFF are indeed particularly important in the development and the maintenance of autoimmune and B cell lymphoproliferative diseases. The increased production of IFN- γ in *Lyn*-deficient mice (a well established model of lupus-like autoimmunity)⁴⁵ feeds back on the myeloid cells to further stimulate BAFF release. Furthermore, the reciprocal production of BAFF and IFN- γ establishes an inflammatory loop between myeloid cells and T cells that exacerbates autoimmunity in this model.⁴⁶ Blocking this inflammatory loop by lowering BAFF (using monoclonal antibody treatment) or removing IFN- γ (genetically) prevented nephritis in *lyn*^{-/-} animals. Our results show that $\Delta 4$ BAFF acts as a transcription factor of BAFF gene to encourage BAFF release after IFN- γ stimulation in U937 cells. IFN- γ -induced BAFF release was abrogated by transfecting the cells with si $\Delta 4$ BAFF.

The potent survival function of BAFF in limiting spontaneous cell death of B-CLL cells provides a key context in which to examine the significance of Δ 4BAFF function. The striking effect of BAFF on B-CLL survival^{4,26} raises the possibility that Δ 4BAFF inhibition leads to decreased BAFF expression and corresponding decrease in cell survival. Thereby, B-CLL cells may be more sensitive to spontaneous apoptosis. Δ 4BAFF expression, observed in B-CLL cells, could explain the mechanism by which some B-CLL cells acquire the ability to express BAFF. Interestingly, transcription factors from the NF- κ B family are highly activated in B-CLL cells.²⁵ Moreover, p65 and p50 increases were found, indicating that p50/p65 heterodimers are functionally active as transcription factors in CLL⁴ and association between Δ 4BAFF and p50 could therefore be promoted in CLL. Although Δ 4BAFF was increased in B-CLL cells, no association was observed between clinical features and levels of expression of Δ 4BAFF in B-CLL cells (data not shown). However, larger numbers of patients must be tested for possible correlations to be found.

The pivotal role of Δ 4BAFF as a transcription factor that controls BAFF expression holds immense promise for the clinic. The potential for Δ 4BAFF to be used as a therapeutic target will require further investigation into the scope of its role and potential as a transcriptional regulator of other genes, with ramifications for disease outcome and treatment strategies aimed at controlling BAFF production in autoimmunity and cancer.

Keywords: B cell activating factor, variant, autoimmunity, chronic lymphocytic leukemia, transcription factor

ONLINE METHODS

Cell and patients

All patients and healthy donors gave informed consent and the study was approved by the ethics committee at the Brest university medical school hospital. B lymphocytes were isolated from tonsil and peripheral blood of healthy donors, centrifugated on Ficoll-Hypaque (PAA laboratories, Pasching, Austria), followed by 2 rounds of rosetting with sheep erythrocytes (TCS Bioscience, Buckingham, UK) and incubated with human B cell enrichment kit (Stem-Cell-Technologies, Grenoble, France) for negative selection. Purity of B lymphocytes was checked by FACS analysis (EPICS®Elite, Beckman-Coulter) after fluorescein-isothiocyanate (FITC) conjugated anti-CD19 mAb staining (Clone J4.119, Immunotech, Marseille, France). B lymphocyte purity was more than 99%. T lymphocytes were isolated from peripheral blood of healthy donors, centrifugated on Ficoll-Hypaque, followed by 2 rounds of rosetting with sheep erythrocytes. Monocytes were isolated from peripheral blood of healthy donors by EasySep®-human-CD14-positive-selection-kit (Stem-Cell-technologies) according to the manufacturer instructions. Polymorphonuclear neutrophils were isolated by dextran (GE-Healthcare, Velizy-Villacoublay, France) sedimentation followed by centrifugation on Ficoll-Hypaque.

Peripheral blood mononuclear cells were obtained from healthy donors, patients with pSS fulfilling the American-European Consensus Group criteria for the disease,⁴⁷ and untreated patients fulfilling the criteria for the diagnosis of CLL.⁴⁸

EC were obtained from salivary gland biopsy specimens of patients suspected to develop a pSS as previously described.²⁴ Control samples consisted of EC from patients who did not meet the criteria for the diagnosis of pSS.

Synoviocytes were obtained from patients with RA⁴⁹ or osteoarthritis as previously described.²³

RAMOS, HeLa (Human epithelial cervical carcinoma) and human monocytic lymphoma U937 cell-line cells were cultured in RPMI 1640 medium (Lonza, Cologne, Germany) supplemented with 10% heat-inactivated fetal calf serum (FCS), 2 mM glutamine (Invitrogen, Cergy-Pontoise, France), 200 U/ml penicillin and 100 µg/ml streptomycin (Panpharma, Fougères, France).

***BAFF* PCR**

50 ng of cDNA was amplified by PCR using Taq DNA polymerase (Promega, Charbonnières, France). Primer pairs used were JT1322/JT1323 and LLP2008/LLP2009.

Rapid amplification of cDNA ends (RACE-PCR)

The 3' transcript ends were amplified using the SMART-RACE-cDNA-Amplification-Kit (Clontech, Mountain view, CA). We used a gene-specific primer for *BAFF* (exon 2). The products were subcloned using the pTrueBlue-ligation-kit (Genomics One, Laval, QC, Canada), and sequenced using the BigDye-Terminator-Cycle-Sequencing-Kit and the ABI-310 Genetic-Analyzer (Applied Biosystems).

Cloning human Δ 4BAFF

50 ng of cDNA from tonsillar B cells were used as template for the amplification of Δ 4*BAFF* using Advantage®2-PCR-Kit (Clontech) according to manufacturer instructions. Δ 4*BAFF* primers used are listed in **Supplementary table 2** (nested PCR).

PCR product was purified in gel with QIAquick-gel-extraction-kit (Qiagen, Courtaboeuf, France).

Transient transfection of Δ 4BAFF

RAMOS cells was transiently transfected with 10 μ g of pIRES2-EGFP (or p Δ 4BAFF-IRES2-EGFP or p Δ 4^[N124→D]BAFF-IRES2-EGFP) or 5 μ g of pEGFP-N1 (or p Δ 4BAFF-EGFP or p Δ 4^[N124→D]BAFF-EGFP) using a V kit VCA-1003 (Lonza), according to kit instructions. The cells were cultured for 24 h or 40 h in supplemented RPMI medium at 37°C with 5% CO₂.

Microarray analysis

Data analysis

After cDNA labeling and hybridization, data were normalized by quantile normalization using Genespring 12.0 (Agilent Technologies). After this preliminary analysis, we kept the three best samples (in quadruplicate) in each condition (9 samples selected). The selected data files (raw and normalized) have been deposited in MIAME-compliant format and are available in the ArrayExpress database (www.ebi.ac.uk/arrayexpress) under number E-MEXP-3595.

Normalized data were grouped in each condition like this: Control (RAMOS transfected with pIRES2-EGFP), Delta4BAFF (RAMOS transfected with p Δ 4BAFF-IRES2-EGFP) and Mutated-Delta4BAFF (RAMOS transfected with p Δ 4^[N124→D]BAFF-IRES2-EGFP) and the average intensity values across replicates were used for visualization and analysis. One-Way ANOVA (corrected p-value cut-off ≤ 0.01) with Benjamini-Hochberg multiple testing correction was used to identify genes whose expression changed

significantly when Δ 4BAFF is overexpressed compared to Control and Mutated-Delta4BAFF.

Functional annotation analysis

DAVID (Database for Annotation Visualization and Integrated Discovery) analysis was used to identify biological functions and pathways that were over-represented by any differentially expressed genes.⁵⁰ Functional annotation clustering was performed to identify relationships between enriched ontologies, thereby enabling the identification of gene subsets associated with similar biological processes.

Real-time PCR

1 μ g of total RNA isolated for microarray was converted to cDNA using SuperScript®II-RT according to the manufacturer's instructions. All amplifications used SYBR-Green®-PCR Master-Mix, except for *TBX21* (Hs00203436), we used TaqMan®-Gene-Expression-Master-Mix (Applied Biosystems). For *miR155* expression assay we used specific protocol : reverse transcription was done by the TaqMan-MicroRNA-reverse-transcription-kit (Applied Biosystems) according to the manufacturer's instructions and Applied Biosystems TaqMan®-assay. Real-time PCR was performed with a 7300 Real-time PCR system (Applied Biosystems) with the following conditions: 50°C for 2 min and 95°C for 10 min followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. All reactions were performed in duplicate. Gene expression level was calculated with the $2^{-\Delta C(T)}$ method.⁵¹ Target gene expression was normalized to *GAPDH* expression. All primers used are listed in **Supplementary Table 2**.

Immunofluorescence and confocal microscopy

Δ4BAFF-pEGFP or pEGFP-*Δ4*^[N124→D]*BAFF* transfected RAMOS cell-line cells were washed in PBS, centrifugated onto glass slides and fixed in 4% paraformaldehyde (Sigma-Aldrich) at 4°C. Mounting medium used is Vectashield (Vector laboratories, Burlingame, CA). GFP staining was observed by confocal microscopy Leica DMR microscope with camera Leica TCS-NT (Leica, Solms, France) using an oil immersion lens (Zeiss, Le Pecq, France) (PL APO, 100x/1,40). Room temperature is 16°C. Acquisition software used is Leica TCS-NT version 1.6.587.

Electrophoresis Mobility Shift Assay (EMSA)

Nuclear proteins (5 μg) were incubated with DIG probe (50 ng/μL). 20X of unlabeled probe were added for competition. For supershift analysis, 1μg of the following Abs was incubated before adding labeled probe in order to determine the specific reactions: mouse anti-BAFF (clone 137314), rabbit polyclonal anti-BAFF, mouse anti-p50 (clone 285412, R&D Systems), rabbit anti-p52 (Cell Signaling Technology, Boston,MA), rabbit anti-p65 (clone C22B4, Cell Signaling Technology), rabbit anti-Rel B (Cell Signaling Technology), rabbit anti-c-Rel (Upstate) and rabbit anti-p300 (N15, Santa Cruz Biotechnology, Santa Cruz, CA). Binding reaction was then performed at room temperature for 20 min.

The DNA-protein complexes (without any dyes) were resolved by electrophoresis on a 4% non-denaturing-PAGE and then electro-blotted to nylon membrane positively charged (Bio-Rad, Marnes-la-coquette, France), for 30 min at 300mA. After washing, the membrane was blocked 30 min at room temperature in 2% ECL blocking agent. After blocking unspecific binding sites on the membrane, anti-DIG-POD Fab fragments (Roche

Applied Science) was added and incubated for 30 min at room temperature. The complex was visualized by chemiluminescence (ECL advance western blotting detection).

Chromatin immunoprecipitation assay (ChIP)

ChIP assays were performed using the protocol provided by MACS Miltenyi Biotec (Auburn, CA) as described previously. Nuclei isolated from 1% formaldehyde-fixed RAMOS stably transfected cells were sonicated for eleven 20-s intervals. After incubation with mouse anti-BAFF mAb (clone 137314), and rabbit anti-BAFF pAb, DNA fragments were purified for PCR according to manufacturer's instructions. Two *BAFF* promoter regions were amplified using the following primers on the GenBank file AF 186114 for *BAFF* gene: region1 located at -1040 and -840 and region 2 located at -681 and -377. *BAFF* promoter upstream sequence was used as control. PCR was performed using 32 cycles of 94°C for 30 sec, 60°C for 1 min and 72°C for 1 min. The PCR product was visualized on a 1.5 % agarose gel. Primers used were listed in **Supplementary Table 2**.

Co-immunoprecipitation of complexes and Western Blot Analysis

Lysates of p Δ 4*BAFF*-EGFP or pEGFP transient transfected RAMOS B cell line cells were analyzed by co-immunoprecipitation using the MACSTM protein A/G microbeads kit and MACSTM Epitope Tag Protein Isolation Kits (Miltenyi Biotec), according to manufacturer's protocol. Anti-p50, p52 and p65 antibodies were used at 1 mg/ml concentration. The eluted immunoprecipitate is collected and analyzed by SDS-PAGE, as described below. Membranes were subsequently immunoblotted with antibodies to GFP-horseradish peroxidase conjugated (1:1000), p50 (1:1000), p52 (1:1000) or p65 (1:1000) antibodies. Bound antibodies were developed with horse-radish-peroxydase secondary

antibodies (GE Healthcare), except anti-GFP which was already coupled with horse radish peroxidase. All membranes were analyzed using a chemiluminescence.

Generation of Δ 4BAFF-deficient cells by siRNA transfection

3×10^6 U937 cells/well in a 6-well plate were transfected with 12.5 pmol of a specific Δ 4BAFF siRNA (Sequence CUAUACAAAAGGUUUUAUtt). BAFF siRNA (Sequence GCCUGAAACACUACCCAAUtt) and mice negative control siRNA were used as controls (Applied Biosystems). Twenty-four hours after transfection, cells were left untreated or treated with 50 ng/mL IFN- γ (R&D Systems). After a 72 h incubation, *BAFF* and *Δ 4BAFF* transcript expression were measured by RT-PCR as previously described. Release of soluble BAFF (17 KDa) was tested in the supernatant by immunoblot, as previously described.

Analysis of B cell survival after Δ 4BAFF inhibition in CLL

B lymphocytes isolated from peripheral blood of six different CLL patients (5×10^6 cells/well in a 6-well plate) were transfected with 12.5 pmol of Δ 4BAFF siRNA and BAFF siRNA as described above. Cells were maintained in supplemented RPMI-1640 medium at 37°C. Transfection efficiency was analyzed on labeled siRNA with silencer siRNA-labeling kit FAM (Applied Biosystems). SiRNA control was used as a transfection control. After 24h of incubation, cells were washed twice with PBS, and stained with propidium iodide to determine cell survival (Immunotech), according to manufacturer's instructions and analyzed by FACS.

Statistical analysis

Data were expressed as mean \pm standard deviation (SD). Comparisons were made the Mann-Whitney's test for unpaired data.

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FIGURE LEGENDS

Figure 1: $\Delta 4BAFF$ is a *BAFF* splice variant lacking exon 4 and is expressed in peripheral blood cells of healthy individuals and in cell line cells.

(a) Various *BAFF* transcripts from healthy donors peripheral blood cells (monocytes, B cells, T cells and neutrophils) and from cell lines (HeLa, U937 and RAMOS) were subject to Reverse transcriptase-PCR analysis. The amplifications were performed using JT1322 and JT1323⁶ (forward exon 2 and reverse exon 5, see upper panel) or LLP2008 and LLP2009 (forward exon 3 and reverse exon 6, bottom panel). (b) 3' rapid amplification of cDNA ends (3' RACE-PCR). The predominant product was 584 bp in length and corresponded to the classical full-length *BAFF*, one longer product was 817 bp ($\phi BAFF$) and the two smaller were 527 bp ($\Delta BAFF$) and 471 bp (unknown variant). Sequencing analysis (c) revealed that this smaller fragment lacked the predicted exon 4 and corresponded to a new *BAFF* variant, named $\Delta 4BAFF$. (d) Exons are represented as boxes and the size of introns (thin line) is listed. Alternative splicing events are shown, and the name of the resulting protein is indicated. (e) The predicted transmembrane domain (underlined), the potential N-linked glycosylation sites (stars), and the natural processing site of hBAFF (arrow) are indicated. Residues not encoded by the $\Delta 4BAFF$ transcript are shown as dashed lines.

Figure 2: $\Delta 4BAFF$ encodes a 21kDa truncated protein with an intact N-glycosylation site that is located in the nucleus.

(a) Whole cell lysates from RAMOS cells (non-transfected: NT), transiently transfected RAMOS cells with pIRES2-EGFP (empty vector: \emptyset), p $\Delta 4BAFF$ -IRES2-EGFP ($\Delta 4$) or

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mutated form of *Δ4BAFF* on N¹²⁴ (*pΔ4^[N124→D]BAFF-IRES2-EGFP*, *Δ4^[N124→D]*) were analyzed by Western blotting using either an anti-BAFF mAb (clone 137314) or a rabbit anti-BAFF pAb, unable to recognize *Δ4BAFF*. Anti-β-actin mAb was used as control. Data are representative of three independent experiments. (b) Prior to SDS-PAGE, some samples were incubated with PNGaseF and analyzed by Western blotting using the anti-BAFF mAb (clone 137314). (c) RAMOS cells were transiently transfected with pEGFP (empty vector: \emptyset), *pΔ4BAFF-EGFP* (*Δ4*) or *pΔ4^[N124→D]BAFF-EGFP* (*Δ4^[N124→D]*) constructs. At 24 hours post transfection, live cells were visualized using Leica TCS-NT confocal microscope. Images represent individual Z sections captured using a x100 objective with oil. In contrast to diffuse localization of EGFP (left panel), nuclear, nuclear membrane and endoplasmic reticulum fluorescence is evident in cells transfected with *pΔ4BAFF-EGFP*. (d) Subcellular fractionation of lysates from RAMOS cells (non-transfected), *pIRES2-EGFP* (empty vector: \emptyset), *pΔ4BAFF-IRES2-EGFP* (*Δ4*) or *pΔ4^[N124→D]BAFF-IRES2-EGFP* (*Δ4^[N124→D]*) transiently transfected RAMOS cells was performed to isolate cytosol (C), membrane and organelles (M/O) and nucleus (N). The enriched different fractions were analyzed by Western-blotting using the anti-BAFF mAb (clone 137314). Nuclear enrichment was tracked using anti-histone H3 pAb and non nuclear compartment was assessed using anti-CD20 pAb and anti-β-actin mAb.

Figure 3: Gene expression profile of *Δ4BAFF* transfected RAMOS B cell and validation using real-time PCR.

(a) Venn diagram to illustrate the overlapping expression of genes within genes differentially expressed between *pΔ4BAFF-IRES2-EGFP(Δ4)*, *pIRES2-EGFP* (empty vector: \emptyset) and *pΔ4^[N124→D]BAFF-IRES2-EGFP* (*Δ4^[N124→D]*) transfected RAMOS cells. (2 [Insert Running title of <72 characters])

way ANOVA, $P < 0.01$). **(b)** Real-time PCR are presented in the column graphs as relative gene expression normalized to *GAPDH*. **(c)** $\Delta 4$ BAFF induces up regulation of full-length BAFF. Whole cell lysates from RAMOS cells (non-transfected: NT), transfected RAMOS cells with PIRE2-EGFP (empty vector: \emptyset), p- $\Delta 4$ BAFF-IRES2-EGFP ($\Delta 4$) or p $\Delta 4^{[N124 \rightarrow D]}$ BAFF-IRES2-EGFP ($\Delta 4^{[N124 \rightarrow D]}$) were analyzed by Western-blotting using either an anti-BAFF mAb (clone 137314) or rabbit anti-BAFFpAb, unable to recognize $\Delta 4$ BAFF. Anti β -actin mAb was used as control and the ratio of the full-length form of BAFF at 32 kD (revealed by the different anti-BAFF Abs) to β -actin were quantified and shown below each lane. Data are representative of three independent experiments.

Figure 4: $\Delta 4$ BAFF acts as a transcription factor of its own gene by binding to the promoter of the *BAFF* gene at its NF- κ B binding region and forms complexes with p50 from the NF- κ B1 pathway.

(a) ChIP analysis within *BAFF* promoter was performed on RAMOS cells (non-transfected: NT) p $\Delta 4$ BAFF-IRES2-EGFP ($\Delta 4$) and p $\Delta 4^{[N124 \rightarrow D]}$ BAFF-IRES2-EGFP stably transfected RAMOS cells. The indicated antibodies (Abs) were used to precipitate chromatin. PCR to detect the various promoter regions of *BAFF* (i.e. CD40 binding region from -681 to -377; NF- κ B binding region from -1040 to -840 and a control region from -1474 to -1261) were performed on the precipitated DNA (GenBank file AF186114). Mouse IgG was used as a non specific control. **(b)** The promoter of *BAFF* is active in p $\Delta 4$ BAFF-IRES2-EGFP stably transfected cells. pEGFP (empty vector: \emptyset), p $\Delta 4$ BAFF-IRES2-EGFP ($\Delta 4$) and p $\Delta 4^{[N124 \rightarrow D]}$ BAFF-IRES2-EGFP ($\Delta 4^{[N124 \rightarrow D]}$) transfected RAMOS B cells were co-transfected with the -1040 to -840 BAFF promoter cloned in the pDsRed-express 1 reporter vector. pDsRed-575 nm emission fluorescence

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was observed by FACS. **(c)** Supershift analysis of $\Delta 4$ BAFF protein binding to the NF- κ B site. Nuclear extracts of RAMOS cells transiently transfected with p $\Delta 4$ BAFF EGFP were incubated with the NF- κ B probe labeled to digoxigenin (DIG) and antibodies (Abs) against BAFF (clone 137314 and rabbit). Samples were analyzed by electrophoretic mobility shift assay. **(d)** Association of $\Delta 4$ BAFF with p50 in transfected RAMOS cells with p $\Delta 4$ BAFF-EGFP. Nuclear extracts were used for immunoprecipitation with Abs to GFP, p50, p65 or p52 and subsequently analyzed by Western blot for GFP, BAFF, p50, p65 and p52 in pEGFP (empty vector: \emptyset) or p $\Delta 4$ BAFF-EGFP-transfected RAMOS B cells ($\Delta 4$).

Figure 5: $\Delta 4$ BAFF is necessary for BAFF secretion by IFN- γ stimulated monocytic overexpressed in cells of autoimmune individuals

(a) U937 cells transfected with $\Delta 4$ BAFF-specific siRNA (si $\Delta 4$ BAFF), BAFF siRNA (siBAFF) or control siRNA were incubated with IFN- γ (50 ng/mL). RT-PCR analysis was performed in order to detect the presence of BAFF, $\Delta 4$ BAFF or GAPDH mRNA (upper panels). Culture supernatants were collected 72 h after IFN- γ stimulation and the presence of soluble BAFF (17 kDa) measured by Western-blotting using an anti-BAFF mAb (clone 137314, lower panel). Data are representative of three independent experiments. **(b)** Various BAFF transcripts from synoviocytes from patients with rheumatoid arthritis (RA) or osteoarthritis (OA) and from epithelial and B cells from patients with primary Sjögren's syndrome were subject to Reverse transcriptase-PCR analysis. The amplifications were performed using LLP2008 and LLP2009 (forward exon 3 and reverse exon 6, bottom panel).

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Figure 6: Δ 4BAFF is overexpressed in B cells from patients with chronic lymphocytic leukemia (CLL) and is necessary for their survival patients.

(a) Whole B-cells lysates from different B-CLL cells and controls were analyzed by Western blotting using either an anti-BAFF mAb (clone 137314) or a rabbit anti-BAFF pAb, unable to recognize Δ 4BAFF. Anti- β -actin mAb was used as control. The form at 21 kDa corresponds to Δ 4BAFF whereas the form at 32 kDa corresponds to the full length BAFF. Anti- β -actin mAb was used as control and the ratios of the different anti-BAFF Abs to β -actin were quantified and shown below each lane. Data are representative of three independent experiments. **(b)** B cells from 6 different CLL patients (white bars) and 6 healthy donors (black bars) were transfected with si Δ 4BAFF, siBAFF or control siRNA and cultured in medium during 24 hours. B-CLL cell survival was quantified by FACS on non transfected cells (Medium) or on GFP-positive cells using propidium iodide staining. Data are represented as mean \pm SD.

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Figure 1

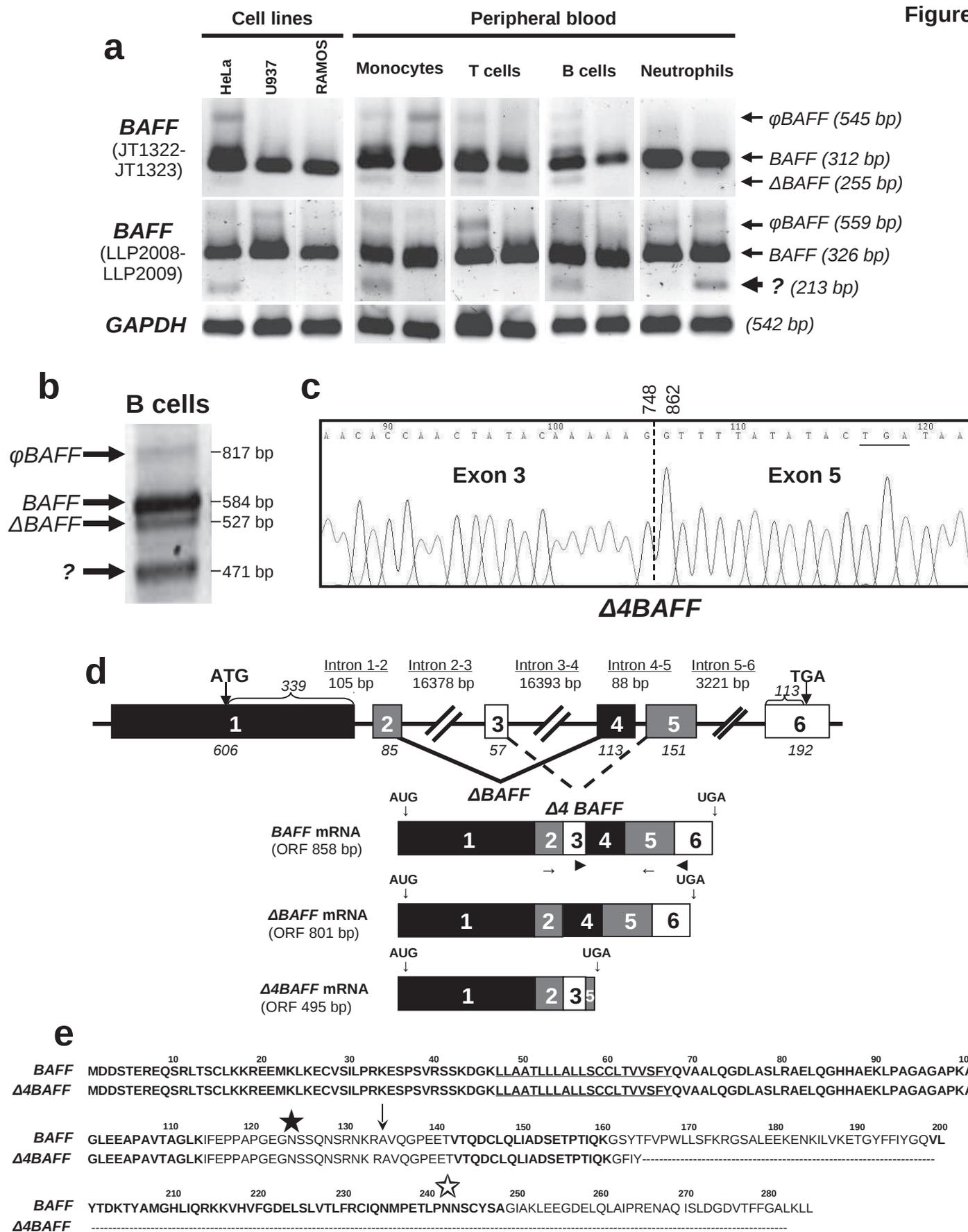


Figure 2

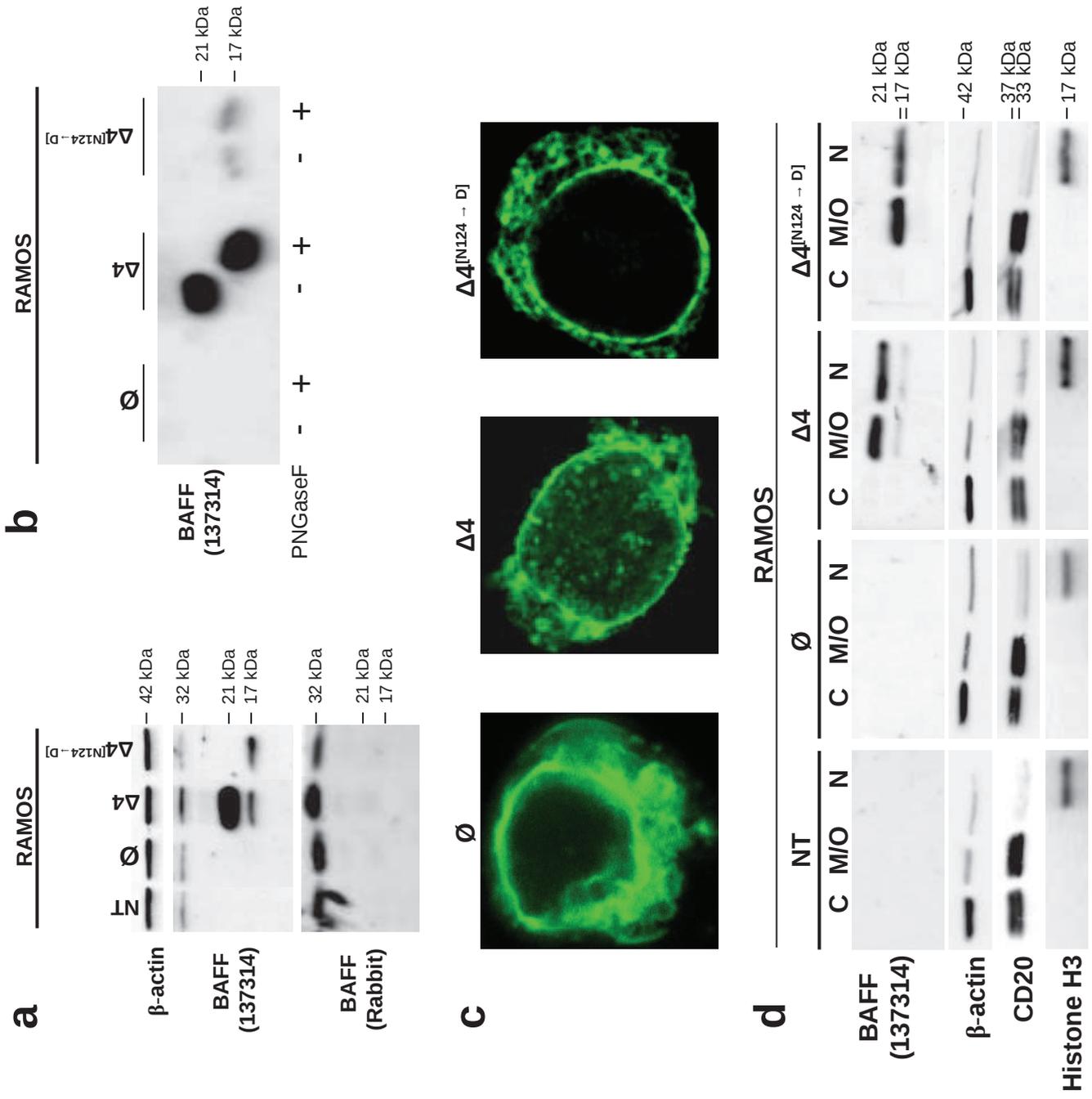


Figure 3

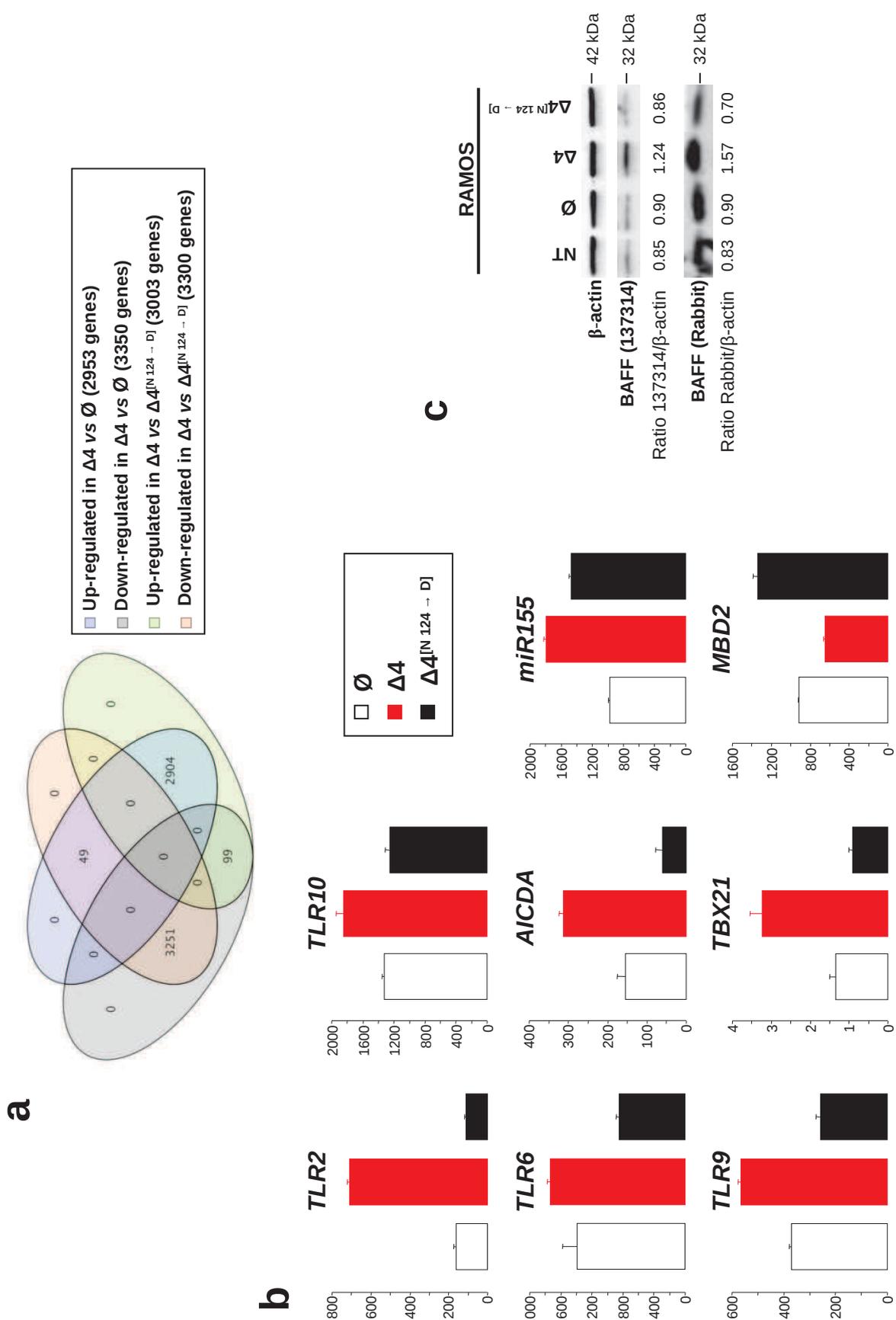


Figure 4

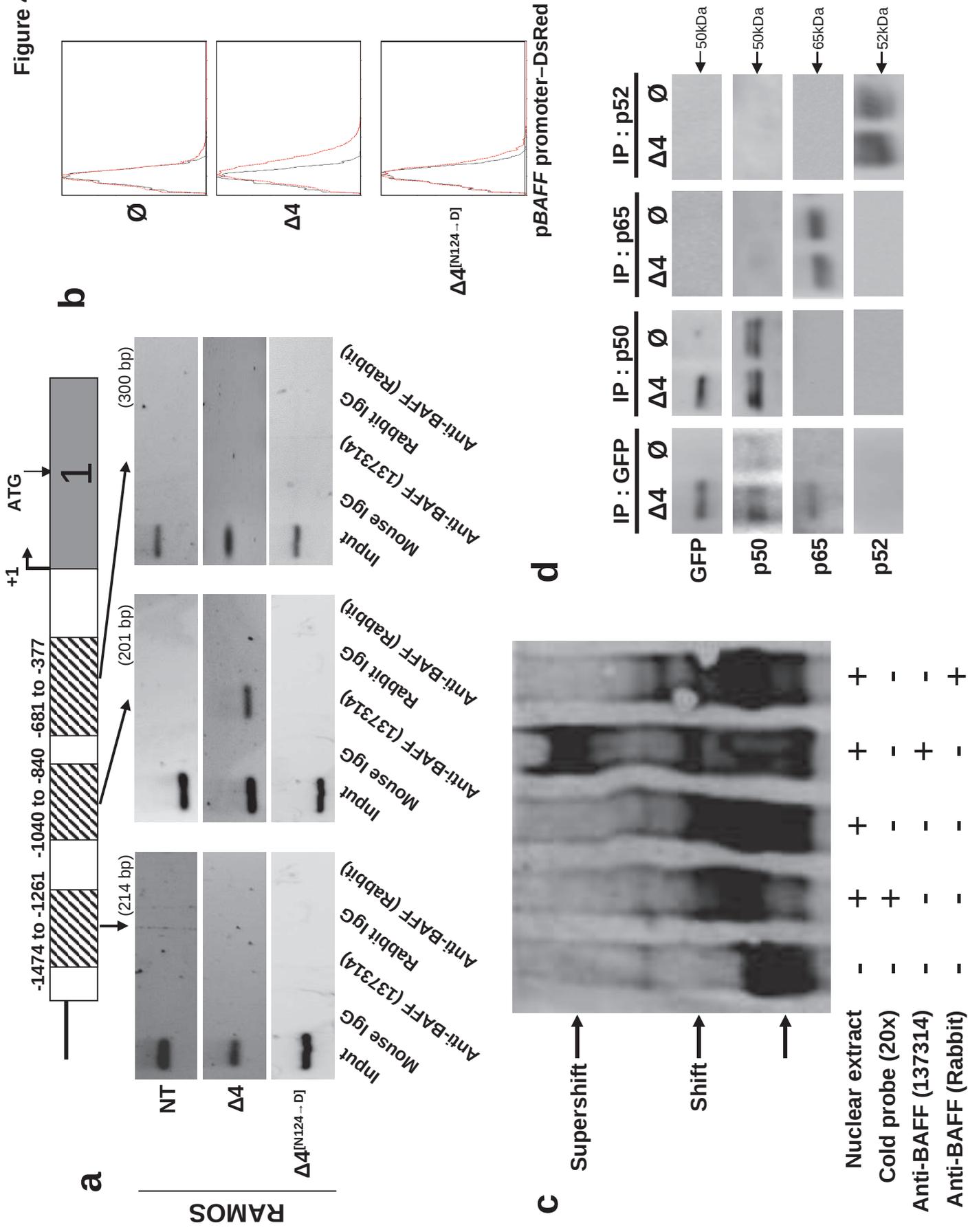


Figure 5

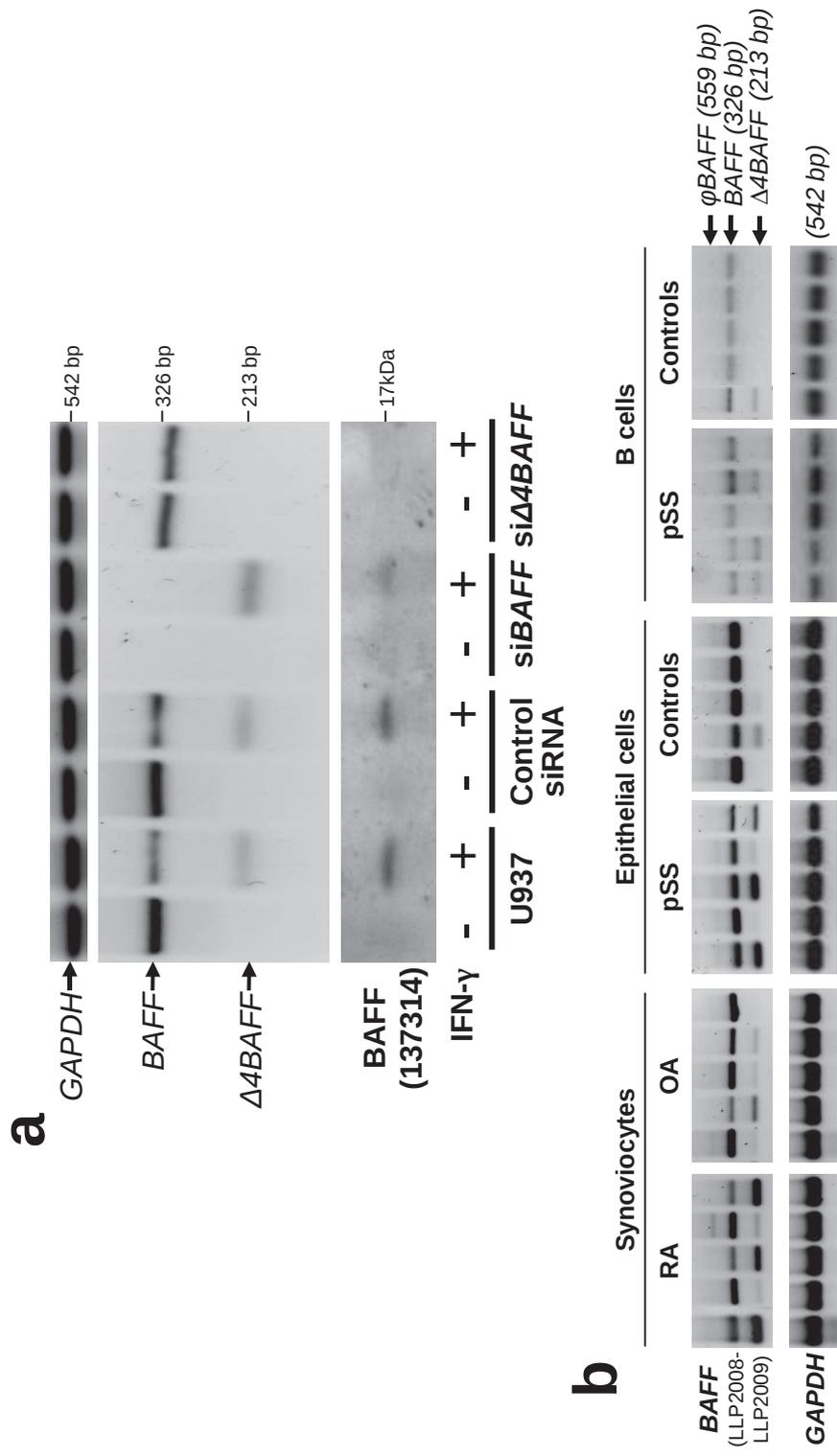
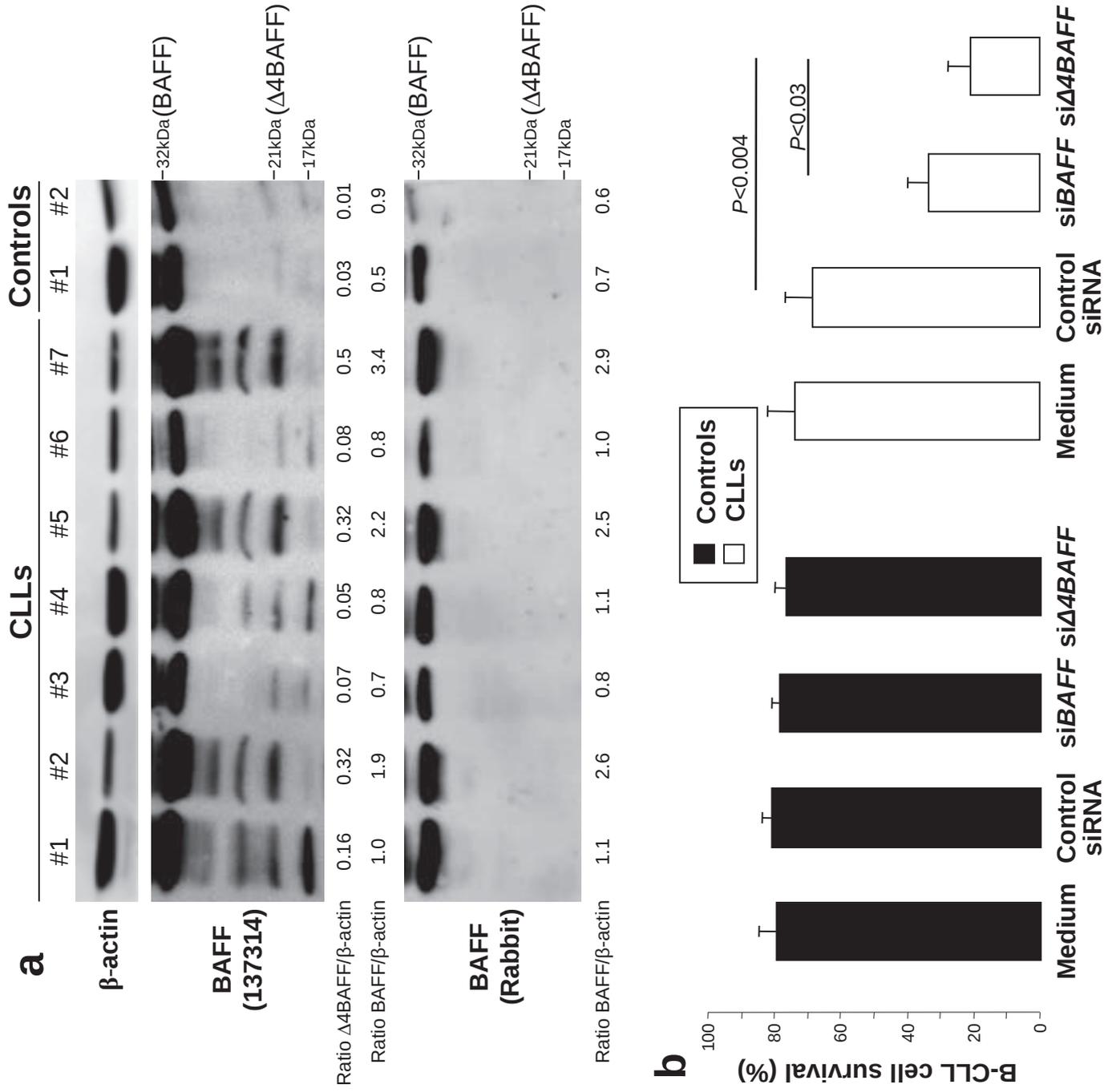


Figure 6



SUPPLEMENTARY INFORMATIONS

Supplementary Methods

RNA extraction and Reverse Transcription

Total RNA was extracted by the RNable method (Eurobio, Les Ulis, France), and reverse-transcribed with SuperScript® II Reverse Transcriptase (RT) according to the manufacturer's instructions (Invitrogen).

Baff PCR

For mouse full-length *Baff*, we used primer pairs mouse *Baff* (exon 5)/mouse *Baff* (exon 6) and mouse $\Delta 5Baff$ (exon 4-6)/mouse $\Delta 5Baff$ (exon 7) for mouse $\Delta 5Baff$. For real-time PCR, all amplifications used SYBR Green® Master Mix (Applied Biosystems, Foster City, CA) in same conditions described in microarray analysis. *Baff* gene expression was normalized to *Hprt* expression. Primers used are listed in **Supplementary Table 2**.

Study of NMD

3×10^6 RAMOS cells/well in a 6-well plate were stimulated with 50 ng/mL IFN- γ (R&D Systems) for 24 h to induce $\Delta 4BAFF$ transcript. After the incubation, cells were left untreated or treated with the translation inhibitor cycloheximide (CHX) (10 μ g/mL). After 2 h incubation, BAFF and $\Delta 4BAFF$ transcript expression were measured by RT-PCR as previously described.

Plasmid construction and site-directed mutagenesis

The p $\Delta 4BAFF$ -IRES2-EGFP and the p $\Delta 4BAFF$ -EGFP were constructed by first PCR amplifying $\Delta 4BAFF$ (primers listed in **Supplementary Table 2**) and then ligated into

pIRES2-EGFP and pEGFP-N1 (both from Clontech) at the XhoI and EcoRI sites (map in **Supplementary Fig.4**).

To study N-linked glycosylation of the new alternative splice variant, p Δ 4BAFF-IRES2-EGFP and p Δ 4BAFF-EGFP were mutated using the Quick-Change Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA). The asparagine 124 (N¹²⁴) was mutated to aspartic acid (D¹²⁴) by substitution of 1 nucleotide (⁴⁶⁹AAC⁴⁷¹ → ⁴⁶⁹GAC⁴⁷¹). The new vectors were called p Δ 4^[N124→D]BAFF-IRES2-EGFP and p Δ 4^[N124→D]BAFF-EGFP respectively.

Stable transfection of Δ 4BAFF in RAMOS cell line

RAMOS cell-line cells were transfected with pIRES2-EGFP, p Δ 4BAFF-IRES2-EGFP or p Δ 4^[N124→D]BAFF-IRES2-EGFP as described in “transient transfection of Δ 4BAFF”. Twenty-four hours after transfection, RAMOS GFP-positive cells, from each condition, were selected by FACS cell sorting. Cells were cultured in RPMI 1640 medium supplemented with G418, a selective antibiotic for Neomycin resistance (PAA laboratories). Once a week, GFP positive cells were sorted by FACS until 3 stably transfected cell lines were obtained. The new cell lines were called: Control (pIRES2-EGFP), Delta4BAFF (p Δ 4BAFF-IRES2-EGFP) and Mutated Delta4BAFF (p Δ 4^[N124→D]BAFF-IRES2-EGFP).

Reporter of Gene Expression

The pBAFFpromoter-DsRed was constructed by first PCR amplifying the BAFF promoter (region 1) and then ligating the purified PCR product into pDsRed-Express1 (Clontech, map in **Supplementary Fig.4**) at the XhoI and HindIII sites.

After transient transfection of pBAFFpromoter-DsRed into 3 stably transfected RAMOS cells, BAFF promoter expression was monitored at 24 hours after transfection using flow cytometry and confocal microscopy.

Western blotting

Equal amounts of protein from cell lysates or subcellular fractionation were separated on 12% SDS–polyacrylamide gel electrophoresis (Bio-Rad, Marnes-la-Coquette, France) in reducing conditions and transferred. Unoccupied sites were blocked by incubation in PBS containing 0.1% Tween-20 and 5% non-fat milk for 1 hr. Membranes were probed with anti-BAFF mAb (clone 137314, R&D systems, Minneapolis, MN) or anti- β -actin mAb (Clone mAbcam 8226, Abcam, Cambridge, MA) overnight at 4°C. Bound antibodies were developed with Horse radish peroxydase-secondary antibodies, (GE Healthcare) and visualized using the enhanced chemiluminescence system: ECL advance western blotting detection (GE Healthcare). The intensity of BAFF was expressed relative to β -actin. For a second development with rabbit polyclonal anti-BAFF (Upstate Biotechnology, Lake Placid, NY), membranes were stripped with Tris HCl buffer pH 7.4 with 2% SDS and β -mercaptoethanol 100mM by heating 30 min at 50°C. Control Abs for the subcellular fractionation were β -actin for cytosolic fraction, CD20 (Thermo Scientific, Fremont, CA) for membrane/organelles fraction and Histone H3 (Abcam) for the nuclear fraction.

Labeling of BAFF promoter oligonucleotides

The double-stranded oligonucleotides encoding the target sequence of NF- κ B family in the BAFF promoter were developed. 10 X DIG DNA Labeling Mix (Roche Applied Science) was used for non-radioactive label of the probe according to manufacturer's instructions. Labeling efficacy was determined by anti-DIG-POD antibody (Roche Applied Science).

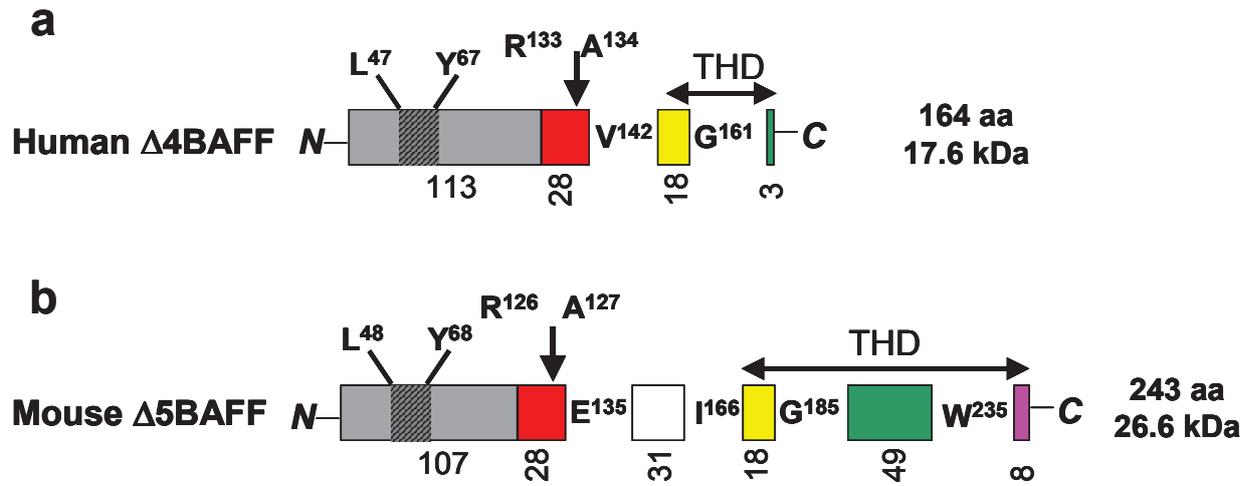
Total lysates, subcellular fractionation

RAMOS GFP-positive cells were selected by FACS cell sorting twenty-four hours post-transfection with either p Δ 4BAFF-IRES2-EGFP and p Δ 4^[N124→D]BAFF-IRES2-EGFP and subjected to total lysis and subcellular fractionation. Total lysates were made with Tris HCl

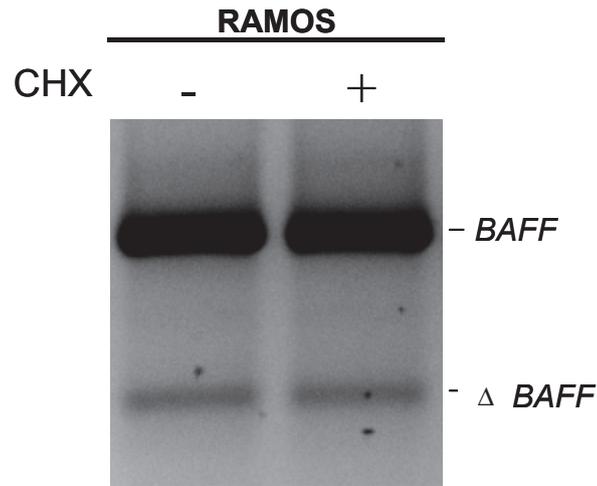
pH 7.5, NaCl 150 mM, Igepal 1%, EDTA 2 mM, NaF 5 mM and proteases inhibitor cocktail (all from Sigma-Aldrich, Saint-Quentin Fallavier, France). Cells were homogenized with this lysis buffer during 1 h at 4°C, and centrifugated 20 min at 500g. Subcellular fractionation was achieved with the ProteoExtract Subcellular proteome extraction kit (Calbiochem Merck Chemicals, Nottingham, UK) according to manufacturer's instructions.

Analysis of glycosylation

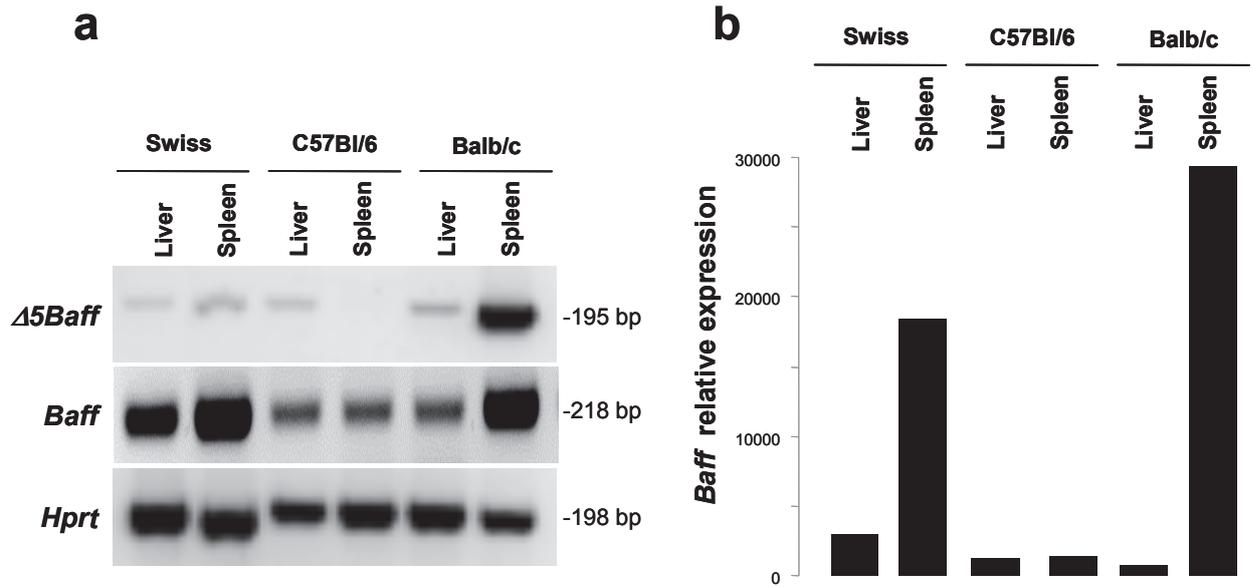
Total lysates were deglycosylated enzymatically. The enzyme used was the peptide N-glycosydase F (Roche Applied science, Meylan, France) according to manufacturer's instructions.



Supplementary Fig.1 : Representation of the 164 and 243 amino acid (aa) domain structure of Δ 4BAFF in humans (a) and Δ 5BAFF in mice (b). The schematic representations show the transmembrane region (hatched area) and the TNF homology domain (THD). Black arrows indicate the cleavage site for furin. Abbreviations: alanine (A), glutamic acid (E), glycine (G), isoleucine (I), leucine (L), arginine (R), tyrosine (Y).



Supplementary Fig.2 : RT-PCR on cDNA from 2-hours cycloheximide (CHX)-treated or untreated RAMOS cell line showed that $\Delta 4BAFF$ transcript did not increase after treatment, indicating that the transcript is stable and not subjected to NMD degradation. RAMOS cells were pre-stimulated with IFN- γ for 24 hours to induce $\Delta 4BAFF$ transcript.



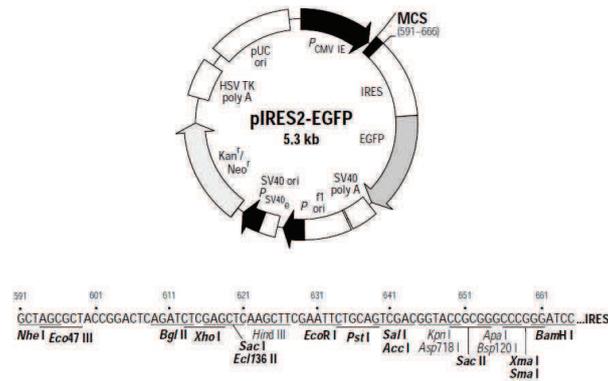
Supplementary Fig.3: Expression and characterization of *Δ5BAFF* transcript in mice. (a) RT-PCR analysis showing that the *Δ5BAFF* alternative splice variant was observed in the spleen from Swiss and Balb/c mice but absent from C57Bl/6 mice. (b) Real-time PCR analysis of the full-length form of *BAFF* from mononuclear cells isolated from the liver and the spleen of different mice strains (Swiss, Balb/c and C57Bl/6). An increase of full-length *BAFF* transcript was observed when *Δ5BAFF* was present.

a

pIRES2-EGFP Vector Information

PT3267-5

Catalog #6029-1

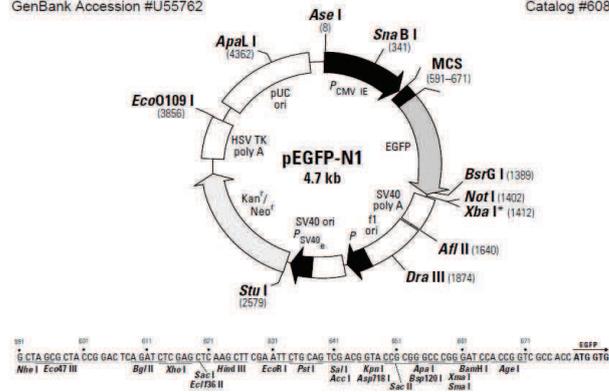
**b**

pEGFP-N1 Vector Information

PT3027-5

GenBank Accession #U55762

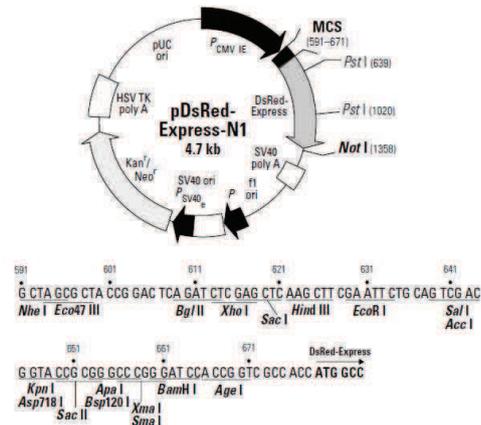
Catalog #6085-1

**c**

pDsRed-Express-N1 Vector Information

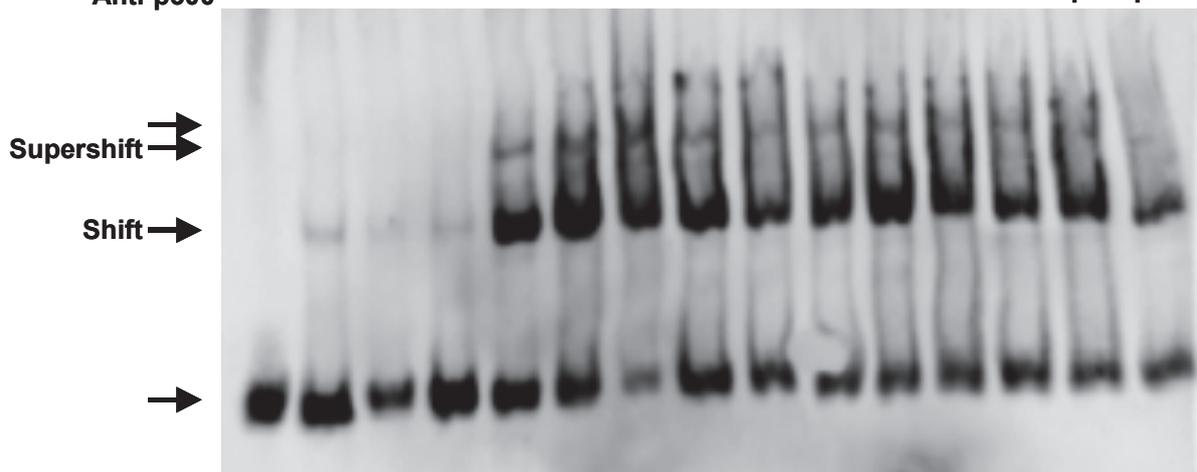
PT3725-5

Cat. No. 632429



Supplementary Figure 4 : Maps of vectors used in this study. (a) pIRES2-EGFP was used to characterize Δ 4BAFF (Western blotting, microarray analysis and ChIP) and to establish the stably transfected RAMOS cell lines. **(b)** pEGFP-N1 was used to observe the subcellular localization of Δ 4BAFF (confocal microscopy) and to confirm its role as a transcription factor for its own parent gene (EMSA and co-immunoprecipitation). **(c)** pDsRed-Express1 vector was used to cloned *BAFF* promoter region 1.

| | | | | | | | | | | | | | | |
|--------------------|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Nuclear extract | - | + | + | + | + | + | + | + | + | + | + | + | + | + |
| Cold probe (20x) | - | - | + | - | - | - | - | - | - | - | - | - | - | - |
| Anti-BAFF (Rabbit) | - | - | - | + | - | - | - | - | - | - | - | - | - | - |
| Anti-BAFF (137314) | - | - | - | - | + | - | + | - | + | - | + | - | + | - |
| Anti-p50 | - | - | - | - | - | + | + | - | - | - | - | - | - | - |
| Anti-p52 | - | - | - | - | - | - | - | + | + | - | - | - | - | - |
| Anti-cRel | - | - | - | - | - | - | - | - | - | + | + | - | - | - |
| Anti-p65 | - | - | - | - | - | - | - | - | - | - | - | + | + | - |
| Anti-p300 | - | - | - | - | - | - | - | - | - | - | - | - | - | + |



Supplementary Fig. 5 : EMSA analysis of Δ 4BAFF and NF- κ B binding to the *BAFF* promoter. Nuclear extracts from RAMOS cells stably transfected with p Δ 4*BAFF*-EGFP were incubated with digoxigenin-labeled BAFF-NF- κ B binding site oligonucleotides. BAFF-NF- κ B cold probe and antibodies (Abs) to BAFF (the monoclonal Ab, clone 137314, able to recognize Δ 4BAFF and the rabbit polyclonal Ab, unable to recognize Δ 4BAFF), p50, p52, cRel, p65 or p300 were added to the binding reaction mixtures. Arrows indicate the digoxigenin-labeled probe, the DNA-protein complex, and the supershifted complexes. Remarkably, anti-p50 and anti-BAFF monoclonal Ab together further shifted the complex.

Supplementary Table 1 : List of selected genes to illustrate the functional annotation analysis (DAVID). $\Delta 4$: RAMOS B cells stably transfected with p $\Delta 4B AFF$ -IRES2-EGFP, $\Delta 4^{[N124 \rightarrow D]}$; RAMOS B cells stably transfected with p $\Delta 4B AFF^{[N124 \rightarrow D]}$ -IRES2-EGFP, \emptyset : RAMOS B cells stably transfected with pIRES2-EGFP.

Up-regulated genes

| | Fold Change absolute | | ProbeName |
|--|-----------------------------------|---|--------------|
| | [$\Delta 4$] vs [\emptyset] | [$\Delta 4$] vs [$\Delta 4^{[N124 \rightarrow D]}$] | |
| IMMUNE SYSTEME (36 genes) | | | |
| Activation-induced cytidine deaminase (AICDA) | 3,51 | 9,09 | A_23_P36641 |
| CD1c molecule (CD1C) | 11,54 | 9,61 | A_23_P51767 |
| CD27 molecule (CD27) | 2,34 | 3,25 | A_23_P48088 |
| CD28 molecule (CD28) | 135,75 | 95,09 | A_23_P91095 |
| Fc fragment of IgG, low affinity IIb, receptor (CD32) (FCGR2B) | 11,48 | 15,79 | A_24_P938284 |
| CD79a molecule, immunoglobulin-associated alpha (CD79A) | 3,18 | 2,8 | A_23_P107735 |
| CD80 molecule (CD80) | 4,84 | 3,88 | A_23_P155632 |
| Interleukin 10 receptor, alpha (IL10RA) | 7,02 | 7,76 | A_23_P203173 |
| Interleukin 17D (IL17D) | 1,85 | 2,04 | A_23_P345692 |
| Interleukin 20 receptor beta (IL20RB) | 5,13 | 3,28 | A_23_P91850 |
| Interleukin 2 receptor, gamma (IL2RG) | 2,61 | 2,91 | A_23_P148473 |
| Interleukin 4 receptor (IL4R) | 1,46 | 2,38 | A_23_P129556 |
| Lymphocyte-specific protein tyrosine kinase (LCK) | 2,55 | 1,85 | A_23_P103361 |
| Protein kinase C, delta (PRKCD) | 2,15 | 1,98 | A_23_P144054 |
| Sphingosine-1-phosphate lyase 1 (SGPL1) | 3,08 | 1,94 | A_23_P75325 |
| SWAP switching B-cell complex 70kDa subunit | 2,76 | 1,77 | A_24_P359165 |
| T-box 21 (TBX21) | 2,62 | 3,83 | A_23_P141555 |
| Transforming growth factor, beta 1 (TGFB1) | 3,9 | 3,88 | A_24_P79054 |
| Toll-like receptor 1 (TLR1) | 2,59 | 2,58 | A_23_P10873 |
| Toll-like receptor 10 (TLR10) | 5,43 | 4,7 | A_23_P33420 |
| Toll-like receptor 2 (TLR2) | 28,46 | 22,67 | A_23_P92499 |
| Toll-like receptor 4 (TLR4) | 3,65 | 3,14 | A_32_P66881 |
| Toll-like receptor 6 (TLR6) | 4,59 | 3,84 | A_23_P256561 |
| Toll-like receptor 9 (TLR9) | 3,57 | 2,82 | A_23_P132654 |
| Tumor necrosis factor receptor superfamily, member 17 (TNFRSF17) | 1,53 | 2,78 | A_23_P37736 |

Tumor necrosis factor (ligand) superfamily, member 15 (TNFSF15)

9,45

9,16

A_32_P158181

PROTEIN LOCALIZATION (102 genes)

| | | | |
|--|------|------|--------------|
| Calreticulin (CALR) | 2,04 | 1,77 | A_23_P67288 |
| Centrosomal protein 250kDa (CEP250) | 2,11 | 2,09 | A_24_P162287 |
| Golgi SNAP receptor complex member 2 (GOSR2) | 2,67 | 2,97 | A_23_P207719 |
| Importin 4 (IPO4) | 3,73 | 3,56 | A_23_P162970 |
| Nucleoporin 160kDa (NUP160) | 2,33 | 2,47 | A_24_P922606 |
| Peroxisomal biogenesis factor 14 | 2,97 | 3,59 | A_24_P921232 |
| Phosphorylated adaptor for RNA export (PHAX) | 2,22 | 2,13 | A_24_P191884 |
| Syntaxin 6 (STX6) | 1,79 | 2,01 | A_24_P639505 |
| Talin 2 (TLN2) | 1,84 | 2,24 | A_24_P347566 |
| Trafficking protein, kinesin binding 2 (TRAK2) | 2,43 | 2,08 | A_24_P122874 |
| Exportin 4 (XPO4) | 2,01 | 1,94 | A_24_P333716 |

RNA PROCESSING (97 genes)

| | | | |
|---|-------|-------|--------------|
| Activation-induced cytidine deaminase (AICDA) | 3,51 | 9,09 | A_23_P36641 |
| Cytoplasmic polyadenylation element binding protein 1 (CPEB1) | 3,88 | 11,49 | A_23_P106322 |
| Heterogeneous nuclear ribonucleoprotein A1-like 2 (HNRNPA1L2) | 1,85 | 2,02 | A_23_P364056 |
| Heterogeneous nuclear ribonucleoprotein L-like | 6,07 | 5,87 | A_24_P916378 |
| MIR155 host gene (non-protein coding) (MIR155HG) | 13,16 | 39,81 | A_32_P108156 |
| Poly(A) polymerase alpha (PAPOLA) | 1,83 | 2,08 | A_23_P376239 |
| Polymerase (RNA) II (DNA directed) polypeptide A, 220kDa (POLR2A) | 2 | 1,86 | A_24_P99679 |
| RNA binding motif protein 15B (RBM15B) | 2,95 | 3,1 | A_24_P945181 |
| Splicing factor 3a, subunit 3, 60kDa (SF3A3) | 2,13 | 2,24 | A_24_P603224 |
| Splicing factor 3b, subunit 3, 130kDa (SF3B3) | 2,82 | 2,6 | A_24_P945396 |
| Small nuclear ribonucleoprotein 25kDa (U11/U12) (SNRNP25) | 2,9 | 3,03 | A_23_P152284 |
| Small nuclear ribonucleoprotein 70kDa (U1) (SNRNP70) | 2,2 | 2,45 | A_23_P4902 |
| tRNA nucleotidyl transferase, CCA-adding, 1 | 2,06 | 2,61 | A_23_P6786 |

TRANSLATION (54 genes)

| | | | |
|--|------|------|--------------|
| Eukaryotic translation elongation factor 1 epsilon 1 (EEF1E1) | 2,16 | 1,97 | A_23_P156842 |
| Eukaryotic translation initiation factor 2B, subunit 3 gamma, 58kDa (EIF2B3) | 2,05 | 1,9 | A_23_P115046 |
| Eukaryotic translation initiation factor 2, subunit 1 alpha, 35kDa (EIF2S1) | 2,27 | 2,08 | A_24_P138556 |
| Eukaryotic translation initiation factor 4 gamma, 2 (EIF4G2) | 2,4 | 2,19 | A_23_P104892 |
| Eukaryotic translation termination factor 1 (ETF1) | 2,11 | 1,98 | A_23_P133582 |
| Ribosomal protein L27a (RPL27A) | 6,01 | 7,5 | A_23_P416305 |
| Ribosomal protein S6 kinase, 70kDa, polypeptide 2 (RPS6KB2) | 2,09 | 1,88 | A_23_P24318 |

REGULATION OF APOPTOSIS (85 genes)

| | | | |
|---|------|------|--------------|
| BH3 interacting domain death agonist (BID) | 2,63 | 2,61 | A_23_P154929 |
| Apoptosis, caspase activation inhibitor (AVEN) | 2,19 | 3,47 | A_23_P100074 |
| Caspase recruitment domain family, member 6 (CARD6) | 2,69 | 1,41 | A_23_P41854 |
| Tumor necrosis factor (ligand) superfamily, member 15 (TNFSF15) | 2,58 | 2,58 | A_23_P94754 |

Down-regulated genes

| REGULATION OF TRANSCRIPTION (306 genes) | Fold Change absolute | | ProbeName |
|---|----------------------|-----------------------------------|--------------|
| | [Δ4] vs [∅] | [Δ4] vs [Δ4 ^(N124→D)] | |
| AT rich interactive domain 3B (BRIGHT-like) (ARID3B) | 2,81 | 2,93 | A_23_P88580 |
| Activating transcription factor 5 (ATF5) | 3,54 | 3,94 | A_23_P119337 |
| BCL6 corepressor | 2,95 | 2,54 | A_23_P405707 |
| CCAAT/enhancer binding protein (C/EBP), beta (CEBPB) | 2,6 | 3,35 | A_23_P411296 |
| CCR4-NOT transcription complex, subunit 4 | 2,06 | 2,3 | A_24_P941922 |
| GATA zinc finger domain containing 1 (GATAD1) | 2,56 | 2,46 | A_24_P35169 |
| HMG-box transcription factor 1 (HBP1) | 3,45 | 7,59 | A_24_P204971 |
| Histone deacetylase 4 (HDAC4) | 4,18 | 5,08 | A_24_P359856 |
| Homeobox A4 (HOXA4) | 5,36 | 5,05 | A_23_P253982 |
| Inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase beta (IKKB) | 2,83 | 2,87 | A_23_P216188 |
| Interferon regulatory factor 9 (IRF9) | 3,68 | 5,27 | A_23_P65442 |
| Jun proto-oncogene (JUN) | 5,84 | 14,54 | A_23_P201538 |
| Methyl-CpG binding domain protein 2 (MBD2) | 2,39 | 3,19 | A_32_P107777 |
| Nuclear receptor subfamily 1, group D, member 2 (NR1D2) | 1,62 | 2,25 | A_23_P302709 |
| Pim-1 oncogene (PIM1) | 4,92 | 5,35 | A_23_P345118 |
| Polymerase (DNA directed), eta (POLH) | 2,01 | 2,27 | A_32_P96692 |
| Polymerase (DNA directed) iota (POLI) | 3,12 | 2,73 | A_23_P306890 |
| SMAD family member 4 (SMAD4) | 2,12 | 2,17 | A_23_P27346 |
| Sterol regulatory element binding transcription factor 2 (SREBF2) | 1,99 | 2,01 | A_23_P419602 |
| Transcription elongation factor A (SII), 2 (TCEA2) | 2,7 | 2,88 | A_23_P147641 |
| TATA element modulatory factor 1 (TMF1) | 2,17 | 2,27 | A_23_P143867 |

IMMUNE RESPONSE (103 genes)

| | | | |
|---|-------|-------|--------------|
| Chemokine (C-C motif) ligand 13 (CCL13) | 3,27 | 1,08 | A_23_P26965 |
| CD22 molecule (CD22) | 1,57 | 2,22 | A_23_P209055 |
| CD48 molecule (CD48) | 2,46 | 2,6 | A_32_P175934 |
| CD55 molecule, decay accelerating factor for complement (Cromer blood group) (CD55) | 2 | 2,25 | A_24_P188377 |
| CD59 molecule, complement regulatory protein (CD59) | 2,03 | 2,6 | A_24_P639441 |
| CD69 molecule (CD69) | 5,14 | 3,57 | A_23_P87879 |
| CD70 molecule (CD70) | 2,29 | 2,43 | A_23_P119202 |
| CD72 molecule (CD72) | 2,03 | 2,67 | A_23_P250245 |
| chemokine (C-X-C motif) receptor 2 (CXCR2) | 15,34 | 12,07 | A_23_P135755 |
| Chemokine (C-X-C motif) receptor 4 (CXCR4) | 2,4 | 2,69 | A_23_P102000 |
| Major histocompatibility complex, class I, A (HLA-A) | 2,75 | 3,03 | A_24_P376483 |
| Major histocompatibility complex, class I, B (HLA-B) | 2,32 | 2,53 | A_23_P125107 |
| Major histocompatibility complex, class II, DO alpha (HLA-DOA) | 2,53 | 2 | A_32_P356316 |
| Major histocompatibility complex, class II, DP beta 1 (HLA-DPB1) | 2,13 | 2,05 | A_24_P166443 |
| Major histocompatibility complex, class II, DQ alpha 1 (HLA-DQA1) | 4,62 | 6,76 | A_24_P196827 |
| Major histocompatibility complex, class I, E (HLA-E) | 3,4 | 3,54 | A_32_P460973 |
| inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase beta (IKKB) | 2,83 | 2,87 | A_23_P216188 |
| Interleukin 11 (IL11) | 1,78 | 2,9 | A_23_P67169 |
| Interleukin 11 receptor, alpha (IL11RA) | 1,88 | 2,37 | A_23_P83277 |
| Interleukin 15 (IL15) | 6,88 | 6,88 | A_23_P29953 |
| Interleukin 22 receptor, alpha 1 (IL22RA1) | 2,12 | 3,05 | A_24_P413479 |
| Interleukin 23, alpha subunit p19 (IL23A) | 9,76 | 16,26 | A_23_P76078 |
| Interleukin 27 (IL27) | 3,06 | 3,21 | A_23_P315320 |
| Interleukin 32 (IL32) | 1,97 | 2,81 | A_23_P15146 |
| Interferon regulatory factor 4 (IRF4) | 2,12 | 2,3 | A_23_P156505 |
| Lymphotoxin alpha (TNF superfamily, member 1) (LTA) | 1,44 | 2,5 | A_23_P156683 |
| Leukocyte receptor tyrosine kinase (LTK) | 1,9 | 2,08 | A_23_P14853 |
| MHC class I polypeptide-related sequence B (MICB) | 2,23 | 2,28 | A_23_P387471 |
| MHC class I polypeptide-related sequence B (MICB) | 2,3 | 2,47 | A_32_P74983 |
| MHC class I polypeptide-related sequence B (MICB) | 2,34 | 2,39 | A_24_P169688 |

| | | | |
|--|-------|------|--------------|
| Nuclear factor, interleukin 3 regulated (NFIL3) | 3,9 | 3,01 | A_23_P32253 |
| Recombination activating gene 1 (RAG1) | 30,21 | 3,44 | A_23_P360744 |
| Recombination activating gene 2 (RAG2) | 58,56 | 7,1 | A_23_P64525 |
| Sphingosine-1-phosphate receptor 4 (S1PR4) | 6,24 | 3,09 | A_23_P119502 |
| Tumor necrosis factor receptor superfamily, member 10b (TNFRSF10B) | 2,32 | 2,55 | A_24_P218265 |
| Tumor necrosis factor receptor superfamily, member 13C (TNFRSF13C) | 1,75 | 2,3 | A_23_P91764 |
| Tumor necrosis factor receptor superfamily, member 14 (TNFRSF14) | 2,83 | 3,7 | A_23_P126908 |
| Tumor necrosis factor (ligand) superfamily, member 9 (TNFSF9) | 2,17 | 2,29 | A_24_P5856 |
| TNF receptor-associated factor 3 (TRAF3) | 2,51 | 2,09 | A_23_P37068 |
| TRAF3 interacting protein 2 (TRAF3IP2) | 2,79 | 3,11 | A_23_P110879 |
| TNF receptor-associated factor 5 (TRAF5) | 1,78 | 2,32 | A_23_P201731 |

CELLULAR HOMEOSTASIS (59 genes)

| | | | |
|---|-------|-------|--------------|
| ORAI calcium release-activated calcium modulator 3 (ORAI3) | 4,14 | 4,14 | A_23_P106898 |
| Phospholipase C, beta 1 (phosphoinositide-specific) (PLCB1) | 11,51 | 35,75 | A_24_P941643 |
| Phospholipase C, delta 4 (PLCD4) | 5,37 | 8,85 | A_23_P385105 |
| Phospholipase C, eta 2 (PLCH2) | 3,2 | 4,23 | A_24_P62469 |
| Phospholipase D family, member 6 (PLD6) | 17,93 | 21,57 | A_23_P360626 |
| Ubiquitin-like modifier activating enzyme 7 (UBA7) | 2,42 | 2,68 | A_23_P21207 |
| Ubiquitin-conjugating enzyme E2B (RAD6 homolog) | 2,27 | 4,62 | A_23_P362415 |
| Ubiquitin specific peptidase 18 (USP18) | 1,92 | 3,01 | A_32_P132206 |
| Ubiquitin specific peptidase 3 (USP3) | 2,39 | 2,42 | A_23_P170467 |
| Calcium channel, voltage-dependent, beta 4 subunit (CACNB4) | 3,93 | 9,49 | A_23_P209227 |
| Chemokine (C-X-C motif) receptor 4 (CXCR4) | 2,4 | 2,69 | A_23_P102000 |
| Chloride channel 6 (CLCN6) | 2,69 | 2,95 | A_24_P302374 |
| Galactose-3-O-sulfotransferase 1 (GAL3ST1) | 11,62 | 19,81 | A_23_P120863 |
| Jun proto-oncogene (JUN) | 5,84 | 14,54 | A_23_P201538 |
| Potassium voltage-gated channel, Isk-related family, member 1 (KCNE1) | 2,07 | 1 | A_23_P154855 |

Supplementary Table 2 : Sequences of primers used in this study

| Name | Sequence | Purpose |
|--|--|--------------------------------|
| <i>BAFF</i> JT1322 (exon 2) sens | GGAGAAGGCAACTCCAGTCAGAAC | PCR |
| <i>BAFF</i> JT1323 (exon 5) antisens | CAATTCATCCCCAAAGACATGGAC | PCR |
| <i>BAFF</i> LLP2008 (exon 3) sens | TTGCAGACAGTGAAACACCAACT | PCR |
| <i>BAFF</i> LLP2009 (exon 6) antisens | TTCATCTCCTTCTTCCAGTTTTGC | PCR |
| <i>GAPDH</i> sens | CTTAGCACCCCTGGCCAAGG | PCR |
| <i>GAPDH</i> antisens | CTTACTCCTTGGAGGCCATG | PCR |
| <i>BAFF</i> (exon 2) sens | CAGCTCCAGGAGAAGGCAACT | 3'-RACE-PCR |
| $\Delta 4$ <i>BAFF</i> sens 1 | CTGTACAGTAGGGGTAGAGATGCAGAAAG | Cloning $\Delta 4$ <i>BAFF</i> |
| $\Delta 4$ <i>BAFF</i> antisens 1 | TTCATCTCCTTCTTCCAGTTTTGC | Cloning $\Delta 4$ <i>BAFF</i> |
| $\Delta 4$ <i>BAFF</i> nested sens | CACAGATAACAGGAAATGATCCATTCCC | Cloning $\Delta 4$ <i>BAFF</i> |
| $\Delta 4$ <i>BAFF</i> nested antisens | TAGATGTCCCATGGCGTAGGTC | Cloning $\Delta 4$ <i>BAFF</i> |
| $\Delta 4$ <i>BAFF</i> sens 2 | CTCAGATCTCGAGAGCCACCATGGATGACTCCAC | Cloning in pIRES2-EGFP |
| $\Delta 4$ <i>BAFF</i> antisens 2 | CGACTGCAGAATTCATTATCAGTATATAAAACCTTTTTGTATAGTTGG | Cloning in pIRES2-EGFP |
| $\Delta 4$ <i>BAFF</i> sens 3 | CTCAGATCTCGAGAGCCACCATGGATGACTCCAC | Cloning in pEGFP-N1 |
| $\Delta 4$ <i>BAFF</i> antisens 3 | CGACTGCAGAATTCCTCGGTATATAAAACCTTTTTGTATAGTTGG | Cloning in pEGFP-N1 |
| <i>BAFF</i> promoter (region 1) sens | GAGACAGAACTAAAGCTCACTATTCTT | ChIP and EMSA |
| <i>BAFF</i> promoter (region 1) antisens | GACCTGTGAGGACTGTTGCA | ChIP and EMSA |
| <i>BAFF</i> promoter (region 2) sens | AGGCAAGGCTGATTCTCCTC | ChIP |
| <i>BAFF</i> promoter (region 2) antisens | GGAAGTGTGGAAGTAAGTCCACTG | ChIP |
| <i>BAFF</i> promoter (upstream) sens | GACTTTAGGGACTCAGGGGAAAG | ChIP |
| <i>BAFF</i> promoter (upstream) antisens | GAAACAAATTACATTTTGGATGC | ChIP |
| <i>BAFF</i> promoter sens | ATCACTCGAGGGGTCTGGAGTTCTCCACTT-TGCAC | Cloning in pDsRed-Express1 |
| <i>BAFF</i> promoter antisens | GACTAAGCTTGACCTGTGAGGACTGTTGCA | Cloning in pDsRed-Express1 |
| <i>TLR2</i> sens | CCACCGTTTCCATGGCCTGTG | Real-time PCR |
| <i>TLR2</i> antisens | GATGAAGTTCTCCAGCTCCTGCACC | Real-time PCR |
| <i>TLR6</i> sens | ATGTGGCAGCTTTCGAGCCT | Real-time PCR |
| <i>TLR6</i> antisens | TTGAACTCATCTTCTGGCAGC | Real-time PCR |
| <i>TLR9</i> sens | TGAAGACTTCAGGCCAACTG | Real-time PCR |
| <i>TLR9</i> antisens | TGCACGGTCACCAGTTGT | Real-time PCR |
| <i>TLR10</i> sens | GTAAGGCTATCAAAGGAGATGTGAGA | Real-time PCR |
| <i>TLR10</i> antisens | GAGGAGAAGCATAATGGACCTTTG | Real-time PCR |
| <i>AICDA</i> sens | CCACTATGGACAGCCTCTTG | Real-time PCR |
| <i>AICDA</i> antisens | CACTGTCACGCCTCTTCACT | Real-time PCR |
| <i>MBD2</i> sens | CCATGGAACACCCAAAGGTCTT | Real-time PCR |
| <i>MBD2</i> antisens | CAGCAGATAAAAGGTCTCATCATT | Real-time PCR |
| <i>GAPDH</i> sens | TGCACCACCAACTGCTTAGC | Real-time PCR |
| <i>GAPDH</i> antisens | GGCATGGACTGTGGTCATGAG | Real-time PCR |
| mouse <i>Baff</i> (exon 5) sens | TTCCATGGCTTCTCAGCTTT | PCR and Real-time PCR |
| mouse <i>Baff</i> (exon 6) antisens | TTCTGAATACATCGGAACAGGG | PCR and Real-time PCR |
| mouse $\Delta 5$ <i>Baff</i> (exon 4-6) sens | CGACTATACGAAAAGTTCTATAC | PCR |
| mouse $\Delta 5$ <i>Baff</i> (exon 7) antisens | TCATCTCCTTCTTCCAGCCT | PCR |
| mouse <i>Hprt</i> sens | AGCTACTGTAATGATCAGTCA | PCR and Real-time PCR |
| mouse <i>Hprt</i> antisens | AGAGGTCCTTTTACCAGCA | PCR and Real-time PCR |

Article 5

Alternative splicing in exon 4 of BAFF is regulated by interferon-gamma and SC35 protein.

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Manuscript in preparation

Alternative splicing in exon 4 of BAFF is regulated by interferon-gamma and SC35 protein.

Based on the effects of the new-described Δ 4BAFF form over the full-length form of BAFF and its main expression in AIDs and CLL, we analyzed **in the fifth article** the mechanisms for Δ 4BAFF induction and regulation in the autoimmune setting.

First, we tested different stimulations already known as positive regulators of BAFF (such IFN- γ and IFN- α) and their effects on Δ 4BAFF induction. The preliminary results showed that IFN- γ favors the Δ 4BAFF expression in different cell lines (including monocytes, B cells and ECs). Finally, to study the mechanisms implicated in the alternative splicing of exon 4, we transfected in RAMOS B cells a minigene construct, centered on exon 4 and this minigene was then co-transfected with coding plasmids for splicing proteins. These experiments allowed us to conclude that a SR protein (SC35) is essential for the constitutive splicing of BAFF. Interestingly, IFN- γ stimulation induced the alternative splicing of the exon 4, inducing an increase of some hnRNPs leading to binding-site competition with SC35 that is finally abnormally located in the perinuclear space. With these results we can conclude that an inflammatory loop between IFN- γ and BAFF pass through the induction of Δ 4BAFF. These last two articles provide an expanded conceptual view of BAFF gene regulation, and contribute to a better understanding of the mechanisms involved in BAFF up-regulation in AIDs, using the model of pSS. We hope that these results may help to improve BAFF-targeted therapy in these conditions.

Alternative splicing in exon 4 of BAFF is regulated by interferon-gamma and SC35 protein

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Introduction

B-Cell Activating Factor belonging to the **TNF-Family (BAFF)** is a potent B cell-survival factor that regulates B-cell homeostasis and differentiation (1). BAFF is involved in the pathogenesis of autoimmune diseases, such as systemic lupus erythematosus (SLE) (2-3) and primary Sjögren's syndrome (pSS) (4), but also in B-cell malignancies such as chronic lymphocytic leukemia (CLL) (5). *BAFF* gene is located on human chromosome 13q14 and contains six exons and five introns (6). The transcript has 1204 bp and 858 bp in the the open reading frame. Previous studies have characterized three different mRNA from *BAFF* gene in humans: the full-length BAFF, a longer variant called ψ BAFF, and an exon-3 lacking isoform (Δ BAFF or Δ 3BAFF) (7). The larger transcript ψ BAFF was identified in the human cells lines HL-60 and U937, but sequencing proved this transcript to be non-functional because of incomplete splicing of intronic sequencing leading to premature termination codons (PTCs). An exon-3 (57 bp) lacking isoform of BAFF, referred to as Δ BAFF, has been identified in many mouse and human myeloid cells (7). Based on its ability to multimerize with full-length BAFF, this truncated variant might restrain the effects of the full-length-cytokine, and thereby negatively regulate B-cell homeostasis (8). Currently, Δ BAFF protein has never been characterized. Recently our group described an additional transcript, which was devoid of the 113 base pair segment encoded by the exon 4, and was called Δ 4BAFF (GenBank accession number HM 636064) (**Le Pottier et al., Nat Genetics 2012; submitted**). The chromatin immunoprecipitation (ChIP) unveiled that the protein was associated with DNA, and, acted as a transcription factor for the full-length form of BAFF. In addition, this new transcript is mainly detected in autoimmune diseases and CLL. However, the mechanisms implicated in Δ 4BAFF induction and up-

regulation in autoimmunity and B-cell malignancies remain unknown.

Alternative splicing is a major mechanism for regulating biological systems. The majority of human genes (up to 94% of genes) express multiple mRNAs via this mechanism (9). These mRNAs can encode functionally diverse protein isoforms or can promote the on:off control of gene expression by introducing PTCs (10). Many genes in the immune system have been shown to undergo alternative splicing (e.g. HLA, TCR ζ -chain, NF-kB) (11-14). Nevertheless, the knowledge of the global involvement of alternative splicing in different aspects of immunity is still limited. The splicing efficiency of individual exons is determined by multiple features involving gene architecture (e.g. the size of the exon, the size of the flanking introns), a variety of *cis*-acting elements within the exons and flanking introns (RNA sequence), and interactions with components of the spliceosome and auxiliary regulatory factors which transiently co-assemble with the spliceosome (15). Splicing efficiency is influenced by a range of *trans*-acting factors as the SR proteins (SR) (16-17) that bind to the positive regulatory motifs known as Exonic Splicing Enhancers (ESEs) (18) and Intronic Splicing Enhancer (ISEs) or factors such as heterologous nuclear ribonucleoproteins (hnRNPs) (19) that bind to negative splicing regulatory motifs known as Exonic Splicing Silencers (ESSs) and Intronic Splicing Silencers (ISSs). In addition, a larger fraction of these alternative splicing events are regulated according to cell-specific pattern or in response to acute stimulation (20).

The effects of different stimulations such as interleukin (IL)-10, interferon (IFN)- α and IFN- γ have showed an increase of BAFF production by monocytes, macrophages and dendritic cells (21). IFN- γ is a highly expressed cytokine in autoimmune diseases and CLL and is involved in their pathogenesis. Although IFN- γ is a potent inducer of BAFF

expression, the relationship between IFN- γ and the activation of BAFF gene expression is not clear. While IFN- γ responses are mainly mediated via Jak-Stat, this pathway is not thought to be involved in the IFN- γ -induced BAFF expression. Instead, protein kinase A/CREB has been reported as the dominant pathway (22). In addition, IFN- γ may modify the global expression pattern of genes, including spliced variants. For example, IFN- γ stimulation is able to regulate different isoforms of human tryptophanyl-tRNA synthetase (TrpRS) *in vivo* through alternative mRNA splicing. The miniTrpRS (produced by alternative splicing) has anti-proliferative and anti-angiogenic activity, but these effects are not seen with the full-length protein (23). In purified rat pancreatic β -cells, Ortis et al. (24) showed that IFN- γ , in association with other cytokines (IL-1 β and TNF- α), modified the expression of more than 20 genes involved in RNA splicing and the induction of changes in alternative splicing of >50% of the cytokine-mediated genes (by exon-array analysis) showing that these cytokines may affect the alternative splicing in a tissue specific manner.

Thus, we hypothesized that IFN- γ induced synthesis of BAFF, may be due to the alternative splicing of BAFF, generating Δ 4BAFF and the subsequent increase of BAFF. The aim of this was to understand the induction and regulation of Δ 4BAFF using as a model a minigene centered on exon 4 of BAFF, in addition to the analysis of *trans*-regulatory factors and the effects of IFN- γ stimulation.

Materials and Methods

Plasmids

In vitro studies of *BAFF* alternative splicing were performed using a minigene construct (mini-baff4 gene) carrying exon 4 (113pb) and the intron 4-5 (88pb). Since the intron 3-4 is very long (16393 pb) only their flanking sequence (about 200 bp) was cloned in a pcDNA3.1 plasmid. Fragments were PCR-amplified with primers including terminal restriction sites useful for cloning. The pCGT7 plasmids bearing cDNAs encoding SR proteins (SC35, SRp20, SRp40, SRp55) and hnRNPA1 were described elsewhere (25).

Cell culture and transfection

RAMOS and human monocytic lymphoma U937 cell-line cells were cultured in RPMI 1640 medium (Lonza) supplemented with 10% heat-inactivated FCS, 2 mM glutamine, 200 U/ml penicillin and 100 µg/ml streptomycin. RAMOS and U937 wells were transiently transfected with 500 ng of mini-baff4 gene and 500 ng of SC35, SRp20, SRp40, SRp55, hnRNPA1 using the V kit VCA-1003 (Lonza), according to manufacturer's instructions. The cells were cultured for 24 h in supplemented RPMI medium at 37°C with 5% CO₂. For siRNA experiments 3 x 10⁶ RAMOS cells/well in a 6-well plate were transfected with 20 nM of a specific SC35 siRNA and 20 nM of GAPDH siRNA as control. All siRNA were purchased from Applied Biosystems and transfected according to the manufacturer's instructions. The efficacy of siRNA to interfere with SC35 was confirmed by RT-PCR.

Tonsillar B cells were obtained from children undergoing routine tonsillectomy. The tissues were minced up, suspended in PBS and filtered to remove tissue fragments and clumps. Tonsil cell suspensions and blood were layered onto Ficoll-Hypaque, and

centrifuged for 30 min at 450 g. Mononuclear cell were incubated with neuraminidase-treated sheep RBC for 1 h at 4° C, and T cell depleted by a second 25-min round of centrifugation.

RNA extraction and RT-PCR

Total RNA was extracted by the RNable method (Eurobio, Les Ulis, France), and reverse-transcribed with Superscript II RNase H-RT according to the manufacturer's instructions (Invitrogen). One μ l of cDNA was amplified by PCR using Taq DNA polymerase (Promega, Charbonnieres, France). Minigene transcripts were analyzed by conventional and semiquantitative RT-PCR, using the primers described in Table 1. In order to quantify the resulting semiquantitative PCR products, 10 μ l of each sample were loaded on 2% agarose gel and run for 1 h. Gels were visualized and the Quantification of band intensities was performed using the *Quantity One*® (Biorad) software.

Real-time PCR

The relative levels of gene expression of SR, hnRNPs and GAPDH (Table 1) were quantified by real time-PCR using SYBR Green Master Mix reagent in an ABI Prism 7000 sequence detection system (Applied Biosystems) according to the manufacturer's instructions. The number of threshold cycles (C_t) was counted using the $2^{-\Delta C_t}$ method, with GAPDH transcripts as an internal control.

Culture stimulations

Tonsil B cells, RAMOS and U937 cells were cultured for 24h with recombinant (rh)

cytokines including rhIFN- α (2b: 2000 U/mL, Immunotools), rhIFN- γ (50 ng/mL, R&D Systems) and rhIL-10 (100 ng/mL, R&D Systems). Control cells incubated in parallel received culture medium without the cytokines. After 24 h stimulation, total RNA, total lysis extracts or confocal microscopy were done according to the specific experiment.

Total lysates and subcellular fractionation

RAMOS cells alone and stimulated for 24 h with IFN- α and IFN- γ were subjected to total lysis and subcellular fractionation. Total lysis were made with Tris HCl pH 7.5, NaCl 150 mM, Igepal 1%, EDTA 2 mM, NaF 5 mM and proteases inhibitor cocktail (all from Sigma-Aldrich). Cells were homogenized with this lyses buffer during 1h at 4°C, and centrifuged 20 min at 500g. Subcellular fractionation was performed with the ProteoExtract Subcellular proteome extraction kit (Calbiochem Merck Chemicals, Nottingham, UK) according to manufacturer's instructions.

Western blotting analysis

Equal amounts of protein from total cell lyses extracts or subcellular fractionation were separated on 10% SDS-polyacrylamide gel electrophoresis (Bio-Rad, Marnes-la-Coquette, France) in reducing conditions and transferred. Unoccupied sites were blocked by incubation in PBS containing 0.1% Tween-20 and 5% non-fat milk for 1 hr. Membranes were probed with anti- β -actin mAb (1:10000), anti-hnRNP C1+C2 mAb [4F4] (1:1000), anti-hnRNP F+H mAb [1G11] (1:1000) all purchased from Abcam, (Cambridge, MA) and rabbit anti-human SC35 (Thermo Scientific) (1:1000), overnight at 4°C. Bound antibodies were developed with Horse radish peroxidase-secondary

antibodies, (GE Healthcare) and visualized using the enhanced chemiluminescence system: ECL advance western blotting detection (GE healthcare). The intensity of each protein was expressed relative to β -actin. Control Abs for the subcellular fractionation were β -actin for cytoplasm fraction and Histone H3 (Millipore) for the nuclear fraction.

Confocal microscopy

RAMOS B cells alone and stimulated with IFN- γ were harvested for 24 h incubation at 37°C with 5% CO₂. After washing twice with phosphate buffered saline (PBS), 250 μ l of Cytofix/Cytoperm solution was added for 20 minutes at 4°C. After 2 washes with 1 mL Perm/Wash solution, the cells were stained with rabbit anti-SC35 antibody and anti-hnRNPC1+C2 and anti-hnRNP F+H mAb for 30 min at 4°C. After washing, cells were incubated with secondary antibodies: anti-mouse IgG-FITC and anti-rabbit-RRX for 30 min 4°C. After two washes, the cells were centrifuged in the cytospin for 1 min at 1500 rpm. Mounting medium used was Vectashield (Vector laboratories, Burlingame, CA). The cellular staining was observed by confocal microscopy Leica DMR microscope with camera Leica TCS-NT (Leica, Solms, France) using an oil immersion lens (Zeiss, Le Pecq, France) (PL APO, 100x/1,40) at room temperature (16°C) and with the software Leica TCS-NT version 1.6.587.

Analysis of predictive functional ESEs

BAFF exon 4 sequences were analyzed to determine potential ESEs able to bind SR proteins using the ESEfinder web resource (26).

Table 1. Primers used to amplify the mini-baff4 gene, SR and hnRNPs proteins.

| | Primer forward (5'-3') | Primer reverse (5'-3') |
|-----------------------|-------------------------------|-----------------------------------|
| Minigene | CAACTTCAAGCTCCTAAGCCACTGC | TAGGATCCGGTCACCAGGAAGTTGGTTAAATCA |
| GAPDH | CTTAGCACCCCTGGCCAAGG | CTTACTCCTTGGAGGCCATG |
| SC35 (SRSF2) | GACCTCCAAGTCCAGATC | TGGATTGGATTCCCTCTT |
| SRp20 (SRSF3) | ATGATTATCGTAGGAGGAGTC | GACGGCTTGTGATTTCTC |
| SRp40 (SRSF5) | TTGCCTCTTATGGTGACTTA | CCTGCTTCTTGACCTACT |
| SRp55 (SRSF6) | AATACGGACCACCTGTTC | GTTACTTCACCTGCTTGTC |
| hnRNPA1 | ACGAAACCAAGGTGGCTATG | TCCCTGTCACTTCTCTGGCT |
| hnRNPA2/B1 | TAGGAGTGGAAGAGGAGGCA | GGTCCTCCTCCATACCCATT |
| hnRNPC (C1/C2) | GCTGCTCTGTTCATAAGGGC | TTTCCTCGGTTCACTTTTGG |
| hnRNPH1 | GTGCAGTTTGCTTACAGGA | CCCCAGGTCTGTCATAAGGA |
| hnRNPLL | GTTTCTGTTTCACCCGTCGT | TCTGCAGCAAATGTCACACA |
| hnRNPL | GGTGGGAGAACTACGATGA | ACCACATAGCTGATGGGTCC |
| hnRNPE1 | GGAAGCATCATTGGGAAGAA | TCTTCCTCCAGCTTGTCGAT |

Results

Splicing of exon 4 of BAFF is present in B cells and monocytes

We have recently described the alternative splicing of the exon 4 of *BAFF* (a new variant called $\Delta 4$ BAFF) which is mainly detected in autoimmune diseases and CLL and acts in association with p50 from the NF- κ B pathway as a transcription factor for its own parent gene. However, the mechanisms implicated in $\Delta 4$ BAFF induction and up-regulation in autoimmunity and B-cell malignancies are unknown. To investigate this alternative splicing event we constructed an exon 4-centered minigene vector (mini-baff4 gene), containing the complete exon 4 of *BAFF* together with the truncated upstream intron 3-4 and the complete downstream intron 4-5 (**Figure 1A**). Following 24 hours-transient cell transfections in several cell lines, minigene-encoded transcripts were analyzed by gel electrophoresis after reverse transcription and PCR with specific primers for the minigene (sense $\alpha 2-3$, antisense $\beta 2$) (**Figure 1A and Table 1**). The ratios of exon 4-lacking to exon4-containing transcripts were then determined by densitometry analysis. As shown in **Figure 1B**, RAMOS B cell-line and the monocytic cell-line U937 presented a weak exon 4 skipping in our minigene construction. In contrast, colonic cancer cell-lines only showed the exon4-containing form (data not shown), suggesting that *trans*-regulatory proteins control the alternative splicing in a cell/tissue-specific manner. Thus, these results showed that B cells and monocytes may express the exon-4-skipped form. In our previous study, we used as cellular model the RAMOS cell-line. As this cell line presented in a constitutive way the mini-baff4 gene exon 4 skipping, we choose this model to study the mechanism regulating the alternative splicing of exon 4.

SC35 promotes BAFF exon 4 inclusion

To elucidate the mechanisms implicated in the exon 4 inclusion we performed a bioinformatics analysis using the web resource ESEfinder (a search tool for SR proin-binding motifs ESE) (26) to determine which SR proteins have predictive-binding sites on *BAFF* exon 4. This analysis showed a predictive binding of SRp55, SRp40 and SC35 proteins to the exon 4 of *BAFF*. In contrast, ASF/SF2 and SRp20 did not present predictive-binding sites to *cis*-elements on the exon 4. Thus, in theory, these 3 SR proteins with predictive binding sites are implicated in the expression of full-length *BAFF* transcript. In order to determine the proteins implicated in exon 4 inclusion/exclusion, we co-transfected the mini-baff4 gene together with 500 ng of each plasmids coding for SC35, SRp40, SRp55 (which showed predictive-binding sites) and SRp20 (hypothetically used as a negative control) into RAMOS cells. The transfected cells were harvested at 24 h and analyzed for the splicing product of *BAFF* exon 4 by RT-PCR. We demonstrated that over-expression of SC35 promotes the exon 4 inclusion (**Figure 2A**). The ratio inclusion/exclusion markedly increased in SC35 co-transfected cells. In contrast, no effect was evidenced with SRp40 and SRp55 co-transfections (proteins that also had positive predictive-binding sites), and the control SRp20.

To confirm the effect of SC35 on the promotion of *BAFF* exon 4 inclusion, we co-transfected the mini-baff4 gene together with a siRNA of SC35 and a control siRNA into RAMOS cells to knock down SC35 expression and then measure the splicing products by RT-PCR. This experiment confirmed that SC35 is important in the exon 4 inclusion, because as compared with control siRNA, the siRNA SC35 suppressed exon 4 inclusion and decreased in a significant manner the ratio of exon 4 inclusion/exclusion (**Figure 2B**).

SC35-dependent splicing enhancers are known to contain UGCNGYY sequence (Shaal TD 1999). In the bioinformatics analysis we found that 2 predictive SC35-dependent splicing enhancers on the *BAFF* exon 4 may potentially bind to SC35 (ESE1: located between 794 and 801 bp; ESE2: located between 825 and 832 bp). The effects of one or both of these predictive ESEs to bind SC35 will be confirmed by directed mutagenesis.

IFN- γ stimulation induces alternative splicing of *BAFF* exon 4

Taken together that $\Delta 4$ BAFF is found mainly in autoimmune diseases, the increased production of BAFF under different stimulations, and the effects of some cytokines on the alternative splicing, we wanted to determine the impact of these on the may induce the exon4-skipping and the expression of $\Delta 4$ BAFF. To address this question, we tested different stimulations such as IL-10, IFN- α , IFN- γ and CD40L already known as inducers of BAFF production by monocytes, macrophages, dendritic cells and B cells. In our first article, we have found that endogenous $\Delta 4$ BAFF transcript is not expressed by RAMOS B cells and U937 conversely to the small exon4-skipping found in our minigene construction. However, in an interesting way, $\Delta 4$ BAFF transcript and full-length BAFF protein was seen in tonsillar B cells after stimulation with IL-10 and IFN- α , only in association with CD40L stimulation, after 24 and 48h of cell-culture (data not shown). In addition, IFN- γ alone was able to induce $\Delta 4$ BAFF-transcript expression at 24h. These results were confirmed in three different cell-lines: RAMOS B cells, monocytic U937 cell-line, and HSG cell-line (**Figure 3A**). Taken together, these results showed that under specific stimulations, exon 4-skipping may be induced. In the autoimmune setting these results are of particular interest, linking the cytokine abnormalities with BAFF-overproduction. To confirm these results, we transfected the

mini-baff4 gene into RAMOS cell line, and after 24 h, the transfected cells were stimulated with IFN- γ . After 24 h stimulation, the cells were analyzed for the splicing product of *BAFF* exon 4 by RT-PCR. **Figure 3B** shows that under IFN- γ stimulation, the ratio inclusion/exclusion markedly decreased, meaning that the exon 4-skipping is increased by this stimulation. In contrast, IFN- α used as a control did not modify the ratio inclusion/exclusion.

BAFF exon 4 inclusion by SC35 is abrogated by IFN- γ stimulation

As IFN- γ induced a marked exon 4-skipping, we hypothesized that this cytokine may influence the normal function of SR proteins, mainly the already involved SC35. To test the effects of IFN- γ on SR proteins function, we co-transfected the mini-baff4 gene together with 500 ng of each plasmids coding for SC35, SRp40, SRp55 and SRp20 into RAMOS cells. The transfected cells were harvested at 24 h and then stimulated with IFN- γ for 24h. Then the cells were analyzed for the splicing product of *BAFF* exon 4 by RT-PCR. In an unexpected manner, IFN- γ completely abrogated the effects of SC35 over-expression, producing a complete exon 4-skipping (**Figure 4A**). These results were confirmed in three different experiments, showing that under IFN- γ stimulation, the over-expression of SC35 has the opposite effect. This interesting notion is not new, because under specific conditions and antagonisms between SR proteins, some of these members may act as hnRNP-like proteins and favor the alternative splicing. On the other hand, IFN- γ stimulation had not implication on the function of others tested SR proteins, confirming that Δ 4BAFF induction by IFN- γ is associated with an abnormal function of SC35. Thus, to understand the role of IFN- γ stimulation over SC35 abnormal function, we hypothesized either that IFN- γ induces

a decrease of SC35 protein or its dysregulation; and/or induces an increase of hnRNPs, which may compete for binding sites with SR proteins. To verify our hypothesis, first we analyzed by real-time PCR the expression of SR proteins before and after IFN- γ stimulation in RAMOS B cells. The transcripts of SR proteins did not change after 24h IFN- γ stimulation (**Figure 4B**), suggesting that the implicated mechanism of the abnormal function of SC35 induced by IFN- γ may be due to a binding-site competition between SC35 and hnRNPs and not by changes in its expression. These findings were also confirmed by Western-Blotting analysis (**Figure 4C**), showing that SC35 proteins levels did not change after IFN- γ stimulation. SR proteins are concentrated within nuclear speckles of cells that correspond to interchromatin granule clusters (IGCs) at the electron microscopic level and shuttle to sites of active transcription to initiate their co-transcriptional splicing activity. In addition, whereas most of SR proteins are confined to the nucleus, some of them (SF2/ASF, SRp20 and 9G8) shuttle rapidly and continuously between the nucleus and the cytoplasm. Thus, we wanted to evaluate if one of the possible explanations to SC35 abnormal function after IFN- γ stimulation may be the shuttling to the cytoplasmic compartment. The analysis of SC35 by western-blotting after subcellular fractioning showed that SC35 remained in the nuclear fraction, (**Figure 4D**). After verify that SC35 protein remains in the nucleus, we analyzed by confocal microscopy the SC35 localization into the nucleus before and after 24 h of IFN- γ stimulation. Staining in cells without IFN- γ shows a speckled peri-nucleolar pattern, classical for SC35 distribution. In contrast, IFN- γ induces a perinuclear aggregation of SC35 (**Figure 4E**). This different distribution may be related to the abnormal SC35 function after IFN- γ stimulation and may be explained by a competitive mechanism between SC35 and hnRNPs for the exon 4 binding-sites.

BAFF exon 4 skipping induced by IFN- γ stimulation is mediated by hnRNPs increasing

To test the effects of hnRNPs in our model, we tested a plasmid coding for the most studied hnRNP, the hnRNPA1. Using co-transfection of the mini-baff4 gene together with 500 ng of hnRNPA1 plasmid we found that overexpression of this protein does not induce changes on the exon 4 ratio inclusion/exclusion (**Figure 2A**). Similarly, after IFN- γ stimulation, there was not variation in the exon 4 ratio inclusion/exclusion into the RAMOS B cells overexpressing hnRNPA1 (**Figure 4A**), showing that this hnRNP is not implicated in the *BAFF* exon 4 alternative splicing. After demonstrating that the abnormal function of SC35 induced by IFN- γ is due to its different location and not to changes in its expression, we wanted to determine if these effects may be explained by a binding-site competition between SC35 and hnRNPs. Firstly, by real-time PCR, we evaluated the transcripts levels of seven of the most studied hnRNPs (**Table 1**) in RAMOS B cells before and after IFN- γ stimulation. The transcript levels of three hnRNPs (hnRNP C1/C2, E1/E2 and H) were elevated 24h after IFN- γ stimulation in our model (**Figure 5A**). In addition, these results confirmed that hnRNPA1 did not vary after IFN- γ stimulation. Western blot analysis using anti-hnRNP C1/C2 and anti-H antibodies showed in total lysis preparation that these proteins increased two times in the presence of IFN- γ stimulation (**Figure 5B**). These results also showed that hnRNP C1/C2 may be the candidate to compete with SC35 in our model, because it increases after IFN- γ stimulation and not by IFN- α stimulation. In the other hand, hnRNPH also increases after IFN- α stimulation. Sub-cellular fractioning analysis showed that the increase of hnRNP C1/C2 after IFN- γ stimulation is presented in all compartments (nuclear and cytoplasmic) (**Figure 5C**). The important increasing of hnRNP C1/C2 was

also evidenced by confocal microscopy in IFN- γ stimulated RAMOS B cells. Further analysis by flow cytometry and confocal microscopy will be done to verify the competition between hnRNP C1/C2 and SC35 in our model after IFN- γ stimulation.

Discussion

Based on the effects of the new-described Δ 4BAFF form over full-length BAFF and their main expression in autoimmune diseases and CLL, we analyzed the mechanisms of induction and regulation of Δ 4BAFF in the autoimmune setting.

Firstly, to study the alternative splicing of the exon 4, we constructed a minigene centered on this exon and then transfected in different cell lines. This approach showed that after 24h of transient transfection, at base line, an alternative splicing of exon 4 was evidenced. In addition, these findings were replicated in a B-cell line (RAMOS) and a monocytes cell-line (U937) showing that at least in these two cellular populations, the role of individual splicing factors may be similar. In contrast, colonic cancer cells do not present alternative splicing of exon 4 after minigene transfection (data not shown), highlighting that the ability of *trans*-regulatory proteins to control alternative splicing is in a cell/tissue-specific manner (27-28).

To identify and demonstrate the *trans* and *cis*-elements implicated in the alternative splicing of the exon 4, we found by biocomputational analysis (RESCUE-ESE, 26) that some members of the SR family may bind to exon 4 and in theory, favor the constitutive splicing of full-length BAFF. Among these members, a predictive binding of SC35 (SFRS2), SRp40 (SFRS5) and SRp55 (SFRS6) to exon 4 of BAFF was found. In contrast, SRp20 (SFRS3) and ASF/SF2 (SFRS1) had not predictive binding sites to

exon 4. Prompted by these analyses, the co-transfection of the minigene plus coding plasmids for splicing proteins showed that SC35 is essential for the constitutive splicing of BAFF. In fact, over-expression of SC35 led to a complete inclusion of exon 4 in RAMOS B cells. The analyses of *cis*-acting elements showed that two ESEs may bind SC35 on the exon 4, controlling the specific usage of alternative exons. The function of these two specific binding sites to SC35 and their role in the constitutive vs. alternative splicing of exon 4 will be confirmed by directed mutagenesis.

SC35 is one of the most important and studied member of the SR family. Adding to their functions as a splicing factor, SC35 affects transcriptional elongation in a gene-specific manner (29). Interestingly, SC35 can regulate its own expression (30) and may be also an auto-antigen. Covini et al. (31) reported that ANAs from patients with hepatocellular carcinoma may recognize splicing factor SC35. In addition, some members of the SR protein family, including SC35, showed reactivity with sera from SLE patients up to 50% of cases and not other AIDs (RA and antiphospholipid syndrome) (32). In our group, preliminary results (data not shown) evidenced, by indirect immunofluorescence (IFI) that there is co-localization between ANAs and anti-SC35 in 54% of pSS patients, and only 11% of SLE patients, confirming that serum samples of patients with AIDs may recognize nuclear SC35 protein. Sensitivity and specificity were 54% and 90%, respectively in pSS patients. Interestingly, six of 9 pSS with no anti-SS-A or anti-SS-B auto-Abs presented anti-SC35 Abs., and this auto-Ab would be a diagnostic marker for pSS. Western-blot and dot blot analyses confirmed this specificity. Currently we are developing an ELISA test to verify and quantify the presence of anti-SC35 in AIDs.

The role of other SR proteins in alternative splicing of immune genes has been

described. For example, ASF/SF2 (SFRS 1) regulates the expression of CD3 ζ protein limiting the production of the alternatively spliced isoform, which is an unstable transcript. This alternatively spliced variant is more expressed in T cells from SLE patients compared to healthy controls (33). Similar evidence for ASF/SF2 has been described in the alternative splicing of CD200 (14). Taken together, these data show that members of SR protein family, may mediate constitutive splicing, limiting the generation of alternative splicing isoforms, which in some cases may have a deleterious role in the normal immune response.

The function of individual splicing factors may be also situation dependent. It has been reported that in mood disorder, patients present a decrease in Glucocorticoid Receptor (GR) alpha, but not GRbeta (produced by alternative splicing), which is associated with an increased expression of SRp20 but not SRp30c RNA (34).

These results show that the function of a specific splicing factor (in our model, SC35 protein) depends of specific cellular/tissue patterns, but also pathological conditions (induced, for instance, for acute stimulations or stress).

IFN- γ and alternative splicing of exon 4 of BAFF

The splicing efficiency of individual exons is determined by multiple including the cell-specific pattern or in response to acute stimulation (20). As discussed above, we tested different stimulations already known as positive regulators of BAFF (such CD40L, IL-10, IFN- γ , and IFN- α) and their effects on Δ 4BAFF induction. Interestingly, under different stimulations, tonsillar B cells express Δ 4BAFF. The induction of Δ 4BAFF was accompanied by increasing on full-length BAFF at the protein level and soluble BAFF.

Although various stimulations induced $\Delta 4$ BAFF transcript, the most reproducible model was obtained by IFN- γ stimulation, after 24 h of cell-culture. Thus, using only this stimulation, we confirmed that IFN- γ favors the $\Delta 4$ BAFF expression in different cell lines (including monocytes, B cells and epithelial cells). In the second part of the article, an important exclusion of exon 4 on the minigene-transfected RAMOS B cells was found after 24h of IFN- γ stimulation, confirming that the induction of $\Delta 4$ BAFF is mediated by inflammatory stimuli.

The effects of IFN- γ on alternative splicing phenomena have been described in other genes. For example, isoforms of human tryptophanyl-tRNA synthetase (TrpRS) *in vivo* are regulated by IFN- γ through alternative mRNA splicing. The miniTrpRS (produced by alternative splicing) has anti-proliferative and anti-angiogenic activity, but these effects are not seen with the full-length protein. An IFN- γ -sensitive promoter was demonstrated to drive the production of miniTrpRS and two new splice variants of TrpRS, showing that this cytokine may explain alternative splicing variants with different effects (35). In addition, IFN- γ modifies the global expression pattern of genes, including spliced variants. Ortis et al. (24) in purified rat pancreatic β -cells showed that IFN- γ in association with other cytokines (IL-1 β and TNF- α) modified the expression of more than 20 genes involved in RNA splicing, and changes in alternative splicing of >50% of the cytokine-mediated genes (by exon-array analysis), showing that these cytokines may affect the alternative splicing in a tissue specific manner.

Interestingly in our study, RAMOS B cells co-transfected with the mini-baff4-gene and SC35 coding plasmid and then stimulated by IFN- γ for 24h showed completely the inverse effect of SC35 plasmid before stimulation (complete exclusion of exon 4), showing that over-expression of SC35 under the effects of IFN- γ stimulation produced

the opposite effect, acting as a hnRNP-like protein. This interesting concept is not new. For instance, individual SR proteins can sometimes have antagonistic effects on splice site selection, as in the case of ASF/SF2 and SC35 in the regulation of β -tropomyosin (36) and human growth hormone mRNA alternative splicing (37). In addition, in a splicing reporter system for the study of spliced tissue factor transcripts (38), the proteins SC35 and SRp40 antagonized the effects of ASF/SF2 and SRp55, thereby promoting the tissue factor exon 5 exclusion. In our study, this antagonistic effect was not evidenced with other SR proteins and hnRNPA1 co-transfections. Thus, to understand the role of IFN- γ stimulation over splicing regulatory proteins, we hypothesized either that IFN- γ induces a decrease of SC35 protein or its dysregulation; and/or induces an increase of hnRNPs. By different techniques we found that SC35 transcript and protein expression are only slightly modified after IFN- γ . However, we evidenced by microscopy that SC35 is differently located in the perinuclear space after IFN- γ stimulation. In fact, the abnormal function of SC35 induced by IFN- γ (due to this different location) may be due to a binding-site competition between SC35 and hnRNPs and not to changes in their expression. The transcript levels of three hnRNPs (hnRNP C1/C2, E1/E2 and H) were elevated 24h after IFN- γ stimulation in RAMOS B cells. Although the hnRNPA1 is the best studied splicing factor of the hnRNP family (39-41), its expression was not modified in B cells after IFN- γ stimulation and no effect was seen in the co-transfection model with hnRNPA1 coding plasmid. In the study by Ortis et al. (24), an exon-array analysis after 24h IFN- γ stimulation showed that hnRNPC is increased at two times. Similar to our results this hnRNP is regulated by IFN- γ stimulation besides the cell type. The relationship between IFN- γ stimulation and hnRNPC deserves to be further studied. Thus, in our model, IFN- γ stimulation induces hnRNPs that binds to silencer sequences inhibing SC35 protein binding to exon 4 ESEs,

forcing a shift of splicing to a distal splicing site, favoring $\Delta 4$ BAFF induction. In fact, differential expression of SR proteins, and their negative regulators, the hnRNPs, will bring about tissue- and cell-type-specific splicing in vivo (40). In immune genes, the hnRNPs are also implicated in regulation of alternative splicing. For example, the hnRNPL-like (hnRNPLL) has been identified as a critical inducible regulator of CD45 alternative splicing, from the larger isoforms known as CD45RA to the shortest isoform, CD45RO express by activated and memory T cells (42). In our study, hnRNPLL was not modified after IFN- γ stimulation. Concluding with our RAMOS B-cell model, the IFN- γ stimulation induced the alternative splicing of the exon 4 of BAFF, inducing an increase of some hnRNPs leading to binding-site competition with SC35 that is finally abnormally located in the perinuclear space. With these results we can conclude that an inflammatory loop between IFN- γ and BAFF pass through the induction of $\Delta 4$ BAFF.

Figures legends

Figure 1: Splicing of exon 4 of BAFF is present in B cells and monocytes. **A.** Exon 4-centered minigene vector (mini-baff4 gene), containing the complete exon 4 of *BAFF* together with the truncated upstream intron 3-4 and the complete downstream intron 4-5 used to study the induction and regulation of $\Delta 4$ BAFF. Following 24 hours-transient cell transfections in cell lines, minigene-encoded transcripts were analyzed by gel electrophoresis after reverse transcription and PCR with specific primers for the minigene (sense $\alpha 2-3$, antisense $\beta 2$). The ratios of exon 4-lacking to exon4-containing transcripts were then determined by densitometry analysis. **B.** RAMOS B cell-line and the monocytic cell-line U937 transfected with mini-baff4 gene presented exon 4 skipping.

Figure 2: SC35 protein promotes the *BAFF* exon 4 inclusion. **A.** RAMOS cell line was cotransfected with the mini-baff4 gene together with 500 ng of each plasmid-coding for SC35, SRp20, SRp40, SRp55 and hnRNPA1. Following 24 hours-transient cell transfections in cell lines, minigene-encoded transcripts were analyzed by gel electrophoresis after reverse transcription and PCR with specific primers for the minigene (sense $\alpha 2-3$, antisense $\beta 2$). The ratios of exon 4-lacking to exon4-containing transcripts were then determined by densitometry analysis. Co-transfection of mini-baff4 gene with SC35 shows a complete inclusion of exon 4 (column 2), showing that this protein is responsible for expression of full-length BAFF transcript including the exon 4. In contrast, SRp20 (which did not have prediction to bind exon 4) has not effect in the exon 4 splice (column 3). Predicted SRp40 and SRp55 proteins did not show effect in the exon 4 splice (column 4 and 5, respectively). In addition, hnRNPA1, the

most studied hnRNP has not also effect in the exon 4 alternative splicing. **B.** SC35 siRNA transfection confirmed that SC35 promotes BAFF exon 4 inclusion. We co-transfected the mini-baff4 gene together with a siRNA of SC35 and a control siRNA into RAMOS cells to knock down SC35 expression and then measure the splicing products by RT-PCR. This experiment confirmed that SC35 is important in the exon 4 inclusion, because as compared with control siRNA, the siRNA SC35 suppressed exon 4 inclusion and decreased in a significant manner the ratio of exon 4 inclusion/exclusion.

Figure 3: IFN- γ stimulation induces alternative splicing *BAFF* exon 4. **A.** IFN- γ stimulation induced endogenous Δ 4BAFF-transcript expression at 24h in three different cell-lines: RAMOS B cells, monocytic U937 cell-line, and HSG cell-line. **B.** IFN- γ stimulation induced exon4-skipping in RAMOS transfected with the mini-baff4 gene. After 24 h stimulation, the cells were analyzed for the splicing product of *BAFF* exon 4 by RT-PCR using the specific minigene primers (Table 1). Under IFN- γ stimulation, the ratio inclusion/exclusion markedly decreased comparing to baseline, meaning that the exon 4-skipping is induced by this stimulation. In contrast, IFN- α used as a control did not modify the ratio inclusion/exclusion, confirming that 24 h IFN- γ stimulation was reproducible in the minigene assay.

Figure 4: BAFF exon 4 inclusion by SC35 is abrogated by IFN- γ stimulation. **A.** We co-transfected the mini-baff4 gene together with 500ng of each plasmids coding for SC35, SRp40, SRp55, SRp20 and hnRNPA1 into RAMOS cells. The transfected cells

were harvested at 24 h and then stimulated by IFN- γ for 24h. Then the cells were analyzed for the splicing product of *BAFF* exon 4 by RT-PCR. IFN- γ completely abrogated the effects of SC35 overexpression, producing a complete exon 4-skipping (Column 2). On the other hand, IFN- γ stimulation had not implication on the function of others tested SR proteins (Columns 3 to 5), confirming that Δ 4BAFF induction by IFN- γ is associated with an abnormal function of SC35. **B.** Real-time PCR of SR proteins in RAMOS B cells before and after IFN- γ stimulation. The four transcripts tested (SC35, SRp20, SRp40 and SRp55) remained stable after 24 h of IFN- γ stimulation, suggesting that dysfunction of SC35 induced by IFN- γ is not related to changes in SC35 expression. **C.** Western-blotting in RAMOS cells total cellular extracts before and 24 h after IFN- γ and IFN- α stimulation. Revelation with a rabbit anti-SC35 antibody showed that SC35 protein levels are not modified after these stimulations, confirming that abnormal function of SC35 in presence of IFN- γ stimulation is not based in changes of protein expression. The quantity of SC35 was calculated based on the SC35/ β -actine ratio. **D.** Sub-cellular fractions of RAMOS cells were tested by Western-blot to verify if SC35 undergoes shuttling into the cytoplasm and this change in the localization may explain the abnormal function under IFN- γ stimulation. This analysis showed that SC35 remained in the nuclear fraction (C: cytoplasm; M: membrane; N: nucleus). **E.** RAMOS cell line was stimulated by IFN- γ for 24 h. SC35 localization was evaluated by anti-SC35 mouse antibody by confocal microscopy. Staining in cells without IFN- γ stimulation shows a speckled perinucleolar pattern. In contrast, IFN- γ induces a perinuclear aggregation of SC35. This different distribution may be related to the abnormal SC35 function after IFN- γ stimulation.

Figure 5: BAFF exon 4 skipping induced by IFN- γ stimulation is mediated by hnRNPs increasing. **A.** The transcripts of seven hnRNPs were evaluated by real-time PCR, in RAMOS B cells before and after IFN- γ stimulation. The transcript levels of three hnRNPs (hnRNP C1/C2, E1/E2 and H) were elevated 24h after IFN- γ stimulation in our model. In contrast, hnRNPA1 expression did not vary after IFN- γ stimulation. **B.** Analysis of hnRNP C1/C2 and hnRNPH by western blot. In total cell extracts, the protein levels of these two hnRNPs were tested by specific antibodies by western blot. Using the hnRNP/ β -actine ratio we determined that both hnRNPs are increased after 24h IFN- γ stimulation. Interestingly, only hnRNP C1/C2 was modified only by IFN- γ stimulation, because hnRNPH levels are similarly increased after IFN- α and IFN- γ stimulation. **C.** Sub-cellular fractioning analysis showed that the increase of hnRNP C1/C2 after IFN- γ stimulation is presented in all compartments (nuclear and cytoplasmic) (C: cytoplasm; M: membrane; N: nucleus).

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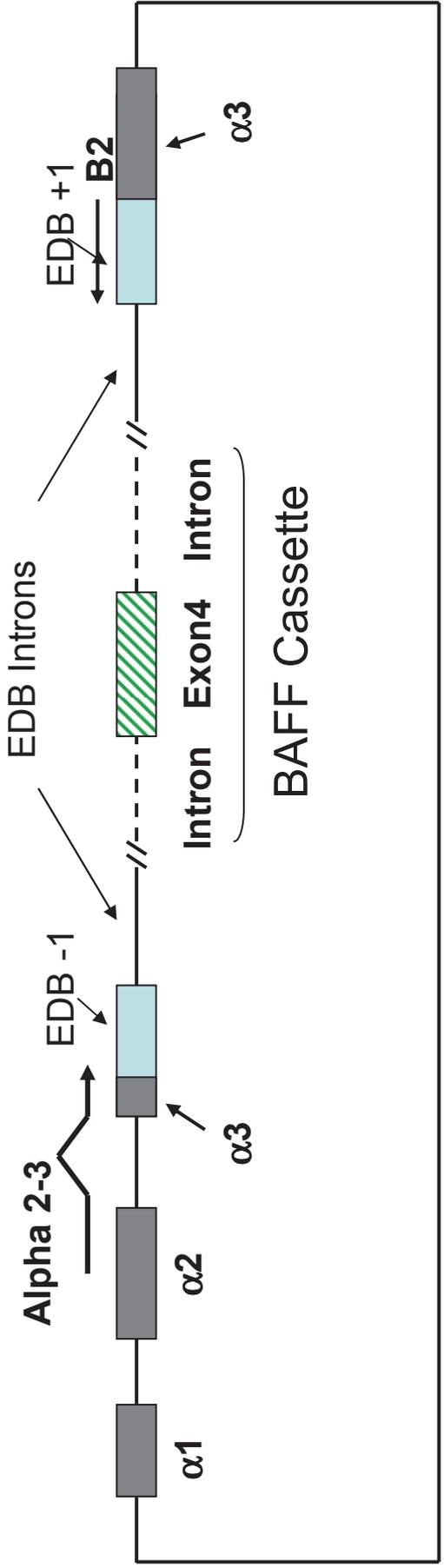
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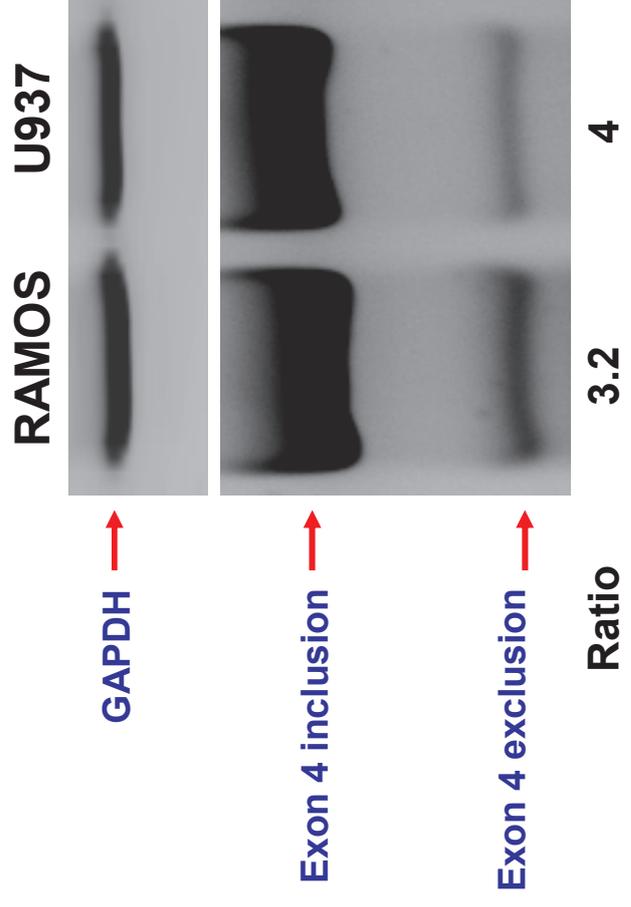
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A.

Figure 1

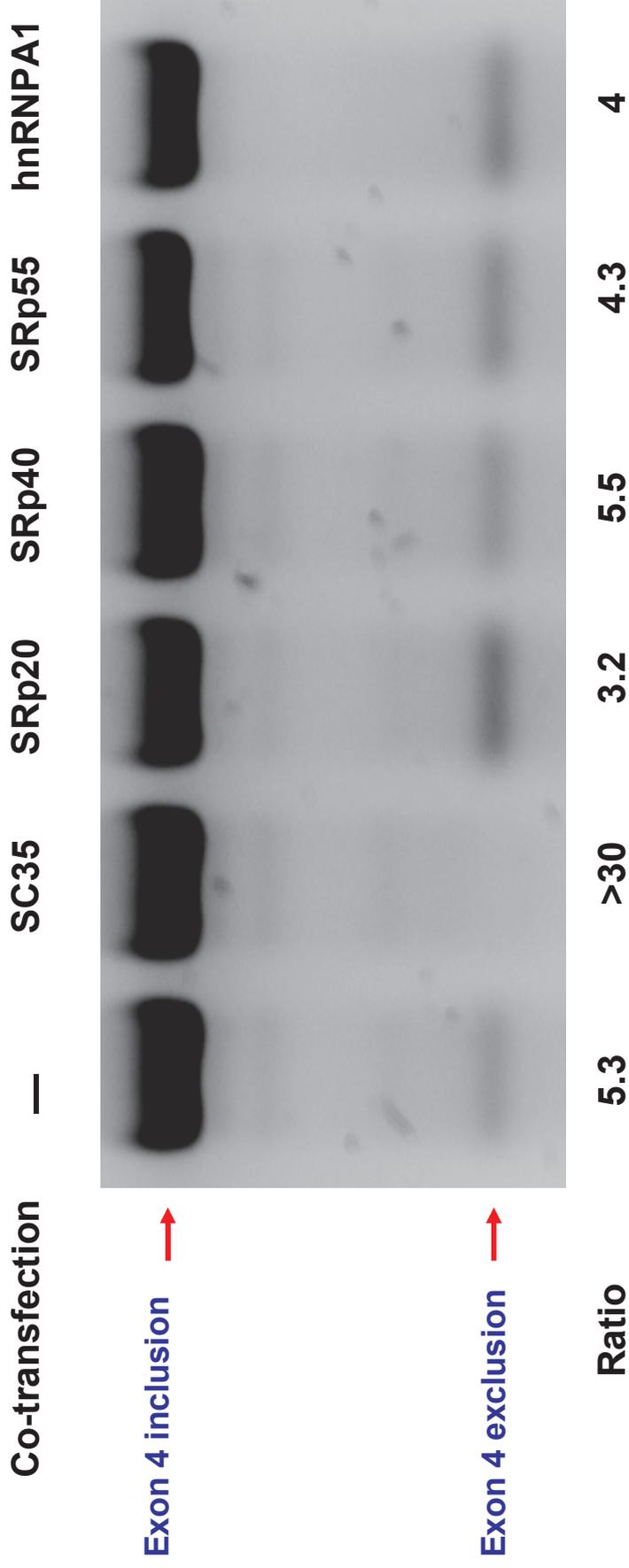


B.



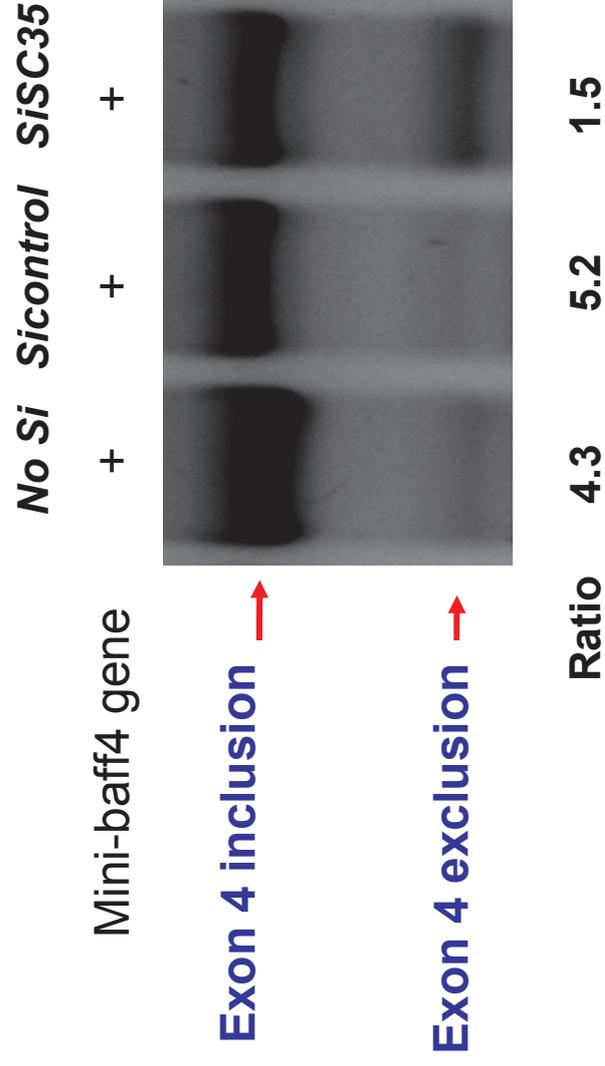
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Figure 2



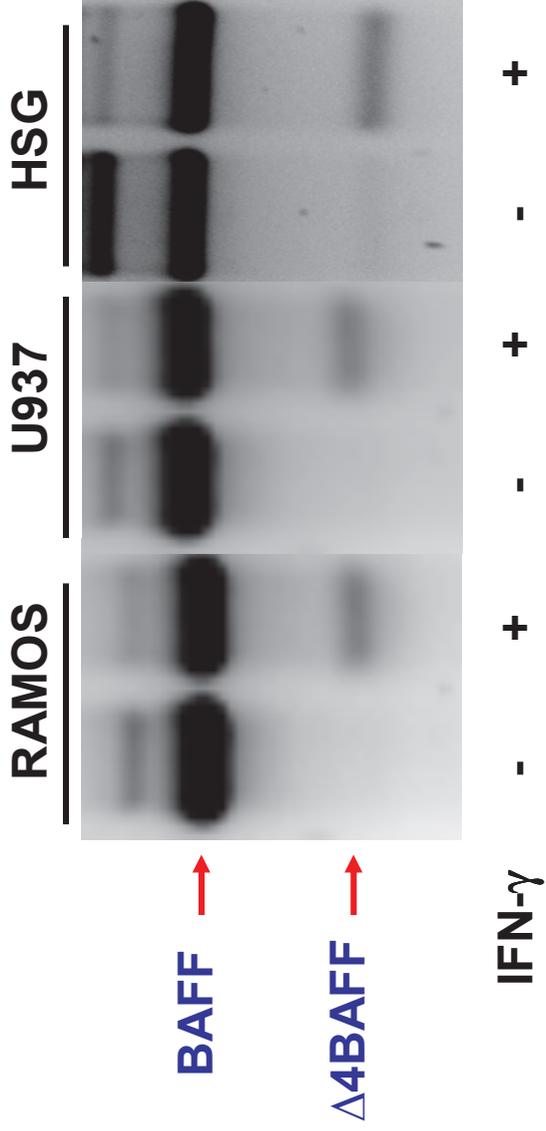
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Figure 2

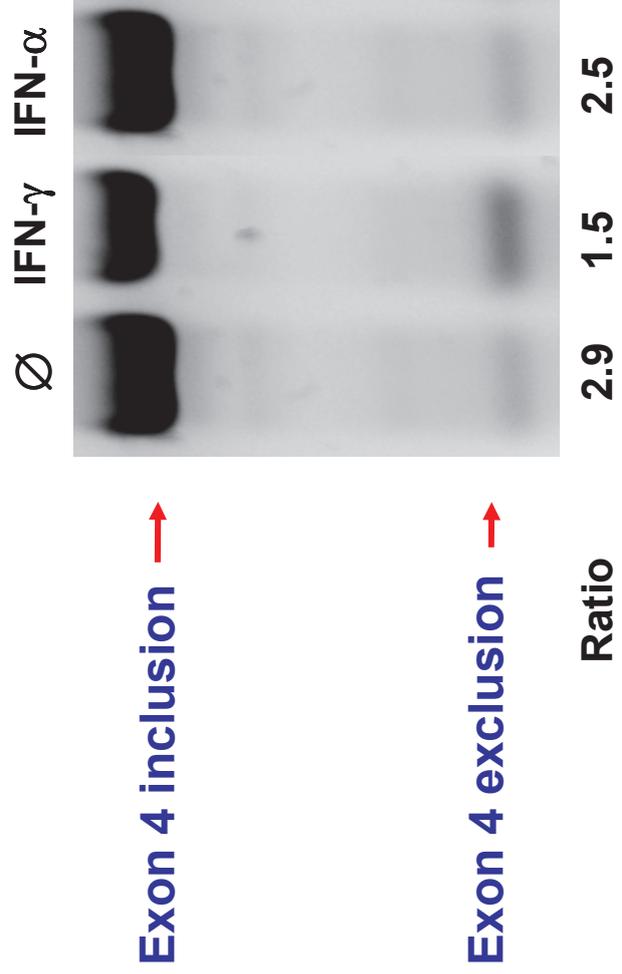


A.

Figure 3



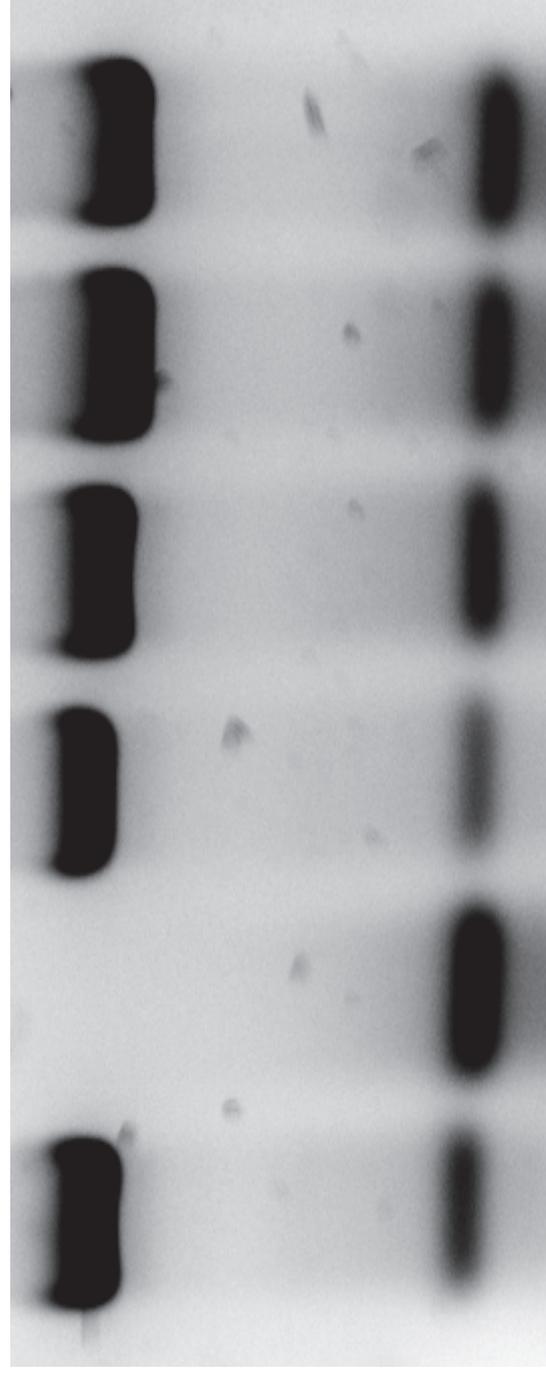
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A.

Figure 4

Co-transfection — SC35 SRp20 SRp40 SRp55 hnRNPA1



Exon 4 inclusion →

Exon 4 exclusion →

Ratio

1.71

1.73

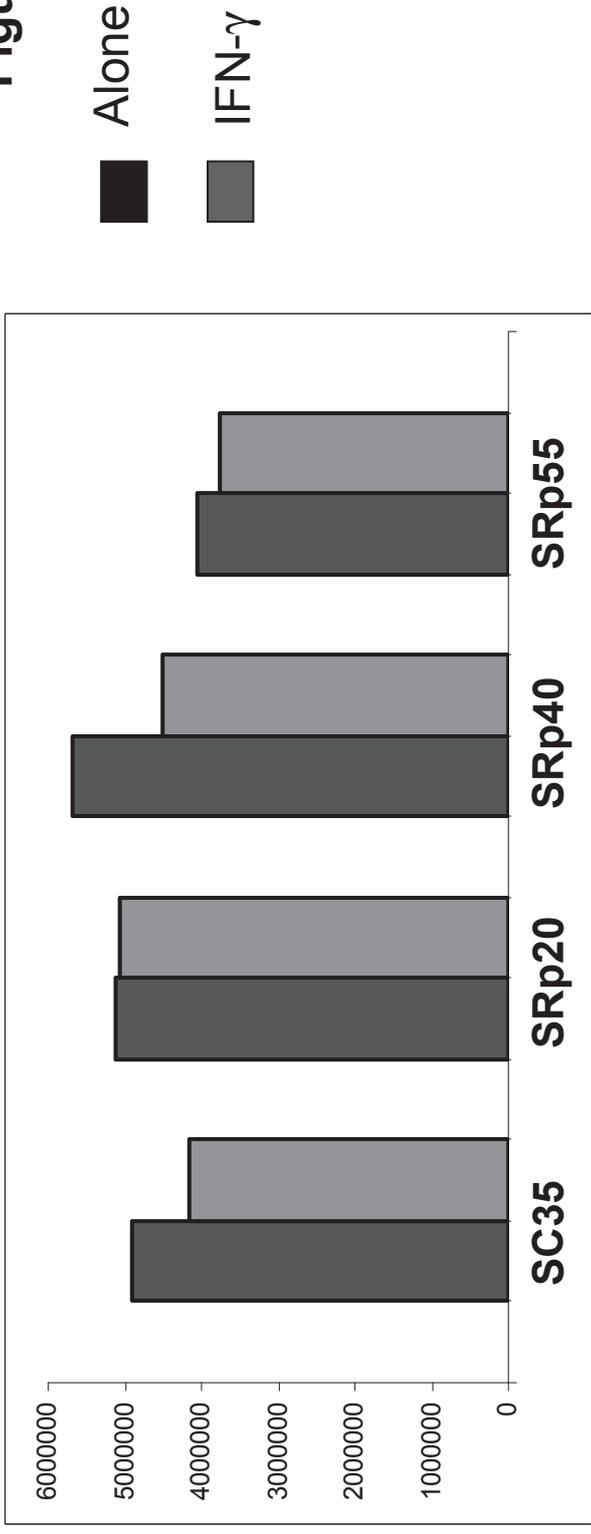
1.19

1.11

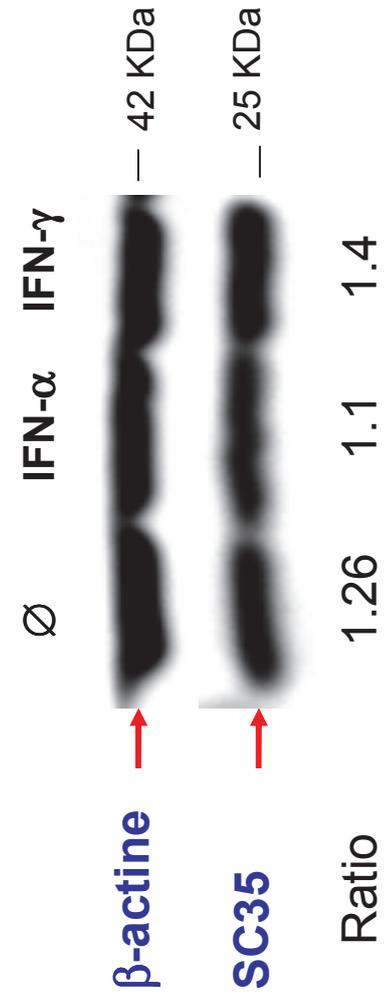
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Figure 4

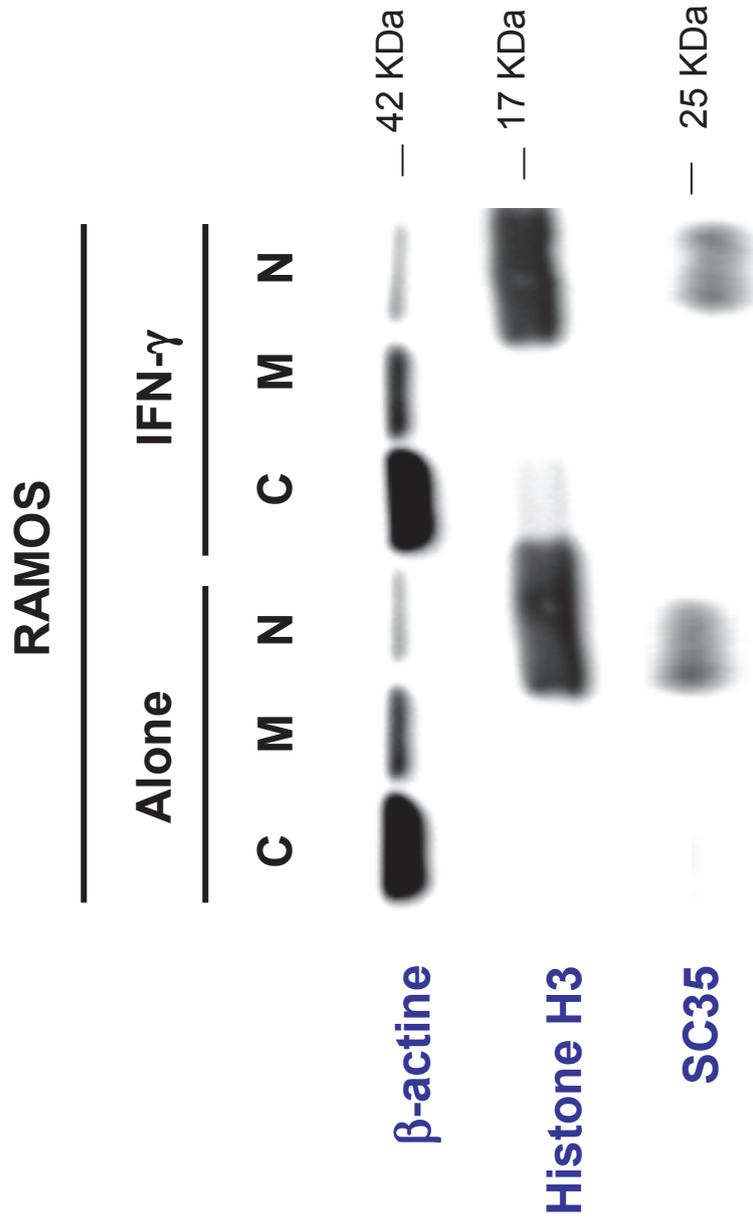


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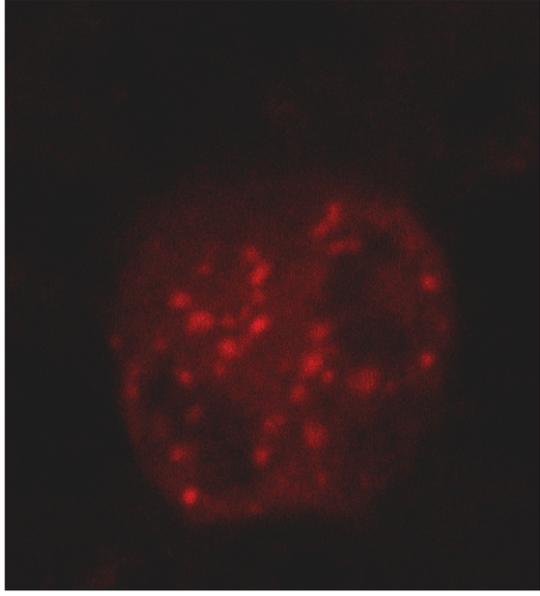
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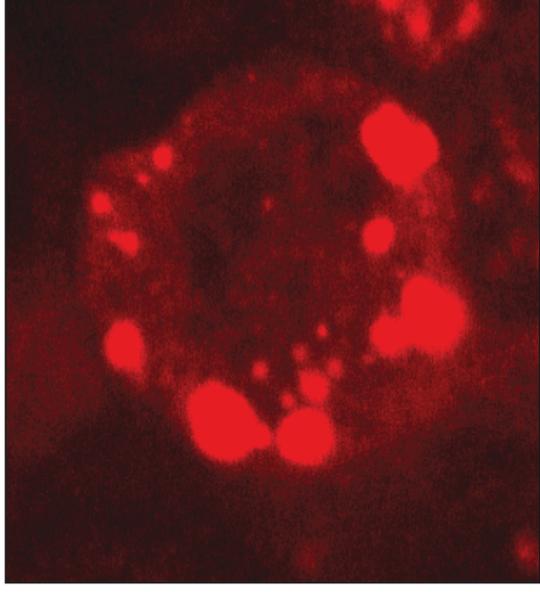


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Figure 4



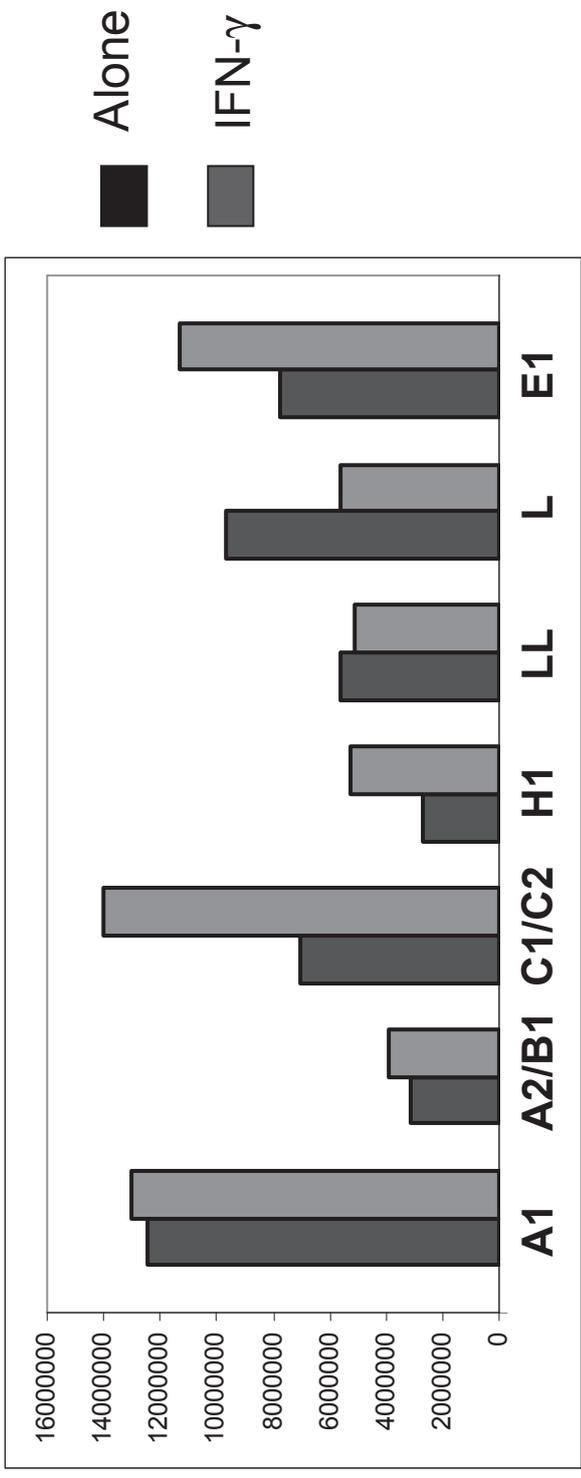
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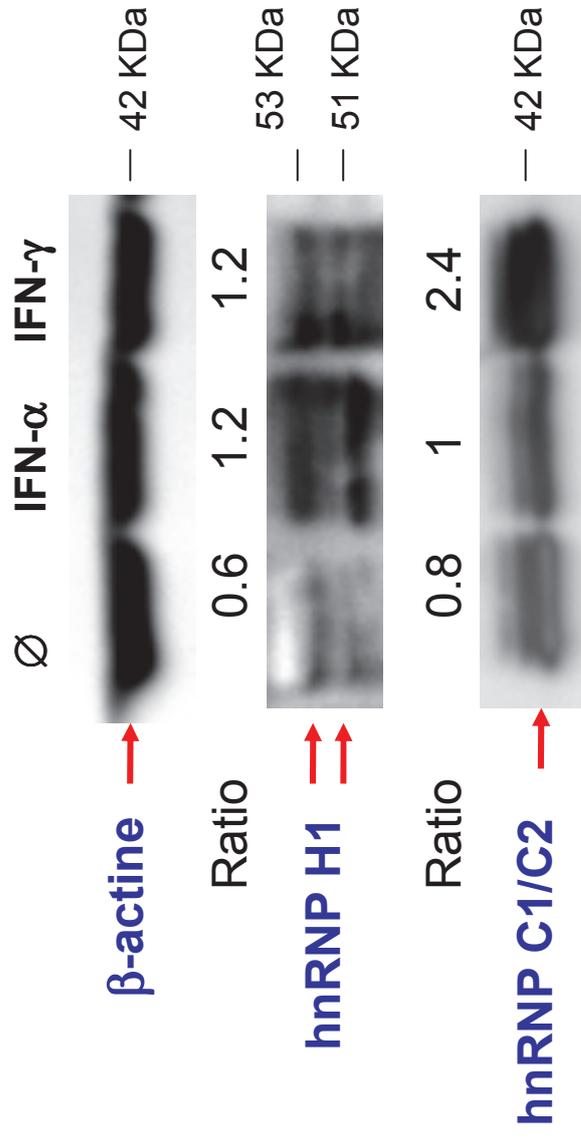
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Figure 5

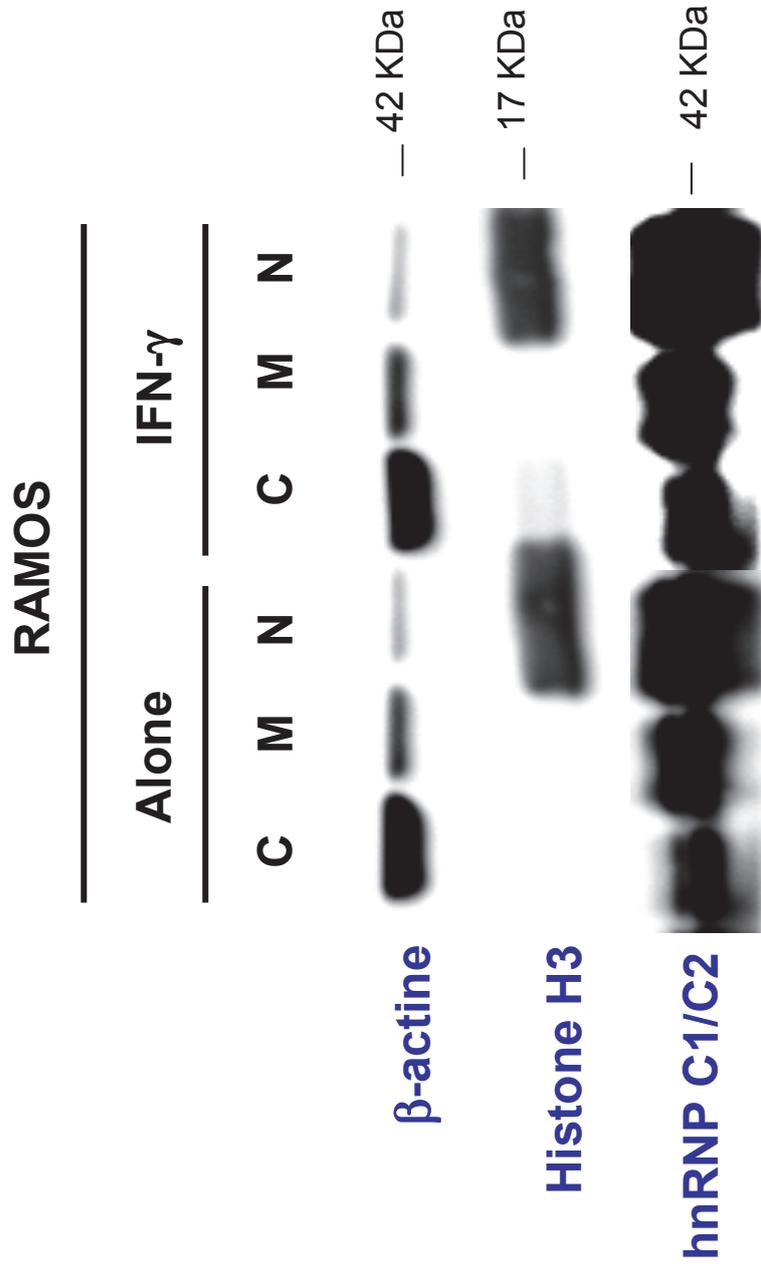


B.



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Figure 5



DISCUSSION

Although many progresses have been done in basic research of pSS, the etiology of this disease remains unknown. Genetic, epigenetic, hormonal, environmental and immunological factors play a role in the development of the disease. The classic immunological concept ascribed a key role to the T-cell. However, recent evidence indicates a major contribution of B cells. In fact, one of the main findings in the pSS understanding has been the study of BAFF in the pathophysiology of the disease.

In this work, we wanted to develop clinical and basic research topics, related to the role of B-cell in the pathogenesis of pSS. The three main subjects will be discussed separately.

A. Skin biopsy in pSS as a routine diagnostic tool

pSS presents as a multifaceted condition with a broad variety of clinical and biological abnormalities. This polymorphism accounts for the delay of 6-7 years in the diagnosis. Thus, the need for an early diagnosis of pSS has become a priority. The AECG criteria are based on laboratory test and MSGs biopsy. However, in many suggestive cases, MSGs biopsy may be non-conclusive. In the clinical spectrum, cutaneous manifestations, including xerosis are common but usually underestimated manifestations in pSS patients. In particular, the skin may function as a diagnostic window on the diseases affecting internal organs and other exocrine tissues. Thus, this analysis conducted us to study the diagnostic utility of skin biopsy in pSS. **In the first article**, we reported that B-cell and T-cell infiltrates in the skin biopsies, may be considered as a new criterion for pSS diagnosis.

In this study the most important finding was that two patients with non-contributive MSGs biopsy had skin infiltrates. In contrast, 10 skin samples from matched healthy volunteers were absolutely devoid of lymphocytes. This result shows that MSG and skin biopsies may be complementary in the study of sicca symptoms and the diagnosis of pSS. The discrepancy between the results of MSG and skin biopsies in pSS patients is not really surprising, since the extent of lymphocyte infiltrates varies between the fields in one MSG sample, between MSG in one biopsy, and between MSG samples in one patient over time (Pennec et al., 1990; Leroy et al., 1992; Pijpe et al., 2007).

Although MSGs biopsy remains the histopathological gold-standard for the SS diagnosis, some authors hold strong reservations regarding this biopsy, arguing that: 1- this site is non-specific; 2- exocrine atrophy is normal as a part of the ageing process. Thus lymphocytic infiltrates has been found in 15% of healthy controls (Radfar et al., 2002). In addition, some patients present some distressing sensory neuropathy in a portion of the mental dermatome, whereas the morbidity of skin biopsy is particularly low. Within the cutaneous infiltrates, T and B cells were described (**Figure 1**). B-cell characterization (CD20+) showed only 1% of CD5 expressing cells, which is a marker for polyspecific auto-Ab producing B lymphocytes. Unexpectedly, B cells were CD27+/IgD-, which are markers of memory B cells. Some B cells were also CD24+ immature B cells resembling to transitional B cells.

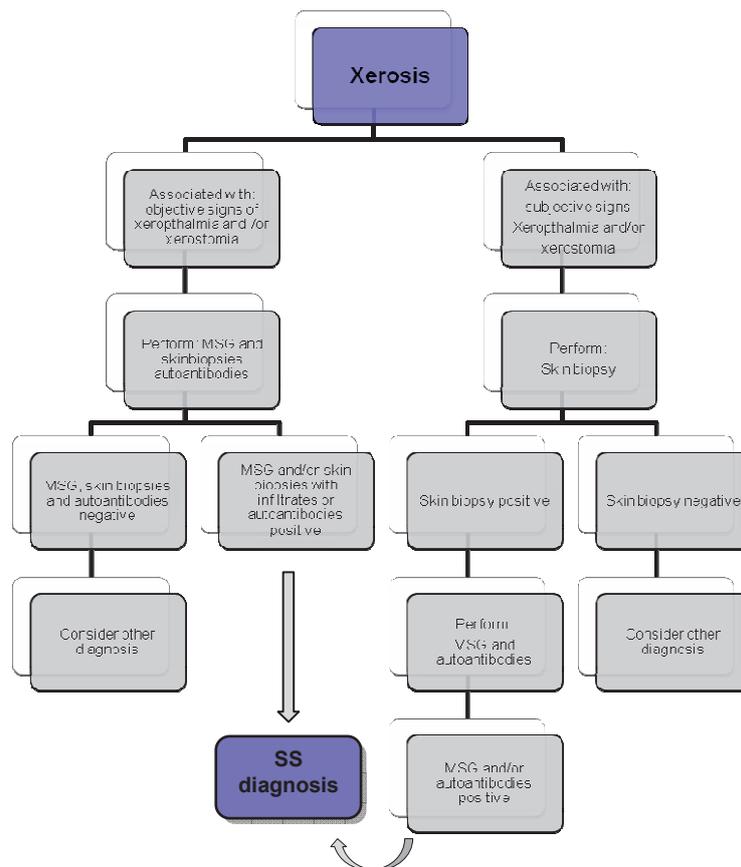
Assuming that homing receptors for the epidermis are specific for T lymphocytes (Sigmundsdottir and Butcher, 2008), the migration of B lymphocytes to the skin may be unlikely. The simplest explanation for their detection among the skin lymphocytes is that one of several of the B-lymphocyte chemotactic factors described in the MSG (Pers et al., 2007) are shared by all exocrine tissues. Consequently, these include also cutaneous sweat glands (Delaleu et al., 2008). Furthermore, we were surprised by our finding that memory B lymphocytes infiltrate not only the MSG (Hansen et al., 2005), but also the skin. The local production of BAFF may explain this finding, and deserves further research. Retention of memory B cells in the exocrine tissues may account, at least in part, for their decrease in peripheral blood. CD5-expressing B cells are not reported to generate memory B cells (Martin and Kearney, 2001), a finding which is consistent with their absence in the B-cell infiltrates from the skin. Among memory B cells, some harbor the CD20+/CD24+ profile, already described in the SGs (Daridon et al., 2006), which is in fact, closer to that of T2 B cells than to that of MZ B cells.

Given the ability of T cells, but not conventional B cells, to home specifically to, and resided preferentially in healthy, as well as in inflammatory (Bos et al., 2005) or malignant (Nashan et al., 2007) skin, our finding that B lymphocytes contribute to the cutaneous infiltrate was highly unexpected. This is, nonetheless, consistent with the likelihood of B cells to play a central role in the pathogenesis of pSS. Thus, the presence of B lymphocytes in the skin biopsy might alert the physician on a routine basis, and be regarded as a clue for the diagnosis of pSS. The view that any exocrine glands are at risk of undergoing the autoimmune assault connotes that the examination

of sweat glands could contribute to the histopathological diagnosis. The emergence of new and potentially active treatments, most notably anti-B lymphocyte monoclonal antibodies may conduct to the development of clinical analysis of skin biopsy in the follow-up.

In clinical practice, the skin of patients suspected to present pSS must be carefully examined and, one step further, the epidermis should be evaluated to search for B cell infiltrates. In **Figure 19**, we proposed a diagnostic flow chart that includes skin biopsy as a possible tool for the diagnosis of pSS. Xerosis may indeed constitute an early sign of pSS before the onset of xerostomia and xerophthalmia.

Figure 19. Proposed flow chart including skin biopsy for pSS diagnosis.



B. The FMS-like tyrosine kinase3 Ligand (FLT3-L), a mediator of B-cell survival, is also a marker of lymphoma in pSS.

Although the physiopathology of pSS remains unclear, the disease has traditionally been ascribed to T cells. Recent evidence, including several works completed in our group, indicates a major contribution of B cells (reviewed by Le Pottier et al., 2007; Youinou et al., 2012 and appendix 5 and 6). B-cell biological abnormalities, such as hypergammaglobulinemia, presence of auto-Abs, and abnormal distribution of mature B lymphocytes in peripheral blood are characteristic of pSS. In addition, the most severe complication in pSS patients is the increased risk of NHL, in 5% of cases. Since FLT3-L and FLT3 play a role in normal B-cell development and hematological malignancies (Lisovsky et al., 1996 ; Antonysamy et Thomson, 2000 ; Bertrand et al., 2000 ; Drexler et Quentmeier, 2004) we evaluated this cytokine and its receptor in patients with pSS. Although this cytokine has been mainly studied in hematological malignancies, studies in AIDs are scarce. **In the second article**, we found that serum levels of FLT3-L were higher in pSS patients compared to controls, and these levels were correlated with abnormal B cell distribution subsets in peripheral blood of patients. In other AIDs, Dehlin et al. (Dehlin et al., 2008) have showed that synovial membrane of RA patients express high levels of FLT3-L and synovial fluid levels of FLT3-L were higher in RA patients compared to those in osteoarthritis patients. However, in this study, serum FLT3-L levels were similar in RA patients and normal controls. In addition, intraarticular administration of a B-cell line that had been transfected with the FLT3-L gene resulted in highly erosive arthritis in a mouse model. This highlighted the effect of FLT3-L in RA.

In our study, as serum FLT3-L levels were elevated in pSS and these higher levels were correlated with the abnormalities of B-cell subset distribution, we hypothesized that Bm2 and Bm2' subsets, which are abnormally increased in peripheral blood of pSS expressed the receptor FLT3. In B lymphocytes, FLT3 expression has been previously described in pre-pro-B cells and pro-B cells, but not in mature B cells (Wasserman et al., 1995). The expression of FLT3 on Bm2 and Bm2' cells was confirmed by different techniques in our study. FLT3 expression, although weak, may be implicated in B-cell survival and proliferation in pSS.

The proliferative effect of FLT3-L stimulation on B cells was confirmed by B-cell culture experiments showing that this cytokine potentiated the proliferation of B cells in addition to IgM stimulation. This additive result was not surprising, since FLT3-L alone has no effect on normal hematopoiesis (Sitnicka et al., 2003; Vosshenrich et al., 2003;

Jensen et al., 2008), but favors the B-cell ontogenesis induced by IL-7 (Hunte et al, 1995; Namikawa et al., 1996). In pSS, this synergy may be due to certain cytokines, including BAFF, which is also largely altered in this pathology. In addition, our results showed that mature B cells produce FLT3-L, thus suggesting the possibility of an autocrine effect.

In MSGs biopsies we documented that infiltrating B lymphocytes also expressed FLT3, whereas ECs produced FLT3-L, suggesting that both molecules are produced in situ. Thus, FLT3-L from ECs would be implicated in B-cell survival in the exocrine infiltrates. Then, to confirm this interaction, we developed a coculture experience, using the HSG-cell line, and we found that anti-FLT3 and anti-FLT3-L Abs blocked the B-cell proliferation induced by the ECs. These results showed indirectly that B-cell survival in MSGs is associated with FLT3-L. The anti-proliferative effects of both anti-FLT3 and anti-FLT3-L Abs suggest a possible rational for therapy in pSS. Currently, many small-molecule FLT3 inhibitors have been developed to treat hematological malignancies, but clinical trials have resulted in limited anti-leukemia effects because of off-target toxicities and drug resistance (Weisberg et al., 2009), targeting mainly the mutated FLT3. Clinical trials are ongoing to improve their clinical efficacy. One of the explanations to low clinical effect of FLT3 inhibition by different tyrosine kinase inhibitors in AML patients, is the increase of FLT3-L after chemotherapy treatment (Sato et al., 2011), mainly in patients with relapsing disease. The development of anti-FLT3 Abs might overcome these difficulties and enhance the anti-leukemia efficacy of FLT3 inhibitors. Recently, Yamamoto et al. (Yamamoto et al., 2011) developed an antagonist Ab, designated A2, able to continuously inhibit FLT3-L-induced phosphorylation of FLT3 and MAPK. Thus, this strategy can be a new approach of targeting FLT3 and FLT3-L therapies, and could be tested in AIDs.

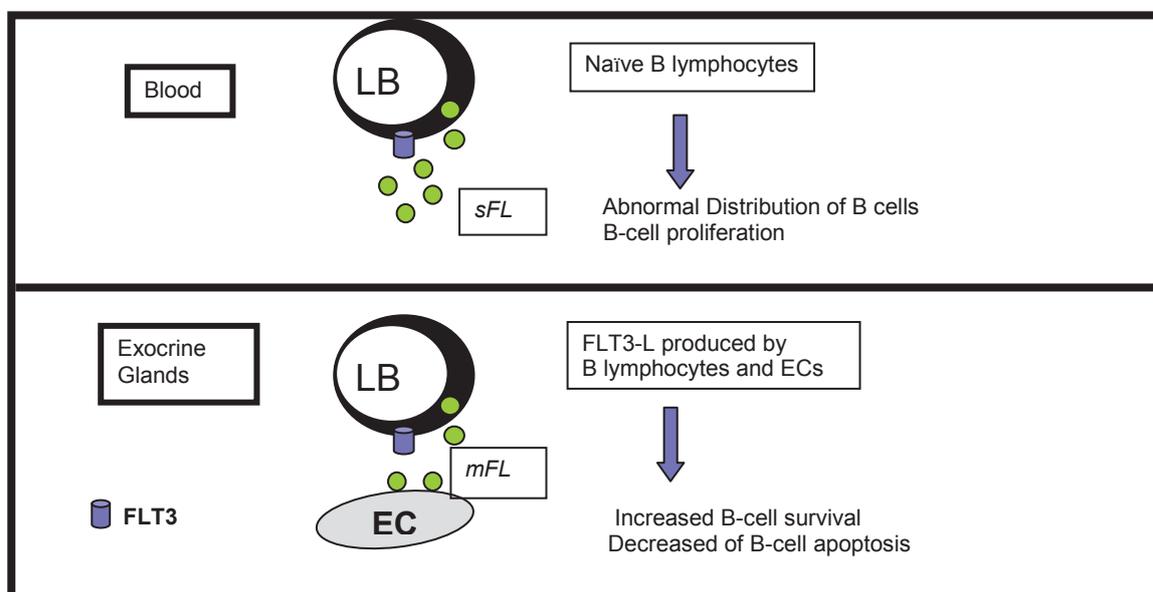
The functions of FLT3-L on differentiation of pre-DCs to mature DCs may explain the benefic effects of FLT3-L targeted therapy in some AIDs in animal models. For example, targeting FLT3 with lestaurtinib may be effective in the treatment of experimental autoimmune encephalomyelitis. Whartenby et al. (Whartenby et al., 2005) showed clinical improvement in this model due to the inhibitory effect in DCs. In the other hand, some authors propose that expansion of DCs induced by FLT3-L may delay progression of AIDs. In the mice model for Type 1 Diabetes (NOD mice), treatment with FLT3-L during early stages was shown to delay diabetes onset, inducing the expansion of myeloid DCs and CD4⁺CD25⁺ Treg cells (Chilton et al., 2004; O'Keefe et al., 2005). However, another study showed that FLT3-L stimulation actually

accelerates autoimmunity when autoreactive CD8 T cells were detectable in blood before treatment in the NOD model (Van Belle et al., 2009). In this case, expansion of DCs and induction of Treg cells by FLT3-L remained intact, but both the number and the functional response of islet-specific CD8 cells were boosted. These inverse results show that FLT3-L may play an important role in the immune tolerance according to the different phases of development of AIDs. Recently, the effects of FLT3-L inhibition with sunitinib (a multi-tyrosine kinase inhibitor) in a mouse model of collagen-induced-arthritis have been described (Dehlin et al., 2011). The antiarthritic effect was achieved by inhibition of DCs, reduction of Abs production, and bone metabolism. This study shows that inhibition of FLT3 is a potent anti-arthritic mechanism reducing antigen presentation, synovial inflammation, and bone resorption. Specific FLT3 inhibitors must be tested in the same pathological context. Based on our results, the **figure 20** summarizes the role of FLT3-L and FLT3 on B-cell hyperactivity in pSS.

Finally, the clinical aspect of FLT3-L in pSS was evaluated. Interestingly, higher serum levels of FLT3-L were correlated with high disease activity scores measured by ESSDAI and two patients with lymphoma presented high levels of FLT3-L. **In the third article**, in order to confirm that FLT3-L levels may be a marker of lympho-proliferation in patients with pSS, we evaluated serum levels of FLT3-L in 369 patients enrolled in the Frech cohort of pSS (ASSESS). In this article, history of lymphoma was significantly associated with higher levels of FLT3-L. In addition, risk factors for lymphoma development such as purpura, low C4, lymphocytopenia, low IgM and high levels of β 2-microglobulin were associated with higher levels of FLT3-L whereas enlargement of parotid glands, lymphadenopathy and splenomegaly were not. Among all the biological markers already described, FLT3-L was the most significantly associated with lymphoma. In this study, levels of FLT3-L remained high in treated patients with lymphoma. In hematological malignancies higher FLT3-L levels are associated with advanced disease before treatment and residual disease after treatment. Retzlaff et al. showed that before treatment, serum levels of FLT3-L correlate with prognostic variables and high grade lymphoma subtype in patients with primary gastro-intestinal NHL (Retzlaff et al., 2002). In ALL, FLT3-L treatment renders cell-lines and primary leukemia cells resistant to chemotherapy agents (Furuichi et al., 2007), showing that high levels of FLT3-L may contribute, at least in part, to persistent minimal residual disease in BM. Thus, persistent high levels of FLT3-L after lymphoma treatment may indicate minimal residual disease, B-cell activation and probably high relapsing risk. In our study, three patients with relapsing disease after chemotherapy presented high levels of FLT3-L. This observation needs to be confirmed in the

prospective follow-up of our cohort. Higher levels were also associated with previous MALT type lymphoma, which is the most characteristic lymphoproliferative disorder in patients with pSS, compared to other types such as DLBCL and MZ lymphomas.

Figure 20. Implication of FLT3-L and FLT3 in B-cell abnormalities in pSS. FLT3-L is elevated in serum of pSS patients and it is correlated with an abnormal B-cell profile in the peripheral blood. FLT3 is expressed by Bm2 and Bm2' (subsets of B cells altered in the disease). In exocrine glands, FLT3-L produced by ECs and in an autocrine manner by B cells favors the survival and proliferation of B-cell in inflammatory infiltrates. The role of FLT3-L over the B-cell proliferation may explain the clinical evolution to B cell lymphoma observed in some patients. Targeting FL may open new strategies in SS patients' therapy.



An alternative explanation to our results would be that FLT3-L is a marker of activity and that patients with pSS and lymphoma have a higher activity of the disease which persists even after cure of lymphoma. In favor of this hypothesis, FLT3-L was correlated with the ESSDAI. Moreover, this correlation occurred even if we excluded the 3 patients with active lymphoma at evaluation, these 3 latter patients having obviously an important increase of the ESSDAI score due to active lymphoma (addition of 12 points) (Seror et al., 2010).

In conclusion, routine evaluation of FLT3-L in pSS may be useful to evaluate clinical severity and activity of the disease. The follow-up of the ASSESS cohort will allow to

evaluate if the baseline level of FLT3-L will be also predictive of occurrence of incident cases of lymphoma.

C. Identification and study of the new variant of BAFF, Δ 4BAFF as a transcription factor of its own gene, and their role in autoimmunity (including pSS)

Elevated expression of BAFF, contributes to the expansion of low-affinity self-reactive B cells during the establishment of tolerance. However, mechanisms leading to BAFF over-expression in AIDs such as SLE or pSS and in lymphoproliferation such as CLL are not fully understood. **In the fourth and fifth articles**, we studied the role and regulation of the new variant of BAFF, called Δ 4BAFF in humans and Δ 5BAFF in mice, which has been recently described in our laboratory. Interestingly, this new variant is mainly detected in AIDs (including PBMC and ECs from pSS patients), and may explain the excess of BAFF in pSS patients. In the fourth article, we confirmed that Δ 4BAFF is a transcription factor that enhances expression of its own parent gene. These findings are of particular interest because BAFF overexpression is a central driver in these AIDs. In pSS, BAFF is associated with B-cell tolerance breakdown and auto-Ab production but also with the abnormal distribution of B cells subsets and B-cell hyperactivity (Varin et al., 2010). In SGs, a minority of B cell clusters represent ectopic GCs cells, while the majority presents features consistent with T2-type and MZ-like B cells (Le Pottier et al., 2009). Interestingly, both types of B-cell clusters include autoreactive B cells and BAFF is associated with expansion of T2 B cells and MZ-like B cells in the SGs (Daridon et al., 2006). Finally, BAFF plays a major role in B-cell repopulation after their depletion by rituximab in pSS (Daridon et al., 2007). Elevated expression of Δ 4BAFF transcript was not systematically associated with an elevated expression of the classical BAFF transcript in synoviocytes from RA or ECs from pSS patients. This could be explained by degradation mechanism. For instance, the NMD mechanism, an integral quality control machinery that initiates active degradation of nonsense mRNAs in order to maintain high fidelity gene expression is activated when translation terminates more than 50-55 nucleotides upstream of the last intron-exon junction (Nagy and Maquat, 1998; Singh and Lykke-Andersen, 2003). Although Δ 4BAFF seems to be a good candidate for NMD degradation, our results using cycloheximide inhibition showed that Δ 4BAFF mRNA is not subject to NMD. Consequently, mechanisms leading to alternative splicing, for regulating protein

expression in a cell-specific manner or in response to precise environmental cues, may favor the alternative splicing of exon 4 under specific stimulations. Consideration must be given to the differential expression of $\Delta 4$ BAFF in explaining the intriguing issues of serum concentrations of BAFF that remain within, or below the normal range in some patients with AIDs (Pers et al., 2005) and why estimates fluctuate with changes in inflammatory activity, extent of the disease and classification criteria chosen by the investigators for pSS, RA or CLL.

The $\Delta 4$ BAFF counterpart splice variant was observed in splenocytes from Balb/c and SWISS mice. BAFF gene in mice possesses seven exons. We demonstrated that exon 5, which shares significant sequence identity with exon 4 in humans, was spliced out. The resulting $\Delta 5$ BAFF protein was also truncated as the splicing event generates a stop codon further upstream in exon 7. However, the $\Delta 5$ BAFF variant was not observed in C57Bl/6 mice. This difference may be due to the mouse strains used. Balb/c mice have been shown to present increased susceptibility to several intracellular pathogens when compared to C57Bl/6 mice (Autenrieth et al., 1994). Splenocytes from Balb/c mice showed a constitutive increased production of IL-10 when compared with C57Bl/6 mice (Roque et al., 2007). Interestingly, splenocytes from C57Bl/6 mice stimulated with IL-10 were induced to produce $\Delta 5$ BAFF. Consequently, IL-10, a cytokine strongly associated with inflammatory response could also promote $\Delta 5$ BAFF expression leading to BAFF production in such circumstances.

In this study, we also observed that N-glycosylation of $\Delta 4$ BAFF was not only required for nuclear entry but also for promoter binding. The importance of the N-glycosylation status for BAFF has been already described, particularly for the Δ BAFF variant (Gavin et al., 2003), but also for the full-length form of BAFF in the serum of patients with AIDs (Le Pottier et al., 2009). In this respect, a number of conflicting results have cast doubt on the reliability of the ELISA presently in use for its quantification, most of them being unable to recognize the non-glycosylated form of BAFF (Le Pottier et al., 2009). Furthermore, N-glycosylation was recently described to be required for the full activation of the transcription factor CREB-H. Unglycosylated or deglycosylated CREB-H was retained in an inactive form in the endoplasmic reticulum and less capable of activating transcription by binding to its promoters (Chan et al., 2010).

We also showed that Δ 4BAFF needs p50 as a cofactor to enter to the nucleus and to bind to BAFF promoter. This cooperation between Δ 4BAFF and p50 appears to be important in regulating BAFF expression in normal health, CLL and AIDs. The interactions of NF- κ B dimers or monomers with heterologous transcription factors through direct binding has been already described and profoundly influences transcriptional responses (Oekinghaus et al., 2011). NF- κ B p50 lacks a transactivation domain and therefore usually form a heterodimer to be transcriptionally active (Ghosh et al., 1998). In vitro studies have shown that p50 can associate with other transcriptional activators such as Bcl-3 (Fujita et al., 1993) or p300 (Deng et al., 2003) to activate transcription. NF- κ B p50 can also form a complex with the transcriptional co-activator CREB to activate IL-10 transcription in macrophages (Cao et al., 2006).

Interestingly we also found that Δ 4BAFF is a transcription factor for hundreds of genes, implicated mainly in the immune response and alternative splicing (microarray results). Among the up-regulated genes after Δ 4BAFF tranfection, we found the Activation-induced cytidine deaminase (*aid*), TLRs and miR155 (a miR implicated in B-cell maturation and Ig production). In an interesting manner, these genes have been also reported to be regulated by NF- κ B pathway, especially the p50 component. For instance, Dedeoglu et al. (Dedeoglu et al., 2004) have described that B cells from p50 deficient mice were impaired in their ability to up-regulate *aid* in reponse to CD40L and IL-4 stimulation. In addition, Carmody et al. (Carmody et al., 2007) reported that by blocking the ubiquitination of p50, Bcl-3 stabilizes the p50 complex that inhibits gene transcription induced by TLRs stimulation, maintaining the innate immune homeostasis. The promoter of the BIC gene encoding the miR155 contains two putative NF- κ B sites able to bind in vitro the NF- κ B proteins p50 and p65 in nuclear extract from MC3 cells (Gatto et al., 2008). This evidence shows that some genes that are up-regulated in our Δ 4BAFF model are also up-regulated by p50 expression, indicating probably a synergistic mechanism between Δ 4BAFF and p50 to bind these promoters. Further investigation of the role of Δ 4BAFF and its interaction with p50 in the contexts of both autoimmunity and B cell leukemia could provide novel therapies targeted at modulating its function.

We also explored the effects of IFN- γ on BAFF expression by U937 monocytes to see if the transcription factor Δ 4BAFF regulates this expression. Once inflammation is initiated, IFN- γ acts to induce BAFF production by monocytes (Nardelli et al., 2001),

intensifying the inflammation process and AIDs. The links between IFN- γ and BAFF are indeed particularly important in the development and the maintenance of AIDs and B-cell lymphoproliferative diseases. The increased production of IFN- γ in *Lyn*-deficient mice (a well established model of lupus-like autoimmunity) (Xu et al., 2005) feeds back on the myeloid cells to further stimulate BAFF release. Furthermore, the reciprocal production of BAFF and IFN- γ establishes an inflammatory loop between myeloid cells and T cells that exacerbates autoimmunity in this model (Scapini et al., 2010). Blocking this inflammatory loop by lowering BAFF (using monoclonal antibody treatment) or removing IFN- γ (genetically) prevented nephritis in *lyn*^{-/-} animals. Our results showed that Δ 4BAFF acts as a transcription factor of BAFF gene to encourage BAFF release after IFN- γ stimulation in U937 cells. IFN- γ -induced BAFF release was abrogated by transfecting the cells with si Δ 4BAFF.

Using B-CLL cells as a model we showed that this new variant favors the production of BAFF and B-cell survival. The potent survival function of BAFF in limiting spontaneous cell death of B-CLL cells provides a key context to examine the significance of Δ 4BAFF function. The striking effect of BAFF on B-CLL survival (Novak et al., 2002; Kern et al., 2004) raises the possibility that Δ 4BAFF inhibition leads to decreased BAFF expression and corresponding decrease in cell survival. Thereby, B-CLL cells may be more sensitive to spontaneous apoptosis. Δ 4BAFF expression, observed in B-CLL cells, could explain the mechanism by which some B-CLL cells acquire the ability to express BAFF. Interestingly, transcription factors from the NF- κ B family are highly activated in B-CLL cells (Munzert et al., 2000). Moreover, p65 and p50 increases were found, indicating that p50/p65 heterodimers are functionally active as transcription factors in CLL (Kern et al., 2004) and association between Δ 4BAFF and p50 could therefore be promoted in CLL. Although Δ 4BAFF was increased in B-CLL cells, no association was observed between clinical features and levels of expression of Δ 4BAFF in B-CLL cells (data not shown). However, larger numbers of patients must be tested for possible correlations to be found.

The pivotal role of Δ 4BAFF as a transcription factor that controls BAFF expression holds immense promise in the clinical setting. The potential for Δ 4BAFF to be used as a therapeutic target will require further investigation into the scope of its role and

potential as a transcriptional regulator of other genes, with ramifications for disease outcome and treatment strategies aimed at controlling BAFF production in AIDs and cancer.

Induction and regulation of Δ 4BAFF expression

Based on the effects of the new-described Δ 4BAFF form over the full-length form of BAFF and its main expression in AIDs and CLL, **in the fifth article** we analyzed the mechanisms for Δ 4BAFF induction and regulation in the autoimmune setting.

Firstly, to study the alternative splicing of the exon 4, we constructed a minigene centered on this exon and then transfected in different cell lines. This approach showed that after 24h of transient transfection, at baseline, an alternative splicing of exon 4 was evidenced. In addition, these findings were replicated in a B-cell line (RAMOS) and a monocytes cell-line (U937) showing that at least in these two cellular populations, the role of individual splicing factors may be similar. In contrast, colonic cancer cells do not present alternative splicing of exon 4 after minigene transfection (data not shown), highlighting that the ability of *trans*-regulatory proteins to control alternative splicing is in a cell/tissue-specific manner (Lee et al., 2002; Ladd et al., 2004).

To identify and demonstrate the *trans* and *cis*-elements implicated in the alternative splicing of the exon 4, we found by biocomputational analysis (RESCUE-ESE, Fairbrother et al., 2004) that some members of the SR family may bind to exon 4 and in theory, favor the constitutive splicing of the full-length form of BAFF. Among these members, a predictive binding of SC35 (SFRS2), SRp40 (SFRS5) and SRp55 (SFRS6) to exon 4 of BAFF was found. In contrast, SRp20 (SFRS3) and ASF/SF2 (SFRS1) had not predictive binding sites to exon 4. Prompted by these analyses, the co-transfection of the minigene plus coding plasmids for splicing proteins showed that SC35 is essential for the constitutive splicing of BAFF. In fact, over-expression of SC35 led to a complete inclusion of exon 4 in RAMOS B cells. The analyses of *cis*-acting elements showed that two ESEs may bind SC35 on the exon 4, controlling the specific usage of alternative exons. The function of these two specific binding sites to SC35 and their role in the constitutive vs. alternative splicing of exon 4 will be confirmed by directed mutagenesis.

SC35 is one of the most important and studied member of the SR family. Adding to their functions as a splicing factor, SC35 affects transcriptional elongation in a gene-specific manner (Lin et al., 2008). Interestingly, SC35 can regulate its own expression. This is accomplished via a feedback loop that connects the level of SC35 protein to the productive splicing of the SC35 transcript. The SC35 protein binds to its own pre-mRNA uncovering ss downstream of the termination codon. When these alternative ss in the 3'UTR are used, the normal termination codon becomes a PTC based on the 50 nucleotide rule. NMD then degrades the transcript, down-regulating the expression of SC35. In this way, SC35 appears to auto-regulate its own production (Sureau et al., 2001). Interestingly, SC35 may be also an auto-antigen. Covini et al. (Covini et al., 1997) reported that ANAs from patients with hepatocellular carcinoma may recognize splicing factor SC35. In addition, some members of the SR protein family, including SC35, showed reactivity with sera from SLE patients up to 50% of cases and not other AIDs (RA and antiphospholipid syndrome) (Neugebauer et al., 2000). In our group, preliminary results (data not shown) evidenced, by indirect immunofluorescence (IFI), that there is co-localization between ANAs and anti-SC35 in 54% of pSS patients, and only 11% of SLE patients, confirming that serum samples of patients with AIDs may recognize nuclear SC35 protein. Sensitivity and specificity were 54% and 90%, respectively in pSS patients. Interestingly, six of 9 pSS with no anti-SS-A or anti-SS-B auto-Abs presented anti-SC35 auto-Abs, and this auto-Ab would be a diagnostic marker for pSS. Western-blot and dot blot analyses confirmed this specificity. Currently we are developing an ELISA test to verify and quantify the presence of anti-SC35 auto-Abs in AIDs.

The role of other SR proteins in alternative splicing of immune genes has been described. For example, ASF/SF2 (SFRS 1) regulates the expression of CD3 ζ protein limiting the production of the alternatively spliced isoform, which is an unstable transcript. This alternatively spliced variant is more expressed in T cells from SLE patients compared to healthy controls (Moulton and Tsokos, 2010). Similar evidence for ASF/SF2 has been described in the alternative splicing of CD200 (Chen et al., 2010) which is an important glycoproteins that prevents inflammatory disorders, graft rejection and AIDs (Gorczynski et al., 2004). A truncated form resulting from alternative splicing is characterized as a functional antagonist to full-length CD200. Taken together, these data show that members of SR protein family, may mediate constitutive splicing, limiting the generation of alternative splicing isoforms, which in

some cases may have a deleterious role in the normal immune response.

The function of individual splicing factors may be also situation dependent. It has been reported that in mood disorder, patients present a decrease in Glucocorticoid Receptor (GR) alpha, but not GRbeta (produced by alternative splicing), which is associated with an increased expression of SRp20 but not SRp30c RNA (Watanuki et al, 2008). These results show that the function of a specific splicing factor (in our model, SC35 protein) depends of specific cellular/tissue patterns, but also pathological conditions (induced, for instance, for acute stimulations or stress).

IFN- γ and alternative splicing of exon 4 of BAFF

The splicing efficiency of individual exons is determined by multiple features involving gene architecture (i.e. the size of the exon), the *cis*-acting elements within the exons and flanking introns (RNA sequence), and interactions with components of the spliceosome and auxiliary regulatory factors which transiently co-assemble with the spliceosome. In addition, a larger fraction of these alternative splicing events are regulated according to cell-specific pattern or in response to acute stimulation (Black, 2003). As discussed above, we tested different stimulations already known as positive regulators of BAFF (such CD40L, IL-10, IFN- γ , and IFN- α) and their effects on Δ 4BAFF induction. Interestingly, under different stimulations, normal tonsilar B cells express Δ 4BAFF. The induction of Δ 4BAFF was accompanied by increasing on full-length BAFF at the protein level and soluble BAFF. Although various stimulations induced Δ 4BAFF transcript, the most reproducible model was obtained by IFN- γ stimulation, after 24 h of cell-culture. Thus, using only this stimulation, we constated that IFN- γ favors the Δ 4BAFF expression in different cell lines (including monocytes, B cells and ECs). In the second part of the fifth article, an important exclusion of exon 4 on the minigene-transfected RAMOS B cells was found after 24h of IFN- γ stimulation, confirming that the induction of Δ 4BAFF is mediated by inflammatory stimuli.

The effects of IFN- γ on alternative splicing phenomena have been described in other genes. For example, isoforms of human tryptophanyl-tRNA synthetase (TrpRS) *in vivo* are regulated by IFN- γ through alternative mRNA splicing. The miniTrpRS (produced by alternative splicing) has anti-proliferative and anti-angiogenic activity, but these

effects are not seen with the full-length protein. An IFN- γ -sensitive promoter was demonstrated to drive the production of miniTrpRS and two new splice variants of TrpRS, showing that this cytokine may explain alternative splicing variants with different effects (Liu et al., 2004). In addition, IFN- γ modifies the global expression pattern of genes, including spliced variants. Ortis et al. (Ortis et al., 2010) in purified rat pancreatic β -cells showed that IFN- γ in association with other cytokines (IL-1 β and TNF- α) modified the expression of more than 20 genes involved in RNA splicing, and changes in alternative splicing of >50% of the cytokine-mediated genes (by exon-array analysis), showing that these cytokines may affect the alternative splicing in a tissue specific manner.

Interestingly in our study, RAMOS B cells co-transfected with the minigene and SC35 coding plasmid and then stimulated with IFN- γ for 24h showed completely the inverse effect of SC35 plasmid before stimulation (complete exclusion of exon 4). This effect was not evidenced with other SR proteins and hnRNPA1 co-transfections. Thus, to understand the role of IFN- γ stimulation over splicing regulatory proteins, we hypothesized either that IFN- γ induces a decrease of SC35 protein or its dysregulation; and/or induces an increase of hnRNPs. By different techniques we found that SC35 transcript and protein expression are only slightly modified after IFN- γ . However, we constated by confocal microscopy that SC35 is differently located in the perinuclear space after IFN- γ stimulation. In fact, the abnormal function of SC35 induced by IFN- γ (due to its different location) may be explained by a binding-site competition between SC35 and hnRNPs and not by changes in its expression. The transcript levels of three hnRNPs (hnRNP C1/C2, E1/E2 and H) were elevated 24h after IFN- γ stimulation in RAMOS B cells. Although the hnRNPA1 is the most studied splicing factor of the hnRNP family (Caceres et al., 1994; Hanamura et al., 1998; Pollard et al., 2002), its expression was not modified in B cells after IFN- γ stimulation and no effect was seen in the co-transfection model with hnRNPA1 coding plasmid. In the study by Ortis et al. (Ortis et al., 2010), an exon-array analysis after 24h IFN- γ stimulation showed that hnRNPC is two times increased. Similar to our results, this hnRNP is regulated by IFN- γ stimulation besides the cell type. The relationship between IFN- γ stimulation and hnRNPC deserves to be further studied. Thus, in our model, IFN- γ stimulation induces hnRNPs that binds to silencer sequences inhibing SC35 protein binding to exon 4 ESEs, forcing a shift of splicing to a distal splicing site, favoring Δ 4BAFF induction. In

fact, differential expression of SR proteins, and their negative regulators, the hnRNPs, will bring about tissue- and cell-type-specific splicing in vivo (Hanamura et al., 1998). For example, artificially altering the ratio of the SR protein SF2/ASF (SRSF1) to hnRNP A1 can lead to dramatic changes in alternative splicing (Mayeda et al., 1993; Cáceres et al., 1994; Wang et al., 1995).

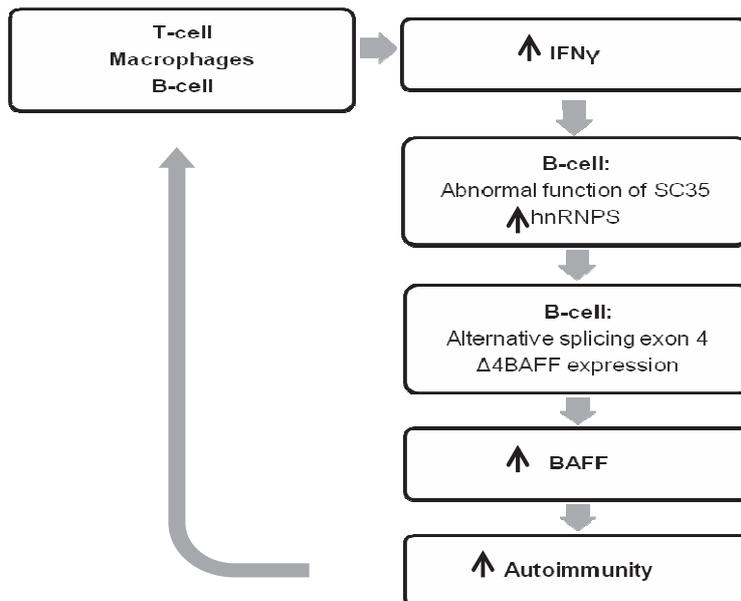
In immune genes, the hnRNPs are also implicated in regulation of alternative splicing. For example, the hnRNPL-like (hnRNPLL) has been identified as a critical inducible regulator of CD45 alternative splicing, from the larger isoforms known as CD45RA to the shortest isoform, CD45RO express by activated and memory T cells (Oberdoerffer et al., 2008). In our study, hnRNPLL was not modified after IFN- γ stimulation.

In an interesting way, recent evidence shows that some hnRNPs may be recognized by auto-Abs in AIDs patients, mainly in pSS (De Beeck et al., 2012). Therefore to understand the relationship between auto-Abs formation to hnRNPs and SR proteins and their effects on the alternative splicing of immune genes would elucidate an additional physiopathological mechanism in AIDs.

Concluding with our RAMOS B-cell model, the IFN- γ stimulation induced the alternative splicing of the exon 4 of BAFF, inducing an increase of some hnRNPs leading to binding-site competition with SC35 that is finally abnormally located in the perinuclear space. With these results we can conclude that an inflammatory loop between IFN- γ and BAFF pass through the induction of Δ 4BAFF. **Figure 21** summarizes our hypothesis about the role of IFN- γ stimulation on Δ 4BAFF induction and subsequently the increasing of BAFF in AIDs.

In conclusion, these last two articles provide an expanded conceptual view of BAFF gene regulation, and contribute to a better understanding of the mechanisms involved in BAFF up-regulation in AIDs, using as model pSS. We hope that these results may help to improve BAFF-targeted therapy in these conditions.

Figure 21. Role of IFN- γ stimulation on Δ 4BAFF induction. IFN- γ produced by activated T cells, macrophages and also B cells in salivary glands induces an abnormal function of SC35 in B cells, accompanied by increasing of hnRNPs (hnRNP C, E and H). These two conditions favor the alternative splicing of exon 4 of BAFF, and induction of Δ 4BAFF. Then, Δ 4BAFF plays its role as transcription factor of BAFF on B cells which is traduced by perpetuation of the autoimmune phenomenon.



General conclusions

Collectively, these results are of clinical and fundamental basic interest in pSS. Of **clinical** interest for all these three points: 1) Memory B cell infiltrates are present in pSS and may be used as a diagnostic and follow-up tool; 2) Levels of FLT3-L are high in pSS and may favor the development of lymphoma. Thus this cytokine can be use as a marker of activity and severity in pSS; 3) the important regulatory role of Δ 4BAFF in the overexpression of BAFF provide an expanded conceptual view of BAFF gene regulation in AIDs, and may open new therapeutic options targeting BAFF in pSS. Of **basic and fundamental research** interest: 1) the accumulation of memory B cells in the skin of pSS highlights the importance of these cells in pSS; 2) FLT3-L proliferation effect may explain survival of infiltrating B cells in exocrine glands of pSS patients. In addition, FLT3 expression by naïve B cells may explain, in part, the abnormal B-cell

distribution seen in peripheral blood of patients; 3) the demonstration that IFN- γ induces $\Delta 4$ BAFF, leading to an increase of BAFF provides an expanded conceptual view of *BAFF* gene regulation, and contributes to a better understanding of the mechanisms involved in BAFF up-regulation in autoimmunity.

Appendix

Appendix 1

Évolution épidémiologique des maladies ostéoarticulaires

Tobón GJ, Saraux A.

La lettre du Rhumatologie 2010; 361: 4-6

Évolution épidémiologique des maladies ostéoarticulaires

Epidemiology of rheumatic diseases

G.J. Tobón*, A. Saroux*

Les études épidémiologiques des maladies ostéoarticulaires portent non seulement sur les indicateurs épidémiologiques de morbidité (la prévalence, l'incidence) et de mortalité, mais aussi sur le rôle des facteurs génétiques et environnementaux dans le risque de développement de certaines d'entre elles. Les plus étudiées sont la polyarthrite rhumatoïde (PR), le lupus érythémateux disséminé (LED), l'arthrose, la goutte et la sclérodermie. D'importants progrès ont été réalisés ces dernières années, et plusieurs facteurs de risque sont maintenant identifiés dans ces pathologies. Les études épidémiologiques ont aussi montré la disparité géographique et l'évolution dans le temps de certaines maladies.

Pour la PR, il existe des disparités régionales, avec une prévalence estimée à 0,81 % au Royaume-Uni (1,16 % chez les femmes et 0,44 % chez les hommes) [1], 0,5 % en Espagne (0,8 % chez les femmes et 0,2 % chez les hommes) [2] et 0,33 % en Italie (0,51 % chez les femmes et 0,13 % chez les hommes) [3]. En France, la prévalence de la PR est de 0,3 % (0,51 % chez les femmes et 0,09 % chez les hommes); elle augmente avec l'âge jusqu'à 75 ans et décroît ensuite (4). Il en découle que la prévalence de la PR est plus importante au Nord du continent. Cette variation géographique est également observée avec l'incidence; elle peut être expliquée par des facteurs génétiques, mais aussi par des facteurs environnementaux, notamment la relation entre le régime alimentaire méditerranéen et la présence de la maladie (5). La tendance à la diminution de l'incidence et de la prévalence de la maladie depuis 1960 et le vieillissement de la population malade plaident en faveur d'une influence de l'environnement. Une étude réalisée aux États-Unis, à Rochester, décrit l'évolution de l'incidence de la PR de 1955 à 1994. Cette étude a confirmé une tendance à la baisse de l'incidence, qui est passée de 62,2/100 000 dans les années 1960 à 32,7/100 000 dans la dernière décennie (6). Cependant, une étude récente a suggéré que l'incidence de la PR pourrait être en hausse après 4 décennies de baisse (7). Cette tendance peut être expliquée par les différences entre les études épidémiologiques, en fonction du schéma de l'étude et des critères de classification de la maladie utilisés (notamment depuis les critères ACR de 1987), mais aussi par des facteurs environnementaux,

comme l'utilisation de contraceptifs ou la thérapie de remplacement hormonal. Une étude récente sur la cohorte ESPOIR suggère que ce traitement pourrait diminuer la sévérité et peut-être l'incidence de la PR chez les femmes qui présentent des allèles HLA DR*01 ou *04, par le biais d'une diminution de la production des anticorps anti-CCP (8). Néanmoins, l'étude de la cohorte WHI (9) n'a pas confirmé d'augmentation ni de baisse de l'incidence de la PR dans le bras traité par estrogène ou estrogène + progestatif par rapport au groupe placebo. Aujourd'hui, de plus en plus d'études démontrent un rôle conjoint de l'environnement et du terrain génétique, avec une interaction gène-environnement responsable de mécanismes biologiques, comme par exemple dans la relation tabac-HLADR-production d'anti-CCP. D'autre part, dans une étude cas-témoins, le risque de développer une PR est plus élevé chez les patients fumeurs possédant 2 copies des HLA à risque (RR = 15,7) que chez les fumeurs qui ne portent pas ces allèles HLA (RR = 2,4) [10].

La mortalité des patients atteints d'une PR est augmentée par rapport à celle de la population générale. Les études réalisées sur les populations ont montré que la mortalité dans la PR est restée stable depuis 3 décennies, surtout parce que la maladie augmentait chez les sujets âgés (> 84 ans) [11], et les patients atteints d'une PR ne présentent pas globalement d'amélioration de la survie, contrairement à la population générale. Cependant, cette tendance à la surmortalité dans la PR pourrait être en cours d'amélioration, peut-être en partie grâce aux effets des biothérapies. Un travail récemment présenté au congrès de l'ACR a montré une diminution de la morbi-mortalité dans une cohorte de la Mayo Clinic aux États-Unis (12). Les patients suivis entre 1995 et 2007 ont été comparés à ceux qui l'avaient été entre 1984 et 1994. Ces analyses ont montré une baisse de la mortalité durant la dernière décennie, indépendamment du sexe et de la positivité du facteur rhumatoïde, avec un HR estimé à 0,67 (IC₉₅: 0,45-0,99; p = 0,049). Cette tendance à la diminution peut être expliquée par la diminution des manifestations extra-articulaires sévères chez les patients examinés durant la dernière décennie, notamment la vascularite rhumatoïde (3,6 % entre 1985 et 1994; 0,6 % entre 1995 et 2007; p = 0,03) [12]. L'incidence de la maladie coronarienne est aussi en baisse chez les patients atteints d'une PR, et ceci peut expliquer la tendance à l'augmentation

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de la survie durant ces dernières années. Les analyses sur ces 2 décennies montrent un HR ajusté sur l'âge et le sexe de 0,59 (IC₉₅: 0,37-0,94; $p = 0,025$) [13].

La prévalence des spondylarthropathies (SpA) – spondylarthrite ankylosante, rhumatisme psoriasique, arthrites réactionnelles, rhumatismes des entérocolopathies et spondylarthropathies indifférenciées – en France est estimée à 0,3 %, avec des chiffres identiques chez les hommes et les femmes, et similaires à ceux de la PR (14). Les prévalences du rhumatisme psoriasique et de la spondylarthrite ankylosante sont respectivement de 0,19 % et 0,08 %. Aux États-Unis, la prévalence des spondylarthropathies est estimée à 0,21 % et celle du rhumatisme psoriasique à 0,25 %. La sévérité de l'atteinte articulaire dans le rhumatisme psoriasique semble moindre aux États-Unis par rapport aux autres séries. Ces différences peuvent néanmoins être expliquées par les méthodes utilisées, notamment selon que les études sont faites en population générale ou dans des centres de référence tertiaires, qui ont plus de chance d'évaluer les patients avec des maladies plus sévères. Les chiffres montrent également que l'incidence du psoriasis et du rhumatisme psoriasique est en augmentation sur les dernières décennies, passant de 3,6/100 000 entre 1970 et 1979 à 9,8/100 000 entre 1990 et 2000 ($p = 0,001$) [15]. En revanche, l'incidence et la prévalence de la spondylarthrite ankylosante est en baisse sur les dernières décennies en Grèce et au Japon.

Les études sur la prévalence du LED ont montré une très forte tendance à l'augmentation pendant 4 décennies. Peu d'études récentes ont été publiées sur l'épidémiologie du LED. Aux États-Unis, sa prévalence varie de 0,4 à 0,8 %, son incidence est de 5,1/100 000, avec des disparités selon l'origine ethnique (1,4/100 000 chez les sujets de type caucasien et 4,5/100 000 chez les sujets noirs), entre 1980 et 1992; alors qu'elle n'était que de 1,51/100 000 entre 1950 et 1979 (16). En France, elle est estimée à 0,04 % et son incidence à 5/100 000. En contraste avec l'importante augmentation du nombre de cas, la survie chez les patients atteints d'un LED a été significativement améliorée grâce à de meilleurs tests immunologiques, à un diagnostic plus précoce, et aux traitements immunosuppresseurs (17).

L'incidence et la prévalence du syndrome de Gougerot-Sjögren primitif (SGSp) est difficile à évaluer dans la population générale, car très peu d'études épidémiologiques sont disponibles sur le sujet et leurs résultats sont très variables du fait de l'hétérogénéité des méthodes de détection (élevée en partant des syndromes secs en population, faible en se limitant aux cas diagnostiqués quel que soit le mode de capture) et des différents critères diagnostiques utilisés. Cette prévalence varie de 0,04 % à 4,8 % à l'échelle mondiale avec, à partir des données disponibles, une prévalence supposée entre

0,5 % et 1 % en Europe. L'incidence du SGSp varie de 3,9 à 5,3/100 000 (18). Du fait du manque d'études épidémiologiques, la progression dans le temps de l'incidence du SGSp reste difficile à définir.

La tendance à la diminution de l'incidence observée dans la PR, n'est pas retrouvée dans la plus fréquente des vascularites, la maladie de Horton. Cette dernière est plus fréquente dans les pays scandinaves et du Nord de l'Europe, avec une incidence de 27/100 000 personnes, et au Nord des États-Unis, où son incidence est évaluée à 18,8/100 000 parmi les personnes âgées de plus de 50 ans (24,4/100 000 chez les femmes et 10,3/100 000 chez les hommes) [19]. Dans les pays du Sud de l'Europe et au Sud des États-Unis, l'incidence est moins importante. Cette maladie est rare chez les Asiatiques. Les études épidémiologiques décrivent une augmentation de l'incidence de la maladie pendant les 2 dernières décennies. Une étude suédoise a montré un doublement de l'incidence de la maladie entre 1977 et 1986, notamment chez les femmes (20). D'autres études ont montré que cette augmentation est accompagnée de pics tous les 7 ans, ce qui pourrait être expliqué par des facteurs environnementaux, notamment les infections. Cependant, cette augmentation est peut-être due davantage à une meilleure reconnaissance clinique qu'à une vraie augmentation de la maladie. Des arguments en faveur de cette dernière hypothèse sont dérivés des études espagnoles qui ont montré une diminution de la maladie de Horton confirmée par anatomopathologie, et c'est elle qui présente aussi des déficits visuels irréversibles, dans la période comprise entre 1981 et 2005 (21). L'impact de la maladie de Horton sur la mortalité reste inconnu, mais des événements cardiovasculaires sont associés à la maladie.

Cette variation géographique est aussi établie pour la pseudopolyarthrite rhizomélique (PPR). L'incidence de la maladie est plus importante dans le Nord que dans le Sud de l'Europe: 112,6/100 000 en Norvège et 12,7/100 000 en Italie (22). Les données concernant l'évolution dans le temps restent peu nombreuses.

Concernant les vascularites à ANCA (maladie de Wegener, micropolyangéite, syndrome de Churg et Strauss), elles ont une incidence de l'ordre de 2/100 000, avec un pic de fréquence entre 65 et 74 ans. Une étude récente réalisée au Royaume-Uni a porté sur l'épidémiologie de la maladie de Wegener entre 1990 et 2005. L'incidence était de 0,84/100 000 (identique chez l'homme et la femme) et stable pendant toute la durée de l'évaluation. En revanche, la prévalence de la maladie a augmenté de 2,88/100 000 (1990) à 6,48/100 000 en 2005 (23), ce qui peut suggérer une amélioration de la survie. Ces tendances sont similaires pour la micropolyangéite et le syndrome de

Churg et Strauss, avec une incidence stable et une prévalence augmentée. les différences géographiques pourraient être liées au manque d'ensoleillement, selon les populations (24).

La goutte est le plus classique des rhumatismes microcristallins de l'adulte et sa fréquence a tendance à s'accroître au cours des dernières décennies dans les pays occidentaux (notamment aux États-Unis, en Finlande et en Nouvelle-Zélande) du fait du vieillissement des populations et de l'évolution des comportements alimentaires (obésité). Sa prévalence est de 9,4 ‰ aux États-Unis et de 14 ‰ au Royaume-Uni et en Allemagne (25, 26).

L'épidémiologie des autres rhumatismes microcristallin n'a été étudiée qu'en termes radiologiques et son évolution au fil du temps reste méconnue.

Concernant les autres maladies ostéoarticulaires, la plus fréquente est l'arthrose. Elle touche toutes les populations et toutes les ethnies ; elle est plus fréquente chez les personnes âgées. Cependant, et selon les articulations touchées, la prévalence de la maladie varie considérablement. Cette différence peut être expliquée par les méthodes diagnostiques employées : la symptomatologie, la clinique ou les radiographies. La charge économique due à la maladie est lourde, et l'évolution épidémiologique montre une tendance

Tableau. Évolution de la prévalence des affections articulaires au cours des dernières décennies.

| Pathologie | Évolution |
|-------------------------|-----------|
| Polyarthrite rhumatoïde | ↘ |
| Spondylarthrite | ↘ |
| Rhumatisme psoriasique | ↗ |
| Maladie de Horton | ↗ |
| Vascularite à ANCA | → |
| Goutte | ↗ |
| Arthrose | ↗ |

à l'augmentation, du fait du vieillissement des populations, mais aussi à cause de l'augmentation de facteurs de risque tels que l'obésité. Le risque cumulé de développer une arthrose du genou est de 44,7 % (IC₉₅ : 48,4-65,2) [27].

Conclusion

Les études épidémiologiques montrent que l'incidence et la prévalence des maladies ostéoarticulaires sont dynamiques, et que les interactions environnementales et génétiques sont complexes. Le **tableau** résume leur évolution dans le temps. Une nouvelle série d'études épidémiologiques donnera plus d'informations concernant l'impact des nouveaux traitements dans ces maladies, et notamment sur la mortalité. ■

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Appendix 2

Neurological manifestations in primary Sjögren's syndrome

Tobón GJ, Pers JO, Devauchelle-Pensec V, Youinou P.

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Review Article

Neurological Disorders in Primary Sjögren's Syndrome

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Sjögren's syndrome is an autoimmune disease characterized by an autoimmune exocrinopathy involving mainly salivary and lacrimal glands. The histopathological hallmark is periductal lymphocytic infiltration of the exocrine glands, resulting in loss of their secretory function. Several systemic manifestations may be found in patients with Sjögren's syndrome including neurological disorders. Neurological involvement ranges from 0 to 70% among various series and may present with central nervous system and/or peripheral nervous system involvement. This paper endeavors to review the main clinical neurological manifestations in Sjögren syndrome, the physiopathology, and their therapeutic response.

1. Epidemiology of Neurological Involvement in Sjögren's Syndrome

Sjögren's syndrome (SS) is a common autoimmune disease (AID) characterized by an autoimmune exocrinopathy [1] involving mainly salivary and lacrimal glands. The histopathological hallmark is periductal lymphocytic infiltration of the exocrine glands, resulting in loss of their secretory function. This disease occurs alone as primary SS (pSS), or in a background of connective tissue diseases as secondary SS (sSS). Even though keratoconjunctivitis sicca (resulting from the involvement of lacrimal glands) and xerostomia (resulting from that of salivary glands) are usually prominent, SS presents as a multifaceted condition with a broad variety of clinical manifestations (i.e., fatigue, arthralgias, Raynaud's phenomenon, interstitial pneumonias, lymphadenopathy, vasculitic urticaria, purpura, renal tubular acidosis, and neurological involvement) and biological abnormalities of B lymphocytes manifests as hypergammaglobulinemia; production of anti-SSA and anti-SSB autoantibodies and of rheumatoid factor; and an increased risk of non-Hodgkin's B-cell lymphoma (NHL) [2, 3].

This polymorphism accounts for the delay in the diagnosis. As a consequence, there is very likelihood that the

prevalence of the disease is far higher than previously estimated [4]. European Community Study Group on diagnostic criteria for SS (2002) is used to classify patients with the disease [5].

Neurological involvement in SS may be manifested in the central nervous system (CNS) and/or peripheral nervous system (PNS). The prevalence of neurological manifestations ranges between 0 and 70% according to the investigators and depending on the recruitments of their clinics, but in general, such complications occur in about 20% of patients [6–12]. This impressive heterogeneity may be explained by the medical department where patients are recruited (i.e., internal medicine versus neurology) [8], the diagnosis criteria for pSS used (before 2002), or the definition of specific neuropathies and the diagnostic test performed to classify the neurological involvement (mainly in asymptomatic patients). Notably, series published before year 2002 included some patients as considered as suffering from pSS without histology and/or antibody evidence. Comparison between these series is impeded by the heterogeneity in the diagnostic criteria.

To illustrate this concern, in a series by Lafitte et al. [8], neurological manifestations in pSS were analyzed in two cohorts from two medical departments (25 patients

from internal medicine and 11 patients from neurology department). Neurological involvement was found in 40% of patients from the internal medicine department. PNS involvement was present in 4 of 25 patients from the internal medicine group, whereas, in the neurology department, there were 10 of 11 patients (mainly axonal sensorimotor/sensory polyneuropathy). CNS involvement occurred in 7/25 patients from the internal medicine department and 4/11 from neurology. Cognitive dysfunction was the most frequent CNS finding. Thus, these results confirmed that neurological involvement in SS varies according to medical department where patients are evaluated.

Selection of patients in the different series is other matter of concern. Most of these series have been constructed retrospectively. For example, Mori et al. [11] reported 92 patients evaluated by neurological symptoms, but the majority of patients (93%) were diagnosed with pSS after neuropathy. Patients were evaluated between 1985 and 2004. Thus, part of patients was diagnosed with the criteria proposed by the Diagnostic Committee of Health and Welfare of Japan (1999) [13]. On the other hand, Gøransson et al. [12] in a cross-sectional study evaluated PNS in 62 pSS patients applying the American-European classification criteria. In this series, 27% of patients presented neuropathy after clinical examination, and 55% had abnormal conduction studies.

Neurological manifestations may precede the sicca symptoms in 40 to 93% of the cases [8, 14]. As described by Mori et al. [11], 93% of patients were diagnosed with pSS after neuropathy symptoms appeared. Patients with pSS and neurological involvement are older than patients without neurological implication [9, 10].

pSS-associated neurological main manifestations are listed in Table 1. PNS involvement in pSS is well characterized, manifested mainly as axonal polyneuropathies (sensory and sensorimotor), trigeminal neuropathy, and small-fiber neuropathy. Distal axonal sensory or sensorimotor polyneuropathy accounts for over 50% of cases of PNS involvement [6, 7, 15]. On the other hand, CNS manifestations are heterogeneous, manifested as focal or diffuse involvement. Most series reported that PNS involvement is more common than CNS disease. However, Delalande et al. reported the same frequency of central and peripheral nervous system involvements [15].

Other aspect to analyze is the severity of evaluated patients. Most of the previous studies have been conducted at reference centers, thus probably patients seen in these studies have a more severe disease. Lopate et al. [16] showed the prevalence of neuropathy in pSS in an outpatient setting. In the outpatient context, they evaluated 22 pSS patients and 10 controls for evidence of neuropathy. Isolated small-fiber neuropathy was found in 45% of cases and none of controls. Large-fiber dysfunction was similar between the two groups. This study highlights the importance of subclinical neuropathy present in many pSS patients that may lead to disability related to painful distal paresthesias and also the clinical differences according to the patient setting.

TABLE 1: Neurological manifestations in primary Sjögren's syndrome.

| Peripheral disorders | Central disorders |
|---|--|
| | Focal |
| | (i) Seizures |
| | (ii) Movement disorders |
| | (iii) Cerebellar syndrome |
| | (iv) Optic neuropathies |
| | (v) Pseudotumor lesions |
| | (vi) Motor and sensory loss |
| | Multifocal disease |
| | (i) Cognitive impairment |
| | (ii) Encephalopathy |
| | (iii) Dementia |
| | (iv) Psychiatric abnormalities |
| | (v) Aseptic meningoencephalitis |
| | Spinal cord dysfunction |
| | (i) Chronic progressive myelopathy |
| | (ii) Lower motor neuron disease |
| | (iii) Neurogenic bladder |
| | (iv) Acute transverse myelitis |
| | Progressive-multiple sclerosis-like syndrome |
| | Central nervous system vasculitic involvement |
| | Trigeminal and other cranial nerves neuropathies |
| | Autonomic neuropathies |
| | Demyelinating polyradiculoneuropathy |
| Axonal polyneuropathies | |
| (i) Symmetric pure sensory peripheral neuropathy | |
| (ii) Symmetric sensorimotor peripheral neuropathy | |
| Sensory ganglioneuropathy | |
| Motor neuropathy | |
| Small-fiber neuropathy | |
| Multiple mononeuritis | |
| Trigeminal and other cranial nerves neuropathies | |
| Autonomic neuropathies | |
| Demyelinating polyradiculoneuropathy | |

2. Pathophysiology

The pathogenic mechanisms responsible for most forms of neurological involvement in pSS are unknown. To explain this involvement, many hypothesis have been considered. Three pathogenic factors may explain the CNS disorders. The first hypothesis is the direct infiltration of the CNS by mononuclear cells [17]. Bakchin et al. [17] reported a patient with ataxia, oculomotor paralysis, seizures, and a large lymphocytic infiltrate at postmortem examination. The second hypothesis is the vascular involvement. The vascular injury may be related to the presence of antineuronal antibodies and anti-Ro antibodies [18]. Finally, Alexander [19–21] suggest that the underlying mechanism of CNS lesion in pSS is the ischemia secondary to small vessel vasculitis.

Several mechanisms are suggested for the development of the involvement of PNS in pSS patients. Vascular or peripheral inflammatory infiltrates with or without necrosis may be

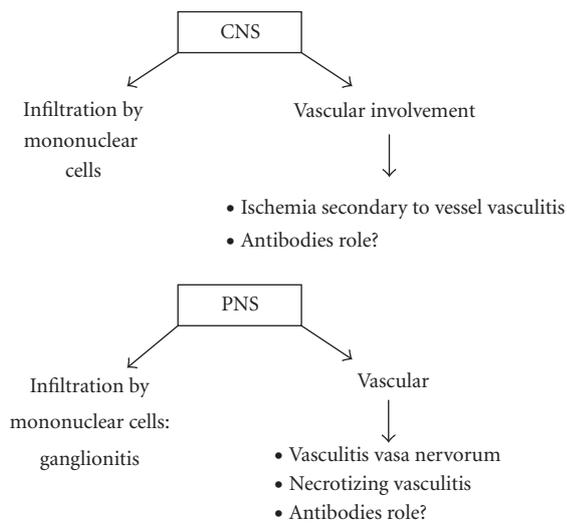


FIGURE 1: Pathophysiological mechanism implicated in the development of central and peripheral nervous system manifestations in primary Sjögren's syndrome.

found [14, 22]. Vasculitis of the vasa nervorum has also been proposed as pathogenic mechanism in PNS involvement [14]. However, others studies have not replicated these findings [15, 23]. In the case of motor neuropathy, necrotizing vasculitis may be found. Lymphocytic infiltration of the dorsal ganglia has been found in some cases of sensory neuronopathy [24]. Antineuronal antibodies have also been described in patients with PNS involvement [25], but the pathological role of these antibodies remains unknown. Antibodies against the type 3 muscarinic receptor have also been described in pSS. These antibodies have shown to be functional, and they are able to inhibit neuron-mediated contraction throughout the gastrointestinal tract. Thus, these antibodies may eventually explain part of the broader autonomic dysfunction found in pSS patients [26]. Figure 1 summarizes the main pathophysiological mechanisms.

3. Central Nervous System Involvement

CNS involvement has not been as well defined as the PNS involvement. Thus, CNS involvement in pSS is controversial, and its prevalence ranges from 0% to 68% [15], according to different series [27–29]. García-Carrasco et al. reported only 1% of CNS involvement (4 patients in a cohort of 400 pSS patients) [29]. SNC involvement varies from diffuse compromise, manifested as cognitive deficits or meningoencephalitis, to focalized compromise, with spinal involvement or optic myelitis. The diagnostic is more difficult compared to PNS involvement, due to unspecific symptoms.

Alexander et al. [18] described CNS manifestations in 20% of pSS patients. The same group [6] showed that 63% of patients presenting CNS involvement had PNS manifestations. Escudero et al. [30] reported that headache is the main CNS complication in pSS. In addition, subclinical tissue injury may be determined by magnetic resonance

imaging (MRI). This method also permits to determine the extension and severity of CNS involvement [27, 31].

Due to high variation in clinical symptoms and signs derived from CNS involvement, some authors propose that these manifestations in pSS are a fortuity association and the link between CNS manifestations and pSS is not well characterized.

3.1. Focal Involvement. Focal encephalic involvement is the main CNS manifestation in pSS [6, 15]. These focal disorders can include motor and sensory loss with hemiparesis, aphasia, dysarthria, seizures, movement disorders, and cerebellar syndrome. Their onset may be acute or insidious or even in a recurring pattern that resembles to multiple sclerosis. Some criteria such as older age, PNS or cranial nerve involvement, spinal cord MRI lesions spanning multiple segments, and cerebral MRI showed cortical brain lesions are characteristic of pSS involvement and rarely seen in multiple sclerosis [27, 28].

Spine cord disorders can include acute or chronic progressive myelopathies, lower motor neuron disease, or neurogenic bladder [15, 32, 33]. Spine complications may be associated with encephalic involvement. In the series by Lafitte et al. [8], myelopathies are reported in 3 of 11 patients with SNC involvement. The clinical picture is often characterized by transverse myelitis [32–34]. Although rare in pSS, acute and chronic myelopathies are frequently severe and life-threatening. These manifestations usually respond poorly to treatment with corticosteroids. Immunosuppressive treatment with cyclophosphamide and steroids has shown some efficacy in patients with progressive disease (see Section 7).

Subacute transverse myelitis with high signal on T2 weighted images and abnormal cerebrospinal fluid (CSF) study (increased protein level and cell count) is a rare but well-described complication in pSS patients [35, 36].

Optic neuropathies have been also described in pSS [37]. This manifestation can be asymptomatic. Alexander [38] reported seven cases of retrobulbar optic neuropathy in pSS patients. Four asymptomatic patients were diagnosed by visual evoked potentials.

Sanahuja et al. [31] described a case of a pSS patient with a large tumefactive brain lesion, who responded well to oral corticosteroid treatment. This lesion, although rarely reported, has to be considered in pSS patients. Differential diagnosis includes lymphoma, glioma, abscesses, metastasis, progressive multifocal leukoencephalopathy, and disseminated encephalomyelitis.

3.2. Diffuse Involvement. CNS involvement can be diffuse, presenting encephalopathy, cognitive dysfunction, dementia, psychiatric abnormalities, and aseptic meningoencephalitis [39–41]. This last complication is characterized by abnormal CSF, with lymphocytic cells and proteins.

Cognitive disturbances of variable severity have been described in pSS patients without mood disorders [8]. Lafitte et al. reported 8 from 36 pSS patients with cognitive dysfunction, characterized by frontal executive dysfunction,

impairment in attention control, intellectual decline, and deterioration of instrumental abilities. Cognitive impairment is not correlated with CSF abnormalities or MRI findings [42, 43]. Malinow et al. [44] described 25 psychiatric abnormalities in 40 pSS patients. Of 16 patients undergoing cognitive function testing, 7 presented mild memory impairment with attention and concentration deficits. Belin et al. [45] evaluated 14 pSS patients with brain MRI, brain 99m Tc-SPECT, and neuropsychological testing. In this series, all patients presented neuropsychological abnormalities, mostly frontal lobe syndrome and memory problems. The neurological involvement was associated with SPECT abnormalities, but not MRI imaging results. Ferreiro et al. reported a patient with diffuse angiographic changes, supporting that an ischemic mechanism caused by CNS vasculitis may be responsible for the clinical presentation in some patients [46].

In conclusion, these studies show the wide range of CNS manifestations that could be associated with pSS. Also, it is important to recognize cognitive problems, which are common in pSS, and cognitive evaluation is a sensible tool sensible to diagnose CNS compromise.

4. Peripheral Nervous System Involvement

As described in epidemiology section, peripheral neuropathy is the most common neurological complication of pSS. It can be present between 20 and 50% of patients when subclinical neuropathy is revealed by a systematic electrophysiological study [47] and clinically from 10 to 32% [6, 14]. In 1962, Kaltreider and Talal [22], described for the first time, the prevalence of neurological involvement in pSS. In this series, 8.3% ($n = 9$) of 109 patients presented neuropathies.

PNS disease includes axonal polyneuropathies (distal axonal sensory and sensorimotor), neuronopathies, mononeuropathies, cranial nerves involvement (mainly trigeminal neuropathy), and autonomic system involvement (Table 1). Axonal polyneuropathies are the most common manifestations of PNS involvement found in 50% of PNS cases [14, 15].

In the series by Gøransson et al. [12], 27% of patients presented peripheral neuropathy and nerve conduction studies were indicative of motor neuropathy in 31% of cases.

4.1. Axonal Polyneuropathies. The axonal polyneuropathies are the most frequent clinical presentation of PNS involvement in pSS. It includes distal sensorimotor and sensory polyneuropathies. Clinical manifestations usually start with distal and symmetric sensitive involvement. Large-fiber sensory dysfunction is evidenced by electrodiagnostic studies.

4.1.1. Sensory Polyneuropathy. Distal sensory polyneuropathy is the most characteristic peripheral involvement in pSS [48]. Sensory neuropathy is characterized clinically by sensitive signs on the lemniscal way, with prevalence on the lower limbs. Manifestations include distal paresthesias and evidence of large-fiber sensory dysfunction on examination and electrophysiological studies. In the series described by

Mellgren et al. [14], 33 pSS patients with neuropathy were evaluated for neurological examinations, electromyography, and nerve conduction studies. Evaluation also included sural nerve biopsy in 11 patients. Thirty-two percent presented exclusive sensory neuropathy. Mori et al. [11] described 18 patients with painful sensory neuropathy and 36 with sensory ataxic neuropathy from one series of 92 pSS with neuropathy, confirming its high prevalence. This manifestation may be related to skin vasculitis but regularly is not associated with other systemic manifestations of pSS.

4.1.2. Sensorimotor and Motor Polyneuropathy. A mixed sensorimotor polyneuropathy, involving large diameter fibers, most commonly axonal, may be present in pSS. The motor neuron involvement (amyotrophic lateral sclerosis syndrome and anterior horn syndrome) is a rare neurological manifestation in pSS [49] and may be associated with CNS involvement [50].

Another manifestation is the acute motor axonal neuropathy (AMAN), a variant seen in nearly 5% of Guillain-Barré syndrome. More than 60% of AMAN patients have antibodies against ganglioside M1 (GM1) [51, 52]. One case described by Awad et al. [53] showed a patient who developed rapidly fulminant AMAN with anti-GM1 antibodies. Anti-SSA antibodies were also elevated, and sialadenitis was evidenced by minor salivary gland biopsy. This patient responded dramatically to intravenous immunoglobulin (IVIg) treatment.

4.2. Sensory Ganglioneuronopathy. Sensory ganglioneuronopathy or sensory ataxic neuropathy produced by posterior spinal roots involvement is manifested as sensory ataxia, and it is characterized by severe impairment of kinaesthetic sensation with no obvious motor involvement [54]. This type of neuropathy may be considered as a subgroup of sensory neuropathy. Physiopathology is probably due to lymphocytic infiltrates on posterior roots and spinal ganglia [11, 24, 54]. In these studies, it has been described lymphocytic infiltrates without vasculitis and degeneration of dorsal root ganglion neuronal cell bodies. Some authors also propose a role of autoantibodies in this manifestation. Among nine patients with pure sensory neuropathy in the study by Delalande [15], four presented clinical and electrophysiological features of sensory ganglioneuronopathy with ataxia. This form of neuropathy is chronic and progressive, occasionally responding to treatment with IVIg [55].

4.3. Small-Fiber Neuropathy. Special mention requires the more recent described small-fiber neuropathy in pSS. About 40% of pSS patients experience chronic neuropathic pain with normal electrodiagnostic studies [56–60]. In these cases, quantification of epidermal nerve fiber density in skin biopsy has been validated as a diagnostic tool of small fiber neuropathy [61]. In the biopsy, the intraepidermal nerve fiber density is calculated. In the article published by Fauchais et al. [60], 14 pSSs with chronic neuropathic pain and normal neurological examination were evaluated. Small

fiber neuropathy was confirmed by skin biopsy in 13/14 cases. Clinical manifestations were mainly distal burning sensation, dysesthesia, prickling, and allodynia, localized in both hands and feet.

In the outpatient cohort described by Lopate et al. [16], 50% of patients with pSS complained of painful distal paresthesias with evidence of small-fiber sensory loss with normal large-fiber function. Most part of these patients has not been diagnosed before, showing that subclinical or mild neuropathy may be present in pSS and can eventually lead to disability.

The physiopathological mechanism is not well studied. Ischemic and vasculitis processes have been implicated in the small-fiber lesions [62]. Proinflammatory cytokines, such as tumor necrosis alpha (TNF- α), have been also implicated, and some clinical improvement has been seen with IVIg therapy [63] and anti-TNF- α [64] in other clinical conditions.

Some reports showed that patients who initially presented with a small-fiber neuropathy later developed a sensory ataxic neuropathy [11], suggesting that small-fiber neuropathy is on a continuum with large-fiber sensory neuropathy.

4.4. Multiple Mononeuropathy. Similar to multiple mononeuropathy in the context of other AID, this complication is rarely seen in pSS [9, 14]. In the series by Mori et al. [11], 11 of 92 patients with pSS-associated neuropathy (12%) were classified with multiple mononeuropathy. Their clinical evolution is generally faster and more invalidating in pSS compared to other diseases. This complication is associated with cutaneous vasculitis and cryoglobulinemia. The multiple mononeuropathy is mainly produced by ischemic mechanisms [65].

4.5. Trigeminal and Cranial Nerves Neuropathies. Often multiple and recurrent cranial nerves neuropathy may be present in pSS. The most common is trigeminal neuropathy, followed by facial and oculomotor nerves involvement [66, 67]. This trigeminal neuropathy presents sensory rather motor involvement. It involves generally the inferior branch of the trigeminal nerve and remains usually clinically unilateral.

Tajima et al. [68] reported the prevalence of trigeminal involvement as high as 50% of patients with cranial nerves compromise. Mori et al. found that 15 of 92 patients (16%) had trigeminal neuropathy with sensory impairment [11]. None presented motor trigeminal involvement. In Delalande serie [15], coclear-vestibular nerve involvement seems to be more frequent (35% of cranial nerve involvement) than trigeminal neuropathy (29%).

4.6. Autonomic Neuropathy. In some patients, autonomic neuropathy may be manifested with Adie's pupils, anhidrosis, fixed tachycardia, and orthostatic hypotension [9, 11, 16, 69]. Autonomic symptoms may be explained by both ganglioneuropathy and vasculitis. Mellgren et al. [14] reported autonomic neuropathy in 6 of 33 patients with pSS (18%). In the series by Andonopoulos et al. [70], autonomic involvement was routinely searched in 32 patients with pSS.

Fifty percent of patients presented autonomic symptoms induced by clinical tests. Most of cases have been reported to be mild [71]. Mori et al. reported 3 of 92 patients with severe autonomic neuropathy [11]. Adie's pupil, associated with autonomic involvement in pSS [72], is presumably caused by neuronitis in the ciliary ganglion cells. Antibodies against acetylcholine receptor have been described in patients with pSS and autonomic symptoms [73].

However, other studies have not shown the increased involvement of autonomic system compared to controls. Niemelä et al. [74] performed a complete evaluation of autonomic functions on 30 pSS patients and 30 controls. They showed no differences between the two groups in any of the test, concluding that the prevalence of autonomic dysfunction in pSS is similar to general population.

4.7. Polyradiculoneuropathy. Acute or chronic polyradiculoneuropathies have been described in patients with pSS [10, 11]. However, the prevalence in pSS seems to be similar in the clinical, physiopathological, and anatomic context to idiopathic polyradiculoneuropathies.

5. Diagnostic of Neurological Involvement in pSS

5.1. Cerebrospinal Fluid. CSF may be useful to classify some manifestations. Lymphocytes may be found in some manifestations usually less of 50 cells/mm³. In aseptic meningoencephalitis, CSF is abnormal with a higher number of lymphocytes, increased level of proteins, and intrathecal synthesis of gamma globulins [75]. The IgG index is increased during periods of disease activity in up to 50% of cases. CSF is also necessary to the differential diagnosis (i.e., infection, multiple sclerosis). Oligoclonal bands (specifically more than three bands) are highly specific of multiple sclerosis diagnosis. These bands have been reported in about 20 to 25% of pSS compared to more than 90% in MS patients [76–78]. The oligoclonal bands are not stable during the course of the pSS and can disappear after treatment with steroids.

5.2. Magnetic Resonance Imaging. MRI abnormalities are common in pSS and usually consist in hyperintense areas in the subcortical and periventricular white matter on T2-weighted and fluid-attenuated inversion recovery (FLAIR) sequences [27, 28]. These lesions are usually less pronounced in pSS than in patients with multiple sclerosis and rarely touch the basal ganglia or the cerebral cortex.

5.3. Nerve Conduction Velocity Studies. Motor and sensory nerve conduction velocity studies are tested in the median, tibial, and sural nerve. These values give characteristic patterns about the specific neuropathy, and they can differentiate the two major types: axonal degeneration and demyelinating. Axonal polyneuropathy is the most frequent pattern seen in pSS PNS involvement.

5.4. Electromyography. Electromyography patterns such as action potential amplitude twice to normal and an increase in duration of action potential may help to differentiate the neuropathies from myopathies. In pSS, electromyography shows a typical pattern of axonal polyneuropathy, with diminution of sensory amplitudes without latency or conduction velocity involvement. Asymptomatic neuropathies can be found by systematic electromyography test [7].

5.5. Sural Nerve Biopsy. Most of the nerve studies in pSS patients with neuropathy have been performed on sural nerve. Mellgren et al. [14] reported vascular or perivascular inflammation of small epineurial vessels in 11 patients with pSS-related neuropathy. In two patients, a necrotizing vasculitis was diagnosed. In this study, axonal degeneration was observed in both sensorimotor and sensory neuropathies. In the study by Griffin et al. [54], most of 12 biopsies showed varying degrees of myelinated fiber loss. Six biopsies had inflammatory infiltrates around epineurial vessels, but necrotizing vasculitis was not evidenced. Cases of multiple mononeuropathy have shown vasculitis in small arteries and arterioles.

5.6. Skin Biopsy. Utility of skin biopsy in the diagnosis of pSS-related neuropathy has been described in the section of small-fiber neuropathy.

5.7. Neuromuscular Biopsy. The utility of neuromuscular biopsy in pSS-related neuropathy has been evaluated [79]. In the study by Terrier et al., 40 pSS patients with neuropathy underwent neuromuscular biopsy. Pathological results (necrotizing vasculitis in 14 patients and lymphocytic vasculitis in 8) were associated with acute-onset neuropathy, multiple mononeuropathy, and sensorimotor involvement, compared to 18 patients without vasculitis on the neuromuscular biopsy. Necrotizing vasculitis was significantly associated with a better outcome and response to immunosuppressive treatment.

5.8. Autonomic Neuropathy Tests. To classify patients with autonomic neuropathy, different test such as Till-table test, gastrointestinal test, thermoregulatory sweat test, or quantitative sudomotor axon reflex test may be used.

6. Biological Markers in Patients with pSS and Neurological Manifestations

Anti-Ro and anti-La seem to be less frequent in pSS patients with neurological involvement (40%) compared to patients without neurological manifestations (60% of positivity). Thus, new markers are necessary in pSS to better classify subpopulations of patients with neurological involvement. Some antibodies have been described as potential serological markers of neurological involvement in pSS. However, their useful application is doubtful. IgA and/or IgG anti-alpha-fodrin antibodies in pSS appear to be common in neurological pSS (64.5% of 31 pSS patients with neurological manifestations) [80]. However, this percentage

was not different from pSS patients without neurological manifestations. Giordano et al. [81] evaluated IgM and IgG anti-GM1 in 30 pSS patients and its relation with peripheral neuropathy. Anti-GM1 antibodies were present in 12 patients (6 with neuropathy and 6 without), thus showing little help to classify pSS patients with peripheral neuropathy. Antineuronal antibodies have also been described in pSS [82], although their pathological role is unknown.

Anti-GW182 antibodies directed against GW182 protein (a protein located in cytoplasmic structures called GW bodies) have been characterized in autoimmune diseases (mainly in pSS) [83]. In this group, 18 sera of 200 patients (9%) with autoimmune diseases were positive for anti-GW182 antibodies. Interestingly, positive patients had mixed motor and/or sensory neuropathy ($n = 9$), pSS with neurological symptoms ($n = 3$), and 6 patients presented SLE or pSS without neurological manifestations. In conclusion, anti-GW182 antibodies may help to classify patients with autoimmune neurological involvement in different AID.

Of special interest, the antitype 3 muscarinic receptor antibodies have been described in pSS. The IgA isotype may be involved in the pathogenesis of autonomic dysfunction and also may be useful as a novel marker in the pSS diagnosis [84]. Their utility to discriminate patients with neurological involvement has to be tested. Table 2 summarizes the antibodies in neurological manifestations in pSS patients.

Some other biological markers have been described in neurological involvement in pSS. Among these markers, patients with sensorimotor neuropathy have higher rates of mixed cryoglobulin compared to pSS without neurological manifestations (57% versus 11%), monoclonal gammopathy (71% versus 17%), and NHL (57% versus 3%). On the other hand, patients with sensory neuropathy show lower prevalence of chronic B-cell activation markers (lower prevalence of antinuclear antibodies, anti-SSA, and anti-SSB) [85]. Therefore, these results demonstrate that the pathophysiological mechanism is different according to polyneuropathy type, and the B-cell activation markers can be useful to classify a number of patients with a more severe disease and risk of lymphoproliferation, accompanying some neurological manifestations.

7. Treatment of Neurological Manifestations in Sjögren's Syndrome

There is no consensus about the specific treatment of neurological involvement in pSS. Generally, corticosteroid therapy is initiated in patients with either CNS or PNS [15, 87]. CNS involvement is usually treated with high corticosteroid dose. In some cases, response to treatment is exceptional. For example, Caselli et al. [88] showed one patient with dementia who markedly improved after corticosteroid treatment. Concerning the treatment of acute and chronic myelopathies, de Seze et al. [86] showed the tolerance and clinical response of a combination regimen of steroids and monthly cyclophosphamide. Fourteen patients (6 with acute and 8 with chronic myelopathies) were evaluated. Tolerance was good, and nine patients improved clinically

TABLE 2: Antibodies in neurological manifestations of primary Sjögren's syndrome.

| Antibody | Clinical association | Reference |
|---------------------------------|--|---|
| Anti-SSA and anti-SSB | Most of studies show lower prevalence of anti-SSA and anti-SSB antibodies in pSS with neurological involvement. In one series, patients with nonataxic sensory neuropathy had lower prevalence of anti-SSA (40% versus 72%) and anti-SSB (15% versus 41%). | Sene et al. [85] |
| Anti-SSA | This paper showed that anti-Ro antibodies were positive in 48% of patients with CNS compared to only 24% of all patients with pSS. However, the anti-SSA antibodies were detected by double immunodiffusion and not by ELISA. | Alexander et al. [18] |
| Anti-alpha fodrin (IgA and IgG) | These antibodies are common patients in pSS. However, there are not differences between patients with or without clinical neurological involvement. | De Seze et al. [86] |
| Anti-GM1 (IgM and IgG) | No differences between pSS patients with or without neurological involvement. | Giordano et al. [81] |
| Antineuronal antibodies | In a large series of patients with neurological disorders ($n = 882$), these antibodies were detected in patients with pSS and neurological involvement, although the specificity has to be defined. Antiganglion neuron antibodies have been also reported. | Murata et al. [25], Vianello et al. [82] |
| Anti-GW182 | Detected in patients with mixed motor and/or sensory neuropathy without pSS and also in neurological involvement in pSS patients. | Eystathioy et al. [83] |

GM1: ganglioside; GW182: protein located in cytoplasmic structures called GW bodies; CNS: central nervous system; pSS: primary Sjögren syndrome.

(including the total 6 patients with acute myelopathy), three patients remained stable, and the other two patients presented moderate progression. Although randomized studies are necessary, this treatment needs to be considered in patients with progressive disease.

Classically, peripheral neuropathy in patients with pSS responds poorly to treatment [11, 15, 87]. Some groups recommend only treating the symptoms according to the severity. In other patients, immunosuppressive therapy based on corticosteroids, cyclophosphamide, azathioprine, and even plasmapheresis has shown only mild success [89–91].

In the series reported by Terrier et al. [79], patients with necrotizing vasculitis have a better response to immunosuppressive treatment, mainly with cyclophosphamide (71% of patients with necrotizing vasculitis showed good response compared to 25% of patients with lymphocytic vasculitis). Griffin et al. reported a treatment based on corticosteroids and associated in some cases with azathioprine, intravenous cyclophosphamide or plasma exchanges [54]. Only one patient with a relapsing course responded to corticosteroid treatment. Mori et al. suggested that corticosteroids are suitable for multiple neuropathy and multiple cranial neuropathy [11].

IVIg has been also reported as a good therapeutic option in some painful sensory neuropathy cases [92] and in radiculoneuropathy. In a recently series of 19 pSS patients with peripheral neuropathy, intravenous immunoglobulin treatment was evaluated [93]. In this study, 8 patients (42%) showed a decrease of the disability Modified Rankin Scale,

corresponding to a clinical improvement. Patients with sensorimotor or nonataxic sensory neuropathy were markedly improved compared to patients with ataxic neuropathy (2/9). The authors concluded that clinical benefits of IVIg treatment depend on the specific clinical subtype.

Caroyer et al. [94] showed improvement in sensory ganglioneuropathy treated with infliximab. However, no controlled trials have shown efficacy of infliximab or others anti-TNF α in pSS-related neuropathy.

Rituximab, an anti-CD20 antibody, may be useful in systemic complications in pSS patients [95, 96] and in some cases of refractory neuropathy. Recently, Mekinian et al. [97] reported 17 patients with pSS and PNS involvement treated with rituximab. Neurological improvement was observed in 11/17 patients (65%) at three months. Best results were observed in patients with cryoglobulinemia or vasculitis-related PNS involvement (9/10 patients improved).

The benefits from treatment with oromucosal IFN- α in pSS have been reported by several groups [98–101]. Due to possible effects on sicca symptoms, Yamada et al. [102] reported three cases of pSS-associated neuropathy treated with oral IFN- α (two patients with sensory ataxic neuronopathy and one patient with axonal sensorimotor neuropathy with demyelinating features). All three patients responded well to IFN- α , improving the neurological symptoms. Sicca symptoms, antibodies titres, and focus score of salivary gland biopsy were also improved. However, the mechanisms whereby IFN- α induces neurological improvement in pSS are uncertain.

In conclusion, neurological manifestations are common in pSS and often precede the diagnosis. The accurate prevalence of these manifestations is difficult to assess, because the heterogeneity of the series. The pathogenic mechanisms responsible for most forms of neurological involvement in pSS remain unknown, but vascular, ischemic, and immunological mechanisms have been described. Controlled and population-based trials are necessary to better characterize the neurological manifestations in pSS and their therapeutic response.

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Appendix 3

Skin biopsy as a routine tool for primary Sjögren's syndrome

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Skin biopsy as a routine diagnostic tool for primary Sjögren's syndrome

Sjögren's syndrome is an autoimmune disease characterized by disruption of epithelial cells and lymphoplasmocytic infiltration of exocrine glands throughout the body, mainly salivary and lacrimal glands. The lymphocytic infiltrates in the minor salivary gland biopsy and the presence of autoantibodies are the gold standards for diagnosis. However, skin dryness is often underestimated. In addition, skin biopsy of patients with Sjögren's syndrome may have lymphocytic infiltrates, and some patients with nonconclusive minor salivary glands may present infiltrates in the skin. We propose to add the question of skin dryness to the symptoms questionnaire designed for patients presenting clinical features of Sjögren's syndrome and to perform skin biopsy as a routine tool in the diagnosis of Sjögren's syndrome, especially in patients with nonconclusive minor salivary glands. The emergence of new and potentially active treatments, most notably anti-B lymphocyte monoclonal antibodies, may conduct to the use of skin biopsy instead of salivary gland biopsy in their follow-up.

KEYWORDS: B lymphocyte • memory B lymphocyte • Sjögren's syndrome • skin biopsy • xerosis

Definition of primary Sjögren's syndrome

Sjögren's syndrome (SS) is an autoimmune and multifaceted condition with a broad variety of clinical manifestations and biological abnormalities [1]. This enormous polymorphism is associated with the delay in the diagnosis. This disease is characterized by disruption of epithelial cells and lymphoplasmocytic infiltration of exocrine glands throughout the body, mainly salivary and lacrimal glands [2]. The consequences of SS, such as xerostomia and xerophthalmia, reflect the loss of secretory ability. SS can present alone as primary SS (pSS) or accompanying other autoimmune diseases, as secondary SS.

Faced with the ever-growing complexity of criteria for classification of patients [3], physicians with an interest in pSS have continued to devise new tests. In particular, the skin may function as a diagnostic window on the diseases affecting internal organs and other exocrine tissues.

■ Dermatologic clinical manifestations of pSS

Cutaneous manifestations of SS include xerosis, immunologic inflammatory manifestations such as vasculitis and other manifestations such as angular cheilitis, eyelid dermatitis and annular erythema [4,5].

Vascular manifestations include flat and palpable purpura, neutrophilic inflammatory vascular disease indistinguishable from

leukocytoclastic vasculitis, mononuclear inflammatory vascular disease and urticarial vasculitis [6].

Xerosis is a common skin manifestation in pSS characterized by roughness, scaling, loss of elasticity and often discomforting sensations of itching and burning [7]. However, this manifestation is underestimated in the clinical follow-up of pSS. The presence of xerosis varies in different series from 23 to 65%. For example, 42 of 62 patients (67%) described by Bloch *et al.* [8] presented cutaneous dryness and 17 patients complained of a decreased sweating; whereas Whaley *et al.* reported only 23% prevalence of xerosis in 171 patients with pSS [9]. An analysis conducted retrospectively by Bernacchi *et al.* showed that xerosis was more frequent in pSS (43%) compared with the secondary form of the disease (26%) [4]. Because xerosis is subjective and often associated with more demanding symptoms, this manifestation can be neglected, which could explain such different results in the prevalence. TABLE 1 shows the percentage of xerosis in different series of pSS and secondary SS. In our recent study 22 pSS patients had a poorer score for skin dryness on the visual analogue scale compared with 22 healthy controls (58.3 ± 10.1 vs 38.9 ± 7.6 ; $p < 0.01$) [10]. Although not reported spontaneously, xerosis was more common in patients (9/22) versus controls (2/22). It seems that clinical xerosis is not associated with other extraglandular manifestations or immunological parameters [4,10].

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Table 1. Xerosis in primary Sjögren's syndrome.

| Author | n | Frequency of xerosis (%) | Ref. |
|-------------------------|----------------------------------|-----------------------------|------|
| Markusse <i>et al.</i> | 46 primary | 56 | [36] |
| Bloch <i>et al.</i> | 62 (23 primary) | 67 | [8] |
| Whaley <i>et al.</i> | 171 | 23 | [9] |
| Bernacchi <i>et al.</i> | 93 (62 primary and 31 secondary) | 56 primary and 26 secondary | [4] |
| Roguedas <i>et al.</i> | 22 primary | 40 | [10] |

The understanding of xerosis is not completely clear. This condition may be due to infiltrate of the eccrine or sebaceous glands or dysfunctional sweating.

Initially, it has been suggested that xerosis in pSS may be due to decreased eccrine sweating. Katayama *et al.* evaluated sweating in 49 patients with pSS [11]. Sweating was induced by mental stimulation such as deep breathing or hand grasping, and was evaluated in controls and patients with other types of dermatitis (atopic dermatitis, psoriasis vulgaris and senile xerosis). A perspirometer continuously recorded sweat volume. Sweating was reported to be significantly reduced in pSS ($p < 0.005$). This result suggested that patients with pSS develop impaired sweating as an exocrine manifestation in addition to xerostomia and xerophthalmia.

In one study, Bernacchi *et al.* conducted a histopathological and functional evaluation of xerotic skin in pSS [7]. Skin barrier function was evaluated by corneometry and evaporimetry to detect stratum corneum water content and transepidermal water loss. In the deltoid area transepidermal water loss values were significantly lower in pSS patients compared with controls ($p = 0.037$). No morphological alterations or reduction in number of sebaceous and sweat glands were demonstrated. On the contrary, four samples from pSS patients selected for immunohistochemical analyses showed a marked staining with Ki67 antibody on the basal and spinous layers. In addition, the final phase of keratinocyte differentiation also appeared altered in pSS patients, with expression of involucrin not only in the granular, but also in the highest part of the spinous layer. The authors conclude that xerosis in pSS was not related to a chronic inflammatory atrophy of sweat glands, although some biochemical alterations of epidermis (increase of epidermal proliferation and perturbation of epidermal differentiation) were present.

Xerosis could be due to the presence of inflammatory infiltrates in the skin. In a case report, Mitchell *et al.* described a 55-year-old

man with pSS who had experienced difficulty in perspiring [12]. The skin biopsy showed a moderate number of eccrine glands and ductal structures in the reticular dermis and the presence of lymphocytes and plasma cells heavily infiltrating the epithelial structures. Eccrine sweat testing was performed by intradermal injection of 0.1 ml of 1:500 metacholine in sterile saline solution and the visualization of sweating was facilitated by bromophenol blue powder. Very few scattered dots were identified, suggesting a marked decrease in eccrine sweating. Whaley *et al.* performed skin biopsy in seven patients, showing a chronic inflammatory process destroying the sweat glands [9]. A nonspecific perivascular lymphocytic infiltrate was also found.

In conclusion, xerosis may be due to biochemical alterations in association with lymphoplasmocytic infiltrates in sweat glands of patients with pSS. In some cases, a chronic inflammatory phenomenon leading to the destruction of sweat glands has been described. Reciprocally, xerosis is common with age in the elderly. Thus, the diagnosis of pSS warrants consideration in elderly patients referred to the dermatologist in order to investigate the etiology of dry skin.

■ Utility of skin biopsy in the diagnosis of pSS

Recently, we have shown the utility of skin biopsy as a routine diagnostic tool in pSS [10]. This study was developed in 22 pSS patients, diagnosed by minor salivary gland (MSG) biopsy and auto-antibodies anti-Ro and/or anti-La, according to the American–European Consensus Group criteria [3]. Of these 22 patients, we disposed of 12 skin biopsies. Axillae region were ideal for the 6-mm punch biopsies of noninvolved skin, given the concomitance of eccrine, apocrine and apoecrine sweat glands [13]. Lymphocytic infiltrates were found in 8 of 12 skin samples (66%). The most important finding was that two patients with noncontributive MSG biopsy had skin infiltrates (FIGURE 1A). By contrast, ten

skin samples from matched healthy volunteers were absolutely devoid of lymphocytes. This result shows that MSG and skin biopsies may be complementary in the study of sicca symptoms and the diagnosis of pSS.

The discrepancy between the results of MSG and skin biopsies in pSS patients is not really surprising, since the extent of lymphocyte infiltrates varies between the fields in one MSG sample, between MSG in one biopsy, and between MSG samples in one patient over time [14–19].

The skin biopsy may serve as an additional factor for the diagnosis of pSS, with supplemental importance in patients with repeatedly negative MSG biopsies. Although MSG biopsy remains the histopathological gold standard for SS diagnosis, some authors hold strong reservations regarding this biopsy, arguing that: this site is nonspecific; and exocrine atrophy is normal as a part of the ageing process. Thus, lymphocytic infiltrates were found in 15% of healthy controls [20]. In addition, some patients present

some distressing sensory neuropathy in a portion of the mental dermatome, whereas the morbidity of skin biopsy is particularly low.

■ Histological findings in skin biopsy

Within the cutaneous infiltrates, T and B cells have been described (FIGURE 1B). Most of the T lymphocytes, defined by the membrane expression of CD3, were positive for CD4 and negative for CD8. B-cell characterization (CD20⁺) showed only 1% CD5-expressing cells, which is a marker for polyspecific autoantibody-producing B lymphocytes. Unexpectedly, B cells were CD27⁺/IgD⁻, which are markers of memory B cells. Some B cells were also CD24⁺ immature B cells, resembling transitional B cells.

Assuming that homing receptors for the epidermis are specific for T lymphocytes [21], the migration of B lymphocytes to the skin may be unlikely. The simplest explanation for their detection among the skin lymphocytes is that one of several of the B-lymphocyte chemotactic

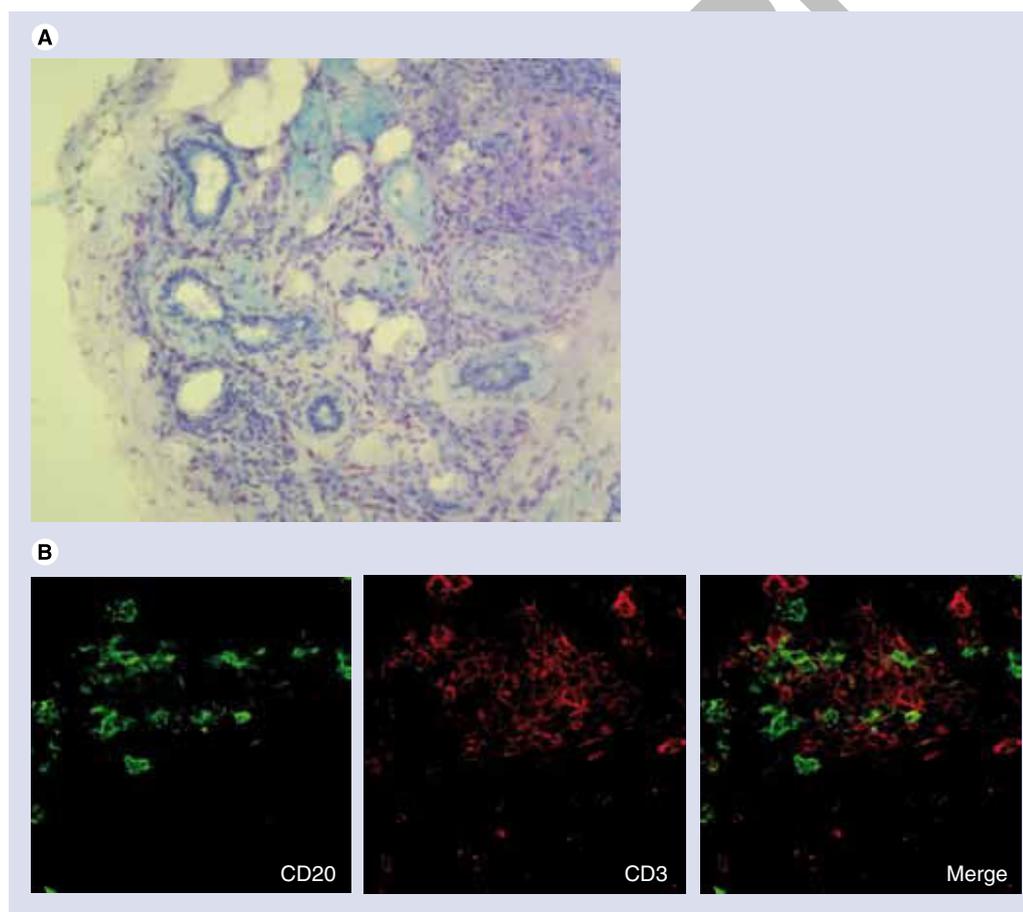


Figure 1. Pathological changes in the cutaneous tissue of patients with primary Sjögren's syndrome. (A) Epidermis infiltrated with mononuclear cells stained with toluidine blue. **(B)** Immunofluorescence analysis of the same skin specimen **(A)** from a patient with primary Sjögren's syndrome shows CD20-positive B lymphocytes (in green) and CD3-positive T lymphocytes (in red) (magnification: $\times 63$).

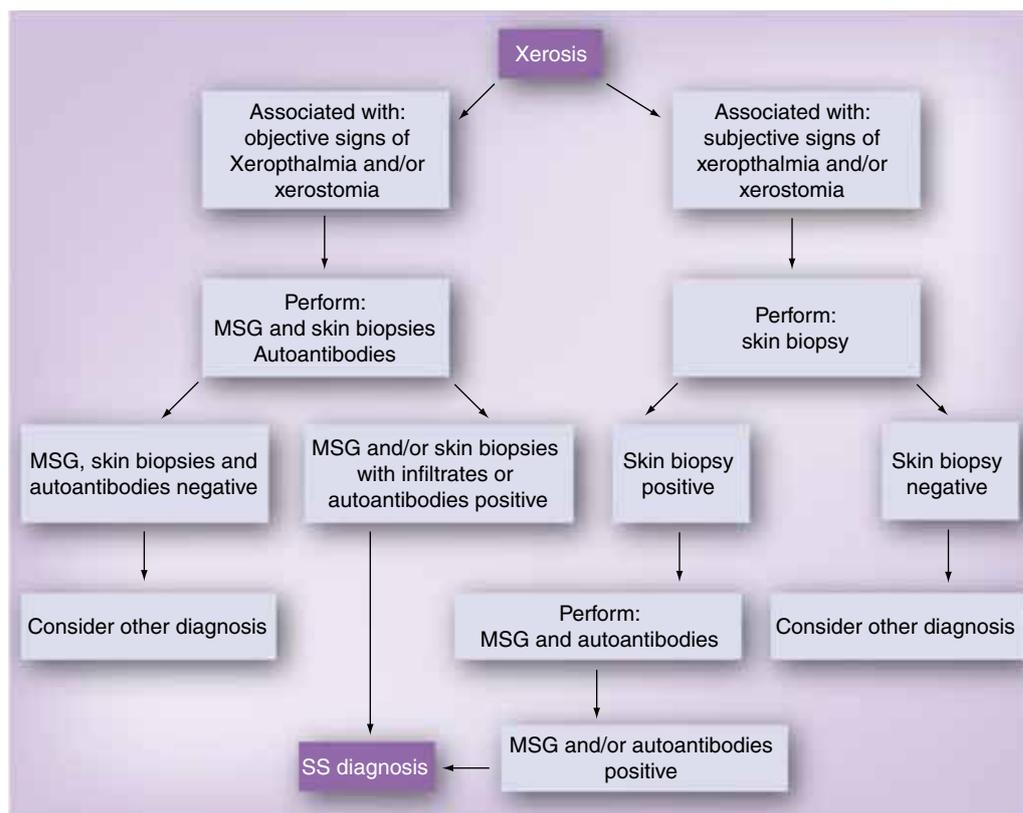


Figure 2. Proposed flow chart including skin biopsy in Sjögren's syndrome diagnosis. MSG: Minor salivary glands; SS: Sjögren's syndrome.

factors described in the MSG [22] are shared by all exocrine tissues. Consequently, these include also cutaneous sweat glands [23]. Furthermore, we were surprised by our finding that memory B lymphocytes infiltrate not only the MSG [24], but also the skin. The local production of B cell activating factor of the TNF family may explain this finding, and deserves further research. Retention of memory B cells in the exocrine tissues may account, at least in part, for their decrease in peripheral blood. CD5-expressing B cells are not reported to generate memory B cells [25], which is consistent with their absence in the B-cell infiltrates from the skin. Among memory B cells, some harbor the CD20⁺/CD24⁺ profile, already described in the salivary glands [26], which is in fact closer to that of transitional type 2 B cells than to that of marginal-zone B cells.

Given the ability of T cells, but not conventional B cells, to home specifically to and reside preferentially in healthy, as well as in inflammatory [27] or malignant [28] skin, our finding that B lymphocytes contribute to the cutaneous infiltrate was highly unexpected. This is, nonetheless, consistent with the hypothesis that B cells play a central role in the pathogenesis of pSS [29,30]. Thus, the presence of B lymphocytes

in the skin biopsy might alert the dermatologist on a routine basis, and be regarded as a clue for the diagnosis of pSS.

■ Alternative tools for pSS diagnosis

B-cell alterations are important in pSS pathogenesis [29,30]. Among major breakthroughs in B-cell abnormalities is the dissection of B-lymphocyte subsets. The subsets of mature B cells (Bm) are defined according to the respective membrane expression of IgD and CD38 into sequential stages from Bm1 through Bm5 [31]. Patients with pSS show a high ratio of increased percentages of blood Bm2/Bm2' cells to decreased percentages of eBm5/Bm5 cells in peripheral blood, compared with other autoimmune diseases and healthy controls [32]. This phenomenon is accompanied by the accumulation of memory B cells in the exocrine glands [33], which was also observed in skin infiltrates in pSS. This ratio, more readily available to the community physician than the MSG biopsy, could provide a new additional criterion for pSS [34]. In combination with skin biopsy, these two criteria may be useful as alternative criteria for the pSS diagnosis. However, an additional evaluation in a multicenter prospective study is needed.

Future perspective

Our clinical recommendation is that skin biopsy may be useful as a tool for the pSS diagnosis. This affirmation is more interesting in the case of noncontributive MSG biopsy. In our previous study, two patients presented skin infiltrates, with normal MSG biopsy. In addition, punch skin biopsy taken in the axillae region is harmless and easy to take at the clinical office. The view that any exocrine glands are at risk of undergoing the autoimmune assault connotes that the examination of sweat glands could contribute to the histopathological diagnosis. The emergence of new and potentially active treatments [35], most notably anti-B lymphocyte monoclonal antibodies, may lead to the use of skin biopsy instead of labial salivary gland biopsy in their follow-up.

In clinical practice, the skin of patients suspected of pSS must be carefully examined and, one step further, the epidermis should be evaluated to search for B-cell infiltrates. In FIGURE 2, we proposed a diagnostic flow chart that includes

skin biopsy as a possible tool for the diagnosis of pSS. Xerosis may indeed constitute an early sign of pSS before the onset of xerostomia and xerophthalmia.

In conclusion, we propose to add the question of skin dryness to the symptoms questionnaire designed for patients presenting clinical features suggesting SS. In addition to the MSG biopsy, skin biopsy should be performed as part of the routine clinical care in these patients. The goal is to improve the diagnosis in early stages of SS.

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Executive summary

Definition of primary Sjögren's syndrome

- Sjögren's syndrome (SS) is an autoimmune and multifaceted condition with a broad variety of clinical manifestations and biological abnormalities.
- Xerostomia and xerophthalmia are the main clinical manifestations.
- Many other organs may be affected.

Skin disease as a manifestation in SS

- Xerosis is a common and underestimated manifestation in SS.
- The presence of xerosis varies in different series from 23 to 65%. Its prevalence seems higher in primary SS patients compared with secondary SS (43 vs 26%).
- Both biochemical alterations and lymphoplasmocytic infiltration of sweat glands may explain the presence of xerosis.

Skin biopsy as a routine tool for the diagnosis of primary SS

- Axillae region is ideal for 6-mm punch biopsies of noninvolved skin, given the concomitance of eccrine, apocrine and apoecrine sweat glands.
- B- and T-cell infiltrates may be present in 66% of primary SS patients. A non-negligible number of patients have skin infiltrates with a minor salivary gland biopsy nonconclusive for the primary SS diagnosis.

Recommendations

- Skin symptoms must be investigated in patients with clinical suspicion of SS.
- Skin biopsy is a simple and safe test, and the results may help SS diagnosis, mainly in patients with nonconclusive minor salivary glands.

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- **Found a higher proportion of xerosis in patients with primary SS compared with healthy controls. In addition, the axilla skin was analyzed by punch biopsy. Lymphocytic infiltration was seen in the skin of eight of the 12 patients tested. Two of them had normal salivary glands biopsy. Most interestingly, B-cell infiltrates were identified in skin infiltrates, so their presence might be a clue to the diagnosis of primary SS. B cells were memory B cells (CD27⁺) and immature B cells (CD20⁺/CD24⁺). The authors suggest that skin biopsies warrant inclusion into the routine clinical care of patients with suspected primary SS.**
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Appendix 4

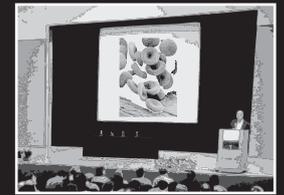
Conference Scene: Updating the highlights of Sjögren's Syndrome

Pers JO, Tobón GJ, Jamin C, Renaudineau Y, Youinou P.

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Conference Scene

Updating the highlights of Sjögren's Syndrome



The 10th International Symposium on Sjögren's Syndrome 1–3 October 2009, Brest, France

The International Symposium on Sjögren's syndrome (SS), launched by Rolf Manthorpe (Sjögren's syndrome Research Center, Sweden) in 1986, is held every 3 years. The main thrust of Pierre Youinou, chairman of the 10th International Symposium on SS, was to avoid superficial discussion whilst trying to cover the entire disease spectrum, and rather to encourage lively debate. Five sessions were organized. These were dedicated to etiology, clinics and biology, pathophysiology, and treatment and epidemiology, through introductions by experts who argued for and against theories based on their own clinical and basic data. The symposium took place in Brest, France, with over 350 participants from 42 countries.

In parallel with guided visits around posters (232 abstracts were submitted), four informal working discussions were organized during lunchtime and in the late afternoon. These sessions focussed on: 'Ultrasonography of the salivary glands' (moderated by A Tzioufas from the University of Athens, Greece, and S Jousse-Joulin from the University of Brest, France); 'Refining the criteria for Sjögren's syndrome' (moderated by T Daniels from the University of California, CA, USA; M Ramos-Casals from Barcelona, Spain, and AL Fauchais from the University of Limoges, France); 'Epigenetics' (moderated by S Gay from the University of Zurich, Switzerland, Y Renaudineau from the University of Brest, France, and Q Lu from the University of Changsha, China) and 'Monitoring the disease under treatment' (moderated by C Vitali from Piombino, Italy; S Bowman from the University of Birmingham, UK, and X Mariette from the Bicêtre hospital, Paris, France). The ultimate aim of these meetings was to reach a consensus and encourage the development of joint projects.

One of the most important innovations was to welcome delegates of 20 National Associations of Patients (such as Finland, India, Japan, New Zealand, Russia, Spain,

UK and the USA) to participate in this meeting, under the auspices of the French Association of patients with SS. They met to figure out what actions can be undertaken to improve the quality of life of the patients all over the world. The Sjögren's associations of patients worldwide have also decided to join and set out an International SS Network.

In this article we present the highlights of the symposium.

Etiology of Sjögren's syndrome

In the first session dedicated to etiology of SS, HM Moutsopoulos (University of Athens, Greece) discussed 'Viruses in terms of pathophysiology'. His lecture referred to epithelial cells as pivotal regulators of autoimmune responses, suggesting that SS is basically an autoimmune epithelitis. Epithelial cells inappropriately express oncogenes (c-Myc and p53) and nuclear autoantigens (La/SSB) that are translocated to the plasma membrane. Most importantly, SS salivary gland epithelial cells (SGECs) appear suitably equipped to recruit and activate immunocytes. They express molecules involved in antigen presentation (MHC, CD40 and B7 costimulatory molecules), lymphoid-cell homing and expansion of immune responses (adhesion molecules, lymphoattractant chemokines and proinflammatory cytokines). Moreover, the expression of functional innate immune receptors such as Toll-like receptors (TLRs)-3, -4, -7 and -9, and CD91 heat shock protein receptor, suggest that SGECs are likely able to directly recognize pathogens and to cross-present antigenic molecules, respectively.

JM Anaya (University of Rosario, Bogota, Colombia) discussed the genetic basis of SS. He recalled that to date, there is no specific gene for primary SS, and all of the polymorphisms associated with primary SS have also been associated with other autoimmune diseases. Ancestral haplotypes *8.1*, *IL-10*, *SSA1*, *IRF5* and

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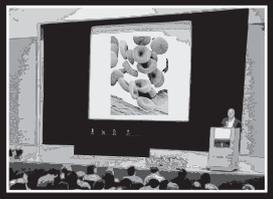
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STAT4 loci have been consistently associated with the disease, although they are not specific for SS. He also reported a meta-analysis to identify common HLA class II alleles contributing to progressive SS (pSS) susceptibility in a worldwide population. Analysis of susceptibility and protective alleles revealed physicochemical differences of critical amino acids located in the antigen-binding groove at DQ- α (DQA1*0502), DQ- β (DQB1*0201) and DR- β (DRB1*0301) chains. Finally, by using a combined approach of gene expression and genome-wide association study, it has been reported that both proapoptotic/antiapoptotic and type I interferon signaling inhibition/stimulation balances occur in SS labial SGEs, and that loci harboring the genes involved in these pathways are candidates for disease susceptibility.

On the sidelines of this etiology session, the creation of an international epigenetic autoimmune group has been proposed to establish gold standards and to launch collaborative studies. S Gay has presented the Swiss epigenetic group and has reviewed the main epigenetic modifications associated with autoimmune diseases (histone modifications, DNA methylations and miRNA). The discussion has mainly concerned the role of microparticles expressing miRNA and their importance as prognostic/diagnostic tools, as cellular activation markers or as effectors owing to their potential role on target cells.

W Brooks (Tampa, FL, USA) discussed his theory regarding the importance of *S*-adenosyl methionine (SAM) degradation by SAM decarboxylase, which may explain, in part, the observed DNA methylation defect in autoimmune diseases. Briefly, overexpression of the putrescine pathway (by virus and/or as a consequence of X-chromosome demethylation) is suspected to increase the activity of the SAM decarboxylase during autoimmune diseases. Following this, Brook's group developed a SAM-decarboxylase inhibitor in order to increase the level of the SAM methylation donor and in turn restore DNA methylation. The discussion has concerned the validation of this theory and the necessity to test the inhibitor(s), not only in cancer models but also in autoimmune models, both *in vitro* (T and B lymphocytes) and *in vivo*. For

example, the nonspecific cellular action of the drug was also mentioned with a risk for developing cancer.

C Selmi (University of Milan, Italy) and E Gershwin (University of California, CA, USA) have presented their work regarding the importance of X-chromosome deletion (monosomy X) in primary biliary cirrhosis (PBC) and scleroderma. A call to study X-chromosome epigenetic modifications between twins that either develop or do not an autoimmune disease (PBC) was presented in order to identify autoimmune-susceptibility genes that could be present among the 15% of X-chromosome genes.

G Illei (National Institute of Health, MD, USA) discussed his results regarding the expression of two miRNAs overexpressed in salivary glands from SS. He reported that two suspected epithelial cell miRNAs (miR-574 and miR-768-3p) may be used to predict the evolution of the disease. Propositions to include more patients and to analyze the different cellular subpopulations in parallel with the blood analysis were suggested.

S Cha (University of Florida, FL, USA) presented her nondiabetic NOD-derived animal model (B6DC), which develops a disease similar to human SS. From her study, she highlighted two miRNAs (150 and 146) to be upregulated in the target tissues as well as in peripheral blood mononuclear cells (PBMCs) from B6DC mice. Consistently, she reported that miR-146 expression was also increased in PBMC and salivary glands from SS patients. Elevated expression of the two miRNAs was detected. A proposition to validate and define gold standards in order to analyze miRNAs (in particular miR-146 and miR-155) in large cohorts with different sample origins was made. Q Lu presented his group and its contribution to the elucidation of aberrant epigenetic regulation including DNA methylation, histone modifications and miRNA in lupus. Propositions to develop an epigenetic bank and to establish gold standards in an epigenetic consortium were put forward. Y Renaudineau presented the contribution of DNA demethylation to the autoreactivity of B cells leading to the overexpression of repressed genes such as human endogenous retrovirus. A proposition to establish gold standards to analyze epigenetic modifications was made.

To conclude this informal working discussion, A Tzioufas and S Bombardieri (University of Pisa, Italy) also reported epigenetic modifications (e.g., methylation, acetylation and sumoylation) of nucleic acid binding proteins (Ro60 and histones). Recognition of these modifications by autoantibodies may reflect an epigenetic disruption during autoimmune diseases. The Brest Epigenetic Task Force was born and has scheduled a meeting in Ljubljana, Slovenia, during the 7th Autoimmunity Congress in May 2010.

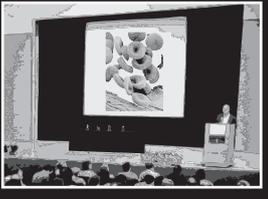
Clinics & biology in Sjögren's syndrome

The debate in Clinics and Biology was dedicated to a new clinical entity, systemic immunoglobulin G4 (IgG4)-related disease. H Takahashi (University of Sapporo, Japan) representing the Japanese Medical society for SS reported that Mikulicz's disease (MD) has long been considered as a manifestation of SS. However, in cases representing a symmetrical and persistent enlargement of the lacrimal and salivary glands compatible with MD, Japanese physicians noticed several clinical differences compared with SS as follows: there was no deflection of female sex differences; extent of xerostomia and xerophthalmia was mild; corticosteroid therapy led to a rapid reduction in glandular swelling and remarkable improvement of secretory function; and no positivity of anti-SSA/B antibodies was observed. In addition, elevated serum levels of IgG4 and abundant infiltration of plasmocytes expressing IgG4 into the lacrimal and salivary glands were reported in MD patients. These are common features of IgG4-related disease, which is a recently proposed clinical entity including autoimmune pancreatitis (AIP), sclerosing cholangitis, Küttner's tumor, retroperitoneal fibrosis and interstitial nephritis. MD often coexisted with IgG4-related diseases such as AIP. Based on those findings, it was proposed that MD be recognized as a new clinical entity of IgG4-related disease. The Japanese Medical Society for SS designed the diagnostic criteria for IgG4-related MD in 2008; persistent symmetrical swelling of two or more pairs of lacrimal and salivary glands, serum level of IgG4 above 1.35 g/l, and infiltration of IgG4⁺ plasmocytes in the tissue (IgG4⁺:IgG ratio

>50%). In the daily clinical setting, it is important to distinguish MD from SS in the patient with swelling of lacrimal and salivary glands, because examinations of the involved organs are different, and early intervention with corticosteroids should be recommended in MD. The etiology of MD and IgG4-related diseases remains to be elucidated. Consequently, it is necessary to accumulate and analyze larger data from patients worldwide. Despite this, a new entity was suggested in Brest as IgG4-related disease.

R Seror (University of Paris VII, France) and C Vitali retrieved the conclusions of the European League Against Rheumatism (EULAR) SS group on the monitoring of SS. A total of 39 SS experts participated in an international collaboration, to develop the EULAR SS Disease Activity Index (ESSDAI), a disease activity index for patients with pSS. Experts identified 12 organ-specific 'domains' contributing to disease activity. For each domain, features were classified into three or four levels according to their severity. A total of 96 real profiles of SS patients with systemic complications, including three successive visits, were abstracted from medical charts and used to generate 702 realistic vignettes. Real patient profiles were scored with the ESSDAI, the SS disease activity index (SSDAI) and the Sjögren's Systemic Clinical Activity Index (SCAI). Each expert, blind to these scores, assessed activity of five real profiles and 20 realistic vignettes using the 0–10 physician global assessment (PhGA), and determined for real profiles whether disease activity improved, worsened or remained stable at visits two and three. It was found that all 12 domains were significantly associated with disease activity in the multivariate model, domain weights ranged from one to six. The ESSDAI scores varied from two to 47 and were significantly correlated with PhGA for both real patient profiles and realistic vignettes. Compared with 57 (59.4%) of the real patient profiles, 468 (66.7%) of the realistic vignettes were considered likely or very likely to be true. In conclusion, the ESSDAI has been designed as a clinical index to measure disease activity in patients with primary SS. Once validated, such a standardized evaluation of primary SS should facilitate clinical research and should be helpful as an





outcome measure in clinical trials. A website has been created to facilitate exchanges with an open-access space to all information on the scores, the recent news on the project and a restricted area for members of the panel with questionnaires for all studies and material for ethical procedures [101].

Pathophysiology of Sjögren's syndrome

ME Gershwin has developed a model for the use of antimicrobial antibodies in defining disease pathogenesis in PBC for other epithelial diseases such as SS. PE Lipsky (National Institute of Health, MD, USA) described the characteristics of circulating B cells with a special emphasis on transitional 1 (T1) B cells (IgD⁺, CD38^{high}) that phenotypically and functionally resemble murine T1 B cells. These cells rapidly die *in vitro* and fail to respond to BCR-mediated stimulation. Furthermore, IL-4 or stromal cells (but not BAFF/BLyS) may be important for T1 B-cell survival and maturation. Systemic lupus erythematosus (SLE) patients have increased proportions of T1 B cells, but not absolute numbers, and this is associated with lymphopenia. T1 B cells have unmutated Ig VH genes and there are no major differences in the VH repertoire of these cells and naive B cells. Gershwin then focused on CD27⁺CD38^{int}IgD⁺ intermediate B cells, which constitute a phenotypically distinct population with characteristics between transitional and naive B cells. CD27⁺CD38^{int}IgD⁺ (intermediate) B cells expressed CD5 as did CD27⁺CD38^{high}IgD⁺ transitional B cells. Since transitional B cells account for only 1–2% of the total circulating B cells, this CD38 intermediate population accounted for the majority of circulating CD5⁺ B cells. Intermediate B cells were significantly increased in cord blood, but were infrequent in normal bone marrow, suggesting peripheral maturation of the intermediate B-cell population. Bone marrow intermediate B cells also expressed CD5, but not pro- or pre-B cells, suggesting the functional role of inhibiting antigen response in immature B cells before full maturation. Pre-naive B cells have acquired some of the functions associated with mature naive B cells, such as plasma cell differentiation upon stimulation with anti-CD40 and

IL-21, or expression of CD86 and the ability to function as antigen-presenting cells for CD4⁺ T cells. These findings also suggest the possibility that under certain circumstances, activation of pre-naive B cells, perhaps during noncognate bystander interactions with activated T cells, could contribute to the functional activation and differentiation of these cells and the emergence of autoimmunity. In addition, SLE and SS patients have increased proportions of pre-naive B cells.

F Mackay (Monash University, Melbourne, Australia) discussed the role of B-cell activating factor (BAFF) in autoimmunity. Indeed, overproduction of BAFF in transgenic (Tg) mice triggers autoimmune disorders similar to SLE and SS, possibly as the result of abnormal self-reactive B-cell survival. Intriguingly, she demonstrated that excess BAFF only mildly affected B-cell immune tolerance and could not prevent deletion of high-affinity self-reactive B cells, but could rescue some low-affinity self-reactive B cells, in particular, marginal-zone B cells. As germinal-center formation and antibody affinity maturation were not essential for disease in these mice, she questioned the real implication of low-affinity autoreactive B cells in driving the disease. Indirect and direct effects of BAFF on T-cell activation, and expansion of the effector T-cell compartment in BAFF Tg mice were then suspected to contribute to nephritis in these animals. Surprisingly, BAFF Tg mice lacking T cells developed the same autoimmune disease as the original BAFF Tg mice. Interestingly, MyD88^{-/-} B cells had impaired autoantibody production in BAFF Tg mice. Overall, this work revealed that autoimmunity in BAFF Tg mice is the result of an abnormal innate B-cell response involving the combined effects of excess BAFF and TLR signaling.

T Defrance (University of Lyon, France) reviewed the functional aspects of the TLR, with a special emphasis on their involvement in autoimmune disorders. He hypothesized an interesting possibility whereby TLR signaling activates the immune response after antigenic stimulation leading to an upregulation of TLR expression, which in turn would facilitate an aberrant autoimmune response following autoantigen encounters.

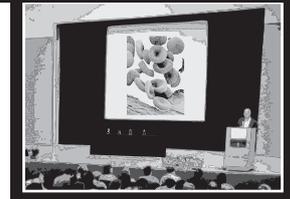
Classification criteria in Sjögren's syndrome

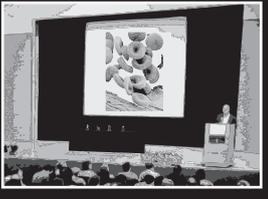
T Daniels discussed the Sjögren's International Collaborative Clinical Alliance (SICCA) that began enrolling participants for baseline examinations at collaborating sites in Argentina, China, Denmark, Japan, UK and USA in 2004. The SICCA cohort includes participants with a broad range of signs or symptoms, ranging from possible early SS to well-established disease. However, to avoid circular reasoning in developing a new definition of SS, no diagnostic labels are used at study entry, even for participants previously diagnosed with SS. Goals of the SICCA project include: establishing an international registry; collecting and storing clinical data and biospecimens; developing standardized classification criteria that are universally applicable; and providing these resources for future studies of SS. All SICCA participants are prospectively enrolled and evaluated using the same protocols to provide uniform data and biospecimen collection from ocular, oral and rheumatologic examinations. Biospecimens include serum, DNA, whole and parotid saliva, tears, conjunctival cells, frozen and paraffin-embedded labial salivary gland (LSG) biopsy specimens and DNA from blood-related controls. By July 2009, SICCA had completed 1447 baseline evaluations. Most participants were reexamined using the same protocols 2 years after their baseline evaluation. The current SICCA natural history data are based on over 300 2-year recall examinations, and will report on changes in physiologic measures such as: salivary and tear production; presence or quantity of serum antibodies; serum complement; LSG histopathology; and development of other organ system diseases or lymphoma.

JP Whitcher (University of California, CA, USA) described the Ocular Sicca Score (OSS), a new simplified quantitative grading system for keratoconjunctivitis sicca (KCS) that was developed by the eight participating ophthalmologists in the SICCA study to analyze the distribution of KCS among the SICCA cohort and its association with other phenotypic characteristics of SS. The ocular exam included an unanesthetized Schirmer test, tear break-up time, ocular surface staining and an external eye examination at the slit lamp. Using

statistical analyses and proportional Venn diagrams, the relationships were examined between an abnormal OSS (>3) and other phenotypic characteristics of SS (LSG biopsies with focal lymphatic sialadenitis focus scores >1 , and positive anti-SSA and/or anti-SSB antibodies). Among 1208 SICCA participants, there were strong associations between abnormal OSS, positive serology and positive LSG focus scores ($p < 0.0001$). Analysis of the overlapping relationships of the three measures identified a large group of participants who had definite KCS but none of the other components of SS. These patients, designated as KCS-only, appear to represent a clinical entity distinct from the KCS associated with SS (SS-KCS). Therefore, Whitcher proposed OSS as a new method for assessing KCS that could be used for diagnosing the ocular component of SS in establishing future classification criteria. Two clinical entities of KCS have been identified, SS-KCS and KCS-only, which may have different etiologies.

Using latent class analysis applied to a wide range of diagnostic tests and phenotypic features to derive a model-based disease classification, S Shiboski confirmed that focal lymphocytic sialadenitis with a focus score greater than or equal to one, anti-SSA/B seropositivity, and an OSS greater than or equal to three, all had high sensitivity against model-based classification criteria. A 2-year follow-up analysis in the SICCA study revealed great stability over time of a working standard with 'two out of three' positive diagnostic tests. During an informal working discussion on 'redefining classification criteria of SS', T Daniels listed the future objectives of the SICCA project: put forward the notion of keeping patient symptoms in the classification criteria versus replacing them with a simplified menu of objective tests; identify groups of patients to participate in clinical trials of new therapeutic agents, or focus laboratory research into the causes or mechanisms of SS; respect and value the opinions of others, and see the SICCA data set as, ultimately, property of the greater scientific community, and generate proposed classification criteria using internal validation (e.g., subsequent data sets and 2-year follow-up data sets); and look forward to the views and suggestions of this and other scientific communities.





AL Fauchais described and discussed a cohort of 419 pSS patients and applied multivariate analysis (detrended correspondence analysis) to identify subgroup characteristics. She observed that a positive LSG focus score (and lymphoma) were independent from other SS components, particularly anti-SSA/B antibodies, hyperglobulinemia and parotid enlargement. Since American–European Consensus criteria exclude pSS patients with negative biopsy and anti-SS-A/B antibodies, she used hyper- γ -globulinemia, rheumatoid factor or positive antinuclear antibodies at the bedside to diagnose pSS, and proposed adding minor autoantibodies as a solution.

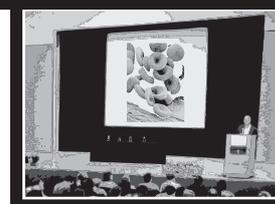
JO Pers (University of Brest, France) evaluated the relevance of the blood B-cell subset profile to the diagnosis of SS. Using flow-cytometry analyses with anti-CD38 and anti-IgD, distribution of mature blood B cells from Bm1 through Bm5 was determined in 161 patients, consisting of 25 who fulfilled the American–European Consensus Group criteria for pSS and 136 who served as disease controls. The percentages of Bm2 and Bm2' cells were increased and those of early Bm5 (eBm5) and Bm5 decreased in pSS patients. The receiver-operating characteristic curves allowed for optimizing a cut-off value of Bm2*Bm2'/eBm5*Bm5 at greater than or equal to five for 88.0% sensitivity and 84.6% specificity. There was admiration for the unique assay, although some concern regarding relatively low sensitivity and small numbers, but it offers good future promise.

An informal working discussion was organized around ultrasonography (US) scoring of salivary glands in SS. S Jousse described the usefulness of US in this indication. Some authors have evaluated abnormal sonoanatomy in pSS while others have studied the vascularization of the gland to demonstrate that pSS glands were in a hyperhemic state. The parotids and submandibular glands were most commonly evaluated using imaging pictures and dynamic studies. They observed length, volume, margins of salivary glands, hypoechoic areas and parenchymal heterogeneity. Sensitivity of US to help clinicians in the diagnosis of pSS ranged from 50 to 70%, and specificity from 70 to 90%. All the experts described US as noninvasive,

inexpensive and easy to perform (i.e., facilities in virtually all hospitals and short time of examination). However, this technique lacks a universally accepted grading scale for the interpretation of the results. Consequently, A Tzioufas proposed the elaboration of an objective grading system, including all possible pictures of SS, and subsequently, their validation in multicenter studies and their correlation with other subjective and objective items of the syndrome in order to define the diagnostic utility of the method. In the future, it would be interesting to have a new non-invasive tool such as US to help clinicians establish the diagnosis of pSS, to use it in the classification criteria and to evaluate the treatment effect.

Immunotherapy of Sjögren's syndrome

A Saraux (cochairman of the symposium from the Brest group) discussed B-depleting trials currently in progress around the world in SS. He highlighted that although many anti-B cell treatments are actually available, no data regarding anti-B treatments other than anti-CD22 (epratuzumab) and anti-CD20 (rituximab) in SS are currently available. In an open-label study, 16 pSS patients (14 women and two men) were scheduled to receive four epratuzumab infusions (360 mg/m² each) at 2-week intervals (Steinfeld group). The most commonly improved parameters were the Schirmer test, nonstimulated whole-salivary flow and visual analog scales (VAS) fatigue score. A clinical response was noted in 53% of patients at 6 weeks and 67% at 32 weeks. This study indicated that epratuzumab holds promise for the treatment of pSS. Human–anti-human antibodies were found in three patients, but were not associated with adverse events. Randomized placebo-controlled trials of epratuzumab are now needed. Subsequently, on the basis of recent evidence concerning the efficacy and safety of the use of anti-CD20 antibodies in various autoimmune diseases, some groups described retrospective cohorts or set up open pilot studies of rituximab in patients with active primary SS. All these publications described improvement of dryness and extraglandular features in approximately 50% of patients, but they pointed

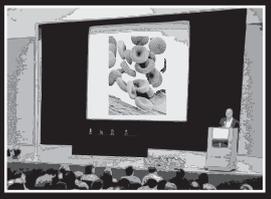


out some adverse events, particularly serum sickness-like reactions, that may occur. Five open-label studies prospectively evaluated rituximab in SS. The first one, by Pijpe *et al.*, conducted in 15 pSS patients with either early pSS or mucosa-associated lymphoid tissue (MALT)-type lymphoma of the salivary glands, suggested an improvement of subjective symptoms and an increase in salivary gland function in patients with residual salivary gland function. However, three patients developed a sickness-like disorder [1].

Saraux then discussed the open-label study he has conducted that consisted of two rituximab infusions (375 mg/m²) 1 week apart in 16 patients with pSS (14 women and two men), who were followed up for 36 weeks. No steroid premedication was used. Patients were eligible if they had active disease with VAS scores greater than 50/100 mm for at least two of the following four variables: global disease activity (including extraglandular manifestations), pain, SICCA symptoms and fatigue. The primary end points were safety and biological effects of rituximab. Secondary end points were variations in the four VAS scores from baseline to week 36. Rituximab treatment induced rapid depletion of all peripheral blood B-cell subsets for at least 1.5 months and depleted salivary gland B cells for at least 12 months. At weeks 12 and 36, significant improvements were noted in the VAS fatigue score, VAS SICCA-symptom score, tender-point count, and SF36 quality-of-life score. In one patient who had pulmonary manifestations, these improved markedly. Mean disease duration was shorter in patients with improvements on at least three of the four VAS scores at any visit than in the other patients. Interestingly, the timing of B-cell repopulation was modulated by BAFF. Pretreatment serum BAFF levels demonstrated a strong negative correlation with time-to-reconstitution. Saraux related an interesting unpublished study kindly sent by M Manoussakis (University of Athens, Greece), in which the authors evaluated 11 patients with type 1 pSS and five patients with pSS-related MALT lymphoma and at least one of the following manifestations: persistent salivary gland enlargement; recurrent cryoglobulinemic or hyperglobulinemic palpable purpura;

vasculitic ulcer(s) resistant to local therapy; peripheral neuropathy (mononeuritis multiplex and/or sensory/mixed polyneuropathy) confirmed by electrophysiologic evaluation; biopsy-proven active proliferative glomerulonephritis with proteinuria (>1 gr/24 h); microscopic hematuria (>10 red blood cells/hpf) and creatinine serum levels less than 4.0 mg/dl; asymptomatic type II cryoglobulinemia; and/or C4 hypocomplementemia (C4 < 15 mg/dl). They evaluated complete, partial or no response to treatment according to these objective criteria of selection. At 6 months, only seven manifestations had a complete response, but response was partial for all patients having mononeuritis multiplex, cryoglobulinemic purpura or proliferative glomerulonephritis. Their conclusion was that rituximab treatment of pSS patients ameliorates certain type I disease-associated clinical features (mononeuritis multiplex, ulcers, cryoglobulinemic purpura and hyperglobulinemic purpura and proliferative glomerulonephritis), and further limits lymphoid neoplastic disease in some patients. However, no evidence of improvement could be observed in fatigue or laboratory aberrations of the disease, although a high rate of infusion-related reactions was observed.

During this 10th International Symposium on SS, several works on rituximab immunotherapy in patients with SS were presented. A Migliore (Rome, Italy) showed the efficacy of rituximab in ten patients with SS associated with rheumatoid arthritis and who were unresponsive to TNF- α inhibitors. In this cohort, rituximab demonstrated significant amelioration for general condition VAS, asthenia VAS and salivary gland secretion. By contrast, Schirmer's test did not change. None of the patients had any adverse events. JJ Scali (Buenos Aires, Argentina) retrospectively evaluated the efficacy and tolerance of rituximab in 31 patients with pSS. A total of 22 patients presented with an improvement in constitutional symptoms, arthritis and myalgias. In addition, subjective improvement in SICCA symptoms was evidenced in 16 patients. No adverse events were related. OA Logvinenko (Moscow, Russian Federation) evaluated the efficacy of rituximab in pSS with severe extraglandular manifestations (eight patients with cryoglobulinemic vasculitis and



glomerulonephritis in one patient) and MALT lymphoma (14 patients). All patients with cryoglobulinemic vasculitis demonstrated improvement in clinical and biologic markers, but relapsed at 6 months. Complete clinical remission of MALT lymphoma was achieved in nine patients with combined therapy and in six patients with monotherapy based on rituximab. Two patients relapsed. H Bootsma (University of Groningen, The Netherlands) presented a randomized, double-blind trial with rituximab compared with placebo in 29 patients with pSS. In the rituximab group, significant improvements were found for the primary end point of secretion of whole saliva, some laboratory parameters (B cells and rheumatoid factor), subjective symptoms and extraglandular manifestations. One patient treated with rituximab developed mild serum sickness-like disease.

In conclusion, in open-label studies and randomized trials, rituximab appeared effective for at least 6–9 months in pSS patients with active disease, improving both the subjective and the objective complaints. The SICCA symptoms abated in some patients, and the objective measures of salivary function improved in patients with early disease. Extraglandular clinical manifestations of pSS responded to rituximab. Retreatment with rituximab resulted in a similarly effective clinical response. Laboratory abnormalities associated with B-cell overactivity also improved. To confirm these promising

results, additional randomized, placebo-controlled trials are ongoing (University of Copenhagen, Rigshospitalet, Denmark; University Medical Centre Groningen, Groningen, The Netherlands; Duke University Medical Center and University of Pennsylvania, USA; and University Hospital, Brest, Ministry of Health, France). Interestingly, a new anti-B-cell therapy study in patients with pSS (the TRACTISS study; S Bowman, Birmingham, UK) has been harmonized with the Tolerance and Efficacy of Rituximab in pSS (TEARS; A Sarau, Brest, France) study for most of the assessment process and entry criteria. Thus, we can consider that a meta-analysis should be achieved once data from both projects are available.

The 11th International symposium on SS will take place in 2011 in Athens, Greece.

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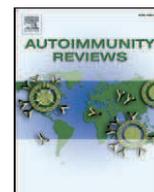
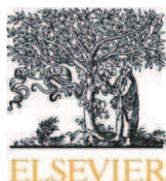
- 101 European League Against Rheumatism. Sjogren's Syndrome Disease Activity Index. www.eularsjogrenactivityindex.com
 - Open-access space to all information on the scores, recent news on the project and a restricted area for members of the panel with questionnaires for all studies and material for ethical procedures.

Appendix 5

B Cell-targeted therapies in Sjögren's syndrome

Tobón GJ, Pers JO, Youinou P, Saraux A.

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B cell-targeted therapies in Sjögren's syndrome

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ABSTRACT

Sjögren's syndrome (SS) or autoimmune epithelitis is characterized by focal lymphocytic infiltrates surrounding the tubular epithelium of exocrine glands and by overactivity of the B-cell population. Although T cells were long considered the main effectors in SS, recent findings indicating a key role for B cells have prompted studies of treatments designed to deplete the B-cell population. Among molecules that can be targeted to achieve B-cell depletion, CD20 and CD22 are surface antigens expressed specifically by B lymphocytes; and the cytokine B-cell-activating factor belonging to the TNF family (BAFF) is a TNF receptor ligand involved in B-cell differentiation, survival, and activation. The aim of this review is to discuss the clinical outcomes of SS patients treated with B-cell depletion.

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1. Introduction

Sjögren's syndrome (SS) is a condition of autoimmune epithelitis that primarily affects females during the fourth and fifth decades of life [1]. The exocrine glands (mainly the salivary and lachrymal glands) exhibit structural lesions characterized by focal lymphocytic infiltrates surrounding the tubular epithelium, and B-cell activity is excessive, resulting in autoantibody production [2]. SS is considered secondary when there is a concomitant systemic autoimmune disease and primary otherwise. The spectrum of SS extends from an organ-specific autoimmune disorder (autoimmune exocrinopathy) to a systemic process that may involve the musculoskeletal system, leading to arthralgia and arthritis [3]. Fatigue is also present and is a major cause of disability. Patients may experience involvement of

internal organs such as the lungs, kidneys, nervous system, and gastrointestinal tract. B-cell overactivity in SS manifests as hypergammaglobulinemia; production of anti-SSA and anti-SSB autoantibodies and of rheumatoid factor (RF); and an increased risk of non-Hodgkin's B-cell lymphoma (NHL) [4,5]. The current treatment consists chiefly in supportive and symptomatic measures aimed at relieving the sicca symptoms. Available drugs improve the dryness, albeit often transiently, but fail to affect the course of the disease. There is a pressing need for disease-modifying drugs to treat patients who have severe organ involvement and factors indicating a high lymphoma risk. Treatments that cause B-cell depletion hold promise as disease-modifying drugs for SS. This review discusses the available clinical data on B-cell depletion used to treat SS.

2. B cells and primary Sjögren's syndrome

Although the pathogenesis of SS remains unclear, the classic concept ascribes a key role to the T cell. However, recent evidence

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indicates a major contribution of B cells [6–8]. B cells produce not only autoantibodies, but also several cytokines, and act as antigen-presenting cells [9]. B-cell infiltrates may constitute germinal centers in the salivary glands [10]. In addition, B-cell activating factor belonging to the TNF family (BAFF), also known as B-lymphocyte stimulator (BLyS), is a cytokine that prevents apoptosis of auto-reactive B cells [11]. Mice transgenic for BAFF develop autoimmune traits reminiscent of primary SS (pSS) [12]. In patients with pSS, circulating BAFF levels are increased and correlate with autoantibody production [13]. B cells may differentiate into two types of effector cells, Be1 producing Th1 cytokines and Be2 producing Th2 cytokines [7]. Moreover, the distribution of mature B-cell subsets from Bm1 through Bm5 in peripheral blood of patients with pSS differs from that in patients with other autoimmune diseases and in controls, being characterized by higher percentages of Bm2 and Bm2' cells [14]. Thus, B cells are involved in the development of SS and therefore hold promise as therapeutic targets.

3. Molecular targets for achieving B-cell depletion

Several B-cell molecules can be targeted (Table 1). The most widely studied target for achieving B-cell depletion is the CD20 antigen (human B-cell-restricted differentiation antigen, Bp35), a hydrophobic transmembrane protein with a molecular weight of approximately 35 kDa found on pre-B and mature B cells [15,16], as well as in over 90% of B-cell NHLs [17]. CD20 is not found on hematopoietic stem cells, pro-B cells, normal plasma cells, or other normal tissues [18]. CD20 regulates one or more early steps in the activation process involved in cell-cycle initiation and differentiation [18] and may serve as a calcium-ion channel [19]. CD20 is neither shed from the cell surface nor internalized upon antibody binding [20]. These characteristics make CD20 a potential target for achieving B-cell depletion.

CD22 is a 135-kDa B-cell restricted type-I transmembrane sialoglycoprotein of the immunoglobulin (Ig) superfamily [21]. CD22 appears intracellularly during the late pro-B-cell stage of ontogeny, shifting to the plasma membrane with B-cell maturation. CD22 is expressed at low levels on immature B cells and at higher levels on mature IgM+ IgD+ B cells but is absent from differentiated plasma cells. The high CD22 expression level in follicular, mantle, and marginal-zone B cells contrasts with the low expression level in germinal B cells [22]. The function of CD22 has not been completely elucidated. However, CD22 acts as a homing receptor for recirculating B cells, due to the affinity of its lectin-like domains for 2,6-linked sialic acid-bearing glycans; and also serves as

a co-receptor that down-modulates the B-cell antigen receptor (BCR) [23,24].

The cytokine BAFF plays a key role in B-cell differentiation, survival, and activation [25]. The BAFF family consists of two ligands, a proliferation-inducing ligand (APRIL) and BAFF; and three membrane receptors, BCMA (B-cell maturation antigen), TACI (transmembrane activator and calcium modulator and cyclophilin ligand interactor), and BAFF-R (also known as BR3). The interactions between ligands and receptors vary: thus, BAFF interacts chiefly with BR3 but can interact with all three receptors, whereas APRIL can interact with TACI and BCMA, but not with BR3 [26]. BAFF enhances B-cell survival, drives B-cell maturation mainly at the early transitional stages, and discontinues humoral tolerance by rescuing autoreactive B cells from apoptosis [27].

CD20, CD22, and BAFF are potential targets for strategies designed to modify B-cell function in pSS, both directly and indirectly (Table 1).

4. Anti-CD20 treatment

Several monoclonal antibodies targeting CD20 are under development. Rituximab, a chimeric monoclonal antibody to CD20 antigen is the only anti-CD20 compound tested in pSS so far.

Rituximab was approved by the FDA in 1997 for the treatment of relapsed or refractory, low-grade or follicular CD20 B-cell NHL. In 2004, rituximab was evaluated for the maintenance treatment of rheumatoid arthritis (RA) [28] and the prevention of graft rejection [29]. Clinical benefits were obtained, although the underlying mechanisms were incompletely understood. Mechanisms currently implicated in the effects of rituximab are complement-dependent cytotoxicity, antibody-dependent cell-mediated cytotoxicity, and induction of B-cell apoptosis.

The first studies of rituximab were conducted using an open-label design. In a retrospective study including 6 patients with pSS treated with rituximab for associated lymphoma ($n=2$) or systemic manifestations ($n=4$), significant improvements in subjective feelings of dryness were recorded in 3 patients, stabilization of diagnostic tests in 2 patients, and improvements in extra-glandular features in 5 patients [30]. Furthermore, 1 of the 2 patients with lymphoma achieved a full remission.

Another retrospective study included 16 pSS patients treated with rituximab for lymphoma ($n=5$) or systemic manifestations ($n=11$) [31]. Only a minority of patients reported relief of dryness. However, the extra-glandular manifestations improved in 9 of 11 patients and the corticosteroid requirements decreased consistently. Rituximab was effective in 4 of the 5 patients with NHL. Adverse events were recorded in 3 (19%) of the 16 patients, including a single patient who developed a mild serum sickness-like reaction with positive human anti-chimeric antibodies (HACAs).

An open-label, 3-month, phase II study in 15 patients with pSS evaluated four rituximab infusions (375 mg/m² once weekly) after pretreatment with prednisolone (25 mg) and clemastine [32]. Of the 15 patients, 8 had disease durations of less than 4 years (early pSS) and 7 had concomitant mucosa-associated lymphoid tissue (MALT) lymphoma of the parotid gland (mean disease duration, 79 months). Tolerability and efficacy of the treatment were assessed 5 and 12 weeks after the first infusion based on both subjective criteria (visual analogue scale [VAS] and quality of life [SF36]) and objective tests. Peripheral B-cell counts dropped within a few weeks and IgM-RF levels fell concomitantly. B-cell counts were normal after 6 to 9 months in the 7 patients for whom long-term follow-up data were available. Rituximab treatment significantly improved the symptoms in the early pSS patients and stimulated submandibular/sublingual salivary gland secretion in the 8 patients with early pSS and in 2 patients with MALT/pSS. Of the 8 patients without MALT lymphoma, 5 received a second course of rituximab (after 9–11 months) due to symptom recurrence. Retreatment produced similar significant improvements in

Table 1

Potential B-cell targets and treatments used for B-cell depletion in patients with Sjögren's syndrome.

| | |
|--------------------------------------|---|
| <i>Direct targeting of B cells</i> | |
| CD-20 antigen | <ul style="list-style-type: none"> • Rituximab (chimeric monoclonal antibody) • Ocrelizumab (humanized monoclonal antibody) • Ofatumumab (human monoclonal antibody) • Veltuzumab (humanized monoclonal antibody) • TRU-015 (engineered protein) |
| CD-22 antigen | <ul style="list-style-type: none"> • Epratuzumab (humanized monoclonal antibody) |
| <i>Indirect targeting of B cells</i> | |
| BAFF | <ul style="list-style-type: none"> • Belimumab (LymphoStat B: fully human monoclonal antibody against BAFF) |
| BAFF receptors | <ul style="list-style-type: none"> • Anti-BR3 • Atacept (IgG Fc fused to the extracellular TACI receptor domain) • Briobacept/BR3-Fc (IgG Fc fused to the extracellular BAFF receptor (BR3) domain) |

BAFF: B-lymphocyte Activator Factor belonging to the TNF family; TACI: transmembrane activator and calcium modulator and cyclophilin ligand interactor.

the salivary flow rate and subjective symptoms as did the initial treatment, with decreases in B-cell counts and IgM-RF levels. Of the 7 MALT/pSS patients, 6 responded initially to rituximab (complete clinical remission in 3 and stabilization in 3). The remaining MALT/pSS patient had progressive MALT lymphoma and severe extraglandular SS 3 months after the first rituximab infusion. Cyclophosphamide was added, which stabilized both the MALT lymphoma and the SS. One of the 6 patients with an initial response experienced a recurrence of MALT lymphoma after 9 months then responded to rituximab re-treatment. Of the 15 patients, 4 (27%) developed HACAs, and 3 of these 4 patients had a serum sickness-like disorder. Interestingly, all patients with HACAs and/or serum sickness-like symptoms belonged to the early-pSS group [32].

In a retrospective study, the effects of rituximab were assessed in 8 patients with SS [33]. All 8 patients were women, their mean age was 37.3 ± 10 years, and their mean disease duration was 100.5 ± 86 months. Of the 7 patients who received more than one rituximab infusion, 4 (57%) responded, exhibiting decreases in parotid gland swelling and complete recovery of the joint involvement, fatigue, and subjective dryness. Adverse effects occurred in 3 patients. In 1 patient, rituximab was stopped during the first infusion and cyclophosphamide was started. In another patient, hypotension developed during the second infusion, which was stopped; nevertheless, this patient experienced substantial improvements that lasted 23 months. One of the patients had RA with secondary SS and experienced complete resolution of the parotid gland swelling and joint involvement for 24 months.

We conducted an open-label study of two rituximab infusions (375 mg/m^2) 1 week apart in 16 patients with pSS (2 men/14 women), who were followed up for 36 weeks [34]. No steroid premedication was used. Patients were eligible if they had active disease with VAS scores greater than 50/100 mm for at least two of the following four variables: global disease activity (including extraglandular manifestations), pain, sicca symptoms, and fatigue. The primary endpoints were safety and biological effects of rituximab. Secondary endpoints were variations in the four VAS scores from baseline to week 36. Rituximab treatment induced rapid depletion of all peripheral-blood B-cell subsets for at least 1.5 months and depleted salivary-gland B-cells for at least 12 months. At week 12, significant improvements were noted in the VAS fatigue score ($P < 0.05$), VAS sicca-symptom score ($P < 0.05$), tender point count (TPC, $P < 0.035$), and SF36 quality-of-life score ($P < 0.01$). At week 36, there were significant improvements in all four VAS scores ($P < 0.05$), in the tender joint count ($P = 0.017$) and TPC ($P = 0.027$), and in the SF36 score ($P < 0.05$). In a patient who had pulmonary manifestations, these improved markedly. Mean disease duration was shorter in the patients with improvements on at least three of the four VAS scores at any visit ($n = 11$) than in the other patients ($n = 5$) (3.8 ± 5.4 versus 30.1 ± 29.5 years; $P = 0.02$). During the first infusion, the first 2 patients experienced mild-to-moderate infusion-related reactions after the rituximab infusion rate was raised to 200 mg/h. The symptoms consisted of fever, headache, fatigue, flush, and pruritus. These symptoms resolved without treatment after the infusion rate was decreased to 100 mg/h. Subsequently, infusions were administered at rates no higher than 100 mg/h during both infusions in all patients. No patient had severe side effects. Interestingly, the timing of B-cell repopulation was modulated by BAFF [35]. Pre-treatment serum BAFF levels showed a strong negative correlation with time to reconstitution ($r = -0.92$, $P < 0.0005$). Consistent with this finding, resistance to rituximab occurred when BAFF was produced by salivary gland epithelial cells or by infiltrating B cells [35,36].

The first double blind, randomized, placebo-controlled study of rituximab in pSS included 18 patients [37]. Patients randomized to active therapy received two 1-g rituximab infusions on days 1 and 15. Each infusion was preceded by 100 mg of methylprednisolone given intravenously. Patients were evaluated monthly for 6 months. The primary efficacy endpoint was the proportion of patients with a greater than 20% improvement in the VAS fatigue score 6 months after therapy.

Full 6-month data were available for 17 of the 18 patients, 8 allocated to rituximab and 9 to the placebo. The VAS fatigue score improved significantly in the rituximab group ($P < 0.001$) but not in the placebo group ($P = 0.147$). The VAS general health score also improved significantly in the rituximab group ($P = 0.021$) but not in the placebo group ($P = 0.96$). Significant between-group differences were found after 6 months in the SF-36 social functioning score ($P = 0.01$) and in the RF decline versus baseline ($P = 0.05$) but not in the immunoglobulin levels or antibody titers. No significant change in the Schirmer test score was found after 6 months. Three serious adverse events occurred in 2 patients in the rituximab group. One patient experienced serum sickness, which responded well to intravenous steroids. The other presented with abdominal pain 4 weeks after the second rituximab infusion then experienced palpitations 3 months after therapy. HACAs were not evaluated. The number of patients was small, and further studies are needed.

In conclusion, in open-label studies and one randomized trial, rituximab seemed effective for at least 6–9 months in pSS patients with active disease, improving both the subjective and the objective complaints. The sicca symptoms abated in some patients, and the objective measures of salivary function improved in patients with early disease. Extraglandular clinical manifestations of pSS responded to rituximab. Retreatment with rituximab resulted in a similar good clinical response. Laboratory abnormalities associated with B-cell overactivity also improved. To confirm these promising results, additional randomized placebo-controlled trials are ongoing (Table 2). The Tolerance and Efficacy of Rituximab in primary Sjögren's syndrome (TEARS) study is a multicenter, randomized, French study started in 2008 in pSS patients with a disease duration shorter than 10 years or extraglandular involvement. Table 3 shows the inclusion criteria.

5. Anti-CD22 treatment

Epratuzumab is a monoclonal antibody to CD22. In an open-label study, 16 pSS patients (14 women/2 men) were scheduled to receive four epratuzumab infusions (360 mg/m^2 each) at 2-week intervals [38]. Among them, 14 received all four infusions without significant adverse reactions, 1 patient received three infusions, and 1 experienced a mild acute reaction to the first infusion that led to discontinuation of the study protocol. Follow-up was 6 months.

Mean disease duration before epratuzumab therapy was 2.9 years, and none of the patients had received prior B cell-targeted therapy. A composite endpoint involving the Schirmer test, nonstimulated whole salivary flow, fatigue, erythrocyte sedimentation rate, and immunoglobulin G (IgG) level was devised to assess the clinical response, defined as a 20% or greater improvement in at least two parameters. The most commonly improved parameters were the Schirmer test, nonstimulated whole salivary flow, and VAS fatigue score. A clinical response as defined for this study was noted in 53% of patients at 6 weeks and 67% at 32 weeks. Peripheral B-cell counts showed mean decreases of 54% and 39% at 6 and 18 weeks, respectively. This study indicates that epratuzumab holds promise for the treatment of pSS. Human anti-human antibodies (HAHAs) were found in 3 patients but were not associated with adverse events. Randomized placebo-controlled trials of epratuzumab are needed.

6. Treatments targeting BAFF and APRIL

Three different classes of BAFF antagonists are at various stages of clinical development (Table 1). Selective BAFF blockers prevent BAFF from interacting with its receptors, leaving APRIL available to interact with TACI and BCMA. Drugs in this class include an antibody to BAFF (anti-BLyS, Belimumab or Lymphostat B) and a fusion protein consisting of human Ig Fc and of the extracellular BAFF-R domain (Briobcept, BAFF-R-Ig, BR3-Fc). Non-selective BAFF blockers abolish the interactions of both BAFF and APRIL with all their receptors. To date, there is a single

Table 2
Completed and ongoing studies of rituximab in patients with primary Sjögren's syndrome declared on Clinical trials.gov.

| Center | Clinical trials.gov identifier/model | Current status | Study completion date | Intervention | Number of patients | Primary outcome measures |
|--|--|-------------------------------------|-----------------------|---|--------------------|--|
| University of Copenhagen, Rigshospitalet, Denmark | NCT00426543 Double blind placebo-controlled | Currently recruiting participants | March 2010 | Rituximab, 1000 mg twice, 14 days apart | 22 | Subjective symptoms (oral and ocular dryness), myalgia/arthralgia and fatigue (6 months) |
| University Medical Centre Groningen, Groningen, The Netherlands, | NCT00363350 Double blind placebo-controlled | Completed | October 2008 | Rituximab, 1000 mg twice, 14 days apart | 30 | Stimulated whole salivary flow rate (48 weeks) |
| Duke University Medical Center and University of Pennsylvania, USA | NCT00101829 Open label | Ongoing but not recruiting patients | August 2009 | Rituximab, 1000 mg twice, 14 days apart | 12 | Grade 3, 4, or 5 toxicity related to rituximab |
| University Hospital, Brest Ministry of Health, France | NCT00740948 Double blind placebo-controlled | Currently recruiting participants | August 2010 | Rituximab, 1000 mg twice, 14 days apart | 120 | 30% improvement on 2 of 4 VAS scores (global disease, joint pain, fatigue, and dryness) |

drug in this class, which is human Ig Fc fused to the extracellular TACI domain (Ataccept, TACI-Ig). Belimumab has been tested in patients with systemic lupus erythematosus (SLE) or rheumatoid arthritis (RA) [39] but produced no evidence of clinical efficacy in either. However, the autoantibody levels decreased. Both studies established the safety and tolerability of belimumab. Given the importance of BAFF in the B-cell abnormalities seen in pSS, clinical trials with anti-BAFF treatments would be of great interest.

7. Conclusions

B cells play a key role in the development of SS. Several molecules found in B-cells can be targeted. Rituximab, a monoclonal antibody to CD20, improved both subjective and objective abnormalities in patients with active pSS. To confirm these promising results, as in systemic lupus erythematosus [40], further randomized placebo-controlled trials are ongoing. Epratuzumab, a monoclonal antibody against CD22, holds promise for the treatment of pSS. In addition, BAFF may be of considerable interest as a treatment target in pSS. Treatments that eliminate the abnormal B cells may be effective in preventing progression toward malignancies and in improving the severe extra-glandular manifestations that are related to B-cell overstimulation (i.e., hypergammaglobulinemia, cryoglobulinemia, hypocomplementemia, arthritis, and vasculitis). The identification of factors that predict the response to B cell-depleting treatments (mainly rituximab) would be valuable for selecting those patients who are most likely to benefit.

Table 3
Inclusion criteria for the TEARS study (Tolerance and Efficacy of Rituximab in Sjögren's syndrome).

| |
|---|
| <ul style="list-style-type: none"> • Age between 18 and 80 years • Meets European criteria issued by the American-European Consensus Group for primary Sjögren's syndrome • Active SS of recent onset; activity is defined as values greater than 50/100 on at least two of four 100-mm visual analog scales (VAS) evaluating global disease, pain, fatigue, and global dryness, respectively; plus at least one of the following: <ul style="list-style-type: none"> ◦ Anti-SSA and/or rheumatoid factor ◦ Cryoglobulinemia ◦ Hypergammaglobulinemia ◦ β2-microglobulin elevation ◦ Hypocomplementemia • One or more active systemic manifestations of SS among the following: <ul style="list-style-type: none"> ◦ Parotid gland enlargement ◦ Cutaneous vasculitis ◦ Pulmonary involvement ◦ Neurological involvement ◦ Renal involvement ◦ Autoimmune cytopenia ◦ Myositis ◦ Arthritis ◦ Lymphadenopathy • Contraception throughout the study and for at least 1 year after the last infusion |
|---|

Take-home messages

- B cells play a key role in the development of pSS and several molecules found in B-cells can be targeted.
- Treatments that eliminate the abnormal B cells may be effective in preventing progression toward malignancies and in improving the severe extra-glandular manifestations that are related to B-cell overstimulation.
- Both rituximab (monoclonal antibody to CD20) and epratuzumab (monoclonal antibody against CD22) hold promise for the treatment of pSS. Further randomized placebo-controlled trials are ongoing.

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Plasmin immunization preferentially induces potentially prothrombotic IgG anticardiolipin antibodies in MRL/MpJ mice

To test the hypothesis, utilizing 2 experimental mouse models, that plasmin is an important autoantigen that drives the production of certain IgG anticardiolipin antibodies (aCL) in patients with antiphospholipid syndrome. **Ede K. et al. (Arthritis Rheum 2009; 60: 31108–17)**. BALB/cJ and MRL/pJ mice were immunized with Freund's complete adjuvant in the presence or absence of human plasmin. The mouse sera were analyzed for production of IgG antiplasmin, IgG aCL, and IgG anti- β 2-glycoprotein I (anti- β 2GPI) antibodies. IgG monoclonal antibodies (mAb) were generated from the plasmin-immunized MRL/MpJ mice with high titers of aCL and these 10 mAb were studied for their binding properties and functional activity in vitro. Plasmin-immunized BALB/cJ mice produced high titers of IgG antiplasmin only, while plasmin immunized MRL/MpJ mice produced high titers of IgG antiplasmin, IgG aCL, and IgG anti- β 2GPI. Both strains of mice immunized with the adjuvant alone did not develop IgG antiplasmin or IgG aCL. All 10 of the IgG mAb bound to human plasmin and cardiolipin. While 4 of the 10 bound to β 2GPI, 3 of 10 bound to thrombin, and 4 of 10 bound to the activated coagulation factor X (FXa). Functionally, 4 of the 10 IgG mAb inhibited plasmin activity, 1 of 10 hindered inactivation of thrombin by antithrombin III, and 2 of 10 inhibited inactivation of FXa by antithrombin III. Plasmin immunization leads to production of IgG antiplasmin, aCL, and anti- β 2GPI in MRL/MpJ mice but leads to production of only IgG antiplasmin in BALB/cJ mice. IgG mAb generated from plasmin-immunized MRL/MpJ mice bind to various antigens and exhibit procoagulant activity in vitro. These results suggest that plasmin may drive potentially prothrombotic aCL in generally susceptible individuals.

Intravenous immunoglobulin G-mediated inhibition of T-cell proliferation reflects an endogenous mechanism by which IgG modulates T-cell activation

Commercial intravenous immunoglobulin G (IVIg) at high doses has therapeutic benefit in autoimmune and inflammatory diseases. It has been shown to inhibit T-cell function but the mechanisms are not clear. Inhibition could result from IVIg processing, donor pooling or intrinsic down-regulatory activity of IgG. To address these points, **MacMillan HF. et al. (Clin Immunol 2009; 132: 222–233)** compared the effects on T-cell activation of IVIg, Fab2 fragment and IgG isolated from single-donor plasma. The authors also investigated the role of accessory cells in the IVIg effects using highly purified T cells stimulated through CD3 and CD28 engagement. T-cell proliferation was evaluated by Oregon Green 488 dye dilution and 3H-thymidine incorporation. IVIg, Fab2 fragment of IVIg and autologous, single donor IgG significantly inhibited T-cell proliferation (35–50%), even in the absence of accessory cells. Depletion of IgG from plasma used for culture significantly increased by (50%) the T-cell proliferation. The addition of physiological concentrations of single-donor, autologous IgG or IVIg to IgG-depleted plasma reduced T-cell proliferation to levels observed in normal plasma. Therefore, donor pooling in IVIg and accessory cells are not required for inhibition of T-cell proliferation by IVIg and the Fab2 region is sufficient to mediate this inhibition. Suppression of T-cell activation by IVIg likely reflects a physiologic, endogenous mechanism of IgG-mediated regulation of T-cell activation.

Appendix 6

Emerging biotherapies for Sjögren's syndrome.

***Tobón GJ, Saraux A, Pers JO, Youinou P.
Expert Opin Emerging Drugs 2010; 15: 269-282.***

Expert Opinion

1. Background
2. Existing treatments and medical need
3. Current research goals
4. Scientific rationale
5. Competitive environment
6. Potential development issues
7. Conclusion
8. Expert opinion

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Emerging biotherapies for Sjögren's syndrome

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Importance of the field: Sjögren's syndrome (SS) is an autoimmune epithelitis. This exocrinopathy is frequently associated with extraglandular complications, and the patients are at risk of developing B cell lymphoma. Given the lack of disease-modifying drugs, and the fact that SS is a quintessential B-cell mediated disease, attention has recently been focused on biotherapies.

Areas covered in this review: Despite negative grounds, TNF- α antagonists have been tested in the disease, and proven not be efficient. However, B-cell depleting therapy using anti-CD20 antibodies such as rituximab, which is a chimeric mAb, has shown promise in the field, while anti-CD22 mAb seems to be less active.

What the reader will gain: New treatments against the B-cell activating factor of the TNF family are about to be tested, or replaced by receptor immunoglobulin decay protein.

Take home message: B-cell depleting therapies seem promising in SS, but no data are, thus far, available on treatments targeting B-cell activating factor of the TNF family.

Keywords: B lymphocyte, BAFF, epratuzumab, etanercept, IFN- α , infliximab, rituximab, Sjögren's syndrome, TNF- α inhibitors

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1. Background

Sjögren's syndrome (SS) is a condition of autoimmune epithelitis that primarily affects females during the fourth and fifth decades of life [1]. SS is considered primary (pSS) when there is a concomitant autoimmune disease such as rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE), and secondary (sSS) otherwise. The spectrum of SS extends from an organ-specific autoimmune disorder (referred to as an autoimmune exocrinopathy) to a systemic process that may involve the musculoskeletal system, leading to arthralgia and arthritis [2-4]. The disease is characterized not only by fatigue, which represents a major cause of disability, but patients may also experience involvement of internal organs such as lungs, kidneys, CNS and peripheral nervous system and the gastrointestinal tract.

Exocrine tissues, most notably lacrimal and salivary glands (SGs), exhibit structural lesions characterized by focal lymphocytic infiltrates surrounding the tubular epithelium, where T cells predominate over B cells [5]. Attention has recently turned to these latter cells originally thought to be restricted to anti-self antibody (Ab) production [6], and since endowed with a prominent role in exacerbation and sustaining of the acquired immune response [4]. B-cell overactivity in SS manifests as hypergammaglobulinemia, the synthesis of a plethora of anti-nuclear Abs [7], such as anti-Sicca Syndrome (SS)A and anti-SSB autoAbs, considerable amounts of serum IgA, IgA-containing immune complexes and elevated levels of IgA with rheumatoid factor (RF) activity [8], coupled with an increased risk ($\times 18.8$) of non-Hodgkin's B-cell lymphoma (NHL) [9]. This polymorphism accounts for the delay in the diagnosis [10,11]. The problem has been overridden by the emergence of new

treatments that have proved effective in RA [12] and SLE [13].
 50 These mAbs present as potentially active in SS.

Although a role of B lymphocytes in SS has been suspected for many years, recent discoveries have unveiled new insights into B-cell derived cytokines, including IFN- γ and IL-4 that modulate the response [14]. They are likely to serve as effectors of some
 55 B-cells functions. Given the kinetics of B-cell generation and the cytokine profile of B lymphocytes, T helper (Th) 1 phenotype may be imprinted by B effectors (Be) 1 cells through their expression of IL-2 and IFN- γ by B cells. This is sustained by an IFN- γ /IFN- γ receptor autocrine loop. In fact, the Th1/
 60 Th2 cytokine balance changes with the progress of the immunopathological lesions [15]. Distinct populations of serum cytokines have also been found [16] to differentiate patients from controls, and from one patient to another, depending on the presence or absence of extraglandular manifestations.

65 **2. Existing treatments and medical need**

The current treatment consists in supportive and symptomatic measures aimed at relieving the sicca symptoms. The key issues for the routine management of SS are systemic treatment for specific extraglandular manifestations and regular assessment by rheumatologists, dentists and ophthalmologists. The good
 70 health of mucosal tissues rests upon maintaining a sustained level of dampness in both the eye and the mouth. In the eyes a variety of moisture protection devices, artificial tears and the use of topical agents including methylprednisolone and cyclosporin may be of great value. A variety of punctual plugs are also available. Dryness of the mouth may be helped by frequent sips of water, artificial saliva and the use of pilocarpine or cevimeline that stimulate the muscarinic receptors [17]. Water is used by many patients, but it is not a good moistener of
 75 the oral mucosa, thus needing very frequent application.

All the available drugs may transiently improve the dryness, but fail to affect the course of the disease. Ultimately, there is a critical need for disease-modifying drugs to treat patients who have severe organ involvement and factors indicating a
 80 high risk of lymphoma.

85 **3. Current research goals**

Immunotherapy in pSS has been unsuccessfully launched with TNF- α inhibitors and IFN- α and is currently being tested with B-cell targeted therapies. By far, treatments that
 90 cause B-cell depletion have yielded the best results as disease-modifying drugs for SS. This review discusses the available clinical data on immunotherapy and focuses on the relevance of B-cell depletion in the treatment of SS.

4. Scientific rationale

95 **4.1 For TNF- α inhibition**

TNF- α , originally described as an antitumor agent, is a pro-inflammatory cytokine produced by several cell

types (viz. monocytes, macrophages, mast and endothelial cells), and is central to the pathogenesis of various autoimmune diseases. 100

TNF- α inhibitors have shown efficacy in the treatment of RA [18], Crohn's disease, ankylosing spondylitis and possibly SLE [19]. Currently, at least four TNF- α inhibitors are available: infliximab (a chimeric mAb), etanercept (a soluble TNF- α receptor fusion construct), adalimumab (a fully
 105 humanized anti-TNF- α mAb) and golimumab (recently approved by the FDA for the treatment of RA). In animal models of SS, TNF- α inhibition has shown promise in suppressing tissue destruction in lacrimal glands [20], or by blocking apoptosis of human epithelial cells by TNF- α induced by their incubation with anti-SSA and anti-SSB Abs [21]. 110

However, TNF- α deletion fails to protect B-cell activating factor of the TNF family (BAFF) transgenic (Tg) mice against autoimmunity [22]. This essential finding establishes that TNF- α is dispensable for BAFF-mediated autoimmune disorders. The observation is telling, because TNF- α inhibitors are irrelevant to the treatment of SS. Supporting this view, BAFF synthesis by RA synoviocytes are negatively regulated by TNF- α [23]. Furthermore, TNF- α deficiency in BAFF-Tg mice led to a high incidence of B cell lymphoma (>35%).
 120 Nonetheless, all these negative grounds notwithstanding, several groups have set out to determine the consequences of TNF- α inhibition in SS patients [24-26].

4.2 For the use of IFN- α

IFN- α has been suggested to play an important role in the pathogenesis of several autoimmune diseases, including
 125 SS [27]. In addition to their antiviral activities, IFN- α has potent immunomodulating effects [28]. Thus, the biologic activity of orally given low doses of IFN- α has been evaluated in pSS. 130

4.3 For depleting B-cells

Although the pathogenesis of SS is getting less and less elusive, the historical concept has long ascribed a key role to T cells. However, evidence is now converging to indicate that the role of B cells in the play is dominant [29]. Not only do they release autoAbs, they also make cytokines [30], act as antigen (Ag)-presenting cells [31], behave as autophagic cells to bridge the gap between innate and adaptative responses [32] and regulate the formation of germinal centers (GCs) through the production of lymphotoxins [33]. In the SGs of patients with SS they
 135 settle as aggregates and conventional GCs that do not exclude autoreactive B cells [34]. In addition, they differentiate into two types of B effectors, Be1 producing Th1-like cytokines and Be2 producing Th2 cytokines [35]. Downstream, the distribution of mature B-cell subsets is defined by their relative expression of IgD and CD38. This blood distribution into Bm1 through Bm5 differs from pSS to normal controls [36], but also from pSS patients to disease controls [11]. This abnormal distribution is characterized by higher percentages of activated Bm2 and GC-founder Bm2 cells and the dramatic 140 145 150

decrease of memory B cells that accumulate in the SGs [37]. Given this number of witnesses for the prosecution against B cells as the main lymphocyte population in SS, they should really plead guilty. As a consequence, they hold promise as therapeutic targets.

5. Competitive environment

5.1 TNF- α inhibitor trials

The first trial was conducted with infliximab, in an open-label pilot of 16 patients with active pSS (Table 1) [24]. Infliximab was infused at the dose of 3 mg/kg and the outcome of the disease evaluated after 14 weeks. Global assessments, erythrocyte sedimentation rate (ESR), salivary flow rate, Schirmer's test, tender joint count, fatigue, dry eyes and dry mouth were all significantly improved, relative to baseline values. These early benefits were claimed to be sustained 1 year later [25]. Subsequently, a multi-center, randomized, double-blind, placebo-controlled trial assigned 103 patients with pSS to receive infliximab 5 mg/kg versus placebo in weeks 0, 2 and 6 [26]. The patients of this TRIPSS, for trial of remicade in pSS were examined over 22 weeks. At weeks 10 and 22, there was no difference between the two groups in the variables tested, such as tender and swollen joints, basal salivary flow rate, Schirmer's test, SGs focus score, C-reactive protein and ESR. However, adverse events were noted in 6 patients from the infliximab-treated group versus 1 from the placebo group. In conclusion, this careful study failed to support the favorable results reported in the pioneering trial.

Similar results have been found with another TNF- α inhibitor, etanercept. Zandbelt *et al.* [38] launched an open-label study to administer etanercept 25 mg s.c. twice a week over a 12-week period to 15 pSS patients, with the point at weeks 18 and 24. Evaluations included fatigue score, serological monitoring, Schirmer's test, rose Bengal cornea staining, tear film breakup time and minor SG biopsy focus score. No functional improvements of the exocrine glands or change in focus score were observed. However, in 4 patients, a decrease in fatigue score was found. In conclusion, the only effect of etanercept was improvement of fatigue in a minority of patients. These results were confirmed to be modest in a randomized, double-blind, placebo-controlled trial that included 28 patients with pSS or sSS, randomized to etanercept 25 mg twice-weekly versus placebo [39]. The primary outcome was an improvement of 20% from baseline values in at least two of three domains (subjective or objective measures of dry mouth, subjective or objective measures of dry eyes, and IgG level or ESR). In the etanercept group, 5 patients felt improvement in the primary outcome compared to 3 in the placebo group at 12 weeks. This difference was not significant. The effect of adalimumab on TNF- α converting enzyme has been investigated on epithelial cells in culture but never used to treat SS patients. In summary, TNF- α inhibitors are ineffective in the treatment of SS.

5.2 IFN- α trials

The benefits from treatment with oromucosal IFN- α in pSS were reported by Ferraccioli *et al.* [40], Shiozawa *et al.* [41], Ship *et al.* [42] and Khurshudian *et al.* [43]. Overall, they showed an increase in stimulated whole saliva flow in a proportion of patients treated with IFN- α 150 IU t.i.d. Shiozawa *et al.* [41] evaluated SG biopsies after IFN- α treatment in nine patients. In this latter group, these patients who experienced a twofold or greater increase in saliva output in response to treatment showed significant histopathologic improvement, including a reduced mononuclear infiltration.

Thereafter in a Phase III study, although unstimulated whole saliva flow was significantly increased in IFN- α -treated patients compared to placebo, the primary endpoints of stimulated whole saliva and subjective oral dryness were not significantly ameliorated [44].

Finally, in one case, it was reported that IFN- α may be useful to treat peripheral neuropathy associated with pSS [45].

5.3 Molecular targets for B-cell depletion

Several B-cell molecules can be targeted (Table 2). For example, CD22 is a 135-kDa B-cell restricted type-I transmembrane sialoglycoprotein of the Ig superfamily [46]. CD22 appears intracellularly during the late pro-B-cell stage of ontogeny, shifting to the plasma membrane with B-cell maturation. It is expressed at low levels on immature B cells, and at higher levels on mature IgM⁺ IgD⁺ B cells but lacks from differentiated plasma cells (PCs). The high CD22 level of expression in follicular, mantle and marginal-zone B cells contrasts with its low level of expression in germinal-center B cells [47]. Although all its functions have not been completely elucidated, CD22 serves as a co-receptor that down-modulates the B-cell Ag receptor and acts as a homing receptor for recirculating B cells, due to the affinity of its lectin-like domains for 2,6-linked sialic acid bearing glycans [48,49].

The most widely studied target for achieving B-cell depletion is the CD20 Ag (human B-cell restricted differentiation Ag), a hydrophobic transmembrane protein with a molecular weight of ~ 35 kDa found on pre-B and mature B cells [50,51], as well as in > 90% of B cells in NHLs [52]. However, CD20 is not found on hematopoietic stem cells, pro-B cells, normal PCs or other normal tissues [53]. CD20 regulates one or more early steps in the activation process involved in cell-cycle initiation and differentiation and may serve as a calcium-ion channel [54]. As CD20 is neither shed, nor internalized upon Ab binding [55], it is an extremely attractive target for achieving B-cell depletion in pSS, but CD22 is also a good candidate (Table 2).

5.3.1 Anti-CD22 trials

Epratuzumab (EPZ) is an anti-CD22 mAb [56]. In an open-label study, 14 women and 2 men with pSS received four 360 mg/m² EPZ infusions at 2-week intervals [57]. Ultimately, 14 individuals received all four infusions without significant

Table 1. TNF- α inhibitors in pSS.

| Drug, dose and administration | Study design | Inclusion criteria | Number of patients | Concomitant medication | Criteria for response | Results | Adverse events | Ref. |
|--|---|---|--------------------|---|--|---|--|------|
| Infliximab 3 mg/kg (i.v.) at weeks 0, 2 and 6 | Single-center, open-label, 14-week-long study | pSS patients, > 18 years of age, with active disease (evaluated by ESR and/or hypergammaglobulinemia) | 16 | DMARDs were not allowed | Improvement in clinical and biological parameters | Global assessments, ESR, salivary flow rate, Schirmer's test, tender joint count, fatigue, dry eyes and dry mouth were all statistically significantly improved in comparison to baseline | One infusion reaction (mild) and two mild respiratory tract infections | [24] |
| Infliximab 3 mg/kg (i.v.) every 8 weeks for 1 year (extension of the study given earlier). A dose of 5 mg/kg were used in two patients because of partial response | Single-center, open-label, 50-week-long study | Of the 16 patients with pSS enrolled in the study mentioned earlier, 10 were chosen. All 10 reported SS symptoms 9 weeks after the third of the three initial infusions | 10 | DMARDs were not allowed | Improvement in clinical and biological parameters | Global assessment, tender joint count, fatigue, dry eyes, dry mouth and ESR improved compared to baseline | Three mild infusion-related events, two uncomplicated infections | [25] |
| Infliximab 5 mg/kg (i.v.) at weeks 0, 2 and 6 vs placebo; follow-up 22 weeks | Randomized, multicenter, controlled trial vs placebo (TRIPSS study) | pSS patients with active disease (evaluated by values > 50 mm on two of three visual analog scales, that involved joint pain, fatigue and sicca symptoms) | 103 | Hydroxychloroquine and corticosteroids (\leq 15 mg/day) were allowed | In two of three scales, > 30% improvement 10 or 22 weeks after treatment in | No improvement in tender joint count, basal salivary flow, Schirmer's test, salivary gland focus score, CRP and ESR | Seven patients presented severe adverse events (two were infusion reactions) | [26] |
| Etanercept 25 mg (s.c.) twice/week for 12 weeks; follow-up 24 weeks | Single-center, open-label, 24-week study | pSS with active disease | 15 | | Improvement in salivary, lachrymal, serological and histological parameters | No increase of salivary or lacrimal gland function And four patients had improved fatigue | No | [38] |
| Etanercept 25 mg (s.c.) twice/week for 12 weeks | Randomized, controlled trial vs placebo | pSS and sSS with active disease | 28 | Long-term medications were allowed | In at least two of the three domains (subjective or objective measures of dry mouth, subjective or objective measures of dryness and IgG level or ESR), > 20% of improvement | In the etanercept group five patients showed improvement compared to three in the placebo group at 12 weeks; this difference was not significant | One atypical injection-site reaction | [39] |

CRP: C-reactive protein; DMARDs: Disease-modifying antirheumatic drugs; ESR: Erythrocyte sedimentation rate; pSS: Primary Sjögren's syndrome; sSS: Secondary Sjögren's syndrome; SWS: Stimulated whole saliva flow; VAS: Visual analog scale.

Table 2. Potential B-cell targets, mAbs and other treatments used for B-cell depletion in patients with SS.**Direct targeting of B cells***CD-20 antigen*

Rituximab (chimeric mAb)

Ocrelizumab (humanized mAb)

Ofatumumab (human mAb)

Veltuzumab (humanized mAb)

TRU-015 (engineered protein)

CD-22 antigen

Epratuzumab (humanized mAb)

Indirect targeting of B cells*BAFF*

Belimumab (LymphoStat B: fully human mAb against BAFF)

BAFF receptors

Anti-BR3

Atacicept (IgG Fc fused to the extracellular TACI receptor domain)

Briobacept/BR3-Fc (IgG Fc fused to the extracellular BAFF receptor, BR3 domain)

BAFF: B-cell activator factor belonging to the TNF family; SS: Sjögren's syndrome; TACI: Transmembrane activator and calcium modulator and cyclophilin ligand interactor.

adverse reactions, 1 received only three infusions and 1 experienced a mild acute reaction to the first infusion that led to discontinuation of the study protocol. Mean disease duration before EPZ therapy was 2.9 years, and none of the patients had received prior B-cell targeted therapy. After 6 months, a composite endpoint involving the Schirmer test, unstimulated whole salivary flow, fatigue, ESR and IgG level was designed to assess the clinical response, defined as a $\geq 20\%$ improvement in at least two parameters. The most frequently improved parameters were the Schirmer test, the unstimulated whole salivary flow and the visual analogue scale (VAS) to score the fatigue. There was a clinical response in 53% of the cases 6 weeks after treatment, and in 67% of the cases 32 weeks after treatment. This study indicates that EPZ holds promise for the treatment of pSS. Human anti-human Abs were found in 3 patients but were not associated with adverse events. Now, there is a need for randomized placebo-controlled trials of EPZ.

5.3.2 Anti-CD20 trials

Several mAbs targeting CD20 are under development. Rituximab (RTX), an anti-CD20 chimeric mAb, was originally approved for the treatment of refractory or relapsed patients with NHL [58]. It is now widely used to treat a range of B-cell malignancies [59]. In addition, due to its efficacy and lack of major known side effects, RTX is successfully used in the clinic to treat patients with autoimmune diseases. Following the seminal work of Edwards and Cambridge in RA [60,61], it is the only anti-CD20 compound thus far tested in pSS (Table 3).

Clinical benefits were obtained, although the underlying mechanisms were incompletely understood. Mechanisms currently implicated in the effects of RTX are complement-dependent cytotoxicity, Ab-dependent cell-mediated cytotoxicity and apoptosis induction. After treatment with RTX, CD20 has been translocated into the lipid rafts of the membrane [62].

The first open-label 3-month Phase II study was undertaken in 15 patients with pSS [63]. These were given four 375 mg/m²/week RTX infusions after prednisolone and clemastine. The disease duration of 8 of the 15 early pSS patients was < 4 years, and 7 suffered from concomitant mucosa-associated lymphoid tissue (MALT) lymphoma of the parotid gland. Tolerability and short-term efficacy of the treatment were determined 5 and 12 weeks after the first infusion, based on objective and subjective criteria, including VAS and quality of life, as assessed by the Short Form 36 (SF36) index. Peripheral B-cell counts dropped within a few weeks and IgM-RF levels fell concomitantly.

B-cell counts were normal after 6 – 9 months in the 7 patients for whom long-term follow-up data were available. RTX treatment significantly improved the symptoms in the early pSS patients and stimulated submandibular/sublingual SG secretion in the 8 patients with early pSS and in 2 patients with MALT/pSS. Of the 8 without MALT lymphoma, 5 received a second course of rituximab (after 9 – 11 months) due to symptom recurrence. Re-treatment produced similar significant improvements in the salivary flow rate and subjective symptoms as did the initial treatment, with decreases in B-cell counts and IgM-RF levels. Of the 7 MALT/SS patients, 6 responded initially to RTX (complete clinical remission in 3 and stabilization in another 3). The remaining patient with progressive MALT lymphoma had developed this complication and severe extraglandular involvement 3 months after the first RTX infusion. Cyclophosphamide was added, which stabilized both the MALT lymphoma and SS. Of the 6 patients with an initial response, 1 experienced a recurrence of MALT lymphoma after 9 months but responded to RTX re-treatment. Of the 15 patients, 4 developed human anti-chimeric Abs (HACAs), and 3 of these 4 patients had a serum sickness-like disorder. Interestingly, all patients with HACAs and/or serum sickness-like symptoms belonged to the early

Table 3. Rituximab in pSS.

| Dose | Study design | Number of patients/ inclusion criteria | Concomitant medication | Criteria for response | Results | Adverse events | Ref. |
|---|--|--|--|--|--|---|------|
| 375 mg/m ² /week for 4 weeks (one patient for 2 weeks) | Multicenter, retrospective analyses | Six patients: four pSS with extraglandular disease, two pSS with MALT | MP (3 patients) | Efficacy on extraglandular involvement, ocular and oral VAS, objective measures (Schirmer's test) | Significant improvements in subjective dryness were recorded in three patients; extraglandular manifestations improved in five patients; one of the two patients with MALT achieved a full remission | Two patients presented infusion related adverse events, one developed a serum sickness | [70] |
| 375 mg/m ² /week for 4 weeks (14 patients), 1 g twice (1 patient), 375 mg/m ² /week for 4 weeks (1 patient) | Retrospective | 16 Patients: 11 pSS with systemic complications, 5 pSS with lymphoma | No premedication was used | Efficacy on extraglandular involvement, ocular and oral VAS, objective measures (Schirmer's test) | Only a minority of patients reported relief of dryness. Extraglandular manifestations improved in 9 of 11 patients. Corticosteroid requirements decreased | Moderate events reported in three patients, one with HACA developed serum sickness | [71] |
| 375 mg/m ² /week for 4 weeks | Prospective, single-center, open-label study | 15 Patients: 8 pSS with early disease (< 4 years), 7 pSS with MALT | Pretreatment with PDN and DMARDs were not allowed | Subjective criteria (VAS and quality of life) and objective criteria | RTX improved the symptoms in the early pSS patients and stimulated submandibular/sublingual secretion in the 8 patients with pSS and in 2 patients with pSS/MALT | Three patients in the early pSS group experienced severe adverse reactions, four had HACA | [63] |
| 375 mg/m ² /week for 4 weeks or 1 g every 2 weeks for two infusions | Retrospective, two-center study | Eight patients: seven pSS with extraglandular manifestations, one sSS with RA | DMARDs were not allowed; pretreatment with steroids and anti-histaminics | Subjective and objective criteria | Of the 7 pSS/MALT patients, 6 responded to RTX (complete remission in 3 and stabilization in 3 patients) | Three patients experienced infusion-related adverse events | [72] |
| 375 mg/m ² /week for 2 weeks | Prospective, single-center, open-label study | 16 Patients with active disease defined by VAS scores > 50/100 mm for at least two of four variables (global disease activity, pain, sicca symptoms and fatigue) | No premedication was used | Primary endpoints were safety and biological effects of RTX. Secondary endpoints were variations in the four VAS scores from baseline to week 36 | Four patients responded, exhibiting decrease in parotid gland swelling and complete recovery of the joint involvement, fatigue and subjective dryness (up to 24 months) | No patients experienced severe adverse events | [66] |

DMARDs: Disease-modifying anti-rheumatic drugs; ESR: Erythrocyte sedimentation rate; HACA: Human anti-chimeric antibodies; MALT: Mucosa associated lymphoid tissue; MP: Methylprednisolone; PDN: Prednisone; pSS: Primary Sjögren's syndrome; RA: Rheumatoid arthritis; RTX: Rituximab; SF36: Short Form 36; SS: Sjögren's syndrome; sSS: Secondary Sjögren's syndrome; SWS: Stimulated whole saliva flow; VAS: Visual analog scale.

Table 3. Rituximab in pSS (continued).

| Dose | Study design | Number of patients/ inclusion criteria | Concomitant medication | Criteria for response | Results | Adverse events | Ref. |
|------------------------------------|--|--|------------------------------------|---|--|--|------|
| Two 1 g infusions on days 1 and 15 | Double blind, randomized, placebo controlled study | 17 Patients with VAS fatigue > 50: 8 patients in RTX group, 9 in placebo | Pretreatment with MP 100 mg (i.v.) | Primary efficacy endpoint was the proportion of patients with a > 20% improvement in the VAS fatigue score 6 months after therapy | The VAS fatigue score improved significantly in the RTX group ($p < 0.001$) but not in the placebo. SF-36 functioning score was different between groups. No significant change in the Schirmer's test was found | Three serious adverse events occurred in two patients in RTX group (serum sickness, abdominal pain and palpitations) | [73] |

DMARDs: Disease-modifying anti-rheumatic drugs; ESR: Erythrocyte sedimentation rate; HACA: Human anti-chimeric antibodies; MALT: Mucosa associated lymphoid tissue; MP: Methylprednisolone; PDN: Prednisone; pSS: Primary Sjögren's syndrome; RA: Rheumatoid arthritis; RTX: Rituximab; SF36: Short Form 36; SS: Sjögren's syndrome; sSS: Secondary Sjögren's syndrome; SWS: Stimulated whole saliva flow; VAS: Visual analog scale.

pSS group. The long-terms effects were described in a different report [64], and, later, the same authors provided histological proof of potential regeneration of the secretor tissues of the SGs [65].

Devauchelle *et al.* conducted a similar open-label study, without steroid premedication, consisting in two 375 mg/m² RTX infusions, 1 week apart, in 14 women and 2 men with pSS, who were followed up for 36 weeks [66]. Patients were eligible if they had active disease with VAS scores > 50/100 mm for at least two of the following four variables: global disease activity (including extraglandular manifestations), pain, sicca symptoms and fatigue. The primary endpoints were safety and biological effects of RTX. Secondary endpoints were variations in the four VAS scores from baseline to week 36. This treatment induced rapid depletion of all peripheral-blood B-cell subsets for ≥ 1.5 months and depleted SG B-cells for ≥ 12 months. At week 12, significant improvements were noted in the VAS fatigue score ($p < 0.05$), VAS sicca-symptom score ($p < 0.05$), tender joint count (TJC, $p = 0.035$) and SF36 quality-of-life score ($p < 0.01$). At week 36, there were significant improvements in all four VAS scores ($p < 0.05$), in the tender joint count ($p = 0.017$) and TJC ($p = 0.027$), and in the SF36 score ($p < 0.05$). In a patient, pulmonary manifestations were markedly improved. Mean disease duration was shorter in the patients with improvements on at least three of the four VAS scores at any visit ($n = 11$) than in the other 5 patients (3.8 ± 5.4 vs 30.1 ± 29.5 years; $p = 0.02$). During the first infusion, the first 2 patients experienced mild-to-moderate reactions when the RTX infusion rate was raised to 200 mg/h. The symptoms consisted of fever, headache, fatigue, flush and pruritus. They resolved without treatment after the decrease of the infusion rate to 100 mg/h. Subsequently, infusions were administered at rates ≤ 100 mg/h during both infusions in all patients. Interestingly, SG measurements and blood inflow responses to salivary stimulation as assessed by ultrasound hold promise as objective noninvasive tools for evaluating RTX effects in patients with pSS ([67], G Valesini pers. commun., and A Tzioufas, pers. commun.).

Depletion and repopulation of B cells in blood and SGs revealed that the timing of B-cell repopulation was modulated by BAFF [68]. In the eight patients whose SGs contained significant numbers of B cells, biopsy specimen were completely devoid of B lymphocytes 4 months after RTX treatment. Pretreatment serum BAFF levels showed a strong negative correlation with time to reconstitution of circulating B cells ($p < 0.0005$). The higher the serum levels of BAFF, the shorter the elapsed time before B-cell repopulation. Consistent with this finding, resistance to RTX occurred when BAFF was produced by infiltrating B cells that were eluted from the SGs and analyzed by regular and reverse transcription polymerase chain reaction of sorted single-cell B lymphocyte preparations [69].

Meanwhile, several retrospective surveys have been completed at quite the same time. One such study of 6 patients

with pSS treated with RTX for associated lymphoma ($n = 2$) or systemic manifestations ($n = 4$) suggested that there were significant improvements in subjective feelings of dryness in 3 patients, stabilization of diagnostic tests in 2 and improvements in extraglandular features in 5 [70]. Furthermore, 1 of the 2 patients with lymphoma achieved full remission.

Another retrospective study by the same group described 5 pSS patients treated with RTX for NHL and 11 for systemic manifestations [71]. Only a minority of patients experienced relief from dryness. However, the extraglandular manifestations improved in 9 of 11 patients and the corticosteroid requirements decreased consistently. RTX was effective in 4 of the 5 patients with NHL. Adverse events were recorded in 3, including 1 who developed a mild serum sickness-like reaction with positive HACAs.

In a third retrospective study, the effects of RTX were assessed in eight patients with SS [72]. All were women; their mean age was 37.3 ± 10.0 years and their mean disease duration 100.5 ± 86.0 months. Among the seven patients who received more than one RTX infusion, four responded, exhibiting decreases in parotid gland swelling and complete recovery of the joint involvement, fatigue and subjective dryness. Adverse effects occurred in three patients. In one patient, RTX was stopped during the first infusion and cyclophosphamide was started. In another patient, hypotension developed during the second infusion, which was stopped. Nevertheless, this patient experienced substantial improvements during 23 months. One of the patients had RA with sSS and experienced complete resolution of the parotid gland swelling and joint involvement for 24 months.

The first double-blind, randomized, placebo-controlled study of RTX in pSS included 18 patients [73]. Patients randomized to active therapy received two 1-g RTX infusions on days 1 and 15. Each infusion was preceded by methylprednisolone 100 mg i.v. Patients were evaluated monthly for 6 months. The primary efficacy endpoint was the proportion of patients with a $> 20\%$ improvement in the VAS fatigue score 6 months after therapy. Full 6-month data were available for 17 of the 18 patients, 8 allocated to RTX and 9 to the placebo. The VAS fatigue score was significantly improved in the RTX group ($p < 0.001$) but not in the placebo group. The VAS general health score was also improved in the RTX group ($p = 0.021$) but not in the placebo group. Significant between-group differences were found after 6 months in the SF-36 social functioning score ($p = 0.01$) and in the RF decline versus baseline ($p = 0.05$) but not in the Ig levels or Ab titers. There were no significant changes in the Schirmer's test scores after 6 months. Three serious adverse events occurred in 2 patients in the RTX group. One patient experienced serum sickness, which responded well to intravenous steroids. The other complained of abdominal pain 4 weeks after the second RTX infusion, and then suffered from palpitations 3 months after therapy. HACAs were not evaluated. In this trial, the number of patients was small, and further studies are needed.

Of interest, MALT [74,75] or marginal zone lymphoma [76,77] complicating SS have been improved by RTX, as documented by position emission tomography. Resistance to RTX has been ascribed to local production of BAFF in mice [78] and humans [79]. These observations converge to demonstrate that BAFF inhibits CD20-mediated and B-cell receptor mediated apoptosis in B lymphocytes [80].

5.3.3 Anti-CD20 ongoing trials

On the occasion of the 10th International Symposium on SS, taking place in Brest, France (1 – 3 October 2009), several ongoing works in patients with SS treated with RTX were presented. Severino *et al.* [81] showed the efficacy of RTX in 10 patients with SS associated with RA and unresponsive to TNF- α inhibitors. In this cohort, RTX induced significant improvements in general condition VAS, asthenia VAS and SG secretion. In contrast, the Schirmer's test values were unchanged. None had adverse events. Moreno-Guzmán *et al.* [82] retrospectively evaluated the efficacy and tolerance of RTX in 31 patients with pSS. A total of 22 benefited from improvement in arthritis and myalgias, and subjective improvement in sicca symptoms was documented in 16 of them. No adverse events were related. Logvinenko *et al.* [83] evaluated the efficacy of RTX in pSS with severe extraglandular manifestations (8 patients with cryoglobulinemic vasculitis, and 1 with glomerulonephritis) and MALT lymphoma (14 patients). All patients with cryoglobulinemic vasculitis showed improvement in clinical and biologic markers, but after 6 months they relapsed. Complete clinical remission of MALT lymphoma was seen in 9 patients with combined therapy and in 6 with RTX monotherapy whereas 2 patients relapsed. Meijer *et al.* [84] presented a randomized, double-blind trial with RTX compared to placebo in 29 patients with pSS. In the RTX group, significant improvements were found for the primary endpoint of secretion of stimulated whole saliva, B-cell count, RF titer subjective symptoms and extraglandular manifestations ($p < 0.04$). Mild serum sickness-like disease was seen in 1 patient treated with RTX.

In conclusion, in open-label studies and randomized trials, there is a trend for efficacy of RTX for $\geq 6 - 9$ months in pSS patients with active disease, improving both the subjective and the objective complaints. The sicca symptoms abated in some patients, and the objective measures of salivary function improved in those with early disease. Extraglandular clinical manifestations of pSS responded to RTX. Re-treatment with RTX resulted in a similar good clinical response. Laboratory abnormalities associated with B-cell over-activity also improved. To confirm these promising results, additional randomized placebo-controlled trials are presently in progress (Table 4).

6. Potential development issues

BAFF, also known as BLyS for B-lymphocyte stimulator, is a cytokine [85] that prevents apoptosis of auto-reactive B cells [86].

Table 4. Completed and ongoing studies of RTX in patients with primary SS declared on clinical trials.gov.

| Center | Clinical trials.gov identifier/model | Current status | Study completion date | Intervention | Number of patients | Primary outcome measures |
|--|--|-------------------------------------|-----------------------|-------------------------------------|--------------------|--|
| University of Copenhagen, Rigshospitalet, Denmark | NCT00426543 Double-blind placebo-controlled | Currently recruiting participants | March 2010 | RTX, 1,000 mg b.i.d., 14 days apart | 22 | Subjective symptoms (oral and ocular dryness), myalgia/arthralgia and fatigue (6 months) Stimulated whole salivary flow rate (48 weeks) |
| University Medical Centre Groningen, Groningen, The Netherlands | NCT00363350 Double-blind placebo-controlled | Completed | October 2008 | RTX, 1,000 mg b.i.d., 14 days apart | 30 | Grade 3, 4 or 5 toxicity related to rituximab |
| Duke University Medical Center and University of Pennsylvania, USA | NCT00101829 Open-label | Ongoing but not recruiting patients | August 2009 | RTX, 1,000 mg b.i.d., 14 days apart | 12 | On two of four VAS scores (global disease, joint pain, fatigue, and dryness), 30% improvement seen |
| University Hospital, Brest Ministry of Health, France | NCT00740948 Double-blind placebo-controlled | Currently recruiting participants | August 2010 | RTX, 1,000 mg b.i.d., 14 days apart | 120 | |

RTX: Rituximab; SS: Sjögren's syndrome.

Mice transgenic for human BAFF develop autoimmune traits resembling SLE [87] and evolving to SS [88]. This is consistent with high serum levels of BAFF in patients with pSS [89], their correlation with autoAb titers [90] and their presence in the SGs [91].

This cytokine influences B-cell differentiation, survival and activation [86]. In addition to BAFF, this TNF family ligands comprises a proliferation-inducing ligand (APRIL), and possess four membrane receptors: BCMA for B-cell maturation Ag; TACI for transmembrane activator and calcium modulator and cyclophilin ligand interactor; BAFF specific BAFF-R (BR3); and APRIL specific polysaccharides. Interactions between ligands and receptors vary. Thus, BAFF interacts chiefly with BR3 but also with TACI and BCMA, whereas APRIL interacts with TACI and BCMA, but not with BR3 [92]. BAFF enhances B-cell survival, drives B-cell maturation mainly at the early transitional stages and discontinues humoral tolerance by rescuing autoreactive B cells from apoptosis. One issue for monitoring treatment with BAFF antagonists is the assay for BAFF. Given the conflicting results, we have developed an in-house-enzyme-linked immunosorbent assay for BAFF [93].

However, treatment of B cells with TACI agonist Ab inhibits proliferation *in vitro* and activation of a chimeric receptor containing TACI intracellular domain induces apoptosis. These results also demonstrate the critical requirement for TACI in regulating B-cell homeostasis [94].

Different forms of BAFF are present in the human serum due to translational modifications. BAFF can indeed be glycosylated or not. The major problem is that glycosylation seems to alter epitope recognition by anti-BAFF Abs. This could also explain why serum concentrations of BAFF vary between studies for a given population of patients.

Three different classes of BAFF antagonists are at various stages of clinical development (Table 2). Selective BAFF blockers prevent BAFF from interacting with its receptors, leaving APRIL available to interact with TACI and BCMA. Drugs in this class include anti-BAFF Ab (Belimumab or Lymphostat B) and a fusion protein consisting of human Ig Fc and of the extracellular BAFF-R domain (Briobcept, for BAFF-R-Ig). Non-selective BAFF blockers abolish the interactions of both BAFF and APRIL with all their receptors. To date, there is a single drug in this class [94], which is human Ig Fc fused to the extracellular TACI domain (Atacept, TACI-Ig). Belimumab [95] has been tested in patients with RA or SLE [96,97]. Both studies established the safety and tolerability of Belimumab. Although the levels of autoAb were decreased, no evidence of clinical efficacy was reported. Differences in the distribution of the forms of BAFF could denote the potential of SLE patients to respond to or resist BAFF antagonist therapy. Here, the difficulty is the incredible heterogeneity of the target molecule [98]. The promise should not be fulfilled [99].

Given the importance of BAFF in the B-cell abnormalities seen in pSS, clinical trials with anti-BAFF treatments that

545 target the forms of BAFF implicated in the pathophysiology
of SS would be of great interest.

7. Conclusion

550 Owing to the key role of B cells in the emergence of SS,
B-cell targeted immunotherapies are the most hopeful treat-
ment in SS. Several molecules found in B-cells can be
targeted. RTX, an mAb to CD20, improved both subjective
and objective abnormalities in patients with active pSS. To
confirm these promising results, as in SLE [100], further ran-
domized placebo-controlled trials are ongoing. EPZ, an
anti-CD22 mAb is promising for the treatment of pSS. In
555 addition, BAFF may be of considerable interest as a treatment
target in pSS. Treatments that deplete abnormal B cells may
be effective in preventing progression towards malignancies
and in improving the severe extraglandular manifestations
that reflect B-cell overstimulation and comprise hyperga-
mmaglobulinemia, cryoglobulinemia, hypocomplementemia,
560 arthritis and vasculitis. Identification of factors that predict
the response to B-cell depleting treatments would be valuable
for selecting those patients who are most likely to benefit.

8. Expert opinion

565 The current treatment of SS consists of supportive and symp-
tomatic measures aimed at relieving the sicca symptoms.
There is a pressing need for disease-modifying drugs to treat
patients who have severe organ involvement and risk factors
to lymphoproliferation. TNF- α inhibitors (infliximab and

etanercept) have been tested in patients with SS. Open- 570
label and randomized studies have confirmed that they are
inefficient in the treatment of SS. The Phase III study using
IFN- α in the treatment of patients with SS did not confirm
the encouraging results of the pilot trials.

As B cells play a key role in the development of pSS, it 575
makes sense to target these cells. Also, treatments that elimi-
nate abnormal B cells are effective in preventing progression
towards malignancies and improving the severe extraglandular
manifestations that are related to B-cell overstimulation.
Both RTX (mAb against CD20) and EPZ (mAb against 580
CD22) hold promise for the treatment of pSS and further
randomized placebo-controlled trials are ongoing.

Finally, given the importance of BAFF in the B-cell 585
abnormalities of pSS, clinical trials with anti-BAFF treat-
ments would be of great interest to target the pathological
forms of BAFF (probably depending on glycosylation) and
to respect the regulatory role of APRIL on its receptor
TACI. Consequently, a double immunotherapy targeting
both BAFF and B cells might be most relevant.

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