The complexity of the BAFF forms and their functional implications

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Summary

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Elevated expression of 'B cell activating factor' (BAFF), a potent B cell survival factor contributes to the expansion of low-affinity self-reactive B cells during the establishment of tolerance. However, mechanisms leading to BAFF over-expression in autoimmune diseases are not understood. We reported the discovery of a new variant for BAFF, Δ4BAFF in humans (in which exon 4 is excised) or Δ5BAFF in mice (in which exon 5 is excised), which acts as a transcription factor of the full-length form of BAFF, and which is preferentially found in cells isolated from patients with autoimmune diseases. When transfected in human B cells, Δ4BAFF upregulates a large number of genes associated with immune response and especially innate immunity and regulation of apoptotis. Furthermore Δ4BAFF acts, in association with p50 from the NF-κB pathway, as a transcription factor for its own parent gene. Another important finding is that Δ4BAFF is an important component of the efficacy of regulatory B cell activity. 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.

We have also tried to understand the complexity of the various forms of BAFF. We observed that epithelial cells expressed BAFF-receptor (BR3) and produce BAFF suggesting autocrine properties. Blocking BR3 results in nuclear translocation of PKCδ promoting epithelial cell apoptosis. Furthermore, only some forms of BAFF are required for epithelial cell survival.

Finally, we studied the consequences of the expression of TLR9 on the B cell surface and demonstrated that TLR9 acts as a co-receptor of the B cell receptor to influence B cell fate independently of CpG binding. We show that CpG activation of B cells, acting synergistically with BCR signals, was inhibited by anti-TLR9 stimulation. Induction of CD25 expression and proliferation of B cells were thus down-regulated by engagement of cell surface TLR9. Overall, our results indicate that TLR9 expressed on B cell plasma membrane might be a negative regulator of endosomal TLR9, and could provide a novel control by which activation of autoreactive B cells is restrained. All these findings contribute to a better understanding on immunopathology of autoimmune diseases with potential applications in therapy.

Résumé

La complexité des différentes formes de BAFF et leurs incidences fonctionnelles

BAFF, «facteur d'activation des lymphocytes B (LB) » contribue à l'expansion des LB autoréactifs de faible affinité lors de la mise en place de la tolérance. Cependant, les mécanismes menant à la surexpression de BAFF dans les maladies auto-immunes ne sont pas compris. Nous avons découvert un nouveau variant de BAFF, Δ4BAFF (dans lequel l'exon 4 est épissé), qui agit comme un facteur de transcription de son propre gène et participe à sa régulation. Ainsi, Δ4BAFF est préférentiellement observé dans les cellules isolées de patients atteints de maladies auto-immunes. De plus, Δ4BAFF régule un grand nombre de gènes associés à la réponse immunitaire innée et à la régulation de l'apoptose. Une autre constatation importante est que Δ4BAFF est un élément clé pour comprendre l'activité des LB régulateurs. Notre travail présente un concept entièrement nouveau suggérant qu'une cytokine peut être régulée par l'activité de l'un de ses variants d'épissage.

Par ailleurs, nous avons observé que les cellules épithéliales expriment le récepteur de BAFF : BR3. Le blocage de BR3 se traduit par la translocation nucléaire de PKCδ et l'apoptose des cellules épithéliales. Par un effet autocrine, nous démontrons que seules certaines formes de BAFF participent à la survie des cellules épithéliales.

Enfin , nous avons étudié les conséquences de l'expression du TLR9 à la surface des LB et démontrons que ce TLR9 membranaire ne fixe pas le CpG et agit comme un co-récepteur négatif du BCR. En effet, l'activation des LB par le CpG capté au niveau endosomal, est inhibée par l'action d'un anticorps anti-TLR9 se fixant au niveau membranaire. Tous ces résultats contribuent à une meilleure compréhension des mécanismes impliqués dans l'immunopathologie des maladies auto-immunes avec des applications potentielles en thérapeutique.

MOTS CLES : BAFF, autoimmunité, variant, syndrome de Gougerot-Sjögren, TLR9

CONTENTS

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ABBREVIATIONS

aa	Amino acids
Ab	Antibody
AID	Activation-induced deaminase
AP-1	Activator protein-1
APC	Antigen presenting cell
APRIL	A proliferation inducing ligand
B10	IL-10 producing B cell
BAFF	B cell activating factor belonging to the TNF family
BAFF-R	BAFF receptor
BCMA	B cell maturation antigen
BCR	B cell receptor
bp	Base pairs
BR3	BAFF-receptor 3
Breg	Regulatory B cell
CD40L	CD40 ligand
CHS	Contact hypersensitivity
CIA	Collagen induced arthritis
CII	Collagen type II
CLL	Chronic lymphocytic leukemia
CSR	Class switch recombination
CVID	Common variable immunodeficiency
DAMPs	Damage associated molecular pattern
DC	Dendric cells
dsRNA	Double strand RNA
EAE	Experimental autoimmune encephalomyelitis
EC	Epithelial cell
ERK1/2	Extracellular signal-regulated kinase ½
ESE	Exonic splicing enhancer
ESS	Exonic splicing silencer
G-CSF	Granulocyte-colony stimulating factor
HSG	Human salivary gland
IKK	IκB kinase
IRAK	IL-1R-associated kinase
IRF	Interferon regulatory factor
ISE	Intronic splicing enhancers
ISS	Intronic splicing silencers
JNK	Jun N-terminal kinase
Kb	kilobase
LPS	Lypopolysaccharide
LRRs	Leucine rich repeats
MAP	Mitogen-activated protein
MEKK3	Mitogen-activated proteine kinase kinase 3
MS	Multiple sclerosis

MyD88	Myeloid differentiation primary response gene 88
MZ	MZ
NEMO	NF-kB Essential Modulator
NFAT	nuclear factor of activated T cells
NKT	Natural killer T
NOD	Non-obese diabetic
ORF	Open reading frame
PAMPs	Pathogen associated molecular pattern
PD-L 1 and 2	programmed death ligands 1 and 2
PKC	Protein kinase C
PKCδ	Protein kinase C delta
PRR	Pattern recognition receptors
pSS	Primary Sjögren's syndrome
RA	Rheumatoid arthris
S1PR1	Sphingosine-1-phosphate receptor 1
SG	Salivary gland
SGEC	Salivary gland epithelial cell
SLE	Systemic lupus erythematosus
SNPs	Single nucleotide polymorphisms
SS	Sjögren's syndrome
ssRNA	Single strand RNA
T1	Transitional type 1 B cell
T2	Transitional type 2 B cell
TACI	Transmembrane activator and calcium modulator ligan interactor
TAK	Transforming growth factor β-activated kinase
TCR	T cell receptor
TCR	T cell receptor
Th1	T helper 1
Th17	T helper 17
THD	TNF homology domain
TIR	Toll-interleukin-1 receptor
TLRs	Toll like receptors
TNF	Tumor necrosis factor
TRAF	TNFR-associated factor
Treg	Regulatory T cell
TRIF	TIR domain-containing adapter inducing IFN-β
UV	Ultraviolet
V	Variable

PREAMBLE

People who treat autoimmune diseases such as systemic lupus erythematosus (SLE), rheumatoid arthritis (RA), and primary Sjögren's syndrome (pSS) dream of finding the diseases' Achilles heel: a protein that plays some causative role, that is required for disease persistence, and that can be targeted therapeutically without causing widespread side effects. Experiments on 'B cell activating factor belonging to the tumor necrosis factor family' BAFF (also known as BlyS), are generating enormous excitement because they suggest that these dreams just might come true. Here, at last, is an example of a molecule that appears to be involved in common human autoimmune diseases, rather than just the ever-so-rare eponymous syndromes. Moreover, experiments in animals suggest that therapies based on antagonizing BAFF may make a real difference clinically. The most promising studies in humans were BLISS-52 and BLISS-76, large phase III studies that demonstrated measurable efficacy for belimumab, a monoclonal antibody against BAFF in SLE.

However, BAFF appears as a very complex molecule and there exists the issues of why the concentration of BAFF remains within normal range in a proportion of patients with autoimmune diseases and why increased BAFF production has been associated with autoantibody by some, but not other patients. This could be due to the existence of different forms of BAFF or posttranslational modifications that may alter its structure and thereby its recognition by the antibodies used.

Because BAFF is a novel therapeutic target, differences in the distribution of the forms of BAFF denote the potential of patients to respond, or to resist BAFF blockade. Reliable indicators for predicting such behavior are therefore becoming increasingly important.

This thesis will deal on the different forms of BAFF and will try to identify their functional implications.

I. INTRODUCTION

1. BAFF

The B cell activating factor belonging to the TNF (Tumor necrosis factor) family (BAFF) also known as BLyS for B-Lymphocyte Stimulator (or TALL-1, THANK, TNFSF13B, zTNF4, CD257), has become significant in B cell biology because this cytokine is responsible for B cell survival and maturation during the early transitional stages. BAFF has also been associated with the control of tolerance and malignancy (Mackay and Schneider 2009).

BAFF's influence is widespread and its effects are as numerous as they are varied. In fact, BAFF offers a range of variants, membrane-bound or soluble, glycosylated or non glycosylated forms, monomer or trimers, homotrimers or heterotrimers, heterotrimers with another TNF APRIL (a proliferation inducing ligand) or heterotrimers with BAFF variants, or even virus-like aggregates of 60 monomers. We will review the complexity of the various forms of BAFF by focusing on the different structural aspects of the molecule (Figure 1). A review entitled "The complexity of the BAFF TNF family members: implications for autoimmunity", was published in the Journal of Autoimmunity and can be found in Appendix 1.

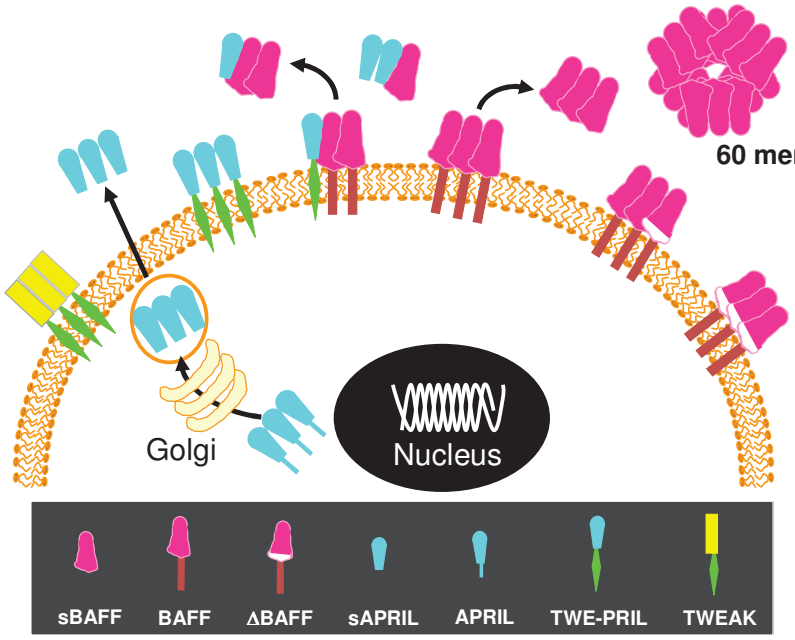


Figure 1 **Different forms of BAFF.**
BAFF offers a bunch of variants: membrane-bound or soluble, monomer or trimers, homotrimers or heterotrimers, herotrimers with APRIL or heterotrimers with TWEAK, or even virus-like aggregates of 60 monomers.

1.1. BAFF forms: from the gene to the protein

1.1.1. Genetics of BAFF

1.1.1.1. BAFF gene an overview

In humans, the BAFF gene is mapped on the chromosome 13 in the q33.3 region. It contains 6 exons and 5 introns corresponding to 39 Kilobase (kb) (Schneider, MacKay et al. 1999) (Figure 2A). The main *BAFF* transcript encoded by the gene contains 1204 base pairs (bp) with an open reading frame (ORF) of 858 bp (Genbank acession number is NM_006573).

In mice, Baff gene is mapped on chromose 8 A1.1 (in contig AC 138397.4.1.246.976) and contains 7 exons and 6 introns corresponding to 31 kb (Figure 2B).

The main *Baff* transcript encoded by this gene contains 1710 bp with an ORF of 930 bp (Genbank accession number is NM_033622).

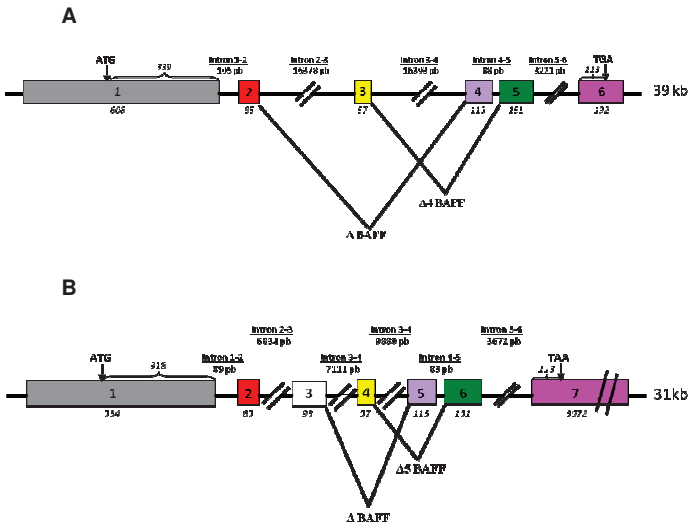
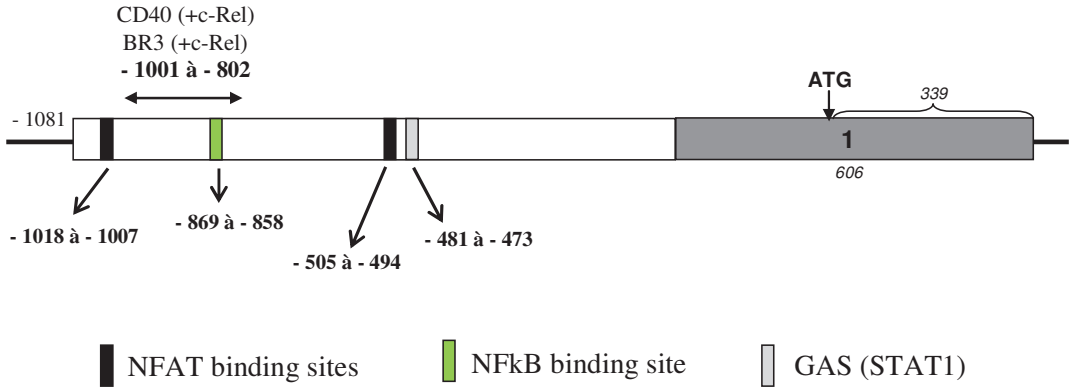


Figure 2 **BAFF genes organization in human (A) and mice (B).**
Exons are represented as boxes and colors are conserved between human and mouse in accordance with exon homology. The size of each exon is indicated below it and the size of each intron are also indicated (thin line). Alternative splicing events are shown and the name of corresponding transcripts is indicated. Bp: base pairs; kb: kilo base (Annexe 1: Lahiri, Pochard et al., 2012).

1.1.1.2. Promoter and transcription factors for BAFF

The promoter of 1020 bp (GenBank accession number AY129225) can be activated by many transcription factors (Figure 3). NFAT (nuclear factor of activated T cells) members (c1 and c2) bind between position -1018 to -1007 and position -505 to -494 on the

BAFF promoter. NF-κB members (p50, p52, c-Rel and to a lesser extent p65) are also reported to bind at a NF-κB binding site (-869 to -858) on the BAFF promoter (Fu, Lin-Lee et al. 2006). Two other transcription factors for BAFF have been described. They belong to the TNF-receptor family and are CD40 and BR3 (BAFF-receptor 3) with a binding at position -1001 to -802 (Lin-Lee, Pham et al. 2006; Fu, Lin-Lee et al. 2009). CD40 and BR3 interact with c-Rel and form a complex on the promoter of BAFF to activate BAFF transcription (Zhou, Pham et al. 2007; Fu, Lin-Lee et al. 2009). In human intestinal epithelial cells (EC), IFN-γ can induce the production of both soluble and membrane-bound BAFF, by JAK/STAT activation pathway and binding of phosphorylated STAT-1 to the IFN-γ activated site (GAS) element at position -481 to -473 on the BAFF promoter (Woo, Im et al. 2013).



GAS: gamma-interferon activated site

Figure 3 Schematic representation of BAFF promoter (human and its transcription factor binding sites.

1.1.1.3. Polymorphism

Many single nucleotide polymorphisms of BAFF have been described and were found to be associated with diseases.

Polymorphism screening by Kawasaki et al. on human *BAFF*, detected four single nucleotide polymorphisms (SNPs) in the promoter, (-1283G→A, -871C→T, -514T→C and -353G→C), one SNP in intron 1 (IVSI-45 C→G), and one rare non-synonymous substitution in the coding region (Kawasaki, Tsuchiya et al. 2002). The -871C→T (rs9514828) SNP has been more extensively studied and was found to increase BAFF transcription in chronic lymphocytic leukemia (CLL) cells (Novak, Grote et al. 2006).

This SNP was associated with risks of non-Hodgkin lymphoma (Novak, Slager et al. 2009), T-cell lymphoma survival (Zhai, Tian et al. 2012), increased risks of chronic idiopathic thrombocytopenic purpura (Abdel-Hamid and Al-Lithy 2011), and primary Sjögren's syndrome (pSS) (Nossent, Lester et al. 2008). Recently, it was reported as exerting a positive influence on rituximab treatment in rheumatoid arthritis (RA) patients (Ruyssen-Witrand, Rouanet et al. 2013). A few more SNPs featuring haplotype block in the 5' regulatory region of the BAFF gene were investigated in Caucasian patients with pSS. Three other SNPs were also identified -2841T→C, -2704T→C, -2701T→A. Disease susceptibility for Ro/La-positive pSS is increased with the CTAT haplotype, but is not associated with the TTTT haplotype. While both haplotypes carry the -871T allele, this allele was not independently associated with disease susceptibility (Nossent, Lester et al. 2008). In RA patients, the TTTT haplotype is linked to the outcome of the rituximab treatment. Patients with this haplotype showed increased positive response in rituximab therapy after anti-TNF therapy had failed to improve the disease condition (Fabris, Quartuccio et al. 2013).

1.1.2. Variants of BAFF

1.1.2.1. Ψ BAFF

Ψ BAFF was identified in the human myeloid cell lines, but its sequencing revealed this transcript to be non-functional because of an incomplete splicing of the intronic sequences leading to the formation of premature stop codon (Gavin, Ait-Azzouzene et al. 2003).

1.1.2.2. ΔBAFF

Discovered in 2003, this isoform lacks the exon 3 of BAFF in humans and exon 4 in mice (Gavin, Ait-Azzouzene et al. 2003). It is co-expressed with BAFF transcript in many myeloid cells. The loss of 57 bp maintains the reading frame. In mice, the junction of exon 3 and 5 results in an additional N-linked glycosylation (N^{155}) (Figure 4). Mouse ΔBaff, which lacks the region between I^{156} and K^{184} and G^{185} is substituted wint an R. This part corresponds to the A-A1 loop, fails to bind to BAFF receptors TACI (transmembrane activator and calcium modulator ligand interactor) and BAFF-R (BAFF receptor), indicating that the skipping of exon 4 during splicing blocks the BAFF function (BAFF receptors have been developed in **1.4 BAFF receptors**). Furthermore, ΔBAFF physically associates with BAFF in disulfide-bounded heteromultimers and these mixed molecules bind poorly to

receptors compared to the homomultimers of BAFF (Gavin, Ait-Azzouzene et al. 2003). The co-expression of BAFF and ΔBAFF on the same cell shows a decrease in the secretion and the cleavage of BAFF on the cell surface (Gavin, Ait-Azzouzene et al. 2003). Thus, ΔBAFF suppresses the BAFF function by competitive co-association, limiting BAFF homotrimerization and BAFF release in the process. The analysis of ΔBAFF transgenic mice reveals that ΔBAFF and BAFF have opposing effects on B cell survival (Gavin, Duong et al. 2005). ΔBAFF transgenic mice have reduced numbers of B cells and T cell-dependent antibody (Ab) responses, but normal pre-immune serum immunoglobulin levels. These normal immunoglobulin levels can be ascribed to either one of these two possibilities: either T cells stimulate BAFF expression in antigen presenting cells (APC) or BAFF acts directly on T cells to affect the immune response (Ng, Sutherland et al. 2004; Ye, Wang et al. 2004). Identical results were obtained in 3H9 mice, in which B cells recognized DNA and chromatin when they expressed some endogenous L chains, by introducing transgenes expressing either BAFF or ΔBAFF (Ota, Duong et al. 2010). Consequently, ΔBAFF seems to play a regulatory role in BAFF production. This production governs the balance between the survival of B cells and the regulation of the immune tolerance threshold. However, in humans, although the Δ*BAFF* transcript was found in some tissues (Krumbholz, Theil et al. 2005), its corresponding protein has not yet been detected.

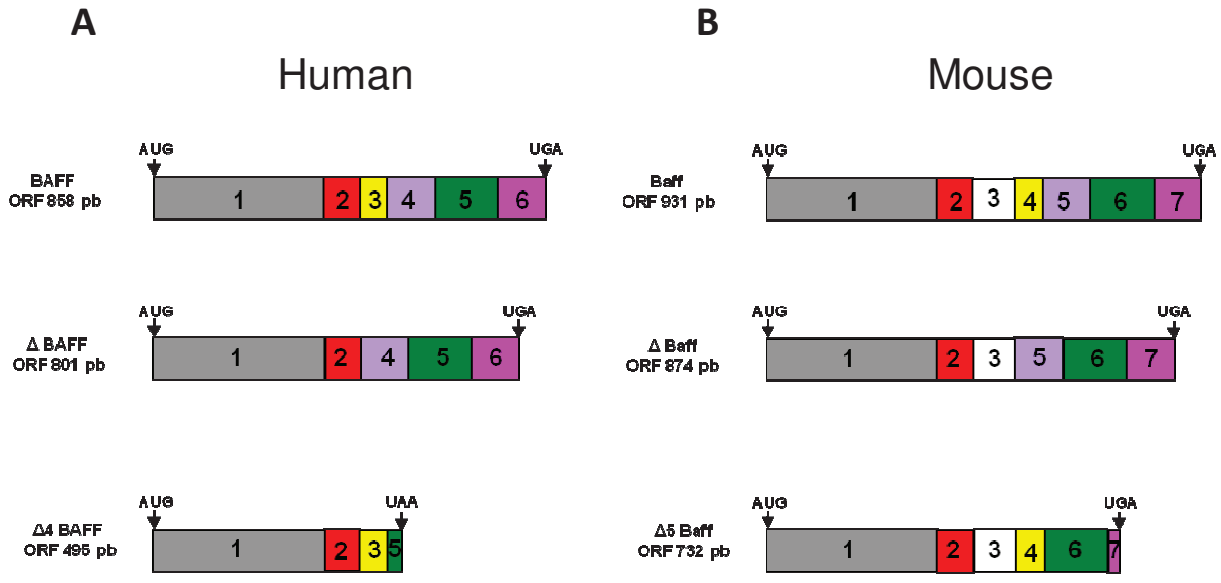


Figure 4 **Schematic representation of *BAFF* and *Baff* transcripts.**
(A)- BAFF, ΔBAFF and Δ4BAFF in human. (B)- Baff, ΔBaff and Δ5Baff in mice. The number of base pairs (bp) in the open reading frame is indicated next to each transcript (Corrected from annexe 1 : Lahiri, Pochard et al., 2012)

1.1.2.3. Δ4BAFF

1.1.2.3.1. Genetics

As previously described BAFF has many variants. We recently identified another functional variant of the BAFF gene in humans and mice (Appendix 2). This new variant lacks exon 4 in humans and exon 5 in mice (which is almost 90% similar to exon 4 in humans). It has a ORF of 495 bp in humans because it lacks 113 bp (encoding the predicted exon 4) hence its designation: Δ4BAFF. Whereas the part between exon 1 and exon 3 is intact, exon 5 starts directly after exon 3 (exon 3's last nucleotide at position 748 is linked to the first base at position 862, within exon 5). During splicing, a new in-frame stop codon (TGA) is generated. In Balb/c and Swiss mice splenocytes the same observation was made, i.e. exon 5 is spliced out. In mice, the splicing process generates a stop codon further in exon 7 resulting in an ORF of 732 nucleotides (Figure 4). In humans, the sites for the N-glycosylation (N124) and furin cleavage are maintained in this variant.

1.1.2.3.2. Protein localization and expression

The protein is localized in the organelles, especially the endoplasmic reticulum. It can also be found on the nuclear membrane and in the nucleus of Δ4BAFF-transfected B cells. Transfection with the unglycosylated $\Delta 4^{[N124 \rightarrow D]}$BAFF leads to a similar localization pattern but, in that case, the protein is no longer present in the nucleus.

1.1.2.3.3. Splicing regulation of Δ4BAFF

Δ4BAFF is induced after INF-γ stimulation. INF-γ stimulation modifies the functions of SR protein SC35 that binds to the positive regulatory motif known as Exonic Splicing Enhancers (ESEs) (Cartegni, Chew et al. 2002) and Intronic Splicing Enhancers (ISEs). We also reported that IFN-γ stimulation was responsible for an increased nuclear expression of heterologous nuclear ribonucleoprotein hnRNP C1/C2 that binds to negative splicing regulatory motifs known as Exonic Splicing Silencers (ESSs) and Intronic Splicing Silencers (ISSs) and consequently favors exon 4 skipping.

One of the objects of our thesis will be to understand the role of this new alternative spliced isoform of BAFF.

1.1.3. BAFF protein

1.1.3.1. Introduction

BAFF is a type II membrane protein (31.2 kDa) that is released as a soluble protein (17.2 kDa) after proteolytic cleavage by furin in the membrane proximal stalk region. Soluble BAFF acts as a cytokine through the regulation of the survival of B cells. BAFF protein can form trimers or 60–mers (virus-like protein structures formed by the association of 20 trimers in basic conditions); depending on the pH. The 60-mers structure is biologically active but its proper function is still unclear. One of the most interesting aspects of the BAFF protein lies in the fact that its expression varies according to its forms. For instance, Shu et al. found in the U937 cell line a BAFF expression at 52 kDa (Shu, Hu et al. 1999) because of the post–translational glycosylation. Tribouley et al. found the presence of full-length BAFF as a 45 kDa protein form (Tribouley, Wallroth et al. 1999), making it more complicated to detect in different autoimmune diseases.

1.1.3.2. Primary structure

Human and murine BAFF are type II transmembrane proteins that contain 285 amino acids (aa) (31.2 kDa) in humans and 309 aa (34.2 kDa) in mice (Figure 5). Some domains are conserved between the two of them:

the transmembrane domain, encoded by exon 1, from L^{47} to Y^{67} for human BAFF and from L^{48} to Y^{68} for mouse;

the cleavage site, encoded by exon 2, after R^{133} for human and after R^{126} for mouse;

the C-terminal domain, encoded by the last 4 exons (3, 4, 5, 6 for human and 4, 5, 6, 7 for mouse), called the TNF Homology Domain (THD) and mainly involved in the organization of the secondary and tertiary structures.

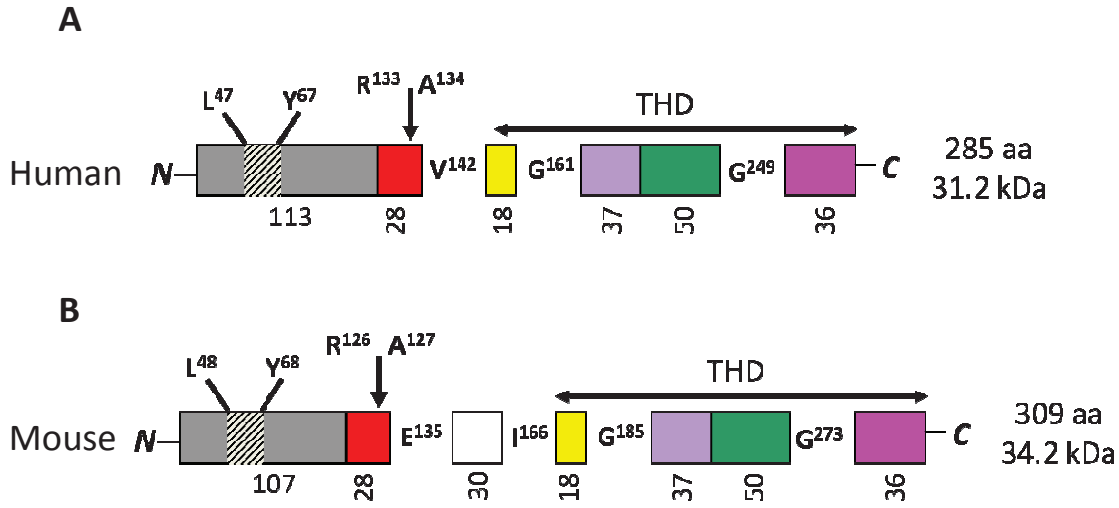


Figure 5 **Schematic representation of BAFF protein in humans (A) and mice (B).**

Color of boxes correspond to exons of genes and transcripts described in figure 1 and figure 3 respectively. When an amino-acid (aa) is encoded by the juntion of 2 exons, its position in the region sequence is indicated (for example: Valine in position 142 in human BAFF is V142). The hatched area represents the transmembrane region. The vertical arrow shows the cleavage site for furin and the horizontal arrow represents the TNF homology domain (THD). The number of aa and predicted molecular weight in kilo Daltons (kDa) for each protein are also indicated (from annexe 1: Lahiri, Pochard et al., 2012).

BAFF is expressed on the membrane and can be cleaved by a furin-like enzyme that belongs to the protein convertase family. This family consensus sequence for cleavage is: $^{R}/_{K}X_{n}R^{\downarrow}$ (n = 0, 2, 4 or 6 and X = any aa but never C and rarely P). The consensus sequence of cleavage for furin is: n = 2 within $RX^{K}/_{R}R\downarrow$. The sequence is $RNKR^{133}$ for human BAFF and $RNRR^{126}$ for mouse (Schneider, MacKay et al. 1999; Gavin, Ait-Azzouzene et al. 2003). Soluble BAFF contains 152 aa (from A^{134} to L^{285}) (17.2 kDa) in humans and 183 aa (from A^{127} to L^{309}) (20.6 kDa) in mice. In humans, the THD is the soluble BAFF. This domain is highly conserved during species evolution because the human soluble BAFF shares a strong homology with porcine BAFF (Guan, Dan et al. 2007), dove BAFF (Lu, Cao et al. 2009), duck BAFF (Guan, Ye et al. 2007) and chicken BAFF (Schneider, Kothlow et al. 2004). The main aa implicated in some receptor interactions are conserved across all species. As a result all these soluble BAFF can stimulate human B cells.

1.1.3.3. Secondary and tertiary structure

The structure of the human soluble BAFF consists of two layered antiparallel β strands that form a typical jellyroll-like β sandwich, like other members of the TNF ligand family (Liu, Xu et al. 2002; Oren, Li et al. 2002). This structure contains 12 strands called: A

(aa146-151), A''' (aa158-160), A'' (aa 163-165), A' (aa 168-174), B' (aa 178-181), B (184-187), C (aa191-201), D (aa 208-215), E (aa 226-235), F (aa 245-253), G (aa 258-262) and H (aa 270-283) that are organized in two β sheets (Karpusas, Cachero et al. 2002; Liu, Xu et al. 2002) (Figure 6). The first anti-parallel sheet forming the jellyroll comprises strands A' , A, H, C and F and the second, strands B', B, G, D and E. Homologies between BAFF and some members of the TNF-L family (TNF-β, TRAIL) occur in β strands C, D, F, G and H which constitute the core of the jellyroll fold, whereas the loop regions AA', CD, DE, EF and GH are the most divergent regions among the TNF family ligands (Liu, Xu et al. 2002; Oren, Li et al. 2002). Soluble BAFF presents characteristic of other TNF-ligand family members:

its structure in loops CD and EF;

the AA' loop has an insertion of two short β strands, A''' and A'', that form a hairpin motif called the "Elbow region". The AA' loop, contains a large insert between strands D and E: the "FLAP region" which is unique to soluble BAFF.

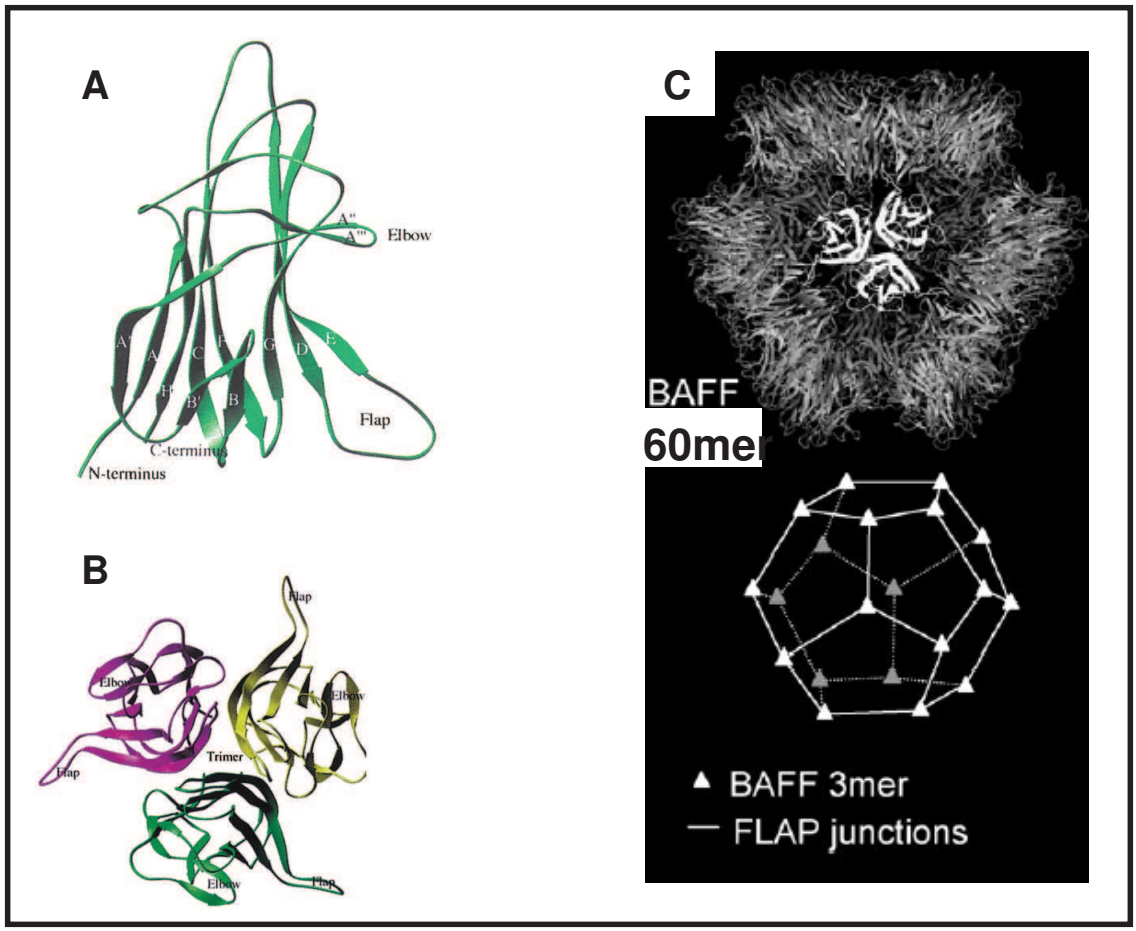


Figure 6 **Secondary, tertiary and quaternary structure of BAFF.**
(A)- Monomer of BAFF with the 12 strands see text for details). (B)- BAFF trimer. (C)- BAFF 60-mers (Bossen and Schneider, 2006; Liu, Xu et al., 2002).

A feature of these three-dimensional structures is common between BAFF, APRIL and TWEAK: a disulfide bridge between C^{232} on strand E and C^{245} on strand F (Bodmer, Meier et al. 2000).

1.1.3.4. *Quaternary* structure

Like all TNF-ligands (Smith and Baglioni 1987), the biological form of BAFF is a trimer. The interface that forms this trimer mainly consists of layered aromatic residues including the F^{194}, Y^{196} and Y^{246} monomers. Two hydrophobic interactions are involved in this BAFF-trimer formation, mediated on one hand by the Q^{144} from each monomer (hydrogen bond) and on the other hand by the three last residues $L^{282,284,285}$ from the C-terminus of three monomers (Liu, Xu et al. 2002). The unique FLAP region (DE loop) of BAFF allows trimer-to-trimer interaction leading to a virus-like assembly of the soluble trimers (Liu, Xu et al. 2002). This structure contains 20 trimers associated with each other by hydrogen bonds and hydrophobic bonds involving 4 residues: Y^{192}, K^{252}, E^{254} and H^{218}. H^{218} from the FLAP region seems decisive for the formation of oligomers. Indeed, when histidine in position 218 is replaced by an alanine, BAFF cannot oligomerize at pH 7 (Cachero, Schwartz et al. 2006). At pH 6.0, BAFF exists only in a trimeric form. At pH 6.5, the ratio oligomers/trimers is 1:2 and 1:1 at pH 7.0. At pH 7.4, only the oligomeric form is present. The oligomeric form can also induce the proliferation of B cells *in vitro* with the same efficiency as the trimeric forms. These structural forms were detected in the supernatants of several cell lines, demonstrating their existence (Cachero, Schwartz et al. 2006). However, their physiological role has not yet been clearly demonstrated.

1.1.4. Glycosylation

BAFF has two potential sites for N-glycosylation formed by two asparagines at position 124 and 242. According to Schneider et al., the complete form of BAFF is N-glycosylated on N^{124} but not on N^{242} (Schneider, MacKay et al. 1999). After treatment with N-glycanase F, the molecular weight of BAFF decreases. The absence of glycosylation on N^{242} could be due to the secondary structure of the protein because this residue is present at the beginning of the strand F. The cleaved form of the soluble BAFF should not be N-glycosylated because the cleavage site is downstream N^{124}. However, another team showed that the cleaved form of BAFF was glycosylated on N^{242} after expression of this soluble form in Pichia pastoris (Diao, Ye et al. 2007). The molecular weight of soluble BAFF was then found to have increased from 17 kDa to 20 kDa.

1.2. BAFF-producing cells

BAFF is mainly expressed by mononuclear cells from blood, spleen and lymph nodes, although low expression exists in the placenta, the thymus and the heart (Moore, Belvedere et al. 1999; Mukhopadhyay, Ni et al. 1999; Schneider, MacKay et al. 1999; Shu, Hu et al. 1999; Alsaleh, Messer et al. 2007; Langat, Wheaton et al. 2008).

The main innate immune cells that express BAFF are monocytes, macrophages, neutrophils and follicular dendritic cells (DC) (Hase, Kanno et al. 2004). Activated T and B cells also produce BAFF (Huard, Arlettaz et al. 2004; Kern, Cornuel et al. 2004; Daridon, Devauchelle et al. 2007).

Other cell types also express BAFF (Mackay, Silveira et al. 2007): stromal cells from the bone marrow (Schaumann, Tuischer et al. 2007), (Ohata, Zvaifler et al. 2005), astrocytes (Krumbholz, Theil et al. 2005), and EC (Daridon, Pers et al. 2006). BAFF was also found in the synovium of patients with rheumatoid arthritis (RA) (Rochas, Hillion et al. 2009).

1.3. BAFF-induced production

Myeloid cells such as monocytes, macrophages and monocyte-derived DC release BAFF after IFN-γ, IFN-α and CD40 ligand (CD40L) stimulations (Litinskiy, Nardelli et al. 2002). In these cells, membrane-bound BAFF can be cleaved by a furin convertase to produce a soluble form (Nardelli, Belvedere et al. 2001). However, neutrophils do not express BAFF on their surface and show a special mechanism for BAFF secretion upon stimulation with G-CSF (granulocyte-colony stimulating factor) and IFN-γ (Scapini, Nardelli et al. 2003). So, whereas BAFF is cleaved at the membrane in other cell types of cells; in neutrophils, it is cleaved intracellularly. Additionally, macrophages, DC and neutrophils synthesize BAFF after IFN-γ and lypopolysaccharide (LPS) stimulation through the production of reactive oxygens (Moon, Lee et al. 2006). On the contrary, IL-4 inhibits the expression of BAFF in monocytes (Nardelli, Belvedere et al. 2001; Scapini, Nardelli et al. 2003). TGF-β upregulates BAFF expression by macrophages. In mouse macrophages, TGF-β has been shown to increase BAFF expression through the TGF-β signalling pathway where Smad3 and Smad4 promoted BAFF promoter activity. In the same study, IFN-γ stimulation further increased TGF-β-induced BAFF expression through the phosphorylation of CREB, and involved the PKA/CREB pathway in the IFN-γ induced BAFF expression (Kim, Jeon et

al. 2008).

In human intestinal EC, IFN-γ induces the production of both soluble and membrane bound BAFF through the JAK/STAT signalling pathway and through the binding of phosphorylated STAT-1 to the BAFF promoter (Woo, Im et al. 2013). SOCS3 plays an important role in the induction of BAFF by gut EC because of the IFN-γ stimulation. Upregulation of SOCS3 blocks the JAK/STAT pathway signalling which in turn suppresses the production of BAFF by IFN-γ stimulation (Do, Choi et al. 2013).

CD40L and anti-IgM stimulations induce BAFF expression in normal human B cells by activating both the NF-kB and the NFAT binding to the BAFF promoter (Fu, Lin-Lee et al. 2006).

1.4. BAFF Receptors

1.4.1. BAFF-R

Discovered in 2001, the BAFF receptor (BAFF-R, TNFRSF13C, or BR3) is specific to BAFF. BAFF-R is expressed in spleen, lymph nodes, peripheral blood leukocytes and thymus. BAFF-R is differentially expressed during B cell ontogeny (Figure 7).

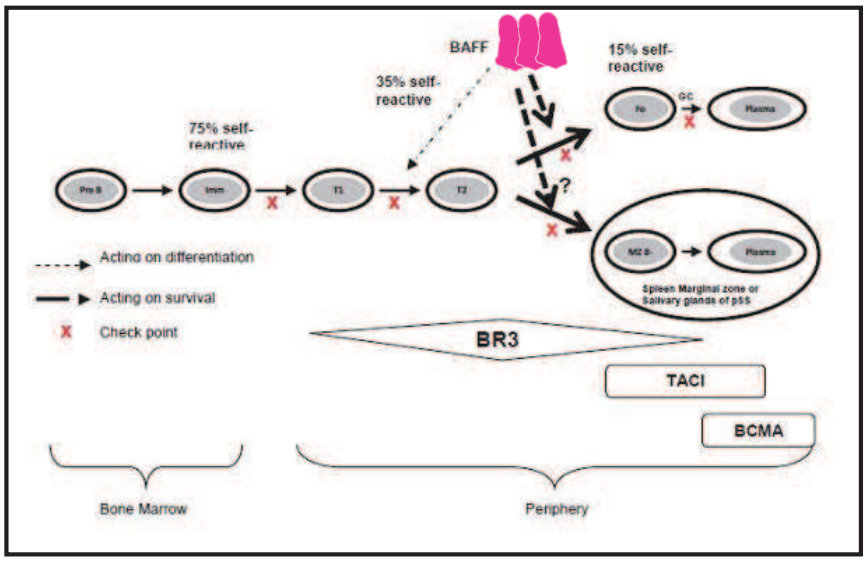


Figure 7 **BAFF receptor expression (BR3, TACI and BCMA) and self-tolerance during B cell ontogenesis.**

Data indicate the proportion of self-reactive B cells at specific B-cell stages bafore or after check points as determined in the anti-HEL/HEL transgenic mouse model. Fo: follicular; GC: germinal center; Imm; immature; MZ: marginal zone; Pre: precursor; T1 or 2: transitionnal type 1 or 2; SS: Sjögren's syndrome and Plasma: plasma cell.

The BAFF-R gene is located on the 22q13.1 chromosome. It is a type III transmembrane protein, in which exon 1 is the ligand-binding domain; exon 2, the transmembrane; and exon 3, the intracellular domain. It is the smallest cysteine-rich domain protein, containing only one Cys-rich ligand binding domain (Thompson, Bixler et al. 2001; Yan, Brady et al. 2001). The murine BAFF-R protein and its human counterpart are about 56% homologous. The BAFF-R protein is present in the cytoplasm, the plasma membrane and the nucleus of B cells (Thompson, Bixler et al. 2001).

In mature B cells, pro-survival signalling is mediated by BAFF-R stimulation then NF-κB signalling pathway activation. In response to various cellular stimuli, the cells activate a series of genes that play a role in the inflammatory and immune responses; under the control of the NF-κB transcription factor. The activation of this regulatory cascade is modulated by the IκB kinase (IKK) complex. The IKK complex contains two kinases and a regulatory subunit NEMO (NF-kB Essential Modulator) which is essential for the kinase activation. The classical activation requires NEMO, whereas the noncanonical pathway is NEMO-independent. The binding of BAFF and BAFF-R results in the activation of both the classic and the noncanonical NF-κB signalling pathways (Figure 8). However, the activation of the alternate NF-κB pathway (which results from the processing of NF-kB2 and the nuclear translocation of p52/Rel B heterodimers) is a major outcome of BAFF-R stimulation (Kayagaki, Yan et al. 2002). The importance of the alternate pathway, downstream of BAFF-R, has been demonstrated *in vivo*. Mice deficient for p52 show a reduction in mature B cells. Bone marrow-derived p52-deficient transitional T1 B cells respond but weakly to BAFF (Claudio, Brown et al. 2002). The identity of the relevant NF-κB regulated genes expressed in response to BAFF-R signalling remains unclear. BAFF induces the upregulation of anti-apoptotic Bcl-2 members such as Bcl-xL or Bcl-2, allowing cells to survive. Bcl-xL (but not Bcl-2) is a well-documented target of the classical NF-κB pathway (Chen, Edelstein et al. 2000). Bcl-2 may be a target of the alternate pathway that is induced by BAFF in B cells. BAFF-R can activate the classical part of the NF-κB pathway through Btk and phospholipase C-γ2 and is able to enhance the signalling pathway (NF-κB1) *via* the phosphorylation of IKKβ and the degradation of IκBα. BAFF-R can also signal through the activation of Akt or through 4E-BP1 *via* Pim-2. BAFF-R can transcriptionally regulate the proliferation of B cells by manipulating the functions of NF-κB/cRel and NF-κB-targeted promoters including BAFF (Fu, Lin-Lee et al. 2009).

BAFF-R also interacts with the TNFR-associated factor (TRAF)-3 and with TRAF-6 in B cells. TRAF-3 is able to negatively regulate the function of BAFF-R by inhibiting BAFF-R mediated NF-κB activation and IL-10 production (Xu and Shu 2002; Hildebrand, Luo et al. 2010). Specific gene deletion of this receptor results in the reduction of transitional and mature B cells in the spleen. In a recent study, BAFF-R deficiency in mice was shown to reduce the development of atherosclerosis by altering the mature B2 cell-dependent immune response (Sage, Tsiantoulas et al. 2012). In another report, Hildebrand et al. showed a subset of patients with non-Hodgkin lymphoma whose BAFF-R gene tail, near the TRAF-3 binding motif, had mutated. This resulted is increased NF-κB1 and NF-κB2 signalling and enhanced production of IgM (Hildebrand, Luo et al. 2010). Warnatz et al. showed that in different Common variable immunodeficiency (CVID) patient, their siblings had mutation in TNFRSF13C. This mutation is due to a homozygous deletion in the TNFRSF13C gene that removes part of the BAFF-R transmembrane domain. These patients showed almost all of the symptoms of CVID patients, including reduced IgG and IgM levels in serum, but showed normal IgA levels (Warnatz, Salzer et al. 2009).

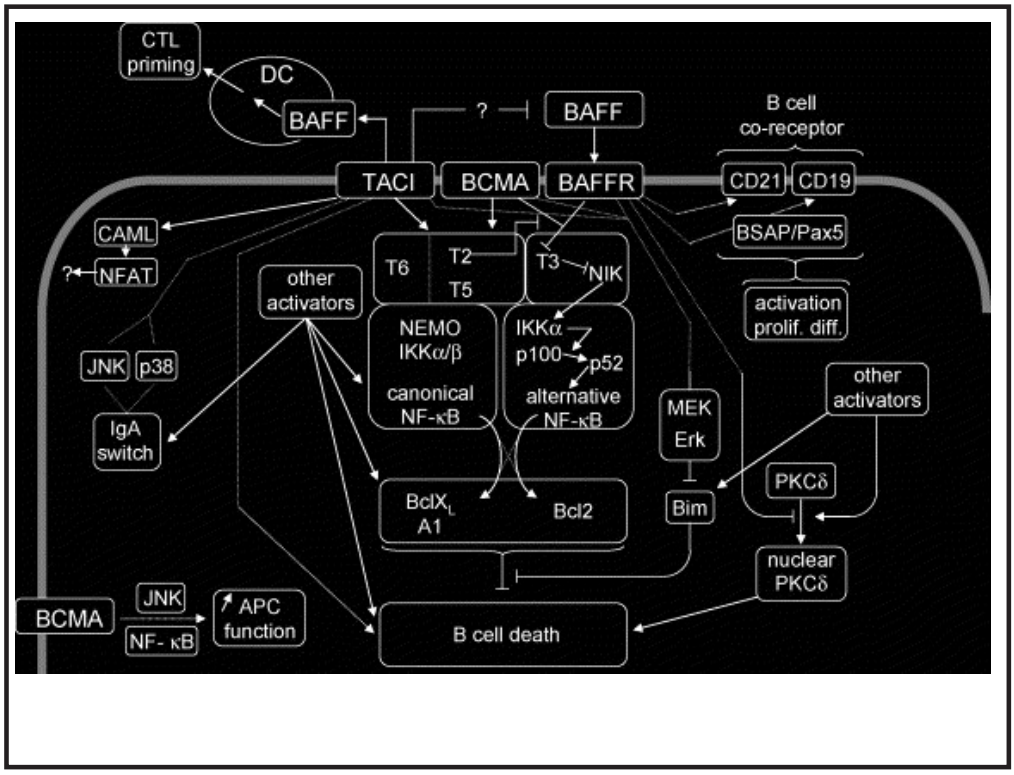


Figure 8 **Signalling pathway through BAFF receptors.**
Molecular events downstream the three BAFF receptors BAFF-R, TACI and BCMA are summarized here (Bossen and Schneider, 2006).

1.4.2. TACI

TACI (transmembrane activator and calcium modulator ligand interactor) is another receptor for BAFF and APRIL that can also bind heparin sulfate proteoglycan syndecan-2 (Bischof, Elsawa et al. 2006). The human TACI gene has 5 exons (Hymowitz, Patel et al. 2005). In humans, this gene can arrange itself into a short form of TACI by skipping exon 2. This variant, just like its normal form, is able to bind BAFF and APRIL (Bossen and Schneider 2006). TACI is a type III transmembrane protein. Conflicting results have been reported regarding the expression of TACI on T cells (Ng, Sutherland et al. 2004; Bossen and Schneider 2006; Mackay and Leung 2006). This receptor is also expressed in mature and transitional B cells, but also in human macrophages and plasma cells (Chang, Arendt et al. 2006). Membrane-bound and oligomeric forms of BAFF can bind to this receptor (Bossen and Schneider 2006). While BAFF-R is able to bind any form of BAFF, TACI cannot bind to soluble BAFF trimers (Bossen, Cachero et al. 2008).

TACI-deficient mice show deficiencies in B cell homeostasis with increased levels of hyper-reactive B cells and an increased splenic B cell component that lead to autoimmunity and lymphoma (Mackay and Schneider 2008). The requisiteness of TACI is obvious for T cell-independent type II humoral immunity as TACI deficient mice do not mount a normal T cell-independent type 2 immune response (von Bulow, van Deursen et al. 2001). TACI can interact with TRAF 2,3,5 and 6; and is able to activate the classic part of the NF-κB signalling pathway and the AP-1, NF-AT transcription factors (Figure 8) (Xia, Treanor et al. 2000). Phosphorylation of the NF-κB inhibitor by the IKK complex allows the cytoplasmic NF-κB proteins p65 and p50 to translocate to the nucleus when their nuclear localization sequence (NLS) is exposed. NF-κB proteins bind to a specific consensus sequence in the DNA and subsequently activate downstream genes.

Studies on TACI knock-out mice lead one to posit that TACI can inhibit/suppress B cell proliferation and activation in normal B cells and maintain immunological homeostasis. These $TACI^{-/-}$ mice develop splenomegaly and accumulate B cells. B cells collected from these mice hyper-proliferate in response to various stimuli. These mice also show increased concentrations of serum Igs and, in response to antigen challenge, increased antibodies. This accumulation of B cells and the hyper-responsiveness of B cells to antigenic challenge, both *in vitro* and *in vivo*, support the fact that TACI plays a critical role in the downregulation of B cell activation and the maintenance of immunological

homeostasis (Yan, Wang et al. 2001; Bossen and Schneider 2006).

TACI is also involved in plasma cell differentiation and survival in response to T-independent type 2 antigens (Mantchev, Cortesao et al. 2007). A reduction in IgA serum leves was observed in TACI-deficient mice (von Bulow, van Deursen et al. 2001). When the mechanism of class-switching induced by TACI was investigated, it was found that the cytoplasmic domain of TACI encompasses a conserved motif that bound myeloid differentiation primary response gene 88 (MyD88), an adaptor protein that activates the NF-κB signalling pathways via a Toll-interleukin-1 receptor (TIR) domain. It was also shown that BAFF and APRIL promoted the recruitment of MyD88 to said conserved cytoplasmic motif of TACI which is distinct from the TIR domain of toll-like receptors (TLRs). Although TACI lacks a TIR domain, it triggers class-switch recombination (CSR) *via* the activation-induced deaminase (AID) (Bing, Siyi et al. 2010). There might be some relation between different TLRs and TACI signalling (TLRs signalling will be developed in **3. Toll-like receptors**). The activation of TLR4 by LPS and the activation of TLR7 and TLR9 by unmethylated CpG DNA can increase TACI expression in B cells (Mackay and Schneider 2008). In CVID patients, there exists a common mutation in the TACI gene (C104R). This mutation is responsible for inhibiting BAFF-binding to TACI (Bacchelli, Buckridge et al. 2007). In another report, the authors described two mutations (S144X and C104R) on the TNFRSF13B gene in CVID patients that result in TACI function loss as well as B cell dysfunction such as class-switching inability. A few other aa substitutions (A181E and R202H) were also reported but, unlike C104R mutation, BAFF ligand-binding was unaffected (Castigli, Wilson et al. 2005; Salzer and Grimbacher 2005).

1.4.3. BCMA

The B cell maturation antigen (BCMA), a type III membrane protein, also binds BAFF and APRIL. This BAFF receptor shares a similar structure with BAFF-R. BCMA contains a short 18-aa sequence analog to BAFF-R and possesses a TRAF binding site (Figure 8). BCMA is located on the 16p13.1 chromosome and contains 3 exons. In 2008, Smirnova et al. discovered three new transcript variants for BCMA: one with partial deletion of exon 1, one with deletion of exon 2 and another one with a new cryptic exon in intron 2 (Smirnova, Andrade-Oliveira et al. 2008). The lack of exon 2 induces the generation of this protein's soluble form, which remains in the cytoplasm because there is no signal peptide. BCMA is highly expressed on long-lived plasma cells, plasmablasts (Avery, Kalled et al.

2003). It is likely that BCMA becomes important after the differentiation of activated B cells because it is expressed by plasmablasts (Avery, Kalled et al. 2003; O'Connor, Raman et al. 2004; Tangye, Bryant et al. 2006).

If we compare the affinities of BCMA to BAFF and APRIL, we notice that BCMA has a lesser affinity with BAFF than it has with APRIL. The binding of BAFF to this receptor activates NF-κB which increases Bcl-2 expression and inhibits the apoptosis. BCMA regulates the expression of CD80, CD86, CD40, MHC-II and ICAM-1 and activates antigen presentation in B cells through a NF-κB dependent manner (Yang, Hase et al. 2005). BCMA can also activate the c-Jun N-terminal kinases (JNK) pathway to induce antigen presentation. Indeed, blocking the JNK pathway inhibits the antigen presentation through BCMA signalling (Bossen and Schneider 2006);(Yang, Hase et al. 2005). BAFF influences the normal development of B cells independently of BCMA. BCMA is not implicated in the survival of B cells until they reached the immature transitional stage. Indeed, BCMA-deficient mice show normal B cell counts just like their wild counterpart (Schiemann, Gommerman et al. 2001). However, BCMA was found to be implicated in the last stage of B cell differentiation and is important for the survival of bone marrow plasma cells, and the survival of plasmablasts (Avery, Kalled et al. 2003; O'Connor, Raman et al. 2004). In B cells of systemic lupus erythematosus (SLE) patients, particularly on memory cells and plasmablasts, the expression of BCMA is significantly higher than control. This increase in BCMA expression is co-related with enhanced CD19 and CD86 expression indicating an activated B cell state (Kim, Gross et al. 2011).

Thus, we can conclude that the expression of BCMA, TACI, and BAFF-R in various cell lines is highly variable and the differential extent to which each one of them co-ordinates with one another in maintaining B cell function is crucial for the immune regulation.

1.5. Functions of BAFF

1.5.1. B cells

BAFF was described as playing different roles in the homeostasis and the activation of B cells through its binding to its different receptors. BAFF-R, TACI and BCMA present different expression pattern during B cell ontongeny (Figure 7). BAFF-deficient mice, or mice in which BAFF activity has been reduced, demonstrate a marked reduction in

the number of peripheral B cells and an abnormal reduction in serum immunoglobulin. Fifty to 75% of the generated B cells in the bone marrow are self-reactive. So, in order to avoid the generation of pathogenic auto-Abs, self-reactive B cells have to be deleted or anergized during successive checkpoints throughout the B-cell development (Gauld, Benschop et al. 2005). In BAFF-deficient models, B cells are stopped at the transitional type 1 (T1) stage, though the release of B cells from the bone marrow remains normal. BAFF is essential for the survival of transitional type 2 (T2) cells that express high levels of BAFF-R. In fact, T2 B cells are mostly dependent on BAFF for their pro-survival activity and BAFF is necessary to prevent T2 apoptosis. This was demonstrated in models where BAFF or BAFF-R are lacking, and where the maturation of MZ or follicular zone B cells is impaired beyond the T1 stage (Schiemann, Gommerman et al. 2001; Mackay, Schneider et al. 2003). In transgenic mice, in which BAFF activity is blocked, the maturation of B cells is stopped between the T1 and T2 stages (Batten, Groom et al. 2000). In addition to a functional B cell receptor (BCR), immature B cells need a BAFF-mediated signal to survive and to become mature. As a result, BAFF-deficient models are unable to provide a humoral antibody response because these models show substantial reduction in mature and MZ B cells and in follicular B cells. Gain-of-function experiments confirm BAFF's ability to promote B cell survival by providing protection against apoptotic signals. Mice harboring BAFF as a transgene, show increased numbers of peripheral blood B cells, along with a spleen and lymph nodes that are greatly enlarged and Payer's patches with increased B cell numbers (Mackay, Woodcock et al. 1999; Gross, Johnston et al. 2000; Khare, Sarosi et al. 2000). Similar results are also obtained when mice are treated with recombinant BAFF (Parry, Riccobene et al. 2001) and in *ex-vivo* B cell cultures (Batten, Groom et al. 2000).

Deletion of BAFF results in the loss of 90% of the mature B cells. B-cell survival by BAFF depends on the NF-κB-mediated upregulation of the anti-apoptotic Bcl-2 family proteins and downregulation of anti-apoptotic proteins. In addition, the inhibition of the nuclear translocation of the pro-apoptotic protein kinase Cδ (PKCδ), appears to be an important mediator of the BAFF-induced survival of B cells (Mecklenbrauker, Kalled et al. 2004). BAFF activates TRAF-3 that is responsible for triggering the NF-κB activation through the induction of both canonical (NF-κB1) and non-canonical (NF-κB2), NF-κB pathways. BAFF-mediated NF-κB induction upregulates various anti-apoptotic proteins, including A1/Bfl-1, Bcl-xL, and Bcl-2, and downregulates the pro-apoptotic protein Bim (Claudio, Brown et al. 2002; Tardivel, Tinel et al. 2004; Zarnegar, He et al. 2004; Craxton,

Draves et al. 2005).

BAFF-R is the key receptor through which BAFF exerts its survival function. Mice with a naturally-occurring mutation on theBR3 gene, or BR3-deficient mice, show severe loss of peripheral B cells and decreased circulating Ig. These results are similar to those obtained with BAFF-deficient mice (Sasaki, Casola et al. 2004; Shulga-Morskaya, Dobles et al. 2004). In addition, neither TACI nor BCMA knock-out mice show impaired B cell survival (Yan, Wang et al. 2001). Moreover, mice that lack both BCMA and TACI possess a normal B cell compartment. This confirms the role of BAFF-R in BAFF-mediated B cell survival (Shulga-Morskaya, Dobles et al. 2004). Act1 is another signalling molecule known to be recruited by BR3 (Qian, Qin et al. 2004). It acts as a negative regulator of BAFF-mediated B cell survival. However, its mechanism of action remains unclear.

Furthermore, BAFF influences class-switching and the secretion of Abs. BAFF enhances B cell response through Pax-5 activation and the high ability of BCR to phosphorylate CD19 (BCR coreceptor) which in turn amplifies BCR signalling (Hase, Kanno et al. 2004). BAFF brings about the AID expression which is required for CSR (Litinskiy, Nardelli et al. 2002; Yamada, Zhang et al. 2005). BAFF produced by DC and macrophages induces switching to IgG, IgA, and IgE isotypes independently of CD40 (Litinskiy, Nardelli et al. 2002). In mice that receive excessive quantities of BAFF, either exogenously or as a transgene, we see an increase in the circulating levels of IgE, IgA, and all of the IgG sub-isotypes, and in IgM as well (Parry, Bouhana et al. 2000; Mackay and Schneider 2009). BAFF-deficient mice fail to develop a proper follicular dendritic cell network and instead build up smaller and unstable germinal centers (GC) in which class-switching and somatic hypermutation still occur, but with diminished IgG and secondary responses (Rahman, Rao et al. 2003).

BAFF controls the activation of the eukaryotic translation initiation factor 4E and induces the phosphorylation of the S6 ribosomal proteins required for translation. This suggests its role in protein synthesis. Indeed, BAFF elevates cell cycle progression proteins such as cyclin D and cyclin E, Cdk4, Mcm2 and 3 (Patke, Mecklenbrauker et al. 2006).

The T cell-independent type II response requires the interaction of BAFF 60-mers, or membrane BAFF, with TACI. The interaction of BAFF with its receptor BAFF-R occurs mainly to that end, since the action of blocking BAFF-R induces an impaired primary

immune response to T dependent antigens with decreased IgG levels. This interaction is also crucial for T cell-dependent IgM responses (von Bulow, van Deursen et al. 2001; Shulga-Morskaya, Dobles et al. 2004).

1.5.2. T cells

Conflicting reports exist regarding the presence of varied BAFF receptors in T cells. The presence of TACI on activated T cells has been demonstrated by some, whereas in other studies, no or minimal expression was found (von Bulow, Russell et al. 2000; Ng, Sutherland et al. 2004). The expression of BAFF-R on T cells is also controversial because according to various reports on activated $CD4^+$ T cells, either increased or decreased expression levels were found (Yan, Brady et al. 2001; Ng, Sutherland et al. 2004) or (Yan, Wang et al. 2001). In contrast, BCMA is not present on T cells. Thus, the role that BAFF plays on T cells is also controversial.

In vitro studies have shown that BAFF can co-stimulate human T cell activation and induce IL-2 secretion (Huard, Schneider et al. 2001). Moreover, higher numbers of activated T cells were detected in BAFF transgenic mice (Mackay, Woodcock et al. 1999). The exogenous stimulation of soluble BAFF increases the percentage of $CD4^+$ T cells in a dose-dependent manner but does not affect $CD8^+$ T cells (Shan, Chen et al. 2006). Both BAFF-transgenic mice $CD4^+$ and $CD8^+$ T cells are activated in the spleen and T cell numbers increase, with effector T cells in greater proportion (Mackay, Woodcock et al. 1999; Shan, Chen et al. 2006). This increases even further with the addition of IL-2 and IFN-γ. This suggests that these cytokines play the role of additive in the BAFF-stimulated proliferation of $CD4^+$ T cells. BAFF also acts as a T helper 1 (Th1) response-promoting cytokine because the stimulation with BAFF induces T cells to secrete more and also speeds up the differentiation into effector T cells. Inhibiting the interaction of BAFF-BAFF-R on T cells can reduce the proliferation of T cells. A defective BAFF-R in T cells is characterized by its inability to respond to BAFF mediated co-stimulation, which indicates that BAFF-R is a main receptor for BAFF, involved in the BAFF mediating stimulation on T cells. However TACI-deficient T cells respond normally to BAFF-mediated co-stimulation (Ng, Sutherland et al. 2004). BAFF stimulation, along with T cell receptor (TCR) engagement, upregulates the anti-apoptotic factor Bcl-2 in activated T cells which may indicate its role as a survival factor (Ng, Sutherland et al. 2004). BAFF increases CD25 on T cell (Ye, Wang et al. 2004).

1.5.3. Macrophage and Dendritic cells

BAFF expression is detected on the surface of human DC and macrophages along with its secretion as a soluble form into culture supernatants. IFNγ, IFNα, IL-10, CD40L, LPS, and peptidoglycan can induce BAFF in these models (Nardelli, Belvedere et al. 2001). Both cells found in the splenic MZ can produce and secrete BAFF (Balazs, Martin et al. 2002). However, the information regarding BAFF production by murine myeloid cell types is more limited and controversial. TLR agonists such as LPS and CpG oligodeoxynucleotides do not induce BAFF secretion by murine DC (Boule, Broughton et al. 2004). However, in another study, the LPS-induced surface expression of BAFF on DC could be observed (Diaz-de-Durana, Mantchev et al. 2006). BAFF induces DC activation and maturation. BAFF activates DC in order to secrete inflammatory cytokines like IL-6, IL-1β, TNF-α and to induce the proliferation of naïve $CD4^+$ T cell and their differentiation into effector CD4 T cells so as to modulate the immune response (Chang, Mihalcik et al. 2008). Unlike APRIL, BAFF secreted by these two cells is more effective to induce the costimulation for B cell proliferation through a BCR-dependent pathway (Craxton, Draves et al. 2005). BAFF from macrophages and DC regulates B cell function by enhancing the proliferation, the antibody secretion and by inducing Ig class-switching. In mice, BAFF affects the maturation of follicular DC and, as in BAFF-deficient mice, the mature follicular dendritic network is affected (Rahman, Rao et al. 2003). BAFF also causes dendritic cells to produce various inflammatory cytokines like IL-6, TNF-α. During this process, BAFF can induce the proliferation of naïve $CD4^+$ T cells and regulate the differentiation of $CD4^+$ T cells into $CD4^+$ Th1 cells in a DC-mediated manner. And finally, BAFF also elevates the activation and maturation of DC and plays an indirect role in modulating the adaptive immune system (Chang, Mihalcik et al. 2008).

1.6. BAFF as a therapeutic target

The importance of BAFF in the mouse model of autoimmunity, the implications of BAFF in the survival of lymphoma cells, and the increased BAFF levels in various diseases have propelled the role of BAFF as a therapeutic target. Several strategies have been developed to block BAFF. Selective inhibition of BAFF is achieved with either soluble BAFF-R or with antibodies to BAFF (Moisini and Davidson 2009).

Selective BAFF blockers prevent BAFF from interacting with its receptors, leaving APRIL free to interact with TACI and BCMA. A clinical program led by Human

Genome Sciences in partnership with GlaxoSmithKline has led to the development of a fully human BAFF-specific monoclonal antibody (belimumab; Lymphostat-B) (Baker, Edwards et al. 2003). A fusion protein consisting of human Ig Fc and of the extracellular BR3 domain (Briobacept, for BAFF-R-Ig) has also been developed. Non-selective BAFF blockers abolish the interactions of both BAFF and APRIL with all their receptors. To date, there is one drug in this class (Seshasayee, Valdez et al. 2003). It is based on human Ig Fc fused to the extracellular TACI domain (Atacicept, TACI-Ig). Atacicept has completed its phase I clinical trial and is now being tested in phase II and III clinical trials in the treatment of SLE, RA and relapsing multiple sclerosis. It yields good results with a good safety profile in the treatment of SLE and RA (Carbonatto, Yu et al. 2008). In a recent study, lupus nephritis patients treated with Atacicept showed an unexpected decrease in serum IgG levels, severe proteinuria and increased rates of infection. These results brought the trials to an end (Ginzler, Wax et al. 2012).

The various secreted form of BAFF, caused by translational modifications and heterogeneity, make it difficult to analyze their concentrations in patients. This has led to ongoing controversies because in some groups of patients the serum concentration of BAFF remains within normal range. Hence, monitoring the BAFF levels before and after treatment remains a thorny issue (Mariette, Roux et al. 2003; Collins, Gavin et al. 2006). Additionally, the glycosylation is also problematic because it seems to alter the epitope recognition by anti-BAFF Abs.

2. REGULATORY B CELL

2.1. History of regulatory B cells

In 1968, Morris et al, first suggested that the suppressive function of B cells was mainly restricted to their ability to produce ‘inhibitory’ Abs (Morris A et al 1968). This finding was then followed by reports linking B cell’s suppressive effect to the induction of tolerance and differentiation of suppressor T cells (L'Age-Stehr, Teichmann et al. 1980; Shimamura, Habu et al. 1984).

In experimental autoimmune encephalomyelitis (EAE) model, mice lacking mature B cells were unable to recover from the disease whereas normal mice could. The poor recovery potential of these B cell-deficient mice proves undeniably that B cells play a role in the suppression of EAE (Wolf, Dittel et al. 1996).

In 2000, Moulin et al. showed that B cells regulate the Th1/Th2 polarization. Indeed B cells promote the production of IL-4 by T cells. In µMT mice (lacking B cells) the production of Th1 cytokines, such as IFN-γ, was increased. B cells modify the antigen-presenting capacity of DC, including the Th1 differentiation through the production of IL-12. Also, IL-10 is involved in the inhibition of IL-12 secretion. In µMT mice, since there is less IL-10 production, an increase of IL-12 production by DC can be observed. This augmentation further regulates the production of IL-4 from T cells (Moulin, Andris et al. 2000). Fillatreau et al. showed in the EAE model that B cells play an important role in the protection against disease. In µMT mice, severe clinical signs were still present whereas B cell-sufficient mice had entered remission. The recovery from EAE needs IL-10 production by B cells that are activated in a T cell-dependent manner. Indeed, chimeric mice (reconstituted with 80% µMT bone marrow + 20% IL-$10^{-/-}$ bone marrow) do not recover from EAE (Fillatreau, Sweenie et al. 2002).

In 1997, Mizoguchi et al. studied the putative pathogenic role of B cells in the development of colitis. They observed an attenuation of colitis in TCR$\alpha^{-/-}$ µMT mice after the administration of purified immunoglobulin from TCR$\alpha^{-/-}$ mice. The amelioration observed in these mice was echoed by an increase in the clearance of apoptotic cells, suggesting an autoantibody-mediated protective mechanism (Mizoguchi, Mizoguchi et al. 1997). In 2002, they reported the presence of regulatory B (Breg) cells expressing CD1d and producing IL-10 that appear after a chronic intestinal inflammation and take part in the suppressive phase of the disease. Transferring Breg cells in diseased mice helps downregulate the inflammation (Mizoguchi, Mizoguchi et al. 2002).

2.2. Development and activation of regulatory B cells

B cells with regulatory properties have been identified in several autoimmune diseases, during inflammation, infection and upon different stimuli. It can be assumed that the development and activation of Breg cells in different models need different activation signals. According to the existing literature, many signals have been reported that induce regulatory properties in B cells and favor the production of IL-10. The main common factors regulating the development and activation of Breg cells include the stimulation through CD40, the engagement of BCR and the TLRs.

2.2.1. CD40 signalling

CD40 belongs to the TNF receptor superfamily found on the surface of B cells, DC, follicular DC and hematopoietic progenitor cells. The CD40–CD40L (CD40L or CD154) interaction is crucial for the development of T cell-dependent immune responses. In the model of chronic colitis, signs of the disease increase as B cells are treated with anti-CD40 blocking Ab. Blocking CD40 decreased B cell's ability to regulate the number of pathogenic $CD4^+$ $TCR\alpha^-$ β+ T cell in diseased mice. The need for CD40 signal is further established when B cells from $CD40^{-/-}$ mice also show decreased regulatory properties (Mizoguchi, Mizoguchi et al. 2000).

At the same time the role of CD40 on arthritis regulation has been evaluated in DBA/1-TCR-β transgenic mice. These mice develop chronic arthritis upon immunization with collagen type II (CII).Treatment of these mice with an agonist of CD40 results in a decrease of the symptom severity as compared with isotype treatment. The therapeutic effects of an anti-CD40 monoclonal Ab are correlated with reduced joint damage and intact bone architecture. Splenocytes of treated mice show an increased production of IL-10 and a decreased production of IFN-γ. All these observations support the notion of a protective role of CD40 in the model of arthritis (Mauri, Mars et al. 2000). B cells stimulated with agonistic CD40 and antigen produce more IL-10 and less IFN-γ, preventing the development of arthritis when transferred in CII-induced arthritis mice showing a B-cell mediated protection (Mauri, Gray et al. 2003). In Mrl/*lpr* mice, the transfer of *in vitro*-stimulated anti-CD40 Ab T2 B cells (T2-like-Bregs), significantly improve renal disease and survival through an IL-10-dependent mechanism (Blair, Chavez-Rueda et al. 2009).

B cells from B6 mice that had recovered from EAE produced IL-10 when stimulated with the autoantigen and anti-CD40 Ab. Bone marrow–chimeric mice in which CD40 deficiency was restricted to the B cell compartment failed to recover from EAE and suffered severe unremitting EAE (Fillatreau, Sweenie et al. 2002).

In humans, peripheral blood B cells expressing CD38 and CD24 can act as Breg cells and suppress the differentiation of Th1 cells. The mechanism involved CD40 stimulation through a pathway partially dependent on IL-10. In SLE patients, the same number of these B cells was observed but with an impaired IL-10 production leading to a defect in the regulatory properties. This absence of regulatory properties in SLE was also

correlated with an abnormal CD40 signalling pathway associated with lower levels of STAT-3 phosphorylation (Blair, Chavez-Rueda et al. 2009).

The role of the CD40-CD40L signalling on the induction of Breg cell properties was further evaluated by our group. Blocking this signalling pathway in the presence of T cells reduces the suppressive effect of B cells on anti-CD3 and anti-CD28 Ab-induced proliferation of T cells. This theory is further strengthened with the evidence that pre incubated B cells with human CD40L transfected murine fibroblast show more regulatory properties on the inhibition of T cell proliferation (Lemoine, Morva et al. 2011).

2.2.2. BCR engagement

Another important signalling pathway leading to the differentiation and activation of regulatory B cells is the engagement of the BCR. Signal transduction through the BCR is functionally interrelated to cell-surface receptors, such as CD19, CD21, CD22, CD40, CD72, and FcγRIIb. CD19 functions as a specialized adapter protein regulating the Src family protein tyrosine kinases, the phosphatidylinositol 3-kinase, and Vav. Thus, it acts as a key molecule for multiple signalling pathways that are crucial for modulating the basal and the BCR-induced signals (Tsubata 1999). Since CD19 is a co-receptor for the BCR signalling, it can be presumed that BCR signalling is one of the factors influencing the production of Breg cells (Watanabe, Fujimoto et al. 2007).

In CD19-deficient mice, T cell mediated inflammation is amplified whereas this inflammation is reduced in CD19 transgenic mice. A subset of $CD5^{+}$ $CD1d^{hi}$ B cells which possesses regulatory capacities reduces T cell-mediated inflammation in CD19 overexpressing mice through IL-10 production (Yanaba, Bouaziz et al. 2008).

BCR engagement also gives protection in the type 1 diabete model. Transfusion of BCR-stimulated B cells protects recipient non-obese diabetic (NOD) mice from type 1 diabete in an IL-10 dependent- manner. B cells produce more IL-10 when receiving a BCR activation signal. The transfer of BCR-stimulated B cells from IL-10 deficient NOD mice failed to confer any protection from type 1 diabetes in recipient NOD mice (Hussain and Delovitch 2007).

One of the effects of BCR stimulation is to increase intracellular Ca^{2+}. The sensor stromal molecule 1 and 2 (STIM1 and STIM2) molecules are essential for the

regulatory properties of B cells because the deletion of these two molecules on B cells increases EAE and decreases IL-10, involving NFAT activation. Although peak IL-10 production is found in B cells isolated from wild-type mice upon simultaneous activation with anti-CD40 and the auto-antigen myelin oligodendrocyte glycoprotein during the recovery phase of EAE, this response is lost in mice with STIM1 and STIM2 deficient B cells (Matsumoto, Fujii et al. 2011).

2.2.3. Toll-like receptors

TLRs are type I transmembrane glycoproteins composed of an extracellular transmembrane and an intracellular signalling domain (Gay and Gangloff 2007). Extracellular TLR domains have reiterated leucine-rich repeat modules bearing pathogen-associated molecular patterns able to recognize a wide range of microbial products. As such, they can alert the host about the presence of danger signals (Medzhitov 2001).

In NOD diabetic mice, B cells which are activated by LPS, a ligand for TLR4, secrete TGF-β. These activated cells can downregulate pathogenic Th1 immunity and confer a delayed onset of the disease (Tian, Zekzer et al. 2001).

The role of TLRs was evaluated in lupus prone Palmerston North (PN) for the initiation of Breg cell properties. Stimulation through TLR9 produces more IL-10 than control in these mice. TLR9-activated B cells downregulate the production of pro-inflammatory cytokines such as IL-12. B cells with regulatory properties display MZ-like B cell phenotypes and control the production of pro-inflammatory cytokines in an IL-10-dependent manner after activation by TLR9 (Brummel and Lenert 2005; Lenert, Brummel et al. 2005).

$CD5^+$ B cells play an important role in regulating inflammation. The absence of $CD5^+$ B cells is associated with the development of a stronger inflammatory response in neonatal mice that become lethally susceptible to CpG challenge. After TLR9 stimulation neonatal B cells effectively control the production of proinflammatory cytokines by neonatal plasmacytoïd and conventional DC, through the secretion of IL-10 (Zhang, Deriaud et al. 2007). Furthermore, during potentially harmful systemic inflammations, once neonatal B cells have been triggered by TLR-, they produce high concentrations of IL-10 and so on prevent optimal IL-12 secretion by neonatal DC, and thus Th1 priming. Although both $CD5^+$ and $CD5^-$ B cell subsets respond to CpG-ODN stimulation, only $CD5^+$ B cells produce IL-10

(Sun, Deriaud et al. 2005).

Another study by Lampropoulou et al. describes the role of TLR-activated B cells on T cell-mediated EAE. In mice with B cell-restricted deficiencies in MyD88, all B cell subsets produce IL-10 when TLR4 and TLR9 are engaged with LPS or CpG respectively. Interestingly, distinct TLR have different roles during EAE. The authors of this study found that mice with B cell-restricted deficiencies in TLR2 and TLR4 develop chronic EAE, while mice carrying TLR9-deficient B cells recover from disease similarly to mice with wild-type B cells. The absence of MyD88 in B cells resulted in a chronic form of EAE and heightened T cell responses of Th1 and T helper 17 (Th17) types, suggesting that B cells facilitate disease resolution by suppressing these pathogenic T cells (Lampropoulou, Hoehlig et al. 2008). Similarly, B cells activated with *Helicobacter felis*, which also signals through TLR2 and MyD88, hinder the development of severe gastric pathology by limiting the Th1 response (Sayi, Kohler et al. 2011). The presence of MyD88 on B cells acts as an important factor for B cells' regulatory functions because TLR engagement on DC or on monocytes induces proinflammatory responses.

In helminth infection, TLR2-stimulated B cells (with Pam3Cys) successfully inhibit CD4+ T cell proliferation and IFN-γ. These cells also inhibit IL-17 production in multiple sclerosis (MS) patients. It is worth noting that this procedure is also IL-10-dependent which highlights the fact that TLRs are required to induce regulatory properties in B cells (Correale and Farez 2009).

Altogether, these data suggest that TLRs are essential for the initiation of regulatory properties in B cells. Consequently, some microbial products could induce B cells suppressive properties.

It is evident that the co-engagement of different stimulations more effectively induces the B cell regulatory properties. IL-10 production by B cells increases when B cells are activated with both anti-CD40Ab and TLR9 stimulations (Lemoine, Morva et al. 2011). This phenomenon clearly indicates that some highly controlled and different signalling pathways are needed for the regulatory properties of B cells.

2.2.4. CD80 and CD86

CD80 and CD86 known ligands are CD28 and CTLA-4, both expressed on T

cells. These two co-stimulatory molecules also have some reported effects on the development and activation of Breg cells. The role of CD86 is evident in a mouse model of inflammatory bowel disease. In this model, the transfer of anti-CD86 mAb-treated B cells reduces the number of infiltrating T cells in the Lamina propria and reduces the development of colitis in recipient TCR-α deficient mice (Mizoguchi, Mizoguchi et al. 2000).

CD80 and CD86 were also reported to have an important role in B cell regulation when B7 deficient mice were used. The co-adoptive transfer of encephalitogenic T cells into chimeric mice (μMT mice reconstructed with B7-deficient mice bone marrow) fails to protect from EAE. Since B7-deficient mice were used, it was not possible to understand the specific role of CD80 and CD86. Moreover B7-deficient B cells show a delayed expression of IL-10 (Mann, Maresz et al. 2007).

2.2.5. Other signals inducing regulatory properties of B cells

Save for the previously mentioned factors there are few other factors that influence the regulatory properties of B cells. Apoptotic cells can provide some endogenous signals which in a direct way increase the production of IL-10 by B cells. The transfer of B cells from apoptotic cell-treated mice provided protection from collagen-induced arthritis (CIA) (Gray, Miles et al. 2007).

The platelet activating factor and serotine are implicated in contact hypersensitivity (CHS) and induce Breg cells after skin exposure to ultraviolet (UV) irradiation. In this model, the authors demonstrated that $CD220^{+}CD19^{+}$ B cells from UV light irradiated mice can confer protection to CHS in recipient mice through the production of increased levels of IL-10 (Matsumura, Byrne et al. 2006).

The initiation of the B cell regulatory properties requires a multistep process. TLRs are needed for the induction phase which is followed by BCR recognition and the CD40 engagement phase (Lampropoulou, Calderon-Gomez et al. 2010).

2.2.6. Phenotype of regulatory B cells

Breg cells were first described as $CD1d^{hi}$ $CD23^{hi}$ $CD21^{int}$. That phenotype appears after the development of chronic intestinal inflammation and these cells were able to suppress the progress of the inflammation and to produce IL-10 (Mizoguchi, Mizoguchi et al. 2002). Then, CD1d appears as an important marker of regulatory B cells, although

controversies persist because both marginal zone (MZ) precursor B cells and MZ B cell are CD1d positive whereas MZ B cells hardly show any regulatory effect. These subsets of IL-10-producing MZ precursor B cells are increased during the remission phase of CIA and show a typical transitional type 2-MZ precursor B cell phenotype: $CD21^{hi}$ and $CD23^{hi}$. In CIA, $AA4^{+}$ $CD21^{hi}CD23^{+}CD24^{hi}IgM^{hi}IgD^{+}CD1d^{+}$ B cells contribute to the remission of the disease (Evans, Chavez-Rueda et al. 2007). Moreover in MRL/*lpr* mice (generating a lupus like disease) transitional type 2-MZ precursor B cells must have a protective role to suppress renal disease and increase survival (Blair, Chavez-Rueda et al. 2009). The transfer of MZ B cells reduces CHS in CD19 deficient mice. Protection was mediated by $CD5^{+}$ and $CD1d^{+}$ cells. This cell population was found to be absent in $CD19^{-/-}$ mice but present in CD19 transgenic mice (Yanaba, Bouaziz et al. 2008).

CD5 positive B cells are a source of IL-10 production. However, as described previously in neonatal conditions, $CD5^{+}$ and $CD5^{-}$ cells presence notwithstanding, only $CD5^{+}$ B cells produce IL-10 (Sun, Deriaud et al. 2005). The $CD5^{+}CD1d^{+}$ IL-10 producing cells (B10 cells) show regulatory properties in the experimental mouse model of EAE and the depletion of this population increases the signs of the disease (Matsushita, Yanaba et al. 2008).

Immature transitional $CD19^{+}CD24^{hi}CD38^{hi}$ B cells have been found in the peripheral blood of healthy individuals and patients with lupus. In healthy individuals, this population produces IL-10 and can regulate the immune response. In lupus, these cells have a deficient production of IL-10 (Blair, Norena et al. 2010). However, in a mouse model of lupus, upon TLR9 activation, these IL-10-producing B cells modulate the T cell-mediated immune response (Lenert, Brummel et al. 2005).

The B10 B cells (IL-10 producing B cells) have also been characterized in humans. These cells are mainly $CD24^{hi}CD27^{+}$ memory B cells and were described in autoimmune disease patients (Iwata, Matsushita et al. 2011). They regulate the $CD4^{+}$ T cell activation (Bouaziz, Calbo et al. 2010).

2.3. BAFF and regulatory B cells

Generally BAFF is viewed as a survival factor for B cell but also as a pro-inflammatory cytokine (Gross, Johnston et al. 2000; Groom, Fletcher et al. 2007). BAFF is also implicated in the T cell-dependent immune reaction (Huard, Schneider et al. 2001; Ye,

Wang et al. 2004). In BAFF-Tg mice where high levels of circulating BAFF are found in the serum (Walters, Webster et al. 2009) increased numbers of circulating $CD4^{+}Foxp3^{+}$ regulatory T cells were described. Because these BAFF-Tg mice harbor more regulatory T cells than control, the T cell effector response is immunocompromised and these mice accept tissue allograft easily.

In another report, the role of BAFF in the induction of B10 B cells was demonstrated (Yang, Sun et al. 2010). The authors reported a population of $CD5^{+}CD1d^{hi}$ B cells which derived mainly from MZ B cells in BAFF-stimulated cells. BAFF activates the transcription factor *AP-1* favoring the increase of IL-10 production by B cells. BAFF-induced $CD5^{+}CD1d^{hi}$ B cells suppress the T cell proliferation and the Th1 cytokine production. Furthermore, BAFF induced $CD5^{+}$ $CD1d^{hi}$ B cells inhibit the CIA development in mice. Injecting BAFF in mice increases the number and frequency of B10 B cells.

However, BAFF-induced Breg cell production was terminated with the use of TACI- Fc. One possible explanation could be that MZ B cells show a different expression of BAFF receptors with a high TACI expression compared to follicular B cells, suggesting that BAFF receptors mediating the B cell regulatory function play different roles (Mantchev, Cortesao et al. 2007).

2.4. Function of regulatory B cells . How do they work ?

Breg cells are key players in the regulation of the immune response. Breg cells exert their regulatory functions during the immune response through several direct and indirect mechanisms. The regulatory function of these B cells is exerted through the production of regulatory cytokines, such as IL-10 and TGF-β. These cytokines have the ability to express inhibitory molecules that suppress pathogenic T cells and autoreactive B cells. IL-10 is one of the cytokines of Breg cells. It is induced after various immune stimulations, such as the TLR pathway activation. However, B cells also require CD40 and BCR ligation to enable further IL-10 production. IL-10 can then directly subdue the harmful immune response by regulating the Th1/Th2 balance and in so doing, decrease the innate cell-mediated inflammatory immune response (Fiorentino, Zlotnik et al. 1991). IL-10 suppresses both the proliferation and the cytokine production (IFN-γ and TNF-α) by Th1 and Th17cells (IL-17). Furthermore, IL-10 also inhibits the TNFα production by monocytes. This inhibition leads to a decreased inflammation. Not only can Breg cells suppress the Th1-

mediated immune responses, they can also convert effector T cells into regulatory T cells (Treg), which leads to a decreased Th1 response and results in immune regulation at the site of inflammation; such as a joint and the central nervous system. It has been demonstrated that endogenous IL-10-producing B cell-deficient mice develop an exacerbated case of arthritis and exhibit an increased frequency of Th1/Th17 pro-inflammatory cells, but a decreased frequency of Treg cells. In line with these findings, B10 cells induced *in vitro* could suppress the Th17 cell differentiation by decreasing the phosphorylation level of Stat3, which subsequently reduces the levels of RORγt, and partially inhibits the Th17 cell population in an IL-10-dependent manner (Yang, Deng et al. 2012). Breg cells are also capable of inhibiting the $CD8^+$ T cell function. This can lead to an impaired clearance of tumors. B cells can also promote DC to not only secrete IL-4 but also to downregulate IL-12, which affects the Th1/Th2 balance. In addition to IL-10-producing Breg cells, TGF-β1-producing Breg cells have been identified in response to LPS stimulation *in vitro*. These B cells can trigger pathogenic Th1 cells to undergo apoptosis through Fas–FasL interactions and/or the inhibition of antigen-presenting cell activity *via* the secretion of TGF-β1.

The mechanisms for regulating the immune response by B cells are also dependent on the promotion of the activation-induced cell death (or apoptosis), which is mediated by death-inducing ligands, such as FasL, TNF-related apoptosis-inducing ligand, programmed death ligands 1 and 2 (PD-L1 and PD-L2), etc. B cells can express FasL and other death-inducing ligands under many circumstances. Both FasL and IL-10 are highly expressed in the $CD5^+$ B-cell population, which indicates that $CD5^+$ B cells may exert regulatory effects through their killing ability. Interestingly, a recent study suggested that B cells can induce the proliferation of Treg cells in the central nervous system during the development of EAE *via* the expression of glucocorticoid-induced TNF receptor ligand rather than IL-10. Breg cells can directly induce the apoptosis of effector B and T cells by Fas-FasL interaction. This leads to a decreased inflammation at the site of infection (Mizoguchi and Bhan 2006).

Apart from the cytokine-mediated and apoptosis mediated immune suppression, B cells can also exert their regulatory effects by cellular interactions. Both B10 and T2-MZ precursor Breg cells share the phenotype high CD1d, which is a MZ B cell marker. CD1d-expressing MZ B cells have been shown to activate invariant natural killer T (iNKT) cells in the presence of DC and help set up peripheral tolerance through the induction

of Treg cells. Moreover, $CD1d^{hi}$ MZ B cells are able of present glycolipids through CD1d. These glycolipids are recognized by iNKT cells, which are cells that are known to play important roles in the autoimmune development. It has been shown that EAE is exacerbated in $CD1d^{-/-}$ mice, which lack iNKT cells. Recently, human transitional B cells ($CD19^{+}CD24^{hi}CD38^{hi}$) were found to play an essential role in the iNKT cell expansion and activation in healthy individuals, but not in SLE patients because transitional B cells from SLE patients are defective in recycling CD1d. Thus, CD1d-expressing Breg cells can also exert their regulatory functions by activating NKT cells (Yang, Rui et al. 2013). In summary, Breg cells can exert their suppressive effects by secreting anti-inflammatory cytokines, such as IL-10 and TGF-β, and by engaging in cell-to-cell contact through the activation of cell death markers or costimulatory molecules. In the following section, the role of Breg cells in various autoimmune diseases, including SLE will be exposed.

3. TOLL-LIKE RECEPTORS

3.1. Introduction

Toll-like receptors represent a family of evolutionary conserved innate immune receptors and are important to mediate the first line of defense against pathogens. These pattern recognition receptors (PRR) recognize an essential component for the survival of the pathogens known as the pathogen-associated molecular pattern (PAMPs). TLRs were first identified in drosophila and noticed for their involvement in embryogenesis. Later, they were seen as playing a major role in fungal infection (Hashimoto, Hudson et al. 1988; Lemaitre, Nicolas et al. 1996). At as of today, 10 TLR subtypes have been identified in humans (Figure 8) and 12 in mice, each one of them possesses its own specific ligands, cellular localization and expression profile (Santegoets, van Bon et al. 2011). TLRs are expressed in various immune cells including DC, natural killer cells, neutrophils, macrophages, B cells and T cells (Zarember and Godowski 2002; Michallet, Rota et al. 2013).

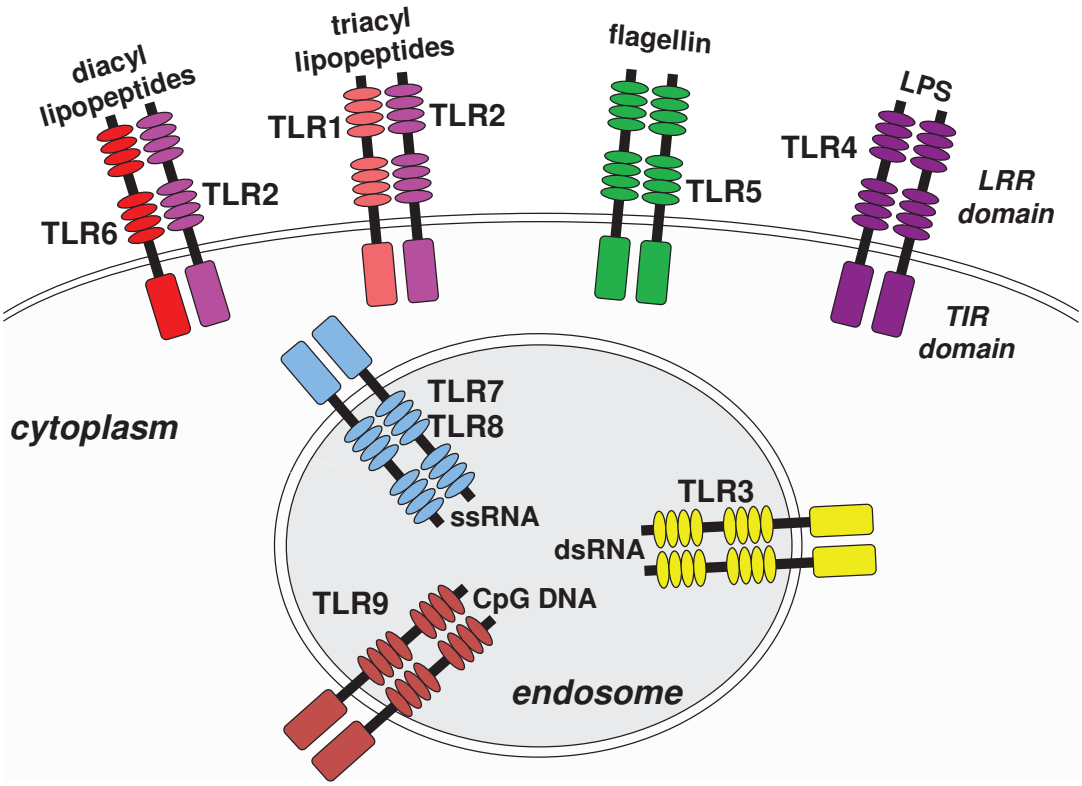


Figure 9 **The different human Toll-like receptors.**
The Toll-like receptors (TLR) are constituted of an extracellular domain enriched in leucines, called Leucine Rich Repeat (LRR) domain, and an intracellular domain containing a conserved region of 200 amino-acid, called Toll/IL-1R (TIR) domain. TLRs are associated as homo- or heterodimers to bind ligands such as proteins, lipids, carbohydrates or nucleotides. They are located either on the plasma membrane (TLR 1, 2, 4, 5, 6, and 10) or on the endosomal membrane (TLR 3, 7, 8, and 9) with their TIR domain present within the cytoplasm allowing the signal transduction. LPS: lipopolysaccharide; ss and dsRNA: single stranded and double stranded RNA; CpG DNA: CpG-enriched DNA.

3.2. Structure

TLRs are type I integral membrane glycoproteins with a trimodular structure. TLRs include a N-terminal ligand recognition domain, a single transmembrane helix, and a C-terminal cytoplasmic signalling domain (Bell, Mullen et al. 2003). The extracellular N-terminal domain consists of leucine rich repeats (LRRs). However, the number of LRRs varies between each TLR. In humans, the number of LRRs varies between 19 and 25 per TLR (20-30 aa in each repeat) folded in β strand and α helix that are linked by a loop (Jin and Lee 2008). When assembled into a protein, multiple consecutive LRRs form a solenoid structure, where the consensus hydrophobic residues point towards the interior to make a stable core and the β-strands align to form a hydrogen-bonded parallel β sheet. These motifs

form a horseshoe structure with both a concave and convex surface, the concave surface being involved in ligand recognition (Botos, Wu et al. 2001). The intracellular signalling domain is called Toll-receptor domain and displays a homology with the IL-1 receptor. It contains approximately 200 aa. This domain is required for the interaction and recruitment of various adapter molecules to activate the downstream pathway (Kumar, Kawai et al. 2009).

3.3. Expression and Ligands

These innate immune receptors recognize a wide variety of microbial molecular motifs, PAMPs. Moreover TLRs can also be activated by damage associated molecular pattern (DAMPs) which are produced in case of aggression or tissue damage. The activation of the TLR signalling pathway initiates innate immune responses. Furthermore, the responses of the innate immune system are important not only to eliminate pathogens but also to develop a pathogen-specific adaptive immunity, thus forming a bridge between innate and adaptive immunity (Pasare and Medzhitov 2004). TLRs are expressed in distinct cellular compartments. TLR1, TLR2, TLR4, TLR5, TLR6, and TLR10 are expressed on the cell surface whereas TLR3, TLR7, TLR8, TLR9 are expressed in the intracellular vesicles such as the endosome and the endoplasmic reticulum. The ligands for TLR family vary from one another.

3.3.1. Bacterial PAMPs recognized by TLRs

TLRs can sense various components of the bacterial cell wall. TLR4, the first identified mammalian TLR, is responsible for sensing bacterial endotoxin LPS (Medzhitov, Preston-Hurlburt et al. 1997). TLR2 recognizes peptidoglycan from gram-positive bacteria. The gram-negative bacterial membrane protein ompA also activates TLR2. Flagellin, the major protein in bacterial flagella is recognized by TLR5. Lipoarabinomannan from mycobacteria is recognized by TLR2 and diacyl or triacyl lipopeptides from bacteria, mycobacteria and mycoplasma are recognized by TLR1/2 or TLR2/6. Genomic DNA from bacteria rich in unmethylated CpG DNA is recognized by TLR9. TLR11, which is exclusively expressed in mice, is associated with sensing uropathogenic bacterial product (Zhang, Zhang et al. 2004).

3.3.2. Viral PAMPs recognized by TLRs

The envelope proteins from viruses such as the respiratory syncytial virus and the mouse mammary tumor virus are recognized by TLR4, TLR2 and TLR6 (Murawski,

Bowen et al. 2009). Furthermore, the hemagglutinin protein of the Measles virus is also recognized by TLR2. Virus nucleic acids are also important PAMPs. The genome of DNA viruses such as the herpes simplex virus and the murine cytomegalovirus contains unmethylated CpG DNA, recognized by TLR9. The genome of RNA viruses contains single strand RNA (ssRNA), which is rich in uridine or uridine/ guanosine and is recognized by TLR7 and TLR8 (in humans only). The double-strand RNA (dsRNA) are sensed by TLR3. Furthermore, the synthetic analog of dsRNA, poly IC, is also recognized by TLR3 (Kumar, Zhang et al. 2006). Guanosine-rich and uridine-rich ssRNA derived from virus, synthetic polyuridine ssRNA acts as ligand for TLR7 (Diebold, Kaisho et al. 2004).

3.3.3. Fungal and protozoal PAMPs recognized by TLRs

TLR2 recognizes phospholipomannans from fungi and specific proteins from protozoa (Akira S. et al. 2006). Lipophosphoglycan and genomic DNA are recognized by TLR2 and TLR9 respectively (Kumar H. et al.2009). TLR2 also detects zymosan, which is found in cell wall of yeast.

3.3.4. Endogenous ligands recognized by TLRs

TLR2 and TLR4 are also implicated in the recognition of endogenous molecules. Among the DAMP, the Heat-Shock Protein 70, 60 and B8 some fragments of the extracellular matrix protein fibronectin, bind to TLR2 and/or TLR4 (Vabulas, Wagner et al. 2002). TLR2 and TLR4 also detect the High-mobility group box 1 protein (Park, Svetkauskaite et al. 2004). Extracellular matrix components, like hyaluronic acid oligosaccharides from damaged cells, also bind TLR4. In addition, oxidized low-density lipoproteins and fatty acids are recognized by TLR4 (Marshak-Rothstein and Rifkin 2007). TLR3 activation by dsDNA and RNA fragments from necrotic cells have been found in RA (Brentano, Schorr et al. 2005).

3.4. Signaling pathways

The recognition of ligands initiates signaling throughout various TIR domains containing adapter molecules like MyD88, TIRAP, TRIF and TRAM (Figure 10). The engagement of TLR1, TLR2, TLR4, TLR5, TLR6, TLR7, TLR8, TLR9 and TLR11, and their respective ligands, recruit MyD88. MyD88, TLR1, TLR2, TLR4 and TLR6 further recruit TIRAP, which serves as a linker adapter between the TIR domain of TLRs and

MyD88. The binding of ligand with TLR3 and TLR4 recruits TRIF.

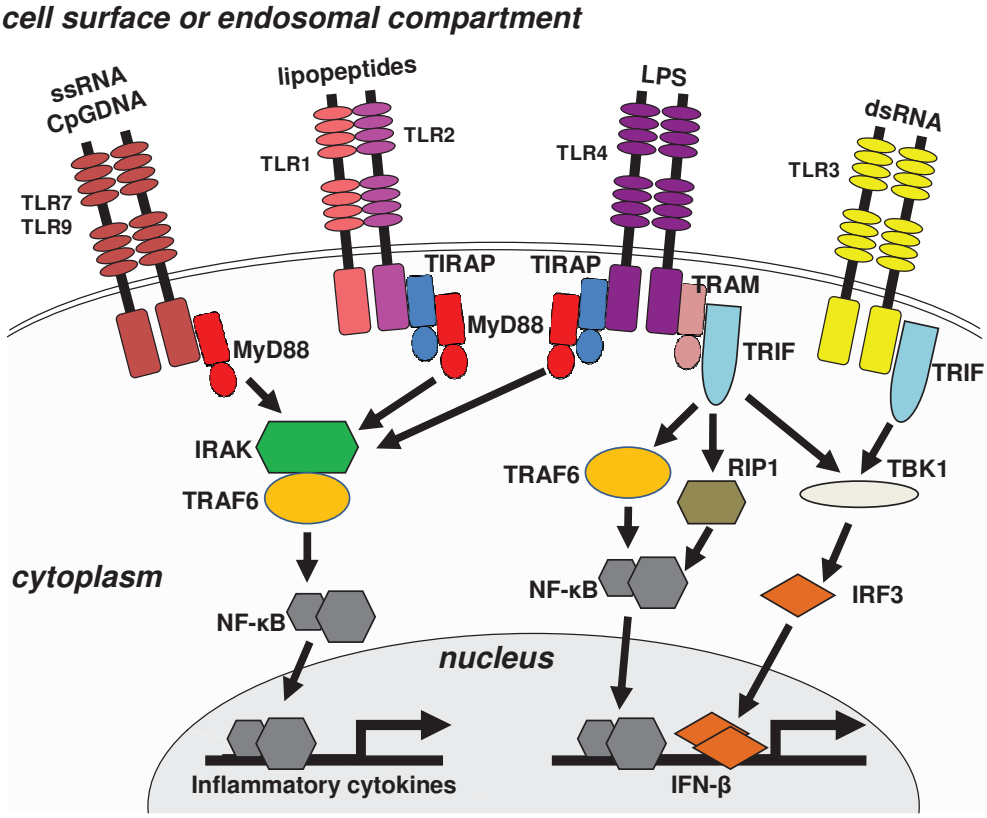


Figure 10 **Toll-like receptors signaling pathways.**
Schematic representation of MyD88-dependent (left) and MyD88-independent (right) cascades following binding of ligands on TLR dimers leading to the production of pro-inflammatory cytokines and type I interferon.

MyD88 is required for the functionality of all TLRs except TLR3. MyD88 enables the recruitment and activation of the IL-1R-Associated Kinase (IRAK)-1 and IRAK-4 leading to the activation of TRAF6 (Ringwood and Li 2008). TRAF6 activates the transforming growth factor *β*-activated kinase-1 (TAK1) via K63-linked polyubiquitination, resulting in the activation of the NF-κB transcription factor (Wang, Deng et al. 2001; Kawai and Akira 2006). Signalling through MyD88 activates some transcription factors of the Interferon-Regulatory Factor (IRF) family, and the activation of the Mitogen-Activated Protein (MAP) kinases such as p38, the JNK, and the extracellular signal-regulated kinase ½ (ERK1/2) which subsequently enables the activation of the AP-1 transcription factor. The subsequent activation of NF-κB and AP-1 induces an inflammatory response through the production of inflammatory cytokines such as TNF-α, IL-6, IL-1β, IL-12 (Kawai and Akira

2006). Moreover, TLR4, TLR7, TLR8 and TLR9 induce an antiviral response by promoting IFNα and IFNβ synthesis. Indeed, MyD88-dependent signalling downstream of TLR7 and TLR9 induce the production of type I IFN (IFN-α and IFN-β). This response is specific to plasmacytoid DC, which express high levels of TLR7 and TLR9 and produce high levels of IFN-α. After activation, the TIR domains of TLR7 and TLR9 recruit a complex consisting of MyD88, IRAK-4, IRAK-1, and TRAF6 (Honda, Yanai et al. 2004), which binds and activates the transcription factor IRF-7, inducing expression of type I IFN (Kawai, Sato et al. 2004).

However, the MyD88-dependent activation of NF-κB can also be induced by the TAK1-independent pathway. Two candidates for this TAK1-independent pathway are the mitogen-activated protein kinase kinase 3 (MEKK3) and the atypical Protein kinase C (PKC) - (Sanz, Diaz-Meco et al. 2000; Yao, Kim et al. 2007).

The signalling pathway triggered by TLR3 is MyD88-independent and requires a TIR domain-containing adapter that induces IFN-β (TRIF) as an adapter molecule. The association of TRIF with the receptor-interacting protein 1 are responsible for the activation of NF-κB (Meylan, Burns et al. 2004). The TLR4-dependent signal transduction can be either MyD88-dependent or MyD88-independent. When TLR4 stimulation begins, at the membrane level, MyD88 comes into play. Then, once TLR4 and its ligand have been internalized in the endosomal compartment, TRIF becomes involved (Kagan, Su et al. 2008). The MyD88-independent signalling that follows the TLR4 stimulation needs the recruitment of TRAM, which is essential for TRIF-TLR4 interaction. TLR4 first induces MyD88 adapter-like/TIRAP-MyD88 signalling at the plasma membrane. Then, after endocytosis into endosomes, TLR4 activates the TRAM-TRIF signalling. Once recruited to the receptor, TRIF interacts with TRAF3 to activate the noncanonical IKKs, TBK1 and IKKε, resulting in the activation of IRF3 and the transcription of IFNβ and IFN-inducible genes (Yamamoto, Sato et al. 2003).

Plasmacytoid DC, produce large amounts of type I IFN in response to TLR7/8 and TLR9 activation (Colonna, Trinchieri et al. 2004). The IRF members play an important role in this regard. The activation of TLR9 and TLR7/8 causes the nuclear translocation of IRF7, a transcription factor that regulates the type I IFN induction in plasmacytoid DC. Following TLR activation IRF7 interacts with MyD88, IRAK1 and TRAF6 to form a signalling complex and to subsequently induce type I IFN production (Honda, Yanai et al.

2004; Kawai, Sato et al. 2004).

3.5. TLRs and B cells

B cell expresses a distinct subset of TLRs. This determines their ability to respond after activation (either exogenous or endogenous). Naïve human B cells express low levels of TLRs, regardless of their activation status, and memory B cells display higher levels of TLR1, TLR6, TLR7, TLR9,TLR10 and low levels of TLR2 (Hornung, Rothenfusser et al. 2002; Mansson, Adner et al. 2006; Agrawal and Gupta 2011).

TLR expression and responsiveness vary in mice B cells. The expression of TLR1, TLR7 and TLR9 is predominant in peritoneal B-1a, B-1b cells and also in splenic follicular and MZ B cells. The expression of TLR2, TLR3, TLR4 and TLR6 is expressed at intermediate levels. The functional analysis of TLR responsiveness shows a proliferative response upon the activation of TLR2, TLR7, TLR9 in follicular and MZ zone B cells. MZ and B-1 B cells display stronger functional responses to TLR ligands than follicular B cells (Oliver, Martin et al. 1999; Genestier, Taillardet et al. 2007). Moreover, MZ B cells show a greater potential to act as APC than follicular B cells in response to TLR stimulations (Oliver, Martin et al. 1999).

Triggering TLR4 induces the proliferation of MZ B cells. Moreover, the activation of TLR2, TLR4, TLR7 and TLR8 induces IgM production and the induction of the Blimp-1 transcription factor by B-1 B cells. Furthermore, TLR9- induced differentiation was found in B-1 and MZ B-cell subsets (Genestier, Taillardet et al. 2007). Murine B cells constitutively express TLR4, and the activation of TLR4 induces proliferation, cytokine secretion and class switch recombination (Bekeredjian-Ding and Jego 2009).

In humans, MZ-like B cells are highly sensitive to TLR stimulation (Bernasconi, Traggiai et al. 2002). Furthermore, the stimulation of human memory B cells by TLR7 and 9 is much more noticeable than the stimulation of naive B cells (Bernasconi, Traggiai et al. 2002; Bekeredjian-Ding, Wagner et al. 2005). However, the human local environment can be a factor for TLR expression and responsiveness. The expression and responsiveness of TLR2, TLR3 and TLR9 are higher in tonsilar B cells than in peripheral blood B cells (Ganley-Leal, Liu et al. 2006; Mansson, Adner et al. 2006).

The expression of TLRs in B cells is regulated by the action of cytokines as

well as by signalling from the BCR. TLR3 and TLR7 are upregulated in murine B cells by IFN-β (Chang, Coro et al. 2007) and by stimulation of the BCR (Sato, Sanjo et al. 2005) The expression of TLR7 in human B cells is also strongly upregulated by type 1 IFN (Bekeredjian-Ding, Wagner et al. 2005).

Studies have showed that TLR signalling interacts with the BCR stimulation or the stimulation by the CD40L (Jain, Chodisetti et al. 2011) for B cell activation. However, co-stimulation through the BCR or CD40L implies specialized roles for different TLRs. BCR or CD40 stimulation in combination with TLRs (TLR3, TLR4 or TLR9) promotes proliferation and activation, whereas others (TLR1/2, TLR2/6, TLR4 and TLR7) promote development into Ab-secreting cells (Boeglin, Smulski et al. 2011). The BCR provides an efficient endocytosis of the intracellular TLR ligands by B cells (Lanzavecchia and Sallusto 2007). The BCR activation also causes intracellular TLRs to move from the early endosomes where they can be found at a basal level to the late endosomes. There, they co-localize with the BCR, its antigen and so, possibly, with microbial DNA (Chaturvedi, Dorward et al. 2008). Although, triggering TLR9 alone can activate naïve B cells (Huggins, Pellegrin et al. 2007; Jiang, Lederman et al. 2007; Bekeredjian-Ding, Doster et al. 2008)

Various cytokines also play an important role in the TLR-induced B cell stimulation. IFN-α amplifies the action of the TLR7 ligands and provides an efficient response even without BCR stimulation (Douagi, Gujer et al. 2009).

After exposure to the TLR9 ligand, B cells induce the expression of various activation markers such as CD69, CD86, CD80 and an increased expression of the MHC class II molecules (Jiang, Lederman et al. 2007; Capolunghi, Cascioli et al. 2008).

TLR activation induces cytokine secretion from B cells. TLR7 and TLR9-stimulated B cells produce different array of cytokines and chemokines such as IL-1α, IL-1β, IL-6, TNF-α, IL-13, IL-10. Different chemokines like MIP-1α, MIP-1β, MCP-1 and IP-10 are also produced. Triggering TLR2 predominantly induces the production of granulocyte macrophage colony stimulating factor (GM-CSF) and G-CSF. However this response occurs more often in memory B cells than in naïve B cells (Agrawal and Gupta 2011).

In mice, different B cell subsets show specialized cytokine secretion profiles. The activation of TLR2, TLR4, and TLR9 induces IL-10, IFN-γ and IL-6 secretion from B cells. However, IL-10 is mainly secreted by MZ and B1 B cells, whereas follicular B cells are

the main source for IFN-γ secretion. Nonetheless, the secretion of IL-6 was found to come from both B-cell subsets (Barr, Brown et al. 2007).

By transferring wild-type, TLR4-deficient, or Myd88-deficient B cells to mice lacking endogenous B cells, it was found that TLR signalling is required in B cells to promote Ab response (Pasare and Medzhitov 2005).

TLRs expressed by B cells are involved in the induction of T-dependent and T-independent isotype class switching. They also play a role in their orientation towards one isotype or another. TLR9 in human B cells participates in the T-dependent class-switching towards the IgG and IgA isotypes while inhibiting the production of IgE (Gantner, Hermann et al. 2003; Ruprecht and Lanzavecchia 2006). In addition, TLRs in B cells enable T-independent class switching towards the IgA, IgG1, IgG2 and IgG3 isotypes in association with IL-10 and BAFF or APRIL cytokines.

Moreover, in mice, the mobilization of TLR9 in B cells facilitates the T-dependent class switching towards the Th1 IgG2a isotype. The transcription factor T-bet is required for this process to occur. In contrast, the activation of TLR9 in B cells inhibits the orientation towards the Th2 IgE and IgG1 isotypes (Liu, Ohnishi et al. 2003; Jegerlehner, Maurer et al. 2007). During influenza virus infection, TLR7 and MyD88 play an important role in regulating Ab production. TLR7 and MyD88-deficient mice show increased levels of influenza-specific IgG1. Moreover, they exhibit decreased IgG2a/c class-switching thereby indicating that TLR7 -and its signalling molecule MyD88- have an impact on the induction of the B cell class-switching to the IgG isotype (Heer, Shamshiev et al. 2007).

TLR signalling also plays an important role in the regulation of the localization and migration of the B-1 and MZ B cells. Although localized in splenic MZ, these cells also circulate between splenic follicles and MZ to capture and transfer blood-borne antigens to follicular DC (Groeneveld, Erich et al. 1985; Martin and Kearney 2002). TLR2, TLR3 and TLR7 ligands promote MZ B cell migration (Rubtsov, Swanson et al. 2008). The sphingosine-1-phosphate receptor 1 (S1PR1) acts as a regulator of MZ B cell retention and upon blocking of the S1PR1, MZ B cells migrate to the follicles. The activation of TLR4 downregulates S1PR1, leading to a reduction in the chemotactic responsiveness of the MZ B cells (Cinamon, Matloubian et al. 2004; Rubtsov, Swanson et al. 2008). The TLR stimulation induces the rapid downregulation of integrin and CD9 leading to the detachment

of B-1 cells from the local matrix of the peritonial cavity. This detachment hints at a mechanism in which B cell-intrinsic TLR signalling can alter the B-1 cell responsiveness, thereby directing the migration of these cells to locations where rapid local antibody responses help limit the pathogen growth (Ha, Tsuji et al. 2006).

3.6. Role of TLRs in autoimmunity

TLRs alert the host at the first signs of microbial infection and activate the initial line of immune defense. However, TLRs also recognize self-epitopes released from dying or damaged cells as well as self-epitopes present at the surface of apoptotic cells or apoptotic bodies. TLR7, TLR8, TLR9 recognize bacterial and viral DNA or RNA, but many studies have shown that these TLRs can also be activated by host RNA, DNA or associated proteins. TLR7 and TLR9 are constitutively expressed in B cells and plasmacytoid DC. Furthermore these cell types are closely linked to disease pathogenesis in different autoimmune diseases like SLE, through the production of autoAbs and IFN-α (Palucka, Banchereau et al. 2002; Jego, Palucka et al. 2003).

Ligands for TLR7, TLR8 and TLR9 must be internalized into the endolysosomes, *i.e.* into the site of these receptors leading to the induction of inflammatory responses in various autoimmune diseases (Marshak-Rothstein and Rifkin 2007). This internalization mechanism was first described using self-reactive B cells from AM14 mouse (expressing a BCR, which recognizes rhumatoid factor-like autoAb with $IgG2a^{a/j}$ specificity). B cells from these mice proliferate upon stimulation with immune complexes formed with IgG2a type monoclonal antibodies and DNA or RNA fragments. The proliferative effect on B cell is ceases with the use of TLR7 or TLR9 inhibitors. Moreover, this phenomenon was not observed in *TLR7* or *TLR9* knock-out mice. The AM14 BCR recognizes –via IgG2- a DNA or RNA fragment that contains an immune complex at the cell surface and allows transport to the endosome, where TLR7 and TLR9 are activated. This suggests a mechanism where immune complex containing DNA or RNA fragments (which may derive from apoptotic or necrotic cells) form a complex with anti-DNA IgG autoantibodies and can stimulate B cells *via* the BCR and TLR9, (Leadbetter, Rifkin et al. 2002; Lau, Broughton et al. 2005) thus affecting the B cell-driven autoimmunity.

FcγRs located on dendritic cells also recognize immune complexes containing DNA or RNA fragments and induce TNF-α production from DC in the presence of GM-CSF.

This pathway requires the presence of FcγRIIIB and interaction with TLR9, because blocking TLR9 or TLR9$^{-/-}$ DCs inhibits the production of TNF-α (Boule, Broughton et al. 2004; Marshak-Rothstein and Rifkin 2007). The serum of SLE patients also triggers the TLR9 function through a mechanism involving FcγRIIa. It also enables the production of IFN-α (Means TK. et al. 2005). Moreover, this production of IFN-α from plasmacytoid DC is dependent on the presence of TLR7. When stimulated with the serum of SLE patients, TLR7$^{-/-}$ plasmacytoid DC do not produce IFN-α, which depends on the presence of anti Sm/RNP Abs (Savarese, Chae et al. 2006).

In a spontaneous lupus model deficiency, TLR7 and TLR9 do not have the same effect on the tissue-specific disease manifestation. TLR7 knock-out mice display less severe nephritis than control whereas TLR9 knock-out mice are severely affected by nephritis and skin disease, suggesting that TLR9 plays a protective role (Christensen, Shupe et al. 2006). However, in chronic graft-versus-host disease, TLR9 knock-out mice shows less nephritis (Ma, Chen et al. 2006).

In case of psoriasis, the antimicrobial peptide LL37 (an endogenous antimicrobial peptide) forms a condensed and aggregated structure with self-DNA and is translocated to the early endocytic compartment of plasmacytoid DC by a mechanism that involves lipid rafts and proteoglycans. This DNA complex activates TLR9 and induces IFN production by plasmacytoid DC (Lande, Gregorio et al. 2007).

Transgenic mice overexpressing TLR7 (Male BxSB mice with Yaa mutation having two copies of the TLR7 gene) display more severe and accelerated lupus than their normal counterparts. The overexpression of TRL7 also develops anti-RNA autoAbs and glomerulonephritis (Subramanian, Tus et al. 2006).

The activation of TLR3 by adding the TLR3 ligand poly(I :C) increases severe glomerulonephritis in Mrl$^{lpr/lpr}$ mice (Patole, Grone et al. 2005). Repeated administrations of LPS, the TLR4 ligand, in lupus-prone mice, accelerates the disease, including the production of autoAbs. Moreover, C57BL/6$^{lpr/lpr}$ mice show a less severe disease compared to their TLR4-producing counterpart. These C57BL/6 lpr/lpr mice show lower AutoAb levels and produce less IFN-γ and IL-6 which results in a decreased renal disease (Hang, Slack et al. 1983; Liu, Yang et al. 2006; Lartigue, Colliou et al. 2009).

In purified plasmacytoid DC from SLE patients, the TLR7 and TLR9

inhibitors block the production of IFN-α, after being stimulated with various viral proteins or anti-ds DNA/anti-RNP immune complexes from SLE patients (Barrat, Meeker et al. 2005).

4. PRIMARY SJÖGREN'S SYNDROME

4.1. Introduction

SS is a systemic multiorgan autoimmune disease with both a chronic and a progressive course. It was named after Henrik Sjögren who, in his 1933 report, described findings of dry mouth and eyes (Igoe and Scofield 2013). The symptoms were linked to the destruction of the exocrine glands and EC (Moutsopoulos 1994). The disease can occur alone (as primary SS: pSS) or in association with other autoimmune disorders (as secondary SS). The disease is characterized by: the lymphocytic infiltration of exocrine glands (lacrimal and salivary) which reduces their secretory function (Kassan and Moutsopoulos 2004), keratoconjunctivitis sicca (*i.e.* consequence of dry eyes), and xerostomia (dry mouth caused by salivary gland (SG) dysfunction). SS then spreads out from an organ-specific autoimmune disorder (referred to as an autoimmune exocrinopathy) to a systemic process involving the musculoskeletal system, the nervous system, the lungs, the kidneys and the blood vessels (Tzioufas and Voulgarelis 2007).

4.2. Epidemiology of the disease

SS primarily affects women in the 4th and 5th decade of their life (9 women for 1 man) (Tzioufas and Voulgarelis 2007). In various epidemiology studies, the prevalence of the disease ranges from 0.1 to 4.8% showing highly heterogeneous results (Mavragani and Moutsopoulos 2010). These results are probably caused by differences in the set of criteria used to establish a diagnostic and the design of the study. As a result, the prevalence and incidence of pSS in the general population remains unclear (Binard, Devauchelle-Pensec et al. 2007).

4.3. Clinical symptoms and diagnosis

pSS usually has a slow course, characterized by non-specific clinical manifestations and a lapse of approximately 6 years till diagnosis (Pavlidis, Karsh et al. 1982).

4.3.1. Sicca syndrome

The keratoconjunctivitis symptoms include a decreased production of tears leading to the destruction of the corneal and bulbar conjunctival epithelium. Sensations like burning, itchiness or the feeling of having sand in one's eyes as well as photosensitivity and bloodshot eyes are common (Al-Hashimi, Khuder et al. 2001).

Additionally, the decreased production of saliva is responsible for xerostomia and ensuing manifestations such as dental caries, oral candidiasis, bacterial sialadenitis, and oral ulcers. Patients report difficulties in swallowing food, the inability to speak continuously, changes in

gustatory perception and a burning sensation in the mouth. Dryness affects the upper respiratory tract and the pulmonary tissue, causing hoarseness, recurrent bronchitis and pneumonitis (Boutsi, Paikos et al. 2000). The enlargement of the SG is also rather common and affects more than half of the patients (Tzioufas and Voulgarelis 2007).

4.3.2. Extraglandular manifestations

The systemic manifestations occur in approximately half of the patients. They include general constitutional symptoms, such as fatigability, low-grade fever, myalgia, arthralgia and the involvement of other organs. Fatigue is one of the most frequent complaints and occurs in around 50% of the cases (Tzioufas and Voulgarelis 2007). Around 20% of patients develop neurological problems linked to the central and peripheral nervous system such as movement disorders, seizures, motor and sensory loss. In some patients, the liver can also be affected with an increase in liver enzymes and stage I primary biliary cirrhosis histopathological lesions. The presence of anti-mitochondrial antibodies in liver samples is evident. B cell lymphoma have also been reported (Mavragani, Moutsopoulos et al. 2006).

The multiple aspects of the disease make it difficult to diagnose, which results in delayed diagnosis, or lack thereof, although an early detection is clinically propitious for therapeutic intervention and disease recovery (Novljan, Rozman et al. 2006).

4.3.3. Classification criteria

As stated earlier, SS does not have a single diagnostic criterion but the diagnosis is generally made through a combination of clinical and laboratory findings. The American-European Community Study Group (AECG) has defined these criteria for diagnosing SS (Vitali, Bombardieri et al. 2002) (Table 1).

Primary SS definition:

a. Presence of any 4 criteria out of 6 and either histopathology (IV) or serology (VI) is positive.

b. Presence of any 3 out of 4 objective criteria (criterion III, IV, V, and VI).

Secondary SS definition: patients with a potentially associated disease (such as another well-defined connective tissue disease), the presence of criterion I or II and positive testing for 2 criteria out of 3 (either III, IV and V) may be considered as an indication of secondary SS (Table 1).

Exclusion criteria for this classification: Past head and neck radiation treatment,

Hepatitis C infection, AIDS, Sarcoidosis, Graft versus Host disease, pre-existing lymphoma, use of anticholinergic drugs (within a time frame inferior to 4 times the drug's half-life).

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 (≤ 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 ≥ 1, defined as a number of lymphocytic foci (which are adjacent to normal-appearing mucous acini and contain more than 50 lymphocytes) per 4 mm^2 of glandular tissue.
V. Test for SGs involvement: a positive result for at least one do the following diagnositc tests: unstimulated whole salivary flow (≤ 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 1. American-European classification criteria for Sjögren's syndrome (Vitali, Bombardieri et al., 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.

4.4. Immunopathology of the disease

4.4.1. Introduction

The histopathologic lesions of the exocrine glands consist of lymphocytic infiltrates. Mild focal infiltrates do not significantly impair the gland organization. However, diffuse severe lesions are associated with a significant loss of the epithelial structure and tissue architecture. The mechanism leading to the accumulation of infiltrating cells is still unclear. These cells interfere with glandular secretion and alter their structure. They secrete cytokines that activate the type 1-IFN regulated pathway like JAK-STAT and produce anti-SSA and anti-SSB autoAbs (Vakaloglou and Mavragani 2011; Yao, Liu et al. 2013). T and B cells form the vast majority of infiltrating mononuclear cells in SG. Most of the infiltrating T cells are $CD4^+$ (50-70% of the total infiltrating T cell population) (Christodoulou, Kapsogeorgou et al. 2010). $CD8^+$ T cells with cytotoxic activity, characterized by their expression of granzymes average 10% of the infiltrating cells. Macrophages, DC, and natural killer (NK) cells amount to only a small portion (5-10%). T cells predominate in mild lesions (up to 60% of total infiltrating mononuclear cells) and B cells predominate in advanced lesions (up to 50% of total infiltrating mononuclear cells). The number of infiltrating T cells and

interdigitating DC is inversely correlated to the stage of the infiltration process (Christodoulou, Kapsogeorgou et al. 2010).

4.4.2. Epithelial cells regulate autoimmune epithelitis

The immunohistochemical analysis of the inflamed SG tissues of SS patients shows that salivary gland EC (SGEC) display high levels of several immunoactive molecules that are known to mediate lymphoid cell homing, antigen presentation, and the amplification of epithelial-immune cell interactions.

Process	Molecule expressed by SGEC		Reference
T cell activation	MHC class-I MHC class-II Costimulatory	HLA-ABC HLA –DR CD80,CD86	(Speight, Cruchley et al. 1989; Manoussakis, Dimitriou et al. 1999) (Kapsogeorgou, Moutsopoulos et al. 2001; Tsunawaki, Nakamura et al. 2002)
B cell survival, maturation and differentiation,	BAFF		(Daridon, Devauchelle et al. 2007)
Immune- cell homing	Adhesion	ICAM1,VCAM,E-selectin	(Tsunawaki, Nakamura et al. 2002)
Innate immunity related	Toll-like receptors	TLR-2,TLR-3,TLR-4,TLR-7,TLR-9	(Spachidou, Bourazopoulou et al. 2007; Zheng, Zhang et al. 2010)
Apoptosis related	Fas, Fas ligand		(Kong, Ogawa et al. 1997)

Table 2. Process involving epithelial cells in pSS

An elevated epithelial apoptosis and an increased expression of apoptosis-related molecules (Fas-FasL, Perforin, granzymes) have been detected in minor SG lesions suggesting that this pathway plays an important role in the disease by destructing EC (Polihronis, Tapinos et al. 1998). EC apoptosis represents a pathway for the generation of autoimmune responses in SS. During apoptosis, the autoantigenic La(SSB) proteins have been shown to be diffusely redistributed into the cytoplasm, whereas both Ro(SSA) and La(SSB) autoantigens are led to the surface apoptotic blebs and bodies (Rosen, Casciola-Rosen et al. 1995). Thus, because of apoptosis, nuclear antigens, such as the autoantigenic Ro(SSA) and La(SSB) ribonucleoproteins, are exposed to the surface which leads to the autoAb responses in SS (Ohlsson, Jonsson et al. 2002). Kong et al. demonstrated that acinar EC from pSS express Fas and FasL. By contrast, the majority of the infiltrating lymphocytes in SS are Fas^+ and $Bcl\text{-}2^+$, but FasL negative, and present a minimal amount of dead cells, particularly in the dense periductal foci due to the presence of anti-apoptotic Bcl-2 (Kong, Ogawa et al. 1997). EC are particularly susceptible to Fas-mediated apoptosis after IFN-γ stimulation, *via* downregulation of the apoptosis inhibitor protein c-FLIP and Bcl-2 (Abu-Helu, Dimitriou et al. 2001). Recently, a report from our group evaluated the role of B cells in inducing EC apoptosis. In co-culture experiments with

the human salivary gland (HSG) cell line and tonsilar B cells, B cells induced the apoptosis of EC. Cell death could not be ascribed to Fas–Fas ligand interactions but required caspase 3 activation and the translocation of the PKC δ into the nucleus of EC. These results suggest a mechanism of B cell induced EC apoptosis (Varin, Guerrier et al. 2012).

Subjected to the environment, EC could be modified into non-professional APC, expressing MHC I & II, CD80, CD86 and CD40. CD80 and CD86 proteins are typically expressed by classic APC and are important for the polarization of naïve T-cells. SGEC-expressing CD86 have been shown to present binding properties denoted by the functional interaction with the stimulating CD28-receptor and reduced binding to the negative regulator of immune responses CTLA-4 (Kapsogeorgou, Moutsopoulos et al. 2001). IFN-γ increases the expression of HLA-II by EC, inducing an important role in their shift towards APC. The functional expression of these immunoreactive molecules indicates that SGEC are, in all likelihood, able to mediate the presentation of antigen peptides and the transmission of activation signals to T cells. The expression of CD40 is also induced by IFN-γ and IL-1β in cultured EC showing the effect of cytokines in the activation of EC (Dimitriou, Kapsogeorgou et al. 2002).

Several TLRs are expressed by EC in SG tissues (TLR1, TLR2, TLR3, TLR4, TLR7) (Kawakami, Nakashima et al. 2007; Spachidou, Bourazopoulou et al. 2007; Zheng, Zhang et al. 2010). In cultured HSG cells, a similar expression pattern has been observed, and TLR ligands increased the ICAM-1 expression and the IL-6 production. TLR signalling in SGEC results in the upregulation of MHC-I, CD54/ICAM-I, CD40, and CD95/Fas proteins expression, and in so doing, link the innate and adaptive immune responses (Spachidou, Bourazopoulou et al. 2007). The constitutive expression of functional TLRs and CD91 molecules by cultured EC suggests that they are implicated in the induction of the local immune response.

EC participate in the release of the Ro/SS-A, La/SS-B autoantigenic proteins through the formation of small vesicles called exosomes in T cells and DC (Thery, Zitvogel et al. 2002; Kapsogeorgou, Abu-Helu et al. 2005).

Not only is BAFF produced by EC in culture from SS-affected patients, the level of BAFF is also increased in the serum and saliva of these patients. The expression level of membrane-bound BAFF does not differ significantly from healthy individuals (Daridon, Devauchelle et al. 2007; Pers, Devauchelle et al. 2007). The production of BAFF from EC also influences the disease by altering B cell differentiation and the formation of ectopic germinal center-like structures (Groom, Kalled et al. 2002; Jonsson, Szodoray et al. 2005). TNF-α is another important cytokine produced by EC that is able to up-regulate the Fas receptor (Matsumura, Umemiya et al. 2002). IFN-γ upregulates

the expression of CD40 in EC which increases the susceptibility of these cells to apoptosis (Ping, Ogawa et al. 2005; Kulkarni, Selesniemi et al. 2006). FLT3-L (FMS like tyrosine kinase Ligand), a cytokine implicated in B cell ontogenesis, is expressed by EC (Tobon, Renaudineau et al. 2010) and contributes to B cell survival in SG infiltrates. Furthermore, CXCL12 and IL-6, also produced by EC, improve the chances of survival of infiltrating auto-Abs-producing plasma cells (Szyszko, Brokstad et al. 2011).

4.4.3. Role of T cells in SS

Various studies have demonstrated that T cells are present in the infiltrates. Mainly $CD4^+$ IFN-γ producing Th1 cells and $CD8^+$ T cells were found. $CD4^+$ Th17 memory cells were also described (Nguyen, Hu et al. 2008; Sakai, Sugawara et al. 2008). The T-cell repertoire is similar in lacrimal and SG from pSS patients. In addition, certain TCR variable (V) region genes (Vα2, Vα11.1, Vα17.1, Vβ2 and Vβ13) are predominantly expressed, suggesting a limited heterogeneity of the infiltrating T cells (Sumida, Kita et al. 1994; Matsumoto, Tsubota et al. 1996; Ohyama, Nakamura et al. 1996). Th1 cells produce IFN-γ, TNF-α and IL-2 which regulates cell-mediated immunity by activating macrophages, natural killer cells and $CD8^+$T cells. IFN-γ induces glandular adhesion molecules such as the vascular cell adhesion molecule-1 (VCAM-1), the α4β1 integrin, the peripheral node addressin, the L-selectin, and the LFA-1, which allows the influx of inflammatory cells into glands (Harris, Haynes et al. 2000; Nguyen, Sharma et al. 2009).

Different clinical manifestations in SS are mediated by the hyperactivity of B cells. Therefore, cytokines produced by Th2 cells are important for maintaining the B cell function. The Th2 cells produce a large array of cytokines including IL-4, IL-5, IL-6 and IL-13. $IL\text{-}4^{-/-}$ mice show a restoration in SG secretion despite the presence of a leukocyte infiltration in exocrine glands and the production of autoAbs (Brayer, Cha et al. 2001; Gao, Killedar et al. 2006). IL-4 is also involved in isotype switching mechanisms and promotes the production of pathogenic IgG1 autoAbs (Gao, Killedar et al. 2006).

The production of IL-17A and IL-17F from Th17 cells can induce the proliferation, maturation and recruitment of neutrophils and can also mediate the local inflammatory response (Kastelein, Hunter et al. 2007). TGF-β, IL-6 and IL-23 inducing Th17 differentiation are present in SG (Nguyen, Hu et al. 2008). High levels of IL-17 in serum and saliva have been reported and IL-17-producing T and EC were found in the inflammatory lesions of SS patients (Ito, Hanabuchi et al. 2008; Nguyen, Hu et al. 2008; Sakai, Sugawara et al. 2008; Katsifis, Rekka et al. 2009). Recently, the expression of IL-17 in the disease initiation has been gaining importance. For example, Jin. et al. showed that the conditional expression of IL-17 in mice induces a SS-like syndrome with decreased

salivary production, changes in cytoplasmic/nuclear patterns to homogenous nuclear staining that match anti-Ro/La Abs (Jin, Kawai et al. 2013).

Foxp3$^+$ Treg cells are seen as playing an important role in controlling autoimmunity. The occurrences of Foxp3$^+$ Treg in minor SG lesions in SS were found to be comparable to those in non-SS sialadenitis controls suggesting that the number of Foxp3$^+$ Treg cells is not defective in SS. However, Foxp3$^+$ T cells circulating in the blood inversely correlate with those infiltrating the SG (Christodoulou, Kapsogeorgou et al. 2008). The fact that there are fewer Treg cells in advanced than in mild SG infiltrates supports the view that DC-derived TGF-β induces Foxp3 in naïve T cells and switches T cell differentiation from the defective Treg cell pathway to a Th17 differentiation pathway in the presence of IL-6 (Bettelli, Carrier et al. 2006; Mangan, Harrington et al. 2006).

4.4.4. Role of B cells in SS

The hyperactivity of B cells is one of the main features of SS. B cells have been shown to produce autoAbs, cytokines and to act as APC (Le Pottier, Devauchelle et al. 2009). An increase in Bm2 (CD38$^+$IgD$^+$)/Bm2' (CD38^{++}IgD$^+$) cells and a decrease in early Bm5 (CD38$^+$IgD$^-$) and Bm5 (CD38$^-$IgD$^-$) cells is a characteristic of the disease (Bohnhorst, Bjorgan et al. 2001; Hansen, Odendahl et al. 2002). Together with a decrease of memory B cells in peripheral blood, memory B cells (CD20$^+$, CD27$^+$) are observed in the SGs of pSS (Hansen, Odendahl et al. 2002). This distribution of B cells can act as a potential diagnostic procedure and our group has shown that a high (Bm2+Bm2')/(eBm5+Bm5) ratio (≥5) is strongly correlated with a diagnosis of pSS compared to RA, SLE patients or healthy controls (Binard, Le Pottier et al. 2009). In addition to this abnormal distribution, the membrane expression of CD72, a transmembrane lectin, that is expressed during B cell maturation, and which can both positively and negatively modulate BCR-mediated signalling, is upregulated in B cells from pSS patients (Smith, Gordon et al. 2004).

A high expression of IgA is common among patients and is associated with rheumatoid factor. Moreover, circulating IgA that contain immune complexes are common and associated with abnormal SG biopsy (Bendaoud, Pennec et al. 1991; Basset, Pers et al. 1997). In addition, elevated levels of BAFF, which prevent the apoptosis of autoreactive B cells, are also found in SS patients.

The analysis of SG in SS-affected patients reveals the presence of T1 and T2 B cells, which implies that B cells play a part in the local production of autoAbs (Daridon, Pers et al. 2006). Furthermore, T2 and MZ-like B cells form aggregates that resemble GC. Although these aggregates look like GC, real GC are less common in primary SS since these B cells aggregates lack the GC B cell-associated CD10 and CD38 markers and also devoid of the AID (Le Pottier, Devauchelle et al.

2009; Guerrier, Le Pottier et al. 2012).

A high percentage of patients displays anti-nuclear Abs like anti-SSA/Ro and anti-SSB/La auto-Abs. This is one of the main criteria for identifying the disease (Salomonsson and Wahren-Herlenius 2003).

Elevated levels of BAFF and APRIL can be found in the serum of patients. These high levels are correlated with the titer of autoAbs (Groom, Kalled et al. 2002; Pers, Daridon et al. 2005). B cells in the SG of patients produce and secrete BAFF (Daridon, Pers et al. 2006). BAFF is critical for the survival of B cells in the periphery. BAFF is also involved in the selection of B-cells by promoting transitional B cells' resistance to apoptosis. BAFF acts as a survival factor for human plasmablasts generated from memory B cells (Avery, Ellyard et al. 2005). The prepotency of memory B cells and activated T cells in the SG (Hansen, Lipsky et al. 2007), coupled with the increased serum levels of IL-10 in SS patients (Avery, Ellyard et al. 2005); (Llorente, Richaud-Patin et al. 1994; Szodoray, Alex et al. 2005), could provide a favorable environment for the production of autoAb-producing plasmablasts.

The local production of BAFF contributes to deleterious effects on activated B cells by raising their expression of CD19 molecules (Hase, Kanno et al. 2004), thus ensuring the survival of B cell aggregates, and Ab isotype-switching both inside and outside GC (Le Pottier, Devauchelle et al. 2009). SS patients are prone to B cell malignancies (Szodoray, Alex et al. 2005). In B cell malignancies, patients show increased serum BAFF levels. Therefore, increased BAFF levels may conduce to B cell malignancy in SS patients. Moreover, BAFF-transgenic mice do not develop anti-SSA/Ro and anti-SSB/La auto-Abs.

4.4.5. Role of BAFF in SS

The implication of BAFF as one of the major cytokines contributing to the pathogenesis of pSS was shown in BAFF-transgenic mice. Mice overexpressing BAFF develop various clinical features of SS, such as inflammation of SG (Mackay, Woodcock et al. 1999). BAFF levels are higher in the serum of SS patients. These high levels are associated with increased production of autoAbs such as anti-SSA, anti-SSB, and rheumatoid factor (Mariette, Roux et al. 2003; Pers, Daridon et al. 2005). Moreover, higher levels of BAFF are present in the SG of diseased patients. Not only is BAFF produced by monocytes, DC and macrophages, it is also produced by B cells and EC which suggests that EC act as a contributor that promotes over-activation of the immune system in pSS (Daridon, Devauchelle et al. 2007; Ittah, Miceli-Richard et al. 2008).

Furthermore, most lymphomas associated with SS find their origin in B cells,

indicating that BAFF plays a role in the pathogenesis of SS (Mavragani, Moutsopoulos et al. 2006). SS patients with lymphoma have higher levels of BAFF in their serum than patients without lymphoma (Gottenberg, Seror et al. 2013; Quartuccio, Salvin et al. 2013). This difference shows the importance of BAFF in the pathogenesis of the disease.

The survival and maturation of B cells depend on the balance between survival signals and death signals. If survival signals -induced by BAFF- are able to counterbalance the pro-apoptotic signals sent by the BCR, then the result will be the survival of B cells. But, overexpression of BAFF may interfere with this equation. Thus, B cells might be able to survive to stronger death signals triggered by autoantigens, which would then lead to the formation of autoreactive B cells. Thus, the local expression of BAFF by EC, by infiltrating T cells and macrophages, could trigger B cell hyperactivation and autoAb production. This BAFF-mediated survival is also evident in the peripheral blood B cells of SS patients. Significantly fewer occurrences of apoptosis were found in the Bcl-2/Bax positive B cell population of SS patients, hinting at the anti-apoptotic and extended survival effects of BAFF in SS patients (Szodoray, Alex et al. 2005). BAFF and the BAFF-receptor are important for the transition of T1 B cells to T2 B cells and for their further maturation. BAFF transgenic mice show an excess of MZ B cells with a high BAFF receptor. The excess of T2 and MZ B cells has also been observed in the SG of SS patients (Mackay, Woodcock et al. 1999; Daridon, Pers et al. 2006).

BAFF is important for the formation of ectopic germinal centers and for setting up follicular DC networks which are capable of retaining immune complexes in the SG of SS patients (Rahman, Rao et al. 2003; Vora, Wang et al. 2003).

IL-6 is one of the cytokine that influences the pathogenesis of SS by participating in the generation and function of Th17 cells (Hsu, Yang et al. 2008; Youinou and Pers 2011). In pSS, the production of IL-6 is partly mediated by BAFF. BAFF produced by monocytes acts in an autocrine fashion to induce the production of IL-6. This BAFF mediated production of IL-6 by monocytes from pSS patients requires the interaction between BAFF and the BAFF-receptor (Yoshimoto, Tanaka et al. 2011).

BAFF also mediates the deleterious effects of activated B cells by upregulating their CD19 expression. SS patients show an increase in naïve Bm2/Bm2' cells in their blood with increased CD19 molecules (Sato, Hasegawa et al. 2000; d'Arbonneau, Pers et al. 2006; Le Pottier, Devauchelle et al. 2009) (Figure 11).

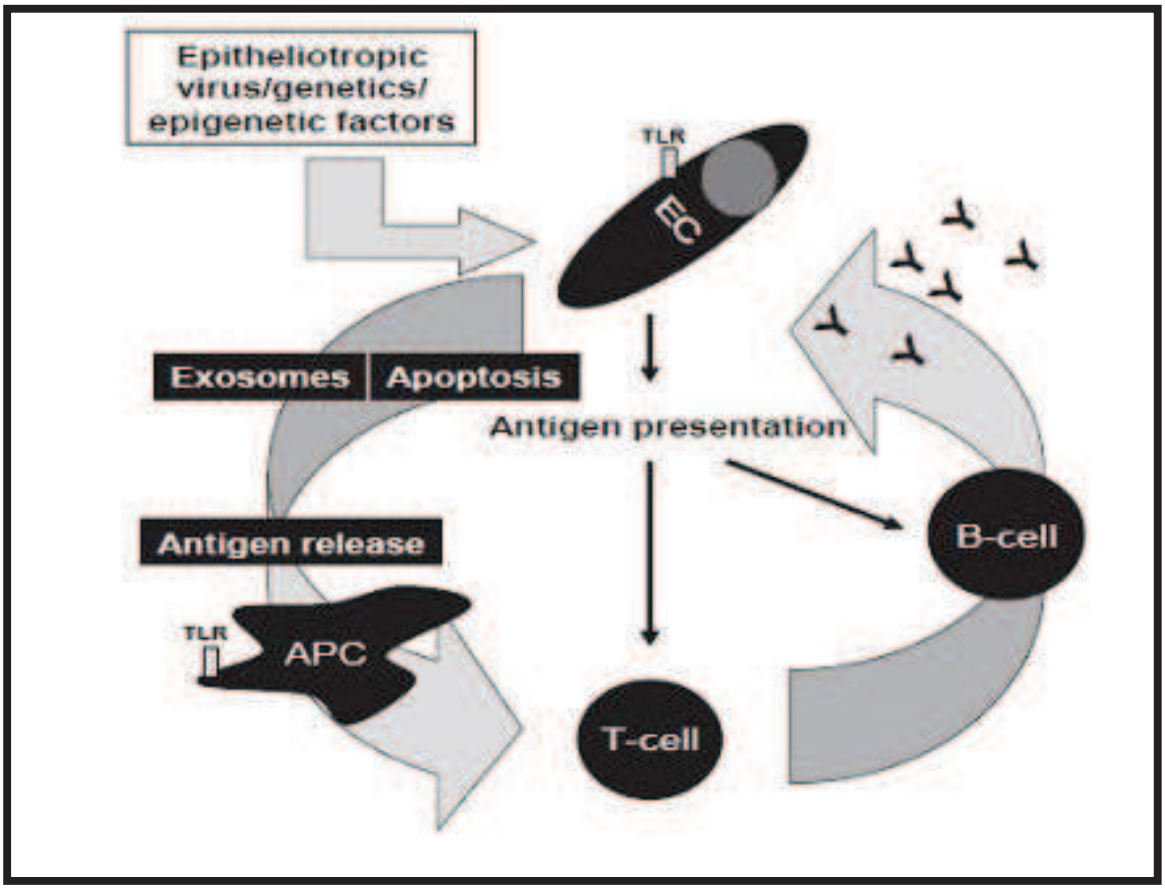


Figure 11 **Pathogenic model of lymphoepithelial lesions in the salivary glands of patients with primary Sjögren's syndrome.**

A vicious circle of aberrant activation of epithelial cells (EC), persistant 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, vesicules that contain intracellular antigens. These vesicules may be captured by antigen-presenting cells, and subsequently the activation of T- and B-cell. An exogeneous infectious agent, such as an epitheliotropic virus may be responsible for the chronic activation of epithelium.

II. ARTICLES

1. SPECIFIC FORMS OF BAFF FAVOR BAFF RECEPTOR-MEDIATED EPITHELIAL CELL SURVIVAL

SS is a slowly progressive, chronic inflammatory process affecting middle-aged persons. It is primarily characterized by the inflammation of the exocrine glands. The disease can appear alone or can be expressed with other systemic disorders. EC play an important role in the pathogenesis of the disease and can present auto-antigens to T and B cells. The B cell activating factor belonging to the TNF family (BAFF) is produced by infiltrating B and T cells in SS patients. An increased level of BAFF is associated with autoantibody production, B cell tolerance breakdown and abnormal distribution of B cell subsets in patients. Among the three BAFF receptors, BR3 is the most specific and the interaction between BAFF and BR3 leads to B cell survival. In this study, our objective was to evaluate the presence of BR3 on EC and the functional outcome of BAFF and BR3 interaction in these cells. We observed that EC from the SG of SS patients express BAFF and BR3 but none of the other BAFF receptors. The HSG cell-line cells resemble the EC of diseased patients and controls, and also express BAFF and BR3. To further evaluate the role of BAFF and BR3, HSG cells were incubated with anti-BR3 blocking antibody and human recombinant BAFF. Blocking BR3 decreased the EC proliferation and induced apoptosis, suggesting that BR3 plays a role in EC survival. However, human recombinant BAFF did not show any significant effect on EC proliferation or apoptosis. To understand the role of BR3 on EC survival, a BR3 specific siRNA inhibitor was used. A decreased survival of HSG cells was observed upon siBR3 transfection, confirming the role of BR3 in EC survival. This survival is PKCδ-dependent because blocking BR3 causes PKCδ to translocate to the nucleus of EC. Furthermore, the neutralization of BAFF with polyclonal rabbit anti-BAFF antibody and mouse monoclonal anti-BAFF antibody leads to different results. The neutralization of BAFF by polyclonal rabbit anti-BAFF antibody reduces the survival of EC, whereas its neutralization with monoclonal anti-BAFF antibody did not show any effect on cell survival. Consequently, some but not all forms of BAFF are involved in EC survival. To investigate this fact, we found that rabbit anti-BAFF Ab can recognize two forms of BAFF produced by EC at 21kDa and at 17kDa, whereas mouse monoclonal anti-BAFF Ab only recognizes the 21kDa form. These results suggest that many forms of BAFF are produced by EC but only few forms take part in EC survival by binding to BR3. Altogether, these results show the importance of BR3 in EC survival. In addition, the effects of the interaction between BR3 and specific BAFF forms are essential for the BR3-mediated survival of EC.

SPECIFIC FORMS OF BAFF FAVOR BAFF RECEPTOR-MEDIATED EPITHELIAL CELL SURVIVAL

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ABSTRACT

Although B cell activating factor (BAFF) and its receptor BR3 are produced and expressed by many cells, their role has been restricted to the lymphocyte lineage. Using various techniques (RT-PCR, indirect immunofluorescence, flow cytometry analysis), we observed the expression of BR3 and the production of BAFF by the human salivary gland cell line, by EC from biopsies of SS patients and their controls, but also by salivary gland EC in culture. To decipher the role of BAFF and BR3 on EC, BAFF and BR3 were neutralized by blocking antibodies or RNA specific inhibitor (siBR3) and epithelial cell survival was analyzed. Blocking BR3 promotes epithelial cell apoptosis *in vitro*. This apoptosis resulted in the nuclear translocation of PKCδ. BAFF neutralization by various anti-BAFF antibodies leads to different effects depending on the antibody used suggesting that only some forms of BAFF are required for epithelial cell survival. Our study demonstrates that BR3 is involved in the survival of cultured EC due to an autocrine effect of BAFF. It also suggests that EC produce different forms of BAFF and that only some of them are responsible for this effect.

Keywords: BAFF, BR3, epithelial cells, salivary glands, Sjögren's Syndrome, protein kinase C δ, glycosilation.

INTRODUCTION

Moutsopoulos defines SS as an auto-immune epithelitis (Youinou 2010) and considers the activation of epithelial cell (EC) as the main immune-pathologic process in the development of SS. ECs express type II HLA in the presence of INFγ (Giroux, Schmidt et al. 2003), thus constituting a pool of antigen-presenting cells (Germain 1994) that are able to present auto-antigens to T and B cells. In SS, ECs produce IFNγ (Hayashi, Arakaki et al. 2009), which in turn has been described to promote the B cell activating factor belonging to the TNF family (BAFF) production (Litinskiy, Nardelli et al. 2002). BAFF was identified as essential for the development and differentiation of B cells (Schneider, MacKay et al. 1999; Huard, Arlettaz et al. 2004). It interacts with three different receptors, BR3 (or BAFF-R), the transmembrane activator and CAML interactor (TACI) and the B-cell maturation antigen (BCMA) (Vincent, Saulep-Easton et al. 2013).

BAFF is produced in lymphoid organs by several cell types (Schneider, MacKay et al. 1999) such as monocytes, macrophages, dendritic cells, T cells and neutrophil polynuclear cells (Nardelli, Belvedere et al. 2001; Huard, Arlettaz et al. 2004). BAFF synthesis can be induced by cytokines such as interferon (IFN)α, IFNγ, interleukin (IL)-10 and CD40L (Litinskiy, Nardelli et al. 2002). Follicular dendritic cells are also a potential source of BAFF (Hase, Kanno et al. 2004) and a weak production of BAFF was observed by activated T cells (Huard, Arlettaz et al. 2004) providing co-stimulatory signals for B-cell selection. Furthermore, bone marrow stromal cells massively express BAFF in order to maintain B cell homeostasis (Gorelik, Gilbride et al. 2003). Finally, production of BAFF is up-regulated in several pathologies. Indeed, BAFF is highly produced by B cells from chronic lymphocytic leukemia patients (Kern, Cornuel et al. 2004) and increased amounts of soluble BAFF have been detected in the serum of patients with myeloma (Moreaux, Legouffe et al. 2004). Likewise, increased concentrations of BAFF were observed in the serum of patients with auto-immune diseases (AID), such as SLE, RA and SS (Pers, Daridon et al. 2005). In SS, infiltrating B and T cells produce BAFF (Daridon, Devauchelle et al. 2007), and more surprisingly, astrocytes in multiple sclerosis secrete BAFF (Krumbholz, Theil et al. 2005).

Clearly, BAFF is essential for the survival of B cells because of its interaction with BR3. (Thompson, Bixler et al. 2001). BR3 signalling activates PI3K as well as non-canonical NF-κB signalling in B cells. Following BAFF engagement, BR3 induces the recruitment of TNFα-associated factor (TRAF)2 and TRAF3, leading to the release of NF-κB-inducing

kinase (NIK). Consequently, NIK phosphorylates IKK1 leading to the processing of p100 into p52 and to the activation of non-canonical NF-κB pathway which then results in B cell survival (Claudio, Brown et al. 2002). BR3 pathway is negatively regulated by Act-1 that has binding sites to TRAF molecules and inhibits TRAF recruitment (Qian, Qin et al. 2004). BR3 stimulation has also been linked to the negative regulation of PKCδ (Mecklenbrauker, Kalled et al. 2004). PKCδ is a target for caspase 3 cleavage that generates an active form of the kinase that operates in the nucleus and contributes to apoptosis (DeVries-Seimon, Ohm et al. 2007). However, nuclear substrates for this pro-apoptotic function of PKCδ have not been identified.

Among BAFF receptors, BR3 is the most specific, mainly expressed by transitional and mature B cells. In SS, we previously observed that BR3 was present on infiltrating B cells but not on T cells (Daridon, Devauchelle et al. 2007). Regulation of the BAFF/BR3 axis in B cells is crucial to prevent autoimmune manifestations (Varin, Le Pottier et al. 2010) . BAFF overexpression in mice transgenic for BAFF, promotes autoimmune-like manifestations such as systemic lupus erythematosus and SS in the presence of high levels of anti-ssDNA and anti-dsDNA autoantibodies, circulating immune complexes, and immunoglobulin deposition in the kidneys. These mice have also vastly increased numbers of mature B cells with high proportion of MZ B cells (Schneider, MacKay et al. 1999).

Act-1 functions as a negative regulator of CD40- and BAFF-mediated B cell survival (Qian, Qin et al. 2004). Mice deficient in Act-1 developed also systemic autoimmune disease with histological and serological features of human SS, in association with systemic lupus erythematosus-like nephritis (Qian, Giltiay et al. 2008). Histological analyses revealed profound lymphocyte infiltration in lacrimal, parotid and submaxillary glands. The majority of the infiltrated B cells displayed a phenotype resembling MZ-like B cells. High titers of anti-SSA/Ro and anti-SSB/La in association with anti-ssDNA and anti-dsDNA were detected in sera of Act-1 deficient mice. These two mouse models emphasize the pivotal role of the BAFF/BR3 axis in B cell tolerance.

Meanwhile, we also demonstrated that BR3 was expressed by ECs without being able to explain the reason for this expression. The aim of the present study was to decipher the role of BAFF and BR3 on ECs in SS.

MATERIAL AND METHODS

Patients and cell line

Salivary gland (SG) biopsies were obtained from 18 patients (3 men and 15 women; ages 32-77 years) fulfilling the American-European Consensus Group criteria (Vitali, Bombardieri et al. 2002) for SS. All had a focus score ≥ 1. Control samples consisted of 15 SG specimens from patients who did not meet the criteria for primary SS (4 men and 11 women: ages 39-74 years), but they had presented sicca symptoms and, as such, had undergone a SG biopsy. All SS patients and controls gave their consent and the study was approved by the Brest CHRU Ethics Committee. To summarize, sections were cut into small fragments and incubated in Supplemented Basal Epithelial Medium (SBEM). SBEM medium contains three volumes of Ham's F12 medium (Invitrogen), one volume of Dulbecco's Modified Eagle Medium (DMEM) (Lonza, Verviers, Belgium), 2.5% of Fetal Calf Serum (FCS) (Eurobio, Courtaboeuf, France), 2 mM of L-glutamine, 10 ng/ml of EGF (Epidermal Growth Factor) (Promega, Madison, WI, USA), 0.5 µg/ml of insulin (Novo-Nordisk, Küsnacht, Switzerland) and 0.4 µg/ml of hydrocortisone (Sigma-Aldrich, Saint-Quentin-Fallavier, France). Cells were incubated at 37°C with 5% CO_2. The HSG cell line was incubated in DMEM, supplemented with 10% FCS, 2mM L-glutamine (Gibco, Invitrogen, Auckland, New Zealand), 1% non-essential amino acids (Sigma-Aldrich, St Louis, MO), 100 IU/ml penicillin (Panpharma, Fougères, France) and 100 µg/ml streptomycin (Panpharma).

Detection of BAFF and its receptors

Total RNA was extracted by the RNAble® method (Eurobio, Les Ulis, France) according to the supplier's instructions. One µg of total isolated RNA was converted to cDNA using SuperScript II reverse transcriptase (Invitrogen), according to the manufacturer's instructions. PCR was performed using GoTaq polymerase (Promega) under the following conditions: initial denaturation at 94°C for 5 mins, followed by 5 cycles at 94°C for 30 secs, 1 min at 61°C and 1 min at 72°C, then 40 cycles at 94°C for 30 secs, 40 secs at 56°C and 1 min at 72°C, and finally a final extension at 72°C for 10 mins. PCR products were separated on a 2% agarose gel (Interchim, Montluçon, France) containing GelRed™ Nucleic Acid gel Stain (Interchim) and analyzed using Quantity One® software (version 4.6.3, Biorad, Marnes-la-Coquette, France). Primers used for PCR are : GAPDH (5'-CTTAGCACCCCTGGCCAAGG-3' and 5'-CTTACTCCTTGGAGGCCATG-3'), BAFF (5'-TTGCAGACAGTGAAACA-CCAACT-3'and 5'TTCATCTCCTTCTTCCAGTTTTGC-3'),

BR3 (5'-CTGGTCCTGGTGGGTCTG-3'and 5'-TCTTGGTGGTCACCAGTTCA-3'), TACI (5'-AGTGGCCTGGGCCGGAG-3' and 5'-CTCCTTGCGGCAGC-TGAGTGAC-3'), BCMA (5'-CTCCTCTAACATGTCAGCGTTATTGTA-ATG-3'and 5'-GTCAATGTTAGCCATGCCCAGGGA-3').

OCT-embedded (Miles, Naperville, IL) SG biopsies were snap-frozen in isopentane (Sigma-Aldrich). 4µm-thick cryostat sections were cut from the blocks and mounted onto poly-L lysine-coated slides (Thermo Scientific, St Herblain, France). The slides were then incubated for 40 mins at room temperature with a rabbit anti-BAFF Ab (Upstate Lake Placid, NY) alone and with fluorescein isothiocyanate (FITC)-conjugated mouse anti-cytokeratin (CK) 18 Ab (Sigma-Aldrich), in combination with either a rabbit anti-BR3 Ab (ProSci, Poway, CA) or a rabbit anti-CD20 Ab (Thermo Scientific). After three washes in PBS, the slides were incubated for another 40 minutes with FITC-conjugated polyclonal donkey anti-rabbit IgG Ab (Jackson ImmunoResearch, West Grove, PA) or tetramethyl rhodamine isothiocyanate (TRITC)-conjugated donkey anti-rabbit IgG Ab (Jackson ImmunoReserarch), in PBS supplemented with 2% donkey serum (Sigma-Aldrich). After five washes, the sections were fixed with 4% cold paraformaldehyde (Sigma-Aldrich) and analyzed with the TCS-NT Leica confocal imaging system (Leica Microsystems, Wetzlar, Germany). FITC-conjugated donkey anti-rabbit IgG Ab and TRITC-conjugated donkey anti-rabbit IgG served as negative controls and did not show any fluorescence.

HSG cells were incubated with a mouse anti-BAFF mAb (R&D System, Minneapolis, MN), a rabbit anti-BR3 Ab, a goat anti-TACI Ab (Peprotech, Rocky Hill, NJ) or a goat anti-BCMA Ab (R&D Systems) for 40 mins at room temperature. After 3 washes in PBS, the cells were incubated with a FITC-conjugated donkey anti-mouse IgG Ab, a TRITC-conjugated donkey anti-rabbit IgG Ab or a TRITC-conjugated donkey anti-goat IgG Ab (Jackson ImmunoResearch) for 30 mins at room temperature. Cells were then analyzed by confocal microscopy. For PKCδ localization, HSG cells were stained with a mouse anti-PKCδ mAb (BD Biosciences, Franklin Lakes, NJ) revealed by a FITC-conjugated donkey anti-mouse IgG Ab (Jackson ImmunoResearch). Cell nuclei were labeled with propidium iodide (PI) for 20 minutes at 4°C. After 3 washes in PBS, HSG cells were observed by confocal microscopy.

After trypsination and washing at 1200 rpm for 10 mins, HSG cells or ECs purified from SG biopsies were incubated with a mouse anti-BAFF mAb, a rabbit anti-BR3 Ab, a goat anti-TACI Ab or a goat anti-BCMA Ab, for 30 mins at 4°C. After 3 washes in PBS, stainings were

revealed with a FITC-conjugated donkey anti-mouse IgG Ab, a FITC-conjugated donkey anti-rabbit IgG or with a FITC-conjugated donkey anti-goat IgG (all from Jackson ImmunoResearch) for 30 mins at 4°C. Corresponding FITC-conjugated isotypes were used as controls and cells were analyzed by flow cytometry (EPICS® XL-MCL, Coulter). Antibodies used for immunofluorescence and flow cytometry analyses are shown in Table 2.

Cell stimulation

HSG cells were removed from the flask using trypsin (PAN-biotech GmbH, Aidenbach, Germany), washed in PBS and incubated on 10-well slides (Thermo Scientific, Pittsburg, PA) the day before the experiment. They were stimulated for 24 hours with a rabbit anti-BR3 (from 1 to 20 µg/ml) Ab or with human recombinant BAFF (hrBAFF, Immunotools, Friesoythe, Germany) (from 25 to 1000 ng). A positive control for apoptosis was used by stimulating cells with 200 µM etoposide (Teva, Paris la Défense, France). The viability of HSG cells was assessed by flow cytometry using FITC-labeled annexin V and PI (Immunotech, Beckman Coulter, Marseille, France).

The day before the experiment, primary ECs purified from SG biopsies were trypsinated, washed in PBS and seeded into a 96-well flat-bottomed culture plate (Nunc, Roskilde, Denmark). ECs were stimulated for 24 hours with 20 µg/ml mouse anti-BAFF Ab or rabbit anti-BAFF Ab. As a negative control, ECs were incubated with mouse (Immunotech, Marseille, France) or rabbit (SouthernBiotech, Birmingham, AL) IgG isotypes. In order to visualize proliferating cells, HSG cells were incubated with a FITC-conjugated mouse anti-Ki-67 mAb (Dako, Glostrup, Denmark) and observed by confocal microscopy.

TdT-mediated dUTP-biotin nick end labelling (TUNEL) assay

After 24 hours of stimulation, ECs were cytospined on glass cover ships and labeled with the TUNEL kit (MEBSTAIN Apoptosis kit II, Immunotech) according to the supplier's instructions. In brief, cells were fixed for 15 minutes at 4°C with 0.1 M NaH_2PO_4 pH 7.4 solution containing 4% PFA. Then, ECs were permeabilized for 15 mins at room temperature with 0.5% PBS-T (Tween 20 and 0.2% BSA). After three washes in distilled water, cells were incubated with TdT solution for 1 hour at 37°C, washed and further incubated for 10 minutes with a blocking solution. Finally, the avidin-FITC solution was incubated for 30 mins, at room temperature, and after 3 washes in PBS, nuclei were labelled with PI for 20 mins at 4°C. The slides were mounted with Vectashield (Vector Laboratories, Burlingame, CA) and analyzed by confocal microscopy.

Cell transfection with siRNA

Short interference RNAs (siRNAs) of BR3 and positive and negative controls (Applied Biosystems, Ambion, Austin, TX) were coupled with FAM fluorescein using the Silencer siRNA labeling kit (Applied Biosystems) according to the supplier's instructions. In brief, 5 µg of 20 µM siRNA were incubated with a labeling buffer, FAM labeling reagent and nuclease-free water for 1 hour at 37°C, in the dark. That mix was then precipitated with 0.1 volume of 5 M NaCl and 2.5 volumes of absolute ethanol (Carlo Erba Reagents, Rodano, Italy) for 1 hour at -20°C. After centrifugation at 14000g for 20 minutes at 4°C, the supernatant was removed and the siRNA pellet was washed with 70% ethanol by a 5 minute centrifugation at 14000g at 4°C. The supernatant was removed and the pellet was dried at room temperature for 10 minutes then re-suspended in nuclease-free water to reach a final concentration of 20 µM.

2.10^4 cells from the HSG cell line were cultured the day before the experiment in a 96-well plate at 37°C in 5% CO_2 atmosphere. Specific siRNAs for BR3 or a control siRNA were incubated for 15 mins with FuGENE HD transfection reagent (Promega) and OptiMEM medium (Gibco) at 3:1 ratio (3 µl FuGENE HD for 1 µg siRNA). FuGENE HD/siRNA mix was then added to the cells. Cell survival was evaluated by flow cytometry after PI labeling. In order to strenghten BR3 gene extinction by siRNA, HSG cells were co-transfected with three different BR3 siRNAs (s225507, s41837, s41838, Ambion Life technologies).

BAFF forms analysis byWestern-blotting

Supernatants of primary EC cultures were concentrated with the Microcon® centrifugal filter device kit (Millipore, Bellerica, MA, USA) before analysis by 13% SDS-PAGE. After electro-blotting (Bio-Rad, Marnes-la-coquette, France), the PVDF membrane was saturated for 1 hour at room temperature with 5% non-fat dry milk. Two primary anti-BAFF Abs were used for staining: mouse anti-BAFF mAb at 1 µg/mL and rabbit anti-BAFF pAb at 2 µg/mL that were revealed with a goat anti-mouse IgG-horseradish Peroxidase (GE Healthcare, Velizy-Villacoublay, France) and a donkey anti-rabbit IgG-horseradish Peroxidase (GE Healthcare), respectively. Membranes were visualized by chemiluminescence using ECL advance Western blotting detection kit (GE Healthcare).

Statistics

All results are expressed as the mean ± standard deviation. Comparisons were made using the Mann-Whitney U test for unpaired data.

RESULTS

Epithelial cells express BAFF and BR3

BAFF expression was observed in the SG biopsies of the 18 SS patients we tested but also in those from the 15 tested control subjects (Figure 1A). Immunofluorescence revealed intense BAFF staining on ductal ECs but also on lymphocyte infiltrating cells in SS patients. The expression of BAFF on the plasma membrane of ECs suggests that these cells produce BAFF, although it may be possible that BAFF has been inserted into its receptor. Thus, we explored the expression of *BAFF* and *BAFF receptors* by RT-PCR in cultured ECs from healthy controls and SS patients. Transcripts for *BAFF* were found both in cultured EC from healthy controls and from SS patients. Among *BAFF receptors*, only *BR3* transcripts were detected (Figure 1B). The presence of BR3 at the protein level was also demonstrated by immunofluorescence on ductal ECs from SG biopsies in controls (Figure 1C, left panel) and SS patients (Figure 1C, right panel) and on infiltrating B cells from SS patients (Figure 1C, middle panel).

To further study the role of BR3 on ECs, we decided to use the HSG cell line after having confirmed that BR3 was also expressed. Using flow cytometry and immunofluoresence analyses, we demonstrated that the HSG cell line displayed the same characteristics, i.e. the expression of BAFF and BR3 on the cell membrane and the absence of TACI and BCMA (Figure 1D).

Blocking BR3 promotes EC apoptosis in vitro

In order to identify the role of BR3 on EC, HSG cells were incubated with hrBAFF or a blocking anti-BR3 Ab for 24 hours. EC proliferation (Ki-67 labeling) and apoptosis (TUNEL method) were evaluated. Increasing concentrations of hrBAFF had no effect on either the EC proliferation (Figure 2A) or on apoptosis (Figure 2B). However, blocking BR3 with increasing concentrations of anti-BR3 Ab induced a strong reduction in HSG cell proliferation (Figure 2A) and an increase in HSG cell apoptosis (Figure 2B). The controls, i.e. HSG cells treated with etoposide, showed absence of proliferation and strong apoptosis.

PKCδ has been implicated in the regulation of apoptotic cell death in ECs (Matassa, Kalkofen et al. 2003). Also, we have recently described that PKCδ translocation inside the nucleus was associated with EC apoptosis in SGs from SS patients (Varin, Guerrier et al. 2012). Thus, we analyzed PKCδ location in HSG cell line incubated with increasing concentrations of anti-

BR3 Ab. BR3 blockade resulted in PKCδ translocation from the cytoplasm to the nucleus (Figure 3), in accordance with the role of PKCδ in EC apoptosis.

To confirm the importance of BR3 in EC survival, HSG cells were transfected with BR3 specific RNA inhibitor (siBR3). The survival of siBR3-transfected cells decreased over time (Figure 4A), compared to HSG cells transfected with the control siRNA (32.0±4.3% vs. 68.1±7.2%, respectively at 72 hours, $p<0.05$). All these results demonstrate the importance of BR3 in the survival of ECs.

Effect of BAFF neutralization on EC survival

To observe the impact of BAFF/BR3 interaction on EC survival, BAFF was neutralized by incubating ECs from healthy controls with a saturating concentration of different anti-BAFF Abs (Figure 4B). Whereas the mouse monoclonal anti-BAFF Ab had no effect (75.4±9.2% of survival vs. 73.5±5.3% of survival in medium), the blockade of BAFF with the polyclonal rabbit anti-BAFF Ab decreased the survival of ECs (31.9±14.0% of survival, $p<0.05$).

Some forms of BAFF are involved in the survival of epithelial cells

We were wondering whether different forms of BAFF were produced by ECs and whether one of these forms preferentially promoted EC survival. The supernatants of ECs were analyzed by Western-blot (Figure 4C). Unlike the mouse anti-BAFF mAb which recognizes only the 21 kilodalton (kDa) form of BAFF, the polyclonal rabbit anti-BAFF Ab also recognizes a supplementary form of BAFF at 17 kDa. Taken together with the above results, these data suggest that only the 17kDa forms of BAFF participate to EC survival by binding to BR3.

DISCUSSION

We had already observed that the majority of ELISA kits available for BAFF were not able to recognize all the forms of BAFF in the serum (Le Pottier, Bendaoud et al. 2009). We indeed observed that only some polyclonal Ab could recognize the 2 forms of BAFF at 28 and 21 kDa, whereas the monoclonal mouse Ab only recognized the 28 kDa glycosylated form. The importance of the glycosylation status has also been described by Shu et al. (Shu, Hu et al. 1999) and Tribouley et al. (Tribouley, Wallroth et al. 1999), who observed several BAFF isoforms with different degrees of glycosylation. These authors had already considered the consequences in terms of oligomerization and receptor-binding affinity. However, this is the first time that a different functional role is described for one particular form of BAFF. A more detailed analysis of the functional 17 kDa form of BAFF is needed to better understand the importance of post-translational modifications in the survival effect that we observed. In this context, it will be determinant to determine what are the forms of BAFF recognized by antibodies used in anti-BAFF immunotherapies. Three inhibitors have been recently used in systemic lupus erythematosus and rheumatoid arthritis: belimumab, tabalumab and atacicept. Belimumab (Benlysta®) is a recombinant fully humanized IgG1-λ monoclonal Ab that blocks the binding of soluble BAFF to its receptors. BAFF forms recognized by belimumab are soluble homotrimers and oligomers of BAFF (60 mers) (Fairfax, Mackay et al. 2012). Tabalumab (LY2127399) is a fully humanized monoclonal Ab that was designed to have neutralizing activity against both membrane-bound and soluble BAFF (Vincent, Saulep-Easton et al. 2013). Tabalumab recognizes soluble homotrimers and oligomers of BAFF but also membrane-bound BAFF. Atacicept is a chimeric recombinant fusion protein comprising the extra-cellular domain of TACI linked to a human IgG1 Fc domain (Fairfax, Mackay et al. 2012). Atacicept could completely block the BAFF/APRIL (a proliferation-inducing ligand) system. APRIL can form with BAFF active heterotrimers (Roschke, Sosnovtseva et al. 2002) which are also recognized by atacicept (Dillon, Harder et al. 2010). This inhibitor could also link homotrimers of BAFF (soluble and membrane-bound), oligomers of BAFF and homotrimers between BAFF and APRIL. Nevertheless, recognition tests for these inhibitors always used ELISA calibrated with recombinant protein and no information is provided regarding glycosylated isoforms binding. It would be more interesting to test these inhibitors by Western blot using sera from patients with autoimmune diseases and healthy volunteers.

BAFF/BR3 interactions leading to EC survival seem to maintain PKCδ outside the nucleus (Figure 3). This pathway has already been described in B cells (Mecklenbrauker, Kalled et al. 2004) where B cell treatment by BAFF prevented PKCδ nuclear localization. This PKCδ is already known as a common intermediate agent for EC apoptosis induced by several drugs (Matassa, Carpenter et al. 2001). The location of PKCδ in the nucleus is necessary for the induction of apoptosis in EC and, under normal conditions, active regulation of the nuclear presence of PKCδ is essential for survival (Reyland 2007). Furthermore, our results have shed light on our previous observations on EC apoptosis induced by co-culture with B cells (Varin, Guerrier et al. 2012). EC apoptosis was also associated with PKCδ translocation into the nucleus. This PKCδ activation was associated with histone H2B phosphorylation on Ser 14 and PARP cleavage. Recently, Park and Kim demonstrated that PKCδ have an other effect on chromatine during apoptosis. PKCδ robustly phosphorylates histone H3 on Ser 10 and expression of catalytically active PKCδ efficiently induces condensed chromatine structure in the nucleus. Collectively, these findings suggest that PKCδ is the kinase responsible for H3 Ser-10 phosphorylation during apoptosis and thus contributes to chromatin condensation together with other apoptosis-related histone modifications (Park and Kim 2012).

Our previous results described an aberrant expression of BAFF in salivary glands of patients with SS (Daridon, Devauchelle et al. 2007). How do ECs undergo apoptosis in salivary glands? Our hypothesis is that infiltrated B cell expressing BR3 could likely compete with EC to capture BAFF. We have already described that infiltrated B cells in salivary glands in SS are phenotypicaly transitional type 2 and MZ-like B cells (Le Pottier, Devauchelle et al. 2009). These subsets of B cells highly express BR3 and require high levels of BAFF for their survival. This hypothesis is in accordance with observations showing that B cell infiltration increases with the severity of tissue lesions (Christodoulou, Kapsogeorgou et al. 2010). Therefore, B cell-induced EC apoptosis could likely be due to a defective signal received by BR3 on EC, leading to PKCδ activation and its nuclear localization.

It is interesting to note that BR3 has recently been detected in microtubule-associated protein 2-positive primary cultured neurons, spinal cord motor neurons and Neuro2a cells, a mouse neuroblastoma cell line (Tada, Yasui et al. 2013). This study revealed that both BAFF and BR3 are expressed on neuronal cells and play a role in neuronal survival. BR3 signals on neurons also appear to be necessary for neuroprotection *in vivo*. BR3 was also already observed on mammary gland ECs during gland involution at the end of lactation (Jung, Bong

et al. 2004). This post-lacteal regression is also accompanied by an acute inflammatory response and authors described an increase in the production of IFNγ and BAFF. The role of BAFF and BR3 was however not demonstrated. This stage is characterized by massive EC apoptosis and tissue remodeling (Strange, Li et al. 1992). Tissue involution is a postnatal process that allows to study physiological cell death. The first stage of this process, starting immediately after weaning, is marked by increased pro-apoptotic factors and can be reversed by re-induction of suckling. So, this mechanism of involution is closely regulated. A recent study demonstrated that PKCδ is involved in EC apoptosis during mammary gland involution (Allen-Petersen, Miller et al. 2010). In this study, they compared early involution between wild-type mice and PKCδ-deficient mice. When PKCδ is deficient, mammary gland involution is delayed. Nevertheless, in view of our findings, transient expression of BAFF could represent a mechanism for the regulation of EC apoptosis during mammary gland involution. Further careful studies may be required to determine the potential role of BAFF-BR3 axis in organs other than the immune system.

ECs express Toll-like receptors (TLRs) especially TLR2, TLR3 and TLR4 in SS (Kawakami, Nakashima et al. 2007). ECs can thus be activated by lipopeptides from gram-positive bacteria, dsRNA from viruses or LPS from gram-negative bacteria, respectively (Guerrier, Le Pottier et al. 2012). ECs from salivary glands highly expressed TLR3 suggesting that they may be strikingly sensitive to stimulation by virus PAMP. Thus, activation of ECs after TLR3 stimulation with the synthetic analogue of viral dsRNA poly-inosinic acid (poly I:C) induces secretion of chimiokines (Li, Jeong et al. 2010) allowing the recruitment of lymphocytes and the production of IL-6 and IFN-β (Manoussakis and Kapsogeorgou 2010). Moreover, *in vitro* stimulation of TLR-3 by poly I:C induced EC to express and secrete high levels of BAFF. Apart from the induction of activation molecules, TLR3 triggering was also found to induce severe detachment of salivary gland ECs from substrate and subsequent induction of apoptosis, a phenomenon suggestive of anoikis (Manoussakis, Spachidou et al. 2010). TLR3 induced EC apoptosis is mediated *via* the PI3K-Akt signalling pathway and induced caspase 3 cleavage (Nakamura, Horai et al. 2013).

CONCLUSION

Our study demonstrates for the first time that BR3 plays a role in the survival of ECs *in vitro*. It also shows the importance of some forms of BAFF (17kDa) in the functional effects we

observed. In addition, the expression of BR3 on ECs was not related to SS since we also detected it in ECs from healthy controls. Finally, our results suggest unexpected effects on the use of anti-BAFF immunotherapy for SS treatment. Indeed, in addition to depriving B cells of their survival factor, anti-BAFF Ab treatment will also deprive EC in BAFF promoting apoptosis. Therefore, it is essential to evaluate the forms of BAFF recognized by Abs that might be used in anti-BAFF immunotherapy to avoid deleterious effects on ECs.

Acknowledgements

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FIGURE LEGENDS

Figure 1 BAFF and BR3 are expressed by epithelial cells (ECs). **A-** BAFF expression was assessed by confocal microscopy using a rabbit anti-BAFF antibody (Ab) developed with fluorescein-isothiocyanate (FITC)-conjugated donkey anti-rabbit Ab on sections of salivary gland (SG) biopsies from Sjögren's syndrome (SS) patients and healthy controls. **B-** Expression of BAFF and BAFF receptor (BR3, TACI and BCMA) genes were assessed by RT-PCR on EC cultured from SG biopsies of patients with SS and healthy controls. **C-** BR3 expression in biopsy sections of SGs from controls and SS patients was determined using rabbit anti-BR3 Ab revealed by tetramethylrhodamine isotiocyanate (TRITC) conjugated donkey anti-rabbit Ab or FITC-conjugated donkey anti-rabbit Ab. Mouse anti-cytokeratin 18 Ab developed with FITC-conjugated donkey anti-mouse Ab and anti-CD20 developed with TRITC-conjugated donkey anti-mouse Ab were used to identify ECs and B cells, respectively. D- The expression of BAFF and its receptors BR3, TACI and BCMA were evaluated by flow cytometry and immunofluorescence on HSG cell line. Representative experiments of 6.

Figure 2 **Blockade of BR3 promotes EC apoptosis in culture. The** HSG cell line was incubated with different concentrations of human recombinant BAFF (hrBAFF) or anti-BR3 blocking antibody (Ab) (anti-BR3). **A-** Proliferation was analyzed by indirect immuno-fluoresence using the Ki-67 labeling. **B-** Apoptotic cells were assessed by the TUNEL method (green labeling) and dead cells by propidium iodide (red labeling). Etoposide was used as a positive control for apoptosis. Results are expressed as mean ± standard deviation of 6 different experiments.

Figure 3 **Blockade of BR3 induces nuclear translocation of PKCδ in epithelial cells.** The HSG cell line was incubated with increasing concentrations of anti-BR3 blocking antibody (Ab) (anti-BR3) and the PKCδ localization was determined by confocal microscopy using a fluorescein-isothiocyanate (FITC)-conjugated anti-PKCδ Ab. The nuclei were labeled in red by propidium iodide (PI). Representative experiments of 6.

Figure 4 **Some forms of BAFF are involved in *in vitro* epithelial cell (EC) survival. A-** The expression of BR3 on the HSG cell line was inhibited with specific RNA inhibitor (siBR3) and EC survival was estimated at 24, 48 and 72 hours after siBR3 transfection. Untransfected or control siRNA-transfected cells were used as controls (mean ± standard deviation of 6 different experiments). **B-** Healthy control ECs were incubated for 24 hours with mouse anti-BAFF or rabbit anti-BAFF antibodies (Abs). EC survival was evaluated by flow cytometry after fluorescein-isothiocyanate-conjugated annexin V and propidium iodide labeling. Mouse and rabbit IgG were used as controls (mean ± standard deviation of 6 different experiments). **C-** EC supernatant was analyzed by Western-blot after migration on SDS-PAGE. The different forms of BAFF were revealed by the mouse and the rabbit anti-BAFF Abs.

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Figure 1

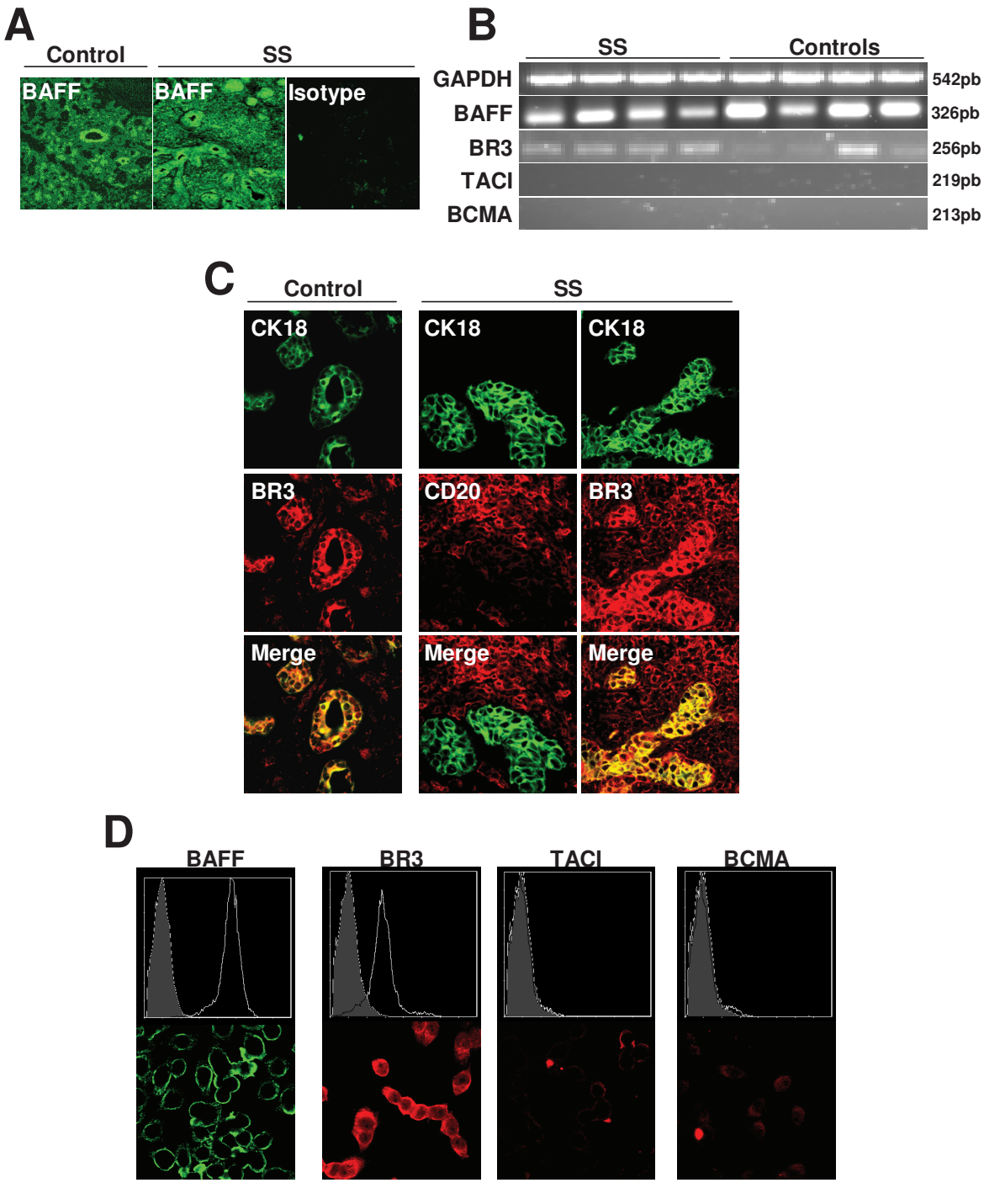
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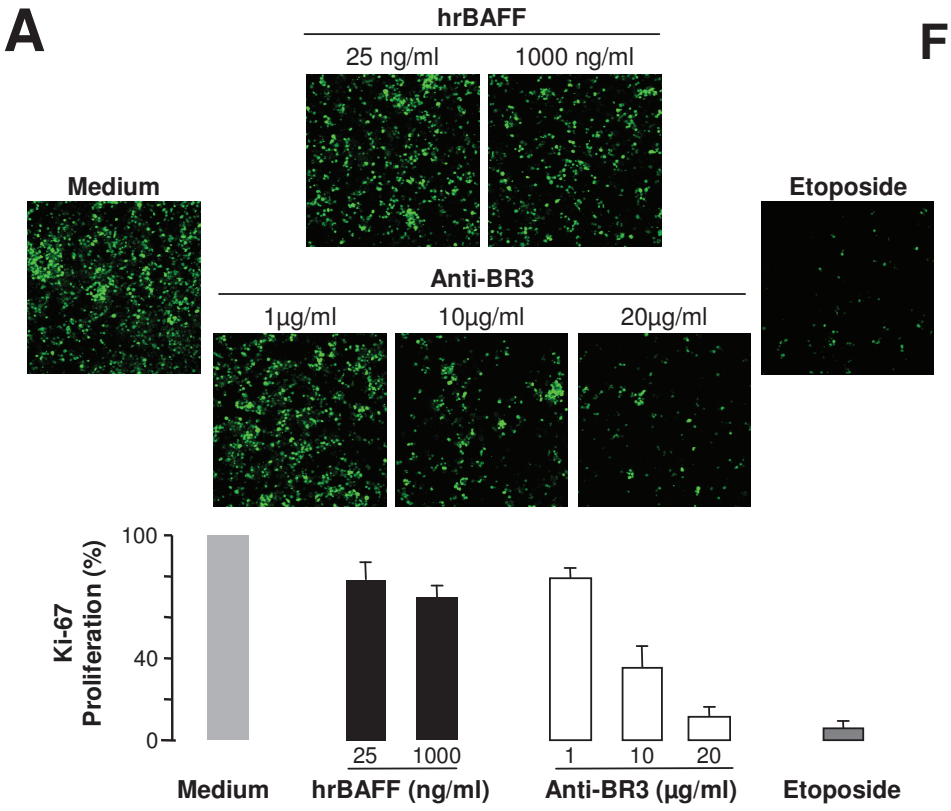
Figure 2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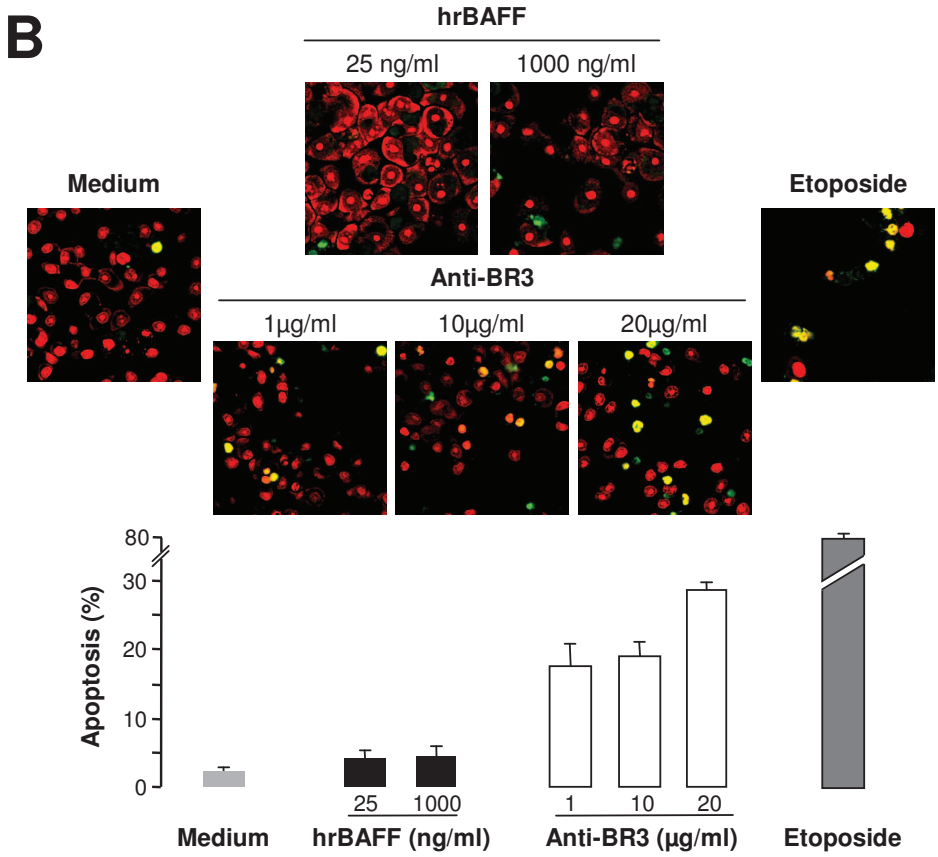
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Figure 3

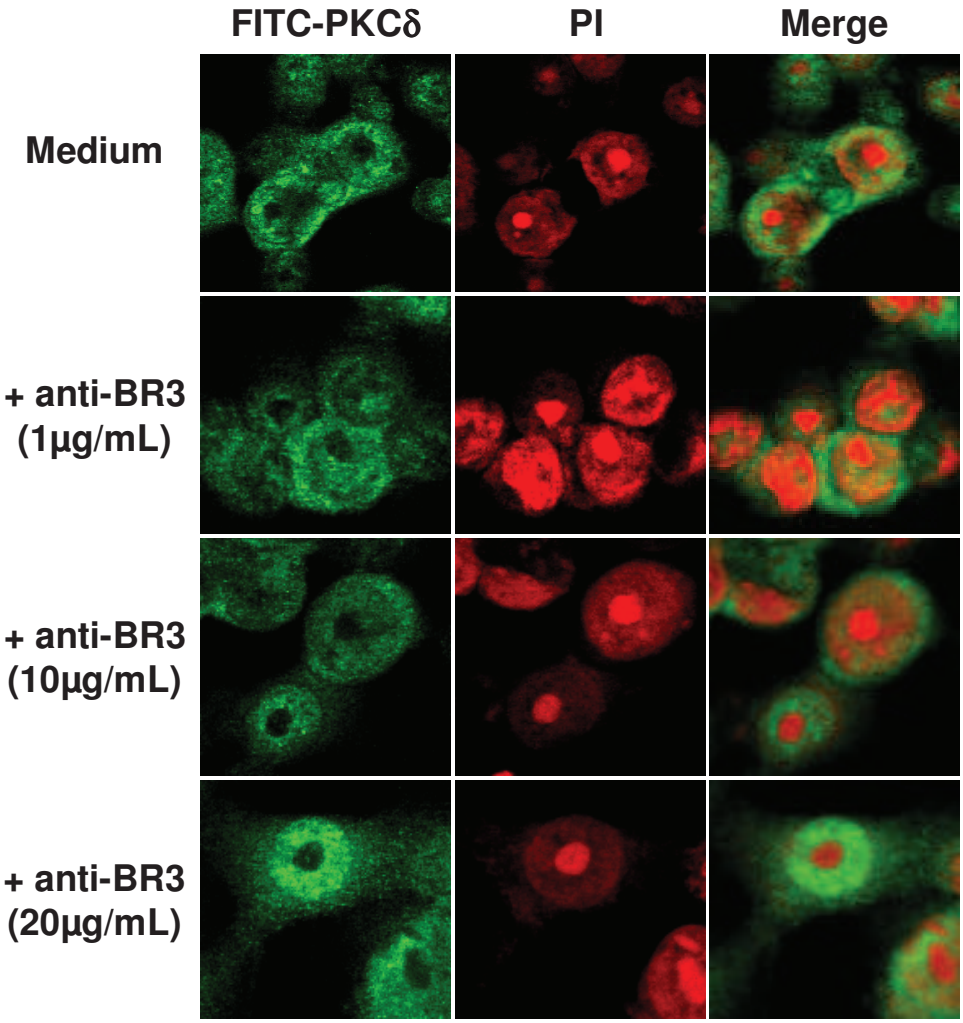
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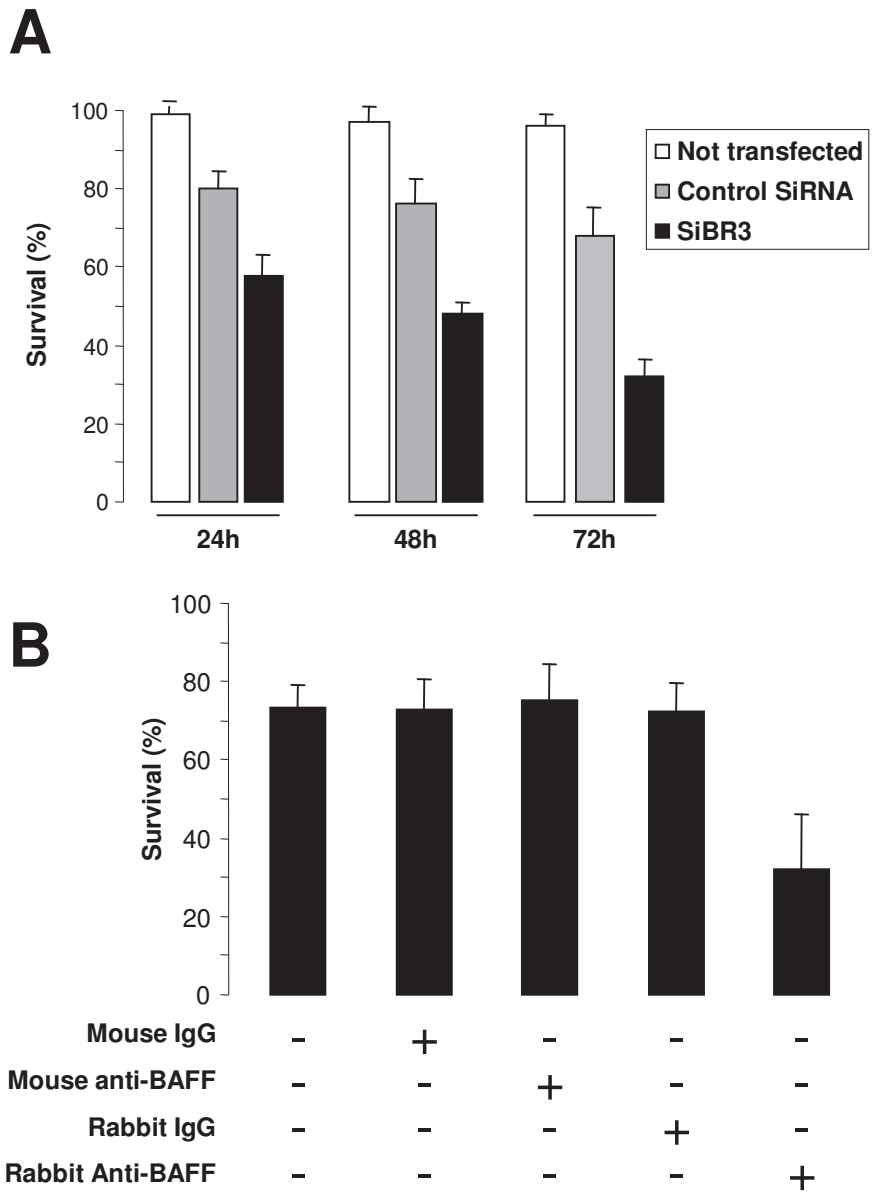
Figure 4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Rabbit IgG - - - + -
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Rabbit Anti-BAFF)

2. DELTA 4 BAFF IS A TRANSCRIPTION FACTOR ENHANCING THE PRODUCTION OF BAFF AND CONTROLLING REGULATORY B CELL FUNCTIONS

Excess of BAFF rescues auto-reactive B cells from apoptosis, thus contributing to the expansion of low-affinity self-reactive B-cells during the establishment of tolerance. We had previously reported (Annexe 2) the discovery of a new transcriptional variant for BAFF, Δ4BAFF in humans in which exon 4 is excised. We demonstrated that this new BAFF spliced isoform lacks also exon 6 and consequently the encoded protein will unlikely form trimers or bind to BAFF receptors. In this study we investigated the functions of Δ4BAFF in B cells. We showed that Δ4BAFF acts as a transcription factor for its own parent gene and modifies the regulatory properties of B cells. We found that Δ4BAFF protein is glycosylated and Δ4BAFF transfection in B cells induces an increased expression of full length BAFF. We also found that Δ4BAFF can bind the 1040-840 region (consensus NF-κB binding site) of the BAFF promoter. Moreover, in gel-shift assay when we incubated nuclear extract of Δ4BAFF transfected B cells with the NF-κB binding probe we observed a DNA-protein complex confirming the binding of Δ4BAFF to the NF-κB binding region. However, we also observed a further shift of the DNA-protein complex with an anti-p50 antibody was associated mouse anti-BAFF antibody. Co-immunoprecipitation experiments confirmed that Δ4BAFF interacts with the NF-κB family member p50, as these two molecules co-immunoprecipitated each other. All these results confirmed the hypothesis that Δ4BAFF can function as a transcription factor for its parent gene and Δ4BAFF associations p50 provides a mechanism by which Δ4BAFF gets translocated to the nucleus. We also did microarray experiment and found that almost 6000 genes have their expression specifically modified by Δ4BAFF transfection in RAMOS B cells. This observation suggests a direct or indirect role of Δ4BAFF as an inducer of gene transcription. Having shown that Δ4BAFF can be induced by CD40 and TLR9 co-stimulation in B cells from healthy controls, and that this co-stimulation was also involved in the induction of efficient regulatory functions in B cells, we hypothesise that Δ4BAFF could be involved in the emergence of Breg capacities. To assess the impact of Δ4BAFF on B cell regulatory property we used Δ4BAFF specific si RNA transfection in B cells. Si Δ4BAFF transfected B cells had a significantly lower suppressive activity on T cell proliferation. We also demonstrated a reduction of both $CD4^+CD25^+FoxP3^+$ regulatory T cells and production of TGF-β which are important for the suppressive activity of regulatory B cells on T cell proliferation. These results suggest that Δ4BAFF, acting as a transcription factor, is responsible for the emergence of regulatory properties in B cells. In

this work we showed that a human cytokine gene can be transcriptionally regulated by the activity of one of its own splice variants and can control B cell regulatory properties.

Delta 4 BAFF is a transcription factor enhancing the production of BAFF and controlling regulatory B cell functions

Short title: Δ4BAFF, a new transcription factor

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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. We have previously reported the discovery of a new transcriptional variant for *BAFF*, *Δ4BAFF* in humans (in which exon 4 is excised) that was induced by interferon-γ. Here we demonstrate that the transfection of glycosylated form of Δ4BAFF in human B cells resulted in upregulation of a large number of genes associated with the innate immune response and regulation of apoptosis. Furthermore, Δ4BAFF acts, in association with p50 from the NF-κB pathway, as a transcription factor for its own parent gene. Finally, Δ4BAFF expression appears to be critical in regulatory B cell function. In the absence of Δ4BAFF, B cells were indeed unable to inhibit T cell proliferation, to produce TGF-β and to induce the expansion of Foxp3 regulatory T 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.

INTRODUCTION

Whereas "B cell activating factor belonging to the tumor necrosis factor family" (BAFF) has an indisputable role in modulating the survival and function of B cells in autoimmune diseases (Mackay and Schneider 2009), mechanisms involved in the regulation of this cytokine remain misunderstood.

BAFF expression requires NF-κB components that act as pivotal transcription factors (Fu, Lin-Lee et al. 2006). NF-κB component, c-Rel, can exert its function on the promoter of BAFF gene by recruiting unusual factors such as CD40 (Zhou, Pham et al. 2007) and BAFF-receptor (Fu, Lin-Lee et al. 2009). Various cytokines such as IL-10, IFN-α and IFN-γ increase BAFF production by monocytes, macrophages and dendritic cells (Litinskiy, Nardelli et al. 2002). Although IFN-γ is a potent inducer of BAFF expression, the relationship between IFN-γ and the activation of BAFF gene expression remains unclear. While IFN-γ response is mainly mediated via Jak-Stat, it seems that the protein kinase A/CREB is the dominant pathway to explain BAFF induction (Kim, Jeon et al. 2008).

The BAFF gene is mapped to human chromosome 13q33.3 and contains six exons in humans (Schneider, MacKay et al. 1999) (chromosome 8 and seven exons in mice). This gene encodes three different mRNAs: the well-characterized full-length *BAFF*, a longer variant called *φBAFF*, and a shorter variant designated *ΔBAFF* (Gavin, Ait-Azzouzene et al. 2003). The larger transcript *φBAFF* 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 (Gavin, Ait-Azzouzene et al. 2003). The smaller transcript

ΔBAFF, 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 (Gavin, Duong et al. 2005). We have recently identified a new transcriptional variant for *BAFF* (**Le Pottier et al, submitted**), *Δ4BAFF* in which exon 4 is excised. We observed that Δ4BAFF was located in the endoplasmic reticulum and the nucleus and that its N-Glycosylation was required for nuclear entry. Furthermore, *Δ4BAFF* expression was induced after IFNγ stimulation and the effects of IFN-γ on alternative splicing phenomena have been already described (Liu, Shue et al. 2004). We demonstrated that IFN-γ stimulation induced an increased expression of heterologous nuclear ribonucleoprotein (hnRNP) C1/C2 able to bind to silencer sequences inhibiting the transacting factor SR protein SC35 (SRSF2) binding to BAFF exon 4 exonic silencing enhancers, forcing a shift of splicing to a distal splicing site favoring *Δ4BAFF* induction.

One report described that treating immune B cells with BAFF was leading to the development of IL-10 secreting B cells with regulatory functions (Yang, Sun et al. 2010), suggesting the involvement of BAFF in the control of regulatory B cells. We have observed that CpG stimulation, along with CD40-CD154 interaction was enhancing the regulatory effect of human B cells on T lymphocytes (Lemoine, Morva et al. 2011) and on dendritic cells (Morva, Lemoine et al. 2012). Regulatory B cells strongly regulate allogenic T cell proliferation through the induction and expansion of the $Foxp3^+$, $CD4^+$, $CD25^+$ regulatory T cells independently of IL-10 secretion (Lemoine, Morva et al. 2011). While regulation of proliferation requires cell-to-cell contact involving CD40 engagement without IL-10, Th1 cell differentiation is dependent on CD80 and CD86 interactions and on the production of IL-10. We hypothesized a pivotal role of Δ4BAFF in the control of BAFF expression and the raise of regulatory functions in B cells.

Here, we report that Δ4BAFF acts, in association with p50 from the NF-κB pathway, as a transcription factor for its own parent gene. Through comparative analysis by microarray, the transfection of Δ4BAFF in RAMOS B cells resulted in differential expression of a large number of genes. The up-regulated genes belong to different families involved in innate immune response and regulation of apoptosis. Furthermore, we observed that Δ4BAFF expression was required for the acquisition of regulatory functions by B cells. All these data contribute to a better understanding of complex physiologic mechanisms involved in B cell survival, as well as in pathophysiology of B cells in diseases.

MATERIEL AND METHODS

CELL AND PATIENTS

All healthy donors gave informed consent and the study was approved by the ethical committee at the Brest university medical school hospital. B lymphocytes were isolated from tonsil of healthy donors, centrifugated on Ficoll-Hypaque (PAA laboratories, Pasching, Austria), followed by 2 rounds of rosetting with neuraminidase-treated sheep erythrocytes (TCS Bioscience, Buckingham, UK). Ultimately, B cells were further purified using the human B cell enrichment kit according to manufacturer instructions (Stem-Cell-Technologies, Grenoble, France). Purity of B lymphocytes was checked by FACS analysis (EPICS®Elite, Beckman-Coulter) using 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) acccording to the manufacturer instructions. Polymorphonuclear neutrophils were isolated by dextran (GE-Healthcare, Velizy-Villacoublay, France) sedimentation followed by centrifugation on Ficoll-Hypaque.

RAMOS B cell line was 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). For *BAFF* and *Δ4BAFF* primer pairs used were LLP2008/LLP2009. Primer pairs used for *Δ4BAFF* specific amplification were Δ4BAFF forward (Exon 3-5) and Δ4BAFF reverse (Exon 5-6) (Supplementary Table 1).

CLONING HUMAN Δ4BAFF

50 ng of cDNA from tonsillar B cells were used as template for the amplification of Δ4*BAFF* as previously described (Le Pottier et al., submitted).

Plasmid construction and site-directed mutagenesis

The construction of p*Δ4BAFF*-IRES2-EGFP and p*Δ4BAFF* –EGFP have been previuously described (Le Pottier et al., submitted).

Transient transfection of Δ4BAFF

RAMOS cells was transiently transfected with 10 µg of pIRES2-EGFP (or p*Δ4BAFF*-IRES2-EGFP or p*Δ4*$^{[N124\rightarrow D]}$*BAFF*-IRES2-EGFP) or with 5 µg of pEGFP (or p*Δ4BAFF*-EGFP or p*Δ4*$^{[N124\rightarrow D]}$*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.

Stable transfection of Δ4BAFF in RAMOS cell line

RAMOS cell-line cells were transfected with pIRES2-EGFP or pΔ4BAFF-IRES2-EGFP or pΔ4[N124→D]BAFF-IRES2-EGFP as described in "transient transfection of Δ4BAFF". Twenty-four hours after transfection, RAMOS GFP positive, in each conditions, were selected

by FACS cell sorting. Cells were cultured in RPMI 1640 supplemented medium with G418, 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-GFP), Delta4BAFF (pΔ4BAFF-IRES2-EGFP) and Mutated Delta4BAFF (pΔ4[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, Mountain View, CA) (map in Supplementary Figure 1) 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 blot analysis of BAFF

Equal amounts of proteins from total cell lyses extracts 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-β-actin mAb (1:10000), anti-BAFF mAb (clone 137314, R&D systems, Minneapolis, MN) or with rabbit polyclonal anti-BAFF (Upstate Biotechnology, Lake Placid, NY) 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, GE healthcare). The intensity of each protein was expressed relative to β-actin.

Microarray analysis

Data analysis

After cDNA labeling and hybridization, data were normalized by quantile normalization using Genespring 12.0 (Agilent Technologies, Les Ulis, France). 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*Δ4BAFF*-IRES2-EGFP) and Mutated-Delta4BAFF (RAMOS transfected with p$\Delta 4^{[N124\rightarrow D]}$*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 Δ4BAFF 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 (Huang da, Sherman et al. 2009). 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 µg 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®Life Technologies, Saint Aubin, France). 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 for15 s and 60°C for 1 min. All reactions were performed in duplicate. Gene expression level was calculated with the 2(-ΔC(T)) method (Schmittgen and Livak 2008). Target gene expression was normalized to GAPDH expression. All primers used are listed in Supplementary Table 1.

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), 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, Meylan, France) 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 -375. *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 1.

Co-immunoprecipitation of complexes and Western Blot Analysis

Lysates of p*Δ4BAFF* -EGFP or pEGFP transiently transfected RAMOS B cell line cells were analyzed by co-immunoprecipitation using the µMACSTM protein A/G microbeads kit and µMACSTM 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 colleted 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 Δ4BAFF-deficient cells by siRNA transfection

3 x 10^6 B cells/well in a 6-well plate were transfected with 12.5 pmol of a specific Δ4BAFF siRNA (Sequence CUAUACAAAAGGUUUUAUtt). Mice negative control siRNA was used as control (Applied Biosystems®Life Technologies).

B cell culture

B cells were cultured for 2 days in 24-well plates in RPMI 1640 medium supplemented as previously described. For stimulation B cells were seeded at 1x10^6 cell/ml on 5x10^5 NIH-3T3 fibroblast transfected with human CD40L gene and treated with mitomycin C (Sigma Aldrich, Saint Quentin Fallavier, France), with or without 0.25 µM CpG-ODN 2006 (Cayla-InvivoGen, Toulouse, France).

Co-culture model between T cells and regulatory B cells

T cells were seeded at $2x10^5$ cell/ml on anti-mouse IgG-Fc (Jackson Immunoresearch, Immunotech) coated 96-well plates in complet RPMI 1640 medium labeled with 5µM CFSE (Molecular probe®Invitrogen) and stimulated with 1g/ml anti-CD3 (Biolegend, London, UK) and anti-CD28 mAbs (Jackson Immunoresearch, Immunotech). B cells transfected with 12.5 pmol of Δ4BAFF siRNA or with mice negative control siRNA, or nontransfected were added to T cells at ratio 1:1 for 4 days. Then, T cell proliferation was evaluated by flow cytometry on FC500 (Beckman Coulter) measuring the decrease in the mean fluorescence intensity (MFI) of CFSE. In co-culture experiments, cells were stained with PE-linked to cyanin 7 (Pc7)-conjugated anti-CD19 mAb (Immunotech), and CFSE MFI analyzed in CD19-negative cells.

For cytokine production, mixed cells from coculture experiments were permeabilized using cytofix/cytoperin permeabilization kit (BD Biosciences, Franklin Lakes, NJ) and stained with Pc7-conjugated anti-CD19 in combination with PE-conjugated TGFβ mAb or FITC-conjugated anti-IL-10 in mAb (both from R&D Systems).

The presence of regulatory T cells was evaluated after permeabilizing cells with transcription factor buffer set (BD BioSciences). T cells were stained with Pc7-conjugated anti-CD4 mAb (Immunotech), FITC-conjugated anti-CD25 mAb (Immunotech) and PE-conjugated anti-FoxP3 mAb (BD BioSciences).

Statistical analysis

Data were expressed as mean ± standard deviation (SD). Comparisons were made with the t-student test or the Mann-Whitney's test for unpaired data.

RESULTS

Δ4 BAFF expression induces up-regulation of full-length BAFF in RAMOS B cells

Interestingly, the transient transfection of Δ4BAFF in RAMOS B cells (using p*Δ4BAFF*-IRES2-EGFP construct) induced an up-regulation of full-length BAFF as shown by the increase in the BAFF (32kDa)/β-actin ratio (Figure 1). The mean ± SD of BAFF (32kDa)/β-actin ratio in RAMOS expressing Δ4BAFF was higher than those transfected with empty vector (pIRES2-EGFP) (1.44 ± 0.11 vs 0.87 ± 0.05 respectively, $P<0.01$, averages of 3 independent experiments). However, this ratio was not increased (0.82 ± 0.07) when RAMOS B cells were transfected with a mutated form of Δ4BAFF at N^{124} (pΔ4$^{[N124\rightarrow D]}$BAFF-IRES2-EGFP) resulting in the expression of unglycosylated Δ4BAFF.

Δ4BAFF acts as a transcription factor of its own parent gene

Since we had previously observed that glycosylated Δ4BAFF was distributed throughout the nucleus and led to the up-regulation of the full-length BAFF expression in RAMOS B cells, we then examined whether Δ4BAFF might function as a transcriptional regulator of the BAFF gene. To test this hypothesis, we performed a 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 –375 known for binding CD40 (Lin-Lee, Pham et al. 2006) and the second primers target a region located at –1040 and –840 chosen because of its capacity to bind NF-κB components (Fu, Lin-Lee et al. 2006). Δ4BAFF 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 (Figure 2A). PCR

analysis using primers upstream of the *BAFF* promoter (located at -1474 to -1261) also did not show Δ4BAFF binding, indicating that the Δ4BAFF protein binds to a specific region within the *BAFF* promoter. To confirm whether Δ4BAFF exerts transcriptional regulatory functions on its own gene, the NF-κB component binding region of the *BAFF* gene promoter within -1040 to -840 was cloned into pDsRed-Express1 reporter plasmid (Supplementary Figure 1), leading to a p*BAFF*promoter-DsRed construct, and analyzed after transient transfection in RAMOS B cells stably transfected with pIRES2-EGFP, p*Δ4BAFF*-IRES2-EGFP or p*Δ4*$^{[N124\rightarrow D]}$*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*Δ4BAFF*-IRES2-EGFP RAMOS B cells (Figure 2B). Together, these results indicate that glycosylated Δ4BAFF acts as a transcription factor of its own parent gene and binds to the NF-κB component binding region of the *BAFF* promoter.

Δ4BAFF forms complexes with the transcription factor p50 from the NF-κB pathway

The NF-κB components binding to the *BAFF* promoter in humans included predominantly p50, p52, c-Rel and to a lesser extent p65 (Fu, Lin-Lee et al. 2006). As a putative NLS sequence was absent, we hypothesized that Δ4BAFF can translocate to the nucleus in association with one of the NF-κB family members. We then synthesized digoxigenin-labelled consensus NF-κB binding oligonucleotides (–1040 to –840) and performed gel shift assays. When nuclear extracts from p*Δ4BAFF*-EGFP-transfected RAMOS B cells were incubated with the NF-κB binding probe, a protein DNA complex was visualized (Figure 2C) 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 Δ4BAFF. Addition of mouse or rabbit immunoglobulins did not supershift the complex (data not shown) and, 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 Figure 2).

Co-immunoprecipitation experiments confirmed that Δ4BAFF interacts with p50 since these two molecules co-immunoprecipitated each other in nuclear lysates from p*Δ4BAFF*-EGFP-transfected RAMOS B cells (Figure 2D). Furthermore, only a weak association between Δ4BAFF and p65 exists since Δ4BAFF coimmunoprecipitate with p65 whereas p65 did not coimmunoprecipitate with Δ4BAFF. Collectively these data suggest that Δ4BAFF and p50 bind together at the NF-κB consensus-binding site of the *BAFF* promoter providing Δ4BAFF with a mechanism to translocate to the nucleus where it can act as a transcription factor for its own parent gene.

Gene expression profile of Δ4BAFF transfected RAMOS B cells

We hypothesized that, since Δ4BAFF protein is distributed throughout the nucleus, Δ4BAFF might function as a transcription regulator. The gene expression profiles of RAMOS B cells stably transfected with pIRES2-EGFP, p*Δ4BAFF*-IRES2-EGFP or p*Δ4*$^{[N124\rightarrow D]}$*BAFF*-IRES2-EGFP was then carried out. 6303 genes were found to be differentially expressed between p*Δ4BAFF*-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*Δ4BAFF*-IRES2-EGFP and p*Δ4*$^{[N124\rightarrow D]}$*BAFF*-IRES2-EGFP transfected RAMOS B cells (P<0.01). A Venn diagram (Figure 3A) showed that 6155 RAMOS B cell genes have their expression specifically modified by Δ4BAFF transfection in comparison to Δ4$^{[N124\rightarrow D]}$BAFF and empty vector (pIRES2-EGFP) transfection. These observations suggested a direct or indirect role of Δ4BAFF as an inducer of gene transcription and the importance of the glycosylation state on N^{124} in the modulation of Δ4BAFF activity.

Gene-lists were analyzed by functional annotation clustering using DAVID. This enabled the identification of common biological charts (Supplementary Table 2). Among the 6155 genes, 2904 genes were up-regulated and 3251 genes were down-regulated by Δ4BAFF-expression compared to empty vector (pIRES2-EGFP) and unglycosylated Δ4$^{[N124\rightarrow D]}$BAFF 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 *Δ4BAFF* transfection in RAMOS B cells (Figure 3B).

Defect in the suppressive activity of si Δ4BAFF transfected-B cells from healthy controls

In order to induce *Δ4BAFF* expression in B cells from healthy controls, tonsilar B cells were seeded 3 days on NIH-3T3 fibroblasts transfected or not with human CD40L gene, with or without CpG-ODN 2006. CD40 and TLR9 co-stimulation was required to induce Δ4BAFF

production (Figure 4A). Interestingly, we had previously reported (Lemoine, Morva et al. 2011) that B cell activation through CD40 and TLR9 engagement leaded to the induction of B cells with highly efficient regulatory functions. Thus, CD40-TLR9-induced regulatory B cells inhibited T cell proliferation through the induction of regulatory $Foxp3^+CD4^+CD25^+$ T cells, and modulated Th1 differentiation through IL-10 production (Lemoine, Morva et al. 2011). To evaluate the impact of Δ4BAFF on regulatory properties of B cells, tonsilar B cells were transfected either with *Δ4BAFF*-specific-siRNA (si *Δ4BAFF*), or control siRNA and co-cultured with CFSE-labelled T cells stimulated with anti-CD3 and anti-CD28 mAbs as previously described (Lemoine, Morva et al. 2011). Efficiency of si *Δ4BAFF* was tested, in RAMOS B cells. As shown in Figure 4B, we confirmed that CD40 and TLR9 co-stimulation induced *Δ4BAFF* expression and observed that *Δ4BAFF* mRNA expression levels were not detectable in cells transfected with si *Δ4BAFF* while the expression levels of BAFF mRNA were not affected. Si *Δ4BAFF* transfected B cells had a significantly lower suppressive activity than si control or untransfected B cells (26.7±8.2% of inhibition of T cell proliferation compared to 49.4±6.2%, $P<0.01$) for untransfected cells and 39.0±9.6% for si control transfected cells, $P<0.01$, Figure 4C).

This result suggests that Δ4BAFF may be responsible for the emergence of regulatory capacities. As we had already demonstrated that regulatory B cells inhibit T cell proliferation by the generation of $CD4^+CD25^+Foxp3^+$ regulatory T cells and the production of cytokines such as IL-10 and TGF-β (Lemoine, Morva et al. 2011), we investigated if the neutralization of *Δ4 BAFF* will have any impact on those factors. As presented in Figure 4D, in comparison to the two control groups, the absence of Δ4BAFF is associated with impaired generation of regulatory T cells (3.7±1.6% obtained in cocultures performed with si *Δ4BAFF* transfected B cells compared

to 22.2±2.6%, P<0.05 with untransfected cells and 24.8±2.9%, P<0.05 with si control transfected B cells). This observation can be linked to the drastic decrease in the production of TGF-β by B cells (Figure 4E) when *Δ4BAFF* is neutralized (9.3±2.4% versus 48.2±10.3%, P<0.04 and 43.6±7.4% P<0.03 with untransfected and si control-transfected B cells, respectively). Interestingly, Δ4 BAFF did not have any direct effect on IL-10 since its neutralization did not modify significantly its production by B cells (Figure 4F) when compared to si control transfected or untransfected B cells. Taking together, all these results, clearly, demonstrate that Δ4 BAFF, acting as a transcription factor, is responsible for the emergence of regulatory properties in B cell through the production of TGF-β.

DISCUSSION

When transfected in RAMOS B cells, Δ4BAFF 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 (Isnardi, Ng et al. 2008; Tili, Croce et al. 2009; Meyers, Ng et al. 2011). Indeed, TLR ligation results in the production of pro-inflammatory cytokines, increased antigen expression, antibody production, proliferation and differentiation in B cells (Huggins, Pellegrin et al. 2007; Jiang, Lederman et al. 2007). We also described an entirely novel function of Δ4BAFF as a transcription factor that enhances expression of its own parent gene. This finding is of particular interest because BAFF overexpression is a central driver in autoimmune diseases and lymphoproliferation disorders and is also associated with B cell tolerance breakdown and autoantibody production (Varin, Le Pottier et al. 2010).

We observed that N-glycosylation of Δ4BAFF was not only required for nuclear entry (**Le Pottier et al**, submitted) but also for promoter binding. The importance of the N-glycosylation status for BAFF has been already described, particularly for the ΔBaff variant (Gavin, Ait-Azzouzene et al. 2003), but also for the full-length form of BAFF in the serum of patients with autoimmune diseases (Le Pottier, Bendaoud et al. 2009). 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 (Le Pottier, Bendaoud et al. 2009). 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 (Chan, Mak et al. 2010).

Cooperation between Δ4BAFF and p50 appears to be important in regulating BAFF expression. The interactions of NF-κB dimers or monomers with heterologous transcription factors through direct binding has been already described and profoundly influences transcriptional responses (Oeckinghaus, Hayden et al. 2011). NF-κB p50 lacks indeed a transactivation domain and therefore usually form a heterodimer to be transcriptionally active (Ghosh, May et al. 1998). However, we found that the Δ4BAFF sequence contains a perfect match between aa145 and aa153 corresponding to the 9aa transactivation domain (9aaTAD) which is common to a large number of yeast and animal transcription factors (Piskacek, Gregor et al. 2007). In vitro studies have shown that p50 can associate with other transcriptional activators such as Bcl-3 (Fujita, Nolan et al. 1993) or p300 (Deng and Wu 2003) to activate transcription. NF-kB p50 can also form a complex with the transcriptional co-activator CREB to activate IL-10 transcription in macrophages (Cao, Zhang et al. 2006). Interestingly, among the genes up-regulated after *Δ4BAFF* transfection, the promoter of the BIC gene encoding miR-155 contains two putative NF-κB sites able to bind *in vitro* the NF-κB proteins p50 and p65 in nuclear extract from MC3 cells (Gatto, Rossi et al. 2008). 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 (Snapper, Zelazowski et al. 1996). Finally by blocking ubiquitinilation of p50, Bcl-3 stabilizes a p50 complex that inhibits TLR gene transcription and limits the strength of the TLR responses (Carmody, Ruan et al. 2007). Consequently, the cooperation between Δ4BAFF and p50 may be an important regulatory mechanism for the transcription of a large number of genes. Further investigation of the role of Δ4BAFF and its interaction with p50 in the context of both

autoimmunity and B cell leukemia could provide novel therapies targeted at modulating its function.

Many data point to the existence of regulatory B-cell subjects. CD40 engagement on B cells appears to be a requisite for the induction of functional Breg cells (Mizoguchi, Mizoguchi et al. 1997; Fillatreau, Sweenie et al. 2002). In systemic lupus erythematosus, stimulation of TLR9 is prerequisite to induce B cell regulation of inflammatory responses (Brummel and Lenert 2005). We have also recently observed that CD40 and TLR9 associated stimulation was the best to induce functional regulatory B cells (Lemoine, Morva et al. 2011). Interestingly, this stimulation was also observed to induce Δ4BAFF expression. We thus hypothesized that regulatory activities of B cells depend on the presence of the transcription factor Δ4BAFF.

Although regulatory B cell efficiency was mainly associated to their production of IL-10 (Mizoguchi, Mizoguchi et al. 2002), then is also strong evidence that part of the immunosuppressive function of B cells is mediated by interactions with other regulatory cell population. Regulatory B cells can induce induce regulatory T cells to regulate T cell-dependent immune responses (Wei, Velazquez et al. 2005). The effectiveness of these regulatory B cells was linked to TGF-β expression by B cells but not IL-10 (Singh, Carson et al. 2008).

IL-10 production was not downregulated after *Δ4BAFF* inhibition by si*Δ4BAFF* in contrast to TGF-β production and regulatory T cell induction. However, neutralizing *Δ4BAFF* in B cells resulted in a significant decrease of the inhibition of T cell proliferation. This result is in accordance with our previous results showing that human regulatory B cells can inhibit the proliferation of T cells through a mechanism independent of IL-10 but through the induction of

Foxp3 regulatory T cells, while the Th1 differentiation is controlled by an IL-10-dependent pathway (Lemoine, Morva et al. 2011).

The pivotal role of Δ4BAFF as a transcription factor that controls BAFF expression and the control of immune response holds immense promise for the clinic. The potential for Δ4BAFF 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.

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Authorship contributions and disclosure of conflicts of interest

JOP, LLP, GT, AL, FM and DSE designed and performed experiments and analyzed the data. Data were additionally analyzed and interpreted by JOP, LLP and PY.

The manuscript was written by JOP and LLP and edited by FM, DSE, GT, AL and PY.

The authors declare no competing financial interests.

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FIGURE LEGENDS

Figure 1: Δ4BAFF expression induces up-regulation of full-length BAFF. Whole cell lysates from RAMOS cells (non-transfected: NT), transfected RAMOS cells with pIRES2-EGFP (empty vector: ø), p***Δ4BAFF***-IRES2-EGFP (Δ4) or ***PΔ4***[N124→D]***BAFF***-IRES2-EGFP (**Δ**4[N124→D]) were analyzed by Western-blotting using either an anti-BAFF mAb (clone 137314) or rabbit anti-BAFFpAb, unable to recognize **Δ4BAFF.** 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 2: Δ4BAFF acts as a transcription factor of its own gene by binding to the promoter of the *BAFF* gene at its NF-κB binding region and forms complexes with p50 from the NF-κB1 pathway. (A) ChIP analysis within *BAFF* promoter was performed on RAMOS cells (non-transfected: NT) p*Δ4BAFF*-IRES2-EGFP (Δ4) and p*Δ4*[N124→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 –375; NF-κB binding region from –1040 to –840 and a control region from –1474 to –1261) were performed on the precipitated DNA (GenBank file AF 186114). Mouse IgG was used as a non specific control. (B) The promoter of *BAFF* is active in p*Δ4BAFF*-IRES2-EGFP stably transfected cells. pEGFP (empty vector: ø), p*Δ4BAFF*-IRES2-EGFP (Δ4) and p*Δ4*[N124→D]*BAFF*-IRES2-EGFP (Δ4[N124→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 emmission fluorescence was observed by FACS. (C) Supershift analysis of Δ4BAFF protein binding to the NF-κB site. Nuclear extracts of RAMOS cells transiently transfected with p*Δ4BAFF*-EGFP were incubated with antibodies (Abs) against BAFF (clone 137314 and rabbit) and the probe labeled to digoxygenin (DIG). Samples were analyzed by electrophoretic mobility shift assay. (D) Association of Δ4BAFF with p50 in transfected RAMOS cells with p*Δ4BAFF*-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: Ø) or p*Δ4BAFF*-EGFP-transfected RAMOS B cells (Δ4).

Figure 3: Gene expression profile of Δ4BAFF transfected RAMOS B cells and validation using real-time PCR. (A) Venn diagram to illustrate the overlapping expression of genes within genes differentially expressed between p*Δ4BAFF*-IRES2-EGFP(Δ4), pIRES2-EGFP (empty vector: ø) and p*Δ4*[N124→D]*BAFF*-IRES2-EGFP (Δ4[N124→D]) transfected RAMOS cells. (2 way ANOVA, $P<0.01$).

(B) Real-time PCR are presented in the column graphs as relative gene expression normalized to *GAPDH*.

Figure 4: Δ4BAFF is induced in regulatory B cells and is required for their functions. (A) Reverse transcriptase (RT)-PCR analysis of *Δ4BAFF* transcript in tonsilar B cells from healthy controls. B cells were seeded on NIH-3T3 fibroblasts transfected or not with human CD40L gene and treated with mitomycin C, with or without CpG-ODN 2006. (B) RAMOS B cells transfected with *Δ4BAFF* si RNA (si*Δ4BAFF*) or control siRNA were stimulated or not through CD40 and TLR9 as described in (A). RT-PCR analysis was performed in order to detect the presence of *BAFF*, *Δ4BAFF* or *GAPDH* mRNA (C) B cells were transfected either with siRNA against Δ4BAFF (Si Δ4) or control siRNA (Si Ctl) or non transfected (NT) and cocultured with anti-CD3 and anti-CD28 antibody-stimulated T cells (ratio 1:1) in the presence of CpG for 4 days. T cell proliferation was evaluated by flow cytometry by measuring CFSE-staining dilution. (D) The presence of regulatory T cells was determined by flow cytometry using PE-Cy7-labelled anti –CD4, PE-labelled anti-FoxP3 and FITC-labelled anti-CD25 antibodies. The production of TGF-β (E) and IL-10 (F) was measured by flow cytometry using PE-Cy7-labelled anti-CD19, FITC-labelled anti-IL-10 and PE-labelled anti-CD19, FITC-labelled anti-IL-10 and PE-labelled anti-TGF-β antibodies. Each experiment was performed 3 times. Student t-test was used for statistical analysis.

Supplementary Table 1: Sequences of primers used in this study.

Name	Sequence	Purpose
BAFF LLP2008 (exon 3) forward	TTGCAGACAGTGAAACACCAACT	PCR
BAFF LLP2009 (exon 6) reverse	TTCATCTCCTTCTTCCAGTTTTGC	PCR
Δ4BAFF (exon 3-5) forward	GACAGTGAAACACCAACTATACAAAAAGGTTTTATATAC	PCR
Δ4BAFF (exon 5-6) reverse	CAGTTTTGCAATGCCAGCTGAA	PCR
GAPDH froward	CTTAGCACCCCTGGCCAAGG	PCR
GAPDH reverse	CTTACTCCTTGGAGGCCATG	PCR
BAFF promoter (region 1) forward	GAGACAGAACTAAAGCTCACTATTCTT	ChIP and EMSA
BAFF promoter (region 1) reverse	GACCTGTGAGGACTGTTGCA	ChIP and EMSA
BAFF promoter (region 2) forward	AGGCAAGGCTGATTCTCCTC	ChIP
BAFF promoter (region 2) reverse	GGAAGTGTGGAAGTAAGTCCACTG	ChIP
BAFF promoter (upstream) forward	GACTTTAGGGACTCAGGGGAAAG	ChIP
BAFF promoter (upstream) reverse	GAAACAAATTACATTTTGGATGC	ChIP
BAFF promoter forward	ATCACTCGAGGGGTCTGGAGTTCTCCACTT-TGCAC	Cloning in pDsRed-Express1
BAFF promoter reverse	GACTAAGCTTGACCTGTGAGGACTGTTGCA	Cloning in pDsRed-Express1
TLR2 froward	CCACCGTTTCCATGGCCTGTG	Real-time PCR
TLR2 reverse	GATGAAGTTCTCCAGCTCCTGCACC	Real-time PCR
TLR6 froward	ATGTGGCAGCTTTCGCAGCCT	Real-time PCR
TLR6 reverse	TTGAACTCATCTTCTGGCAGC	Real-time PCR
TLR9 forward	TGAAGACTTCAGGCCCAACTG	Real-time PCR
TLR9 reverse	TGCACGGTCACCAGGTTGT	Real-time PCR
TLR10 forward	GTAAGGCTATCAAAAGGAGATGTGAGA	Real-time PCR
TLR10 reverse	GAGGAGAAGCATAATGGACCTTTG	Real-time PCR
AICDA forward	CCACTATGGACAGCCTCTTG	Real-time PCR
AICDA reverse	CACTGTCACGCCTCTTCACT	Real-time PCR
MBD2 forward	CCATGGAACTACCCAAAGGTCTT	Real-time PCR
MBD2 reverse	CAGCAGATAAAAGGGTCTCATCATT	Real-time PCR
GAPDH forward	TGCACCACCAACTGCTTAGC	Real-time PCR
GAPDH reverse	GGCATGGACTGTGGTCATGAG	Real-time PCR

Supplementary Table 2: List of selected genes to illustrate the functional annotation analysis (DAVID). Δ4: RAMOS B cells stably transfected with pΔ4*BAFF*-IRES2-EGFP, Δ4$^{[N124\rightarrow D]}$: RAMOS B cells stably transfected with p*Δ4BAFF*$^{[N124\rightarrow D]}$-IRES2-EGFP, Ø: RAMOS B cells stably transfected with pIRES2-EGFP.

Up-regulated genes

	Fold Change absolute		
IMMUNE SYSTEME (36 genes)	**[Δ4] vs [Ø]**	**[Δ4] vs [Δ4$^{[N124\rightarrow D]}$]**	**Probe Name**
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 [Δ4] vs [Ø]	Fold Change absolute [Δ4] vs [Δ4$^{[N124\rightarrow D]}$]	ProbeName
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 (IKBKB)	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 (IKBKB)	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
Jjun 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

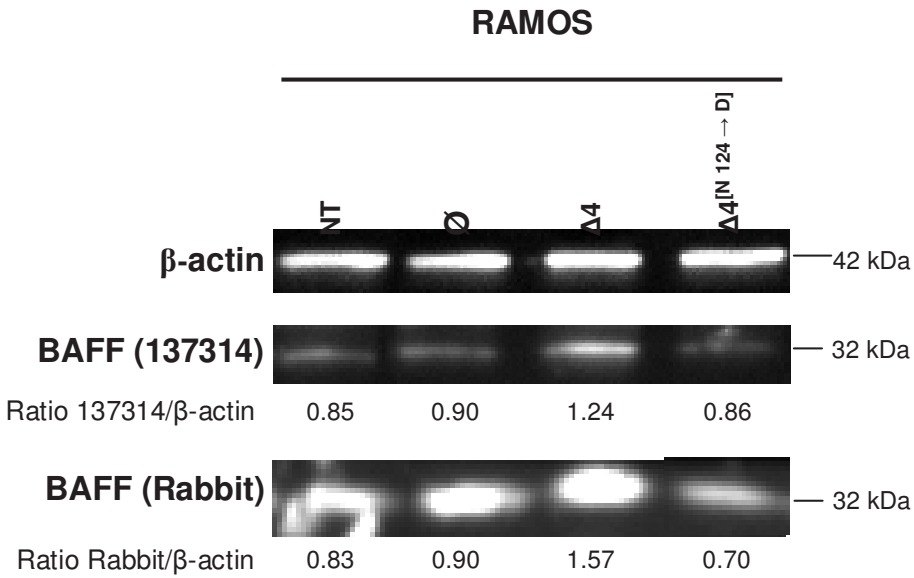

RAMOS
NT
Ø
Δ4
Δ4[N 124 → D]
β-actin
42 kDa
BAFF (137314)
32 kDa
Ratio 137314/β-actin
0.85
0.90
1.24
0.86
BAFF (Rabbit)
32 kDa
Ratio Rabbit/β-actin
0.83
0.90
1.57
0.70

Figure 2

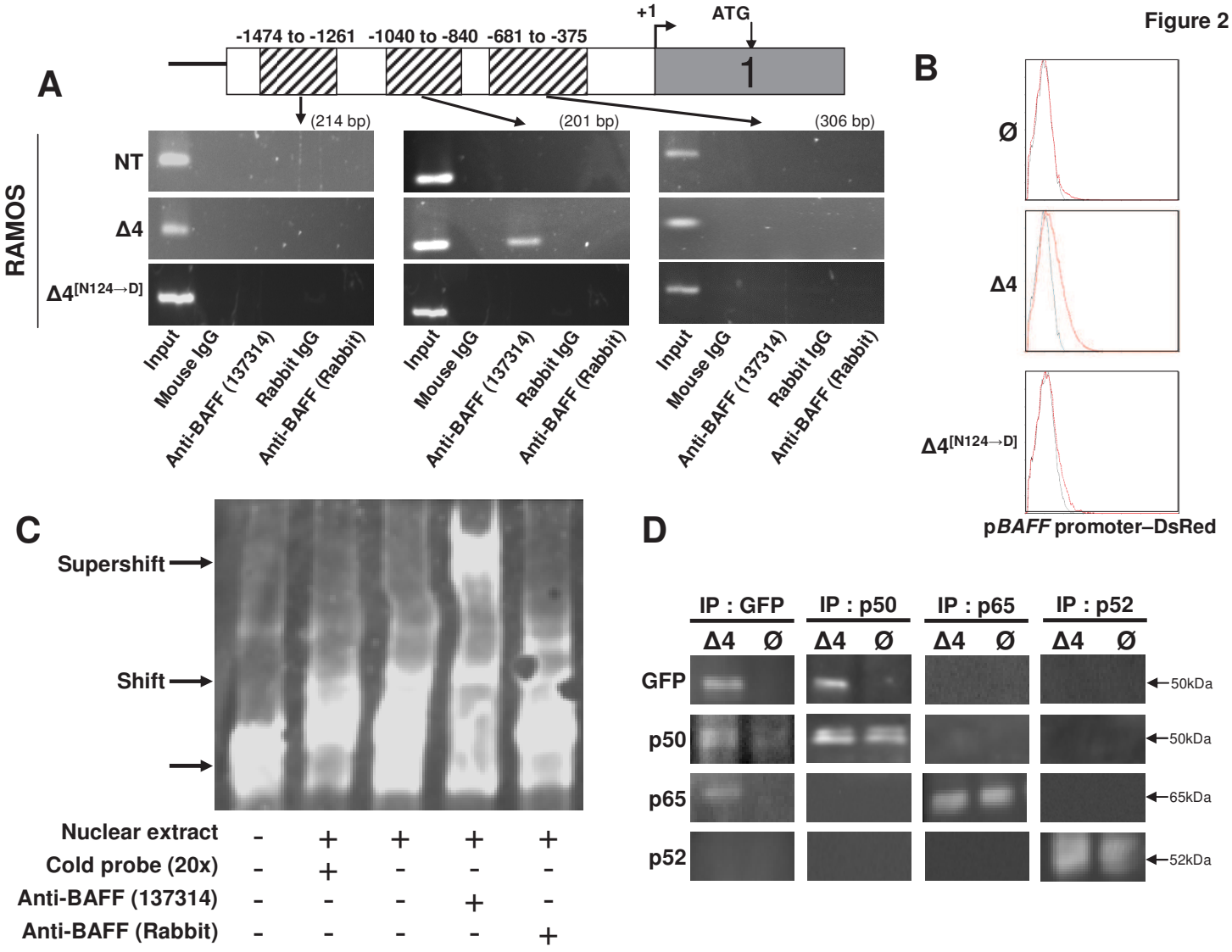

A
+1
ATG
-1474 to -1261
-1040 to -840
-681 to -375
1
(214 bp)
(201 bp)
(306 bp)
RAMOS
NT
Δ4
Δ4[N124→D]
Input
Mouse IgG
Anti-BAFF (137314)
Rabbit IgG
Anti-BAFF (Rabbit)
B
Ø
Δ4
Δ4[N124→D]
pBAFF promoter–DsRed
C
Supershift
Shift
Nuclear extract - + + + +
Cold probe (20x) - + - - -
Anti-BAFF (137314) - - - + -
Anti-BAFF (Rabbit) - - - - +
D
IP : GFP
IP : p50
IP : p65
IP : p52
Δ4 Ø
GFP
p50
p65
p52
50kDa
50kDa
65kDa
52kDa

Figure 3

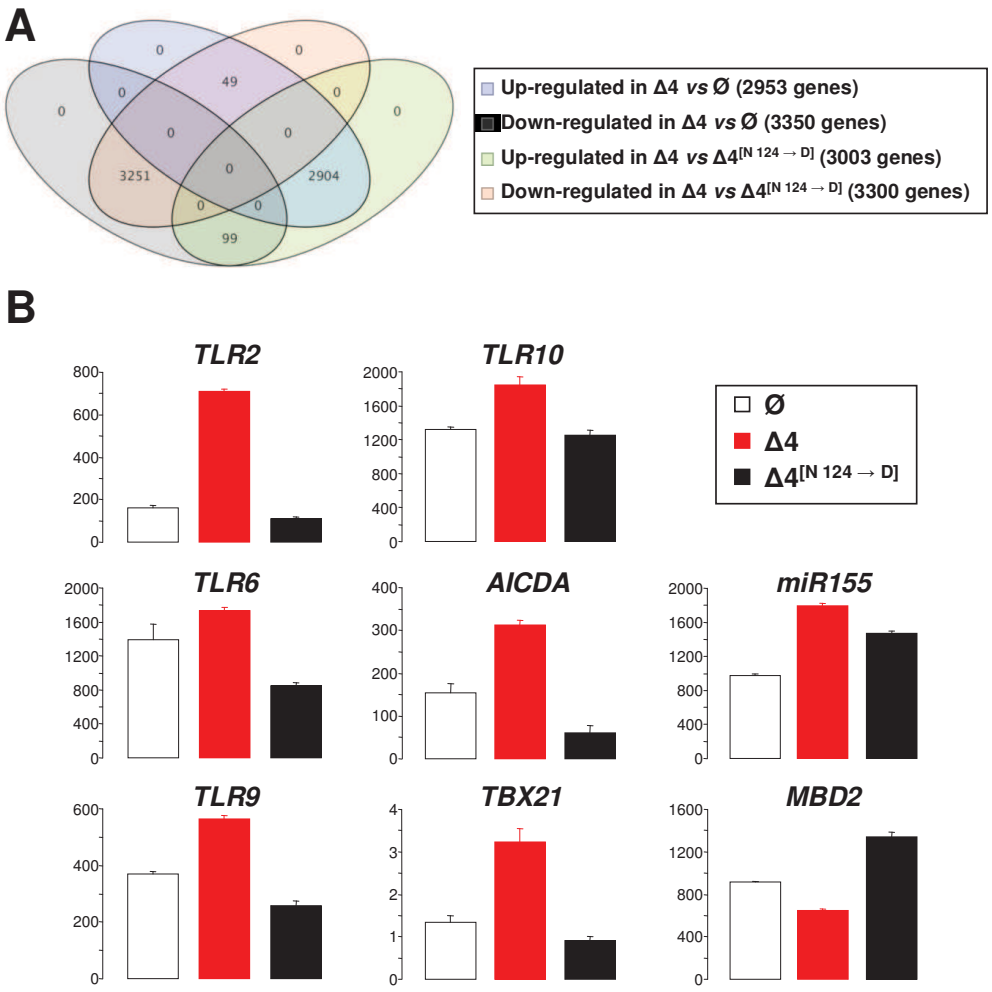
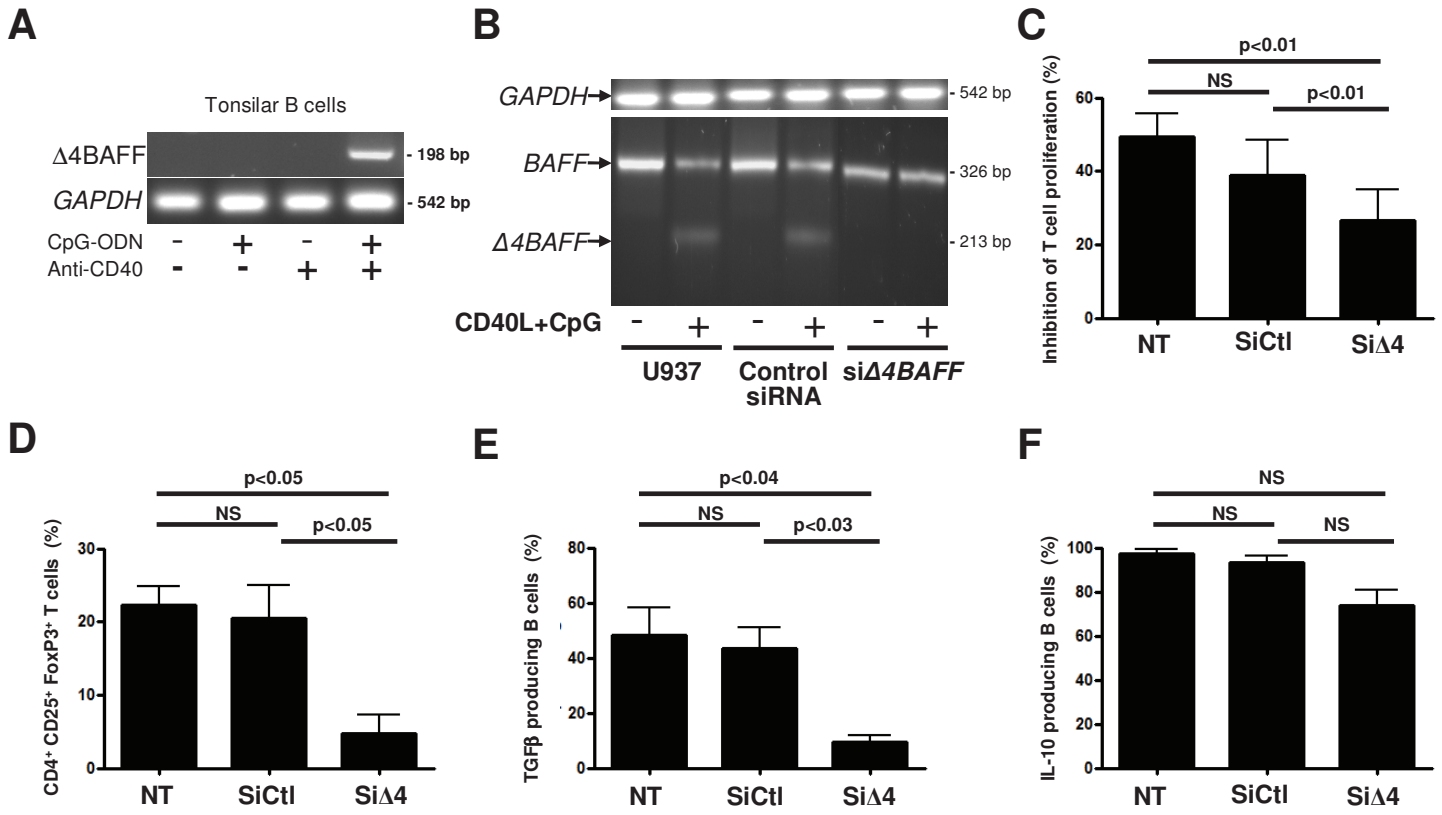
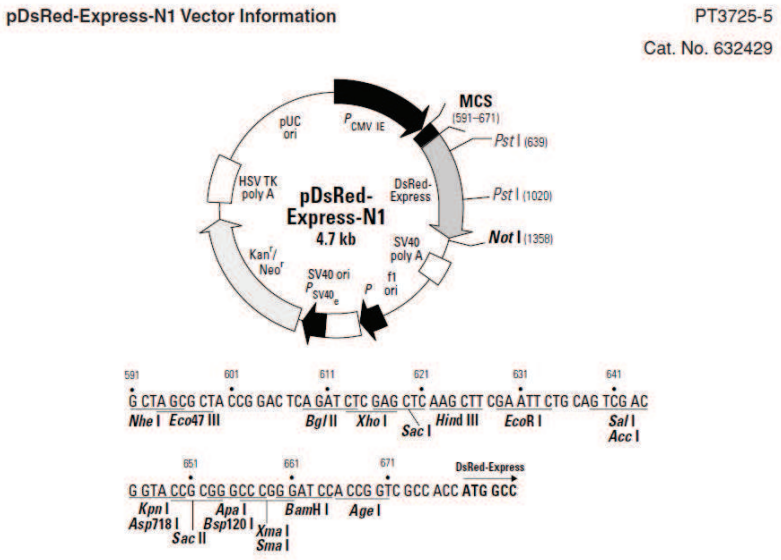
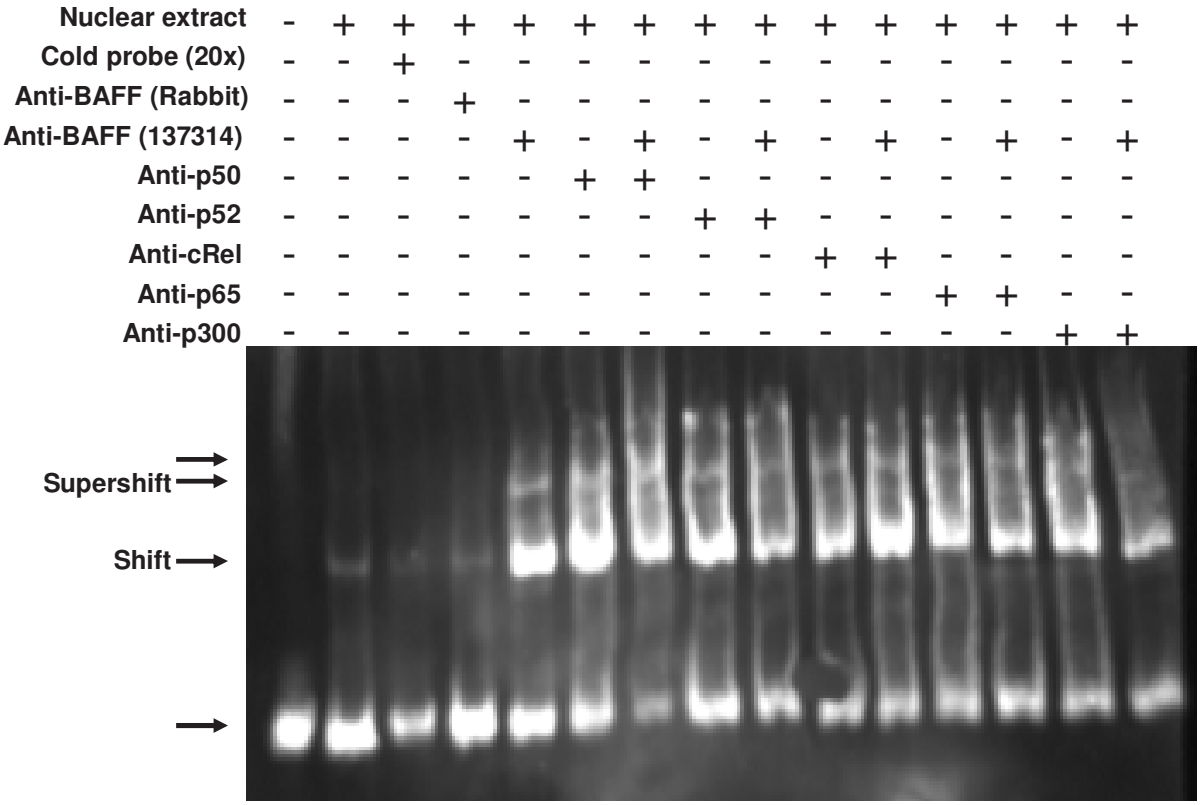

A
0
0
49
0
0
0
0
0
0
3251
2904
0
0
0
99
Up-regulated in Δ4 vs Ø (2953 genes)
Down-regulated in Δ4 vs Ø (3350 genes)
Up-regulated in Δ4 vs Δ4[N 124 → D] (3003 genes)
Down-regulated in Δ4 vs Δ4[N 124 → D] (3300 genes)
B
TLR2
TLR10
Ø
Δ4
Δ4[N 124 → D]
TLR6
AICDA
miR155
TLR9
TBX21
MBD2

Figure 4


A
Tonsilar B cells
Δ4BAFF
GAPDH
- 198 bp
- 542 bp
CpG-ODN - + - +
Anti-CD40 - - + +
B
GAPDH
BAFF
Δ4BAFF
- 542 bp
- 326 bp
- 213 bp
CD40L+CpG - + - + - +
U937
Control siRNA
siΔ4BAFF
C
p<0.01
NS
p<0.01
Inhibition of T cell proliferation (%)
60
40
20
0
NT
SiCtl
SiΔ4
D
p<0.05
NS
p<0.05
CD4+ CD25+ FoxP3+ T cells (%)
30
20
10
0
NT
SiCtl
SiΔ4
E
p<0.04
NS
p<0.03
TGFβ producing B cells (%)
80
60
40
20
0
NT
SiCtl
SiΔ4
F
NS
NS
NS
IL-10 producing B cells (%)
100
80
60
40
20
0
NT
SiCtl
SiΔ4



Supplementary Figure 1 : Map of vector used in this study. pDsRed-Express1 vector was used to cloned *BAFF* promoter region 1.



Supplementary Fig. 2 : EMSA analysis of Δ4BAFF and NF-κB binding to the *BAFF* promoter. Nuclear extracts from RAMOS cells stably transfected with pΔ4*BAFF*-EGFP were incubated with digoxigenin-labeled BAFF-NF-κB binding site oligonucleotides. BAFF-NF-κB cold probe and antibodies (Abs) to BAFF (the monoclonal Ab, clone 137314, able to recognize Δ4BAFF and the rabbit polyclonal Ab, unable to recognize Δ4BAFF), 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.

3. TLR9 EXPRESSED ON PLASMA MEMBRANE ACTS AS A NEGATIVE REGULATOR OF HUMAN B CELL RESPONSE

J Autoimmunity, 2004, in press

Toll like receptors (TLRs) play an important role in the early detection of pathogen associated molecular patterns and are conductive to the activation of the innate immune responses and subsequently, of the adaptive immune system. In this report, we show the presence of TLR9 on the plasma membrane of B cells. The expression of TLR 9 is higher on peripheral blood B cells whereas tonsilar B cells show intermediate levels of expression. TLR 9 activation needs cleavage of its full form. Indeed, experiments showed the presence of cleaved TLR9 on plasma membrane of B cells. The presence of cleaved TLR9 on the plasma membrane suggests that TLR9 could be functionally operant. TLR9 present in the endosomal compartment co-localizes with the BCR leading to B cell hyper response. The complete and cleaved form of TLR9 located on the plasma membrane of B cells co-localize with the BCR in the lipid rafts after BCR stimulation. In contrast, without BCR stimulation, TLR9 is mostly found outside the lipid rafts, suggesting that cell surface TLR 9 could act as a co-receptor of the BCR and may be able to modulate the BCR activation response of B cells. However, the cell surface TLR9 does not bind to the endosomal TLR9 ligand CpG-B. The co-stimulation of BCR and cell surface TLR9 with anti-TLR9 antibody enhanced the phosphorylation profile of the lysate protein which in turn increased the activation of the MAP kinase ERK pathway. The activation of B cells, checked by CD25 expression, was downregulated after anti-TLR9 antibody and BCR co-stimulation in addition with IL-2. The proliferative response of B cells also varies according to the localization of TLR9. The stimulation of the cell surface TLR9 with anti-TLR9 antibody prevents BCR and IL-2 induced proliferation, suggesting that the cell surface TLR9 differs from the endosomal TLR9 and does not act synergistically with the BCR to promote the B cell response. Because signalling cascades can be initiated from the cell surface TLR9, the effects of the cell surface TLR9 were verified on the B cell responses that had been induced by endosomal TLR9 stimulation. The activation of B cells following endosomal TLR9 stimulation was inhibited in the presence of anti-TLR9 Ab, leading to the downregulation of CD25. The synergistic effects of endosomal TLR9 and BCR-induced proliferation were also inhibited when cell surface TLR9 was stimulated; suggesting that cell surface TLR9 plays the role of negative regulator of the endosomal TLR9-induced human B cell response. In this report we described the presence of both full length and cleaved active form of TLR9 on the plasma membrane of human B cells. This cell surface TLR9 acts as a negative regulator of the endosomal TLR9- induced B cell response.

TLR9 EXPRESSED ON PLASMA MEMBRANE ACTS AS A NEGATIVE REGULATOR OF HUMAN B CELL RESPONSE

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Abstract

Toll like receptors (TLRs) are positioned at the interface between innate and adaptive immunity. Unlike others, those such as TLR9, that recognize nucleic acids, are confined to the endosomal compartment and are scarce on the cell surface. Here, we present evidence for TLR9 expression on the plasma membrane of B cells. In contrast to endosomal TLR9, cell surface TLR9 does not bind CpG-B oligodeoxynucleotides. After B cell-receptor (BCR) stimulation, TLR9 was translocated into lipid rafts with the BCR, suggesting that it could serve as a co-receptor for BCR. Nevertheless, stimulation of B cells with anti-TLR9 antibodies did not modify the BCR-induced responses despite up-regulation of tyrosine phosphorylation of proteins. However, CpG-B activation of B cells, acting synergistically with BCR signals, was inhibited by anti-TLR9 stimulation. Induction of CD25 expression and proliferation of B cells were thus down-regulated by the engagement of cell surface TLR9. Overall, our results indicate that TLR9 expressed on the plasma membrane of B cells might be a negative regulator of endosomal TLR9, and could provide a novel control by which activation of autoreactive B cells is restrained.

Key words: B lymphocytes, TLR9, cell surface, CpG-B, stimulation, negative regulator

Research highlights:

- TLR9 is present on the B cell surface
- CpG-B does not bind to plasma membrane TLR9
- Cell surface TLR9 negatively regulates endosomal TLR9-induced B cell response

Introduction

The family of toll-like receptors (TLRs) stands at the junction between innate and adaptive immunity (Abdelsadik and Trad 2011). They are essential in the discrimination between self and non-self. They lead to the development of immune response against a wide variety of pathogens while avoiding abnormal response to endogenous ligands due to the presence of numerous negative regulators (Kawai and Akira 2007).

TLRs are differentially expressed by the different subsets of B cells, conferring a large range of functional responses. Thus, transitional and MZ B cells are highly sensitive to TLR9 stimulation resulting in activation, proliferation and immunoglobulin production (Guerrier, Le Pottier et al. 2012). In these situations, paired BCR and TLR signals up-regulate gene products not induced by BCR or TLR9 alone and can cooperate to facilitate B-cell differentiation (Rawlings, Schwartz et al. 2012). In contrast, follicular B cells are poorly activated due to the presence of regulated events (Meyer-Bahlburg and Rawlings 2012). Identification of these regulatory elements remains a major challenge in view of a control of the TLR9-dependent B cell responses that might be aberrantly activated in autoimmune diseases (Papadimitraki, Bertsias et al. 2007).

Like all TLRs that recognize nucleic acids, TLR9 is confined to the endoplasmic reticulum and to endolysosomes (Barbalat, Ewald et al. 2011). Activation of TLR9 requires the acidification of endosomal compartments that in turn influences direct binding and interaction with its ligand (Rutz, Metzger et al. 2004) and leads to its cleavage, a prerequisite of its activation (Ewald, Lee et al. 2008; Park, Brinkmann et al. 2008). Such intracellular localization and cleavage restrain TLR9 activation to ligands able to reach endolysosomes in sufficient quantities, which is the case for viral and bacterial DNA but normally not the case for self DNA (Barton, Kagan et al. 2006). Thus, a transmembrane TLR9 construct artificially expressed on the cell surface is not functional in its complete form whilst the cleaved mutated form bypasses the requirement of proteolysis and provides sensitivity to mammalian DNA (Mouchess, Arpaia et al. 2011). The intracellular localization and cleavage

requirement prevent the recognition of self DNA and preserve tolerance breakdown (Barton, Kagan et al. 2006).

However, natural cell surface expression of TLR9 has been reported. Intestinal (Lee, Mo et al. 2006) and gastric (Schmausser, Andrulis et al. 2004) epithelial cells have been shown to be able to express TLR9 on their plasma membrane, although the functionality of this TLR9 remains to be clearly established. Moreover, it seems likely that human B lymphocytes can also express cell surface TLR9 (Eaton-Bassiri, Dillon et al. 2004; Dasari, Nicholson et al. 2005; Baiyee, Flohe et al. 2006) but its functional role has not been determined. In the present study, we wish to evaluate the presence of TLR9 on the plasma membrane of human B cells and identify its function on B cell response.

Material and methods

Isolation of B lymphocytes

Cord blood, peripheral blood and tonsils were collected after informed consents had been obtained. Tissues were minced up and filtered to remove fragments and clumps. Cord blood samples, peripheral blood samples and tonsillar cell suspensions were layered onto Ficoll-Hypaque and centrifuged. Mononuclear cells were incubated with neuraminidase-treated sheep red blood cells and T cells depleted by a second round of centrifugation. All preparations were >95% pure B cells.

Flow cytometry

All mAb were purchased from Beckman Coulter, unless otherwise indicated. We used phycoreythrin (PE)-conjugated anti-CD24, PE-cyanin5-conjugated anti-CD38 and biotinylated anti-human TLR9 (Imgenex) revealed using PE-cyanin7-conjugated streptavidin. For the activation response, B cells were stained with fluorescein isothiocyanate (FITC)-conjugated anti-CD25.

For the proliferation assay, B cells were preliminary labeled with 2µM carboxyfluorescein diacetate succinimidyl ester (CFSE) before stimulation and their proliferation evaluated on a FC500 flow cytometer (Beckman Coulter) measuring the decrease in mean fluorescence intensity (MFI) of CFSE.

Cultures of B lymphocytes

B cells were cultured in RPMI1640 medium (Invitrogen Life Technologies) supplemented with 10% heat-inactivated fetal calf serum, 2mM L-glutamine, 200U/ml penicillin and 100µg/ml streptomycin at 2.10^5 cells/well in 96-well culture plates. They were stimulated with 0.25µM CpG-B 2006 (Cayla-InvivoGen), or 10µg/ml anti-IgM-coated beads (BioRad) in the presence of 100U/ml recombinant IL-2 (ImmunoTools) or 10µg/ml anti-TLR9 Abs (clone 26C593.2, Imgenex, or clone eB72-1665, eBioscience) cross-linked on 10µg/ml anti-mouse IgG or anti-rat IgG (Jackson ImmunoResearch Laboratories) coated plates.

Immunofluorescence analysis

B cells were stained with mouse anti-human TLR9 (Imgenenex) revealed with FITC- or tetramethylrhodamine-5,6-isothiocyanate (TRITC)-conjugated donkey anti-mouse

IgG (Jackson). They were co-stained with either rabbit anti-human IgM (Dako) revealed with TRITC-conjugated donkey anti-rabbit IgG (Jackson) or TRITC-conjugated cholera toxin B (CTB, Sigma), or with FITC-conjugated CpG-B (Invivogen). Cells were then fixed in 4% paraformaldehyde, cytospined and analyzed with a TCS-NT confocal imaging system (Leica). Control mouse IgG with either FITC-conjugated or TRITC-conjugated donkey anti-mouse, and control rabbit IgG with TRITC-conjugated donkey anti-rabbit did not reveal background fluorescence.

Isolation of lipid rafts

Based on their insolubility in non-ionic detergent and their low density leading to their separation on a discontinuous sucrose gradient, lipid rafts were isolated from B cell plasma membranes. To this end, tonsillar B cells were first stimulated or not with 10µg/ml rabbit anti-IgM cross-linked with sheep anti-rabbit IgG (Sigma) for 10 minutes at 37°C. After washing at 4°C in TNE buffer (25mM Tris-HCl pH7.5, 140mM NaCl and 1mM EDTA), cells were incubated for 30 minutes in 1% Triton X-100 in TNE buffer containing anti-proteases cocktail (Sigma). One ml of supernatant was mixed with 1ml 85% sucrose, covered with 3ml of 35% sucrose and 1.5ml 5% sucrose, and centrifuged for 17 hours at 180000xg at 4°C. Eleven fractions were collected from the bottom upwards, the latest corresponding to the lipid rafts, and analyzed by Western blot.

Western blot assay

Cell surface expressed proteins were purified using the Cell Surface Isolation Kit (Pierce) according to the manufacturer's instructions. Samples were separated by SDS-PAGE electrophoresis and proteins transferred on polyvinylidene difluoride (PVDF) membranes. After 1 hour of saturation with 5% milk in 0.1% Tween 20 buffer, PVDF membranes were incubated in the presence of either rat anti-TLR9 (Imgenex), rabbit anti-CD20 (Interchim), mouse anti-β-actin (Abcam), rabbit anti-EEA1 (Abcam), horseradish peroxidase (HRP)-conjugated anti-IgM heavy chain (Dako), or biotinylated CTB (Sigma). After washes, HRP-conjugated anti-rat, anti-rabbit or anti-mouse immunoglobulins (all from Jackson), or HRP-conjugated

streptavidin (Amersham) were added, revealed using the ECL Advance kit (GE Healthcare) and membranes analyzed with Quantity One Software (BioRad).

For the activation assay, stimulated cells were lysed and proteins separated as specified above. Detection of phosphorylated tyrosine and phospho ERK were performed using mouse anti-phosphotyrosine (Abcam) and mouse anti-phospho ERK (BD Biosciences), revealed with HRP-conjugated anti-mouse immunoglobulins as above.

Statistical analysis

Data were expressed as mean±SD. Statistical analyses were performed using chi-squared test for comparisons of percentages. Significance was assessed at $P<0.05$.

Results

TLR9 is expressed on B cell plasma membrane

Expression of cell surface TLR9 on non-permeabilized B lymphocytes was determined by flow cytometry (Fig. 1A) and assessed as MFI. Mature B cells and transitional B cells isolated from cord blood expressed low level of TLR9 with a MFI of 1.8±0.1 and 2.2±0.1, respectively. TLR9 expression was elevated on peripheral blood B lymphocytes (MFI of 16.1±1.1) and intermediate on tonsillar B cells (MFI of 6.3±0.7).

To become active, endosomal TLR9 must be cleaved. A soluble fragment is generated in the endosomal lumen which can bind to the transmembrane cleaved form. We looked for a cleavage form of plasma membrane TLR9. Proteins from the surface of B cells were biotinylated, and plasma membranes lysed. Biotinylated proteins were purified on NeutrAvidin column and separated by SDS-PAGE. Western blot analysis using anti-TLR9 mAb revealed a 130kDa band corresponding to the entire form of TLR9 and a supplementary 60kDa band corresponding to the cleaved fragment (Fig. 1B). Western blots were repeated on the whole cell lysates without biotinylation. Densitometric analyses led to determine the ratio of cleaved form (60kDa) of TLR9/entire form (130kDa) of TLR9 (Fig. 1C). It was interesting to note a higher ratio with the biotinylated cell surface proteins indicating that most of TLR9 receptors on the B cell surface are cleaved. Overall, our results suggest that TLR9 can be expressed on the plasma membrane of B lymphocytes and can be cleaved into a potentially active form which constitutes the predominant form on the cell surface. A schematic representation is shown in Fig. 1D.

Role of TLR9 expressed on the plasma membrane

To go further, we wondered if TLR9 expressed on cell surface could be functionally operant. It has been demonstrated that endosomal TLR9 co-localizes with internalized BCR within endosomes resulting in B cell hyper response (Chaturvedi, Dorward et al. 2008). We set out to determine whether cell surface TLR9 co-localized with cell surface BCR. Lipid rafts were fractionated without prior stimulation of the BCR (Fig. 2A). We observed that in fraction 11 enriched in lipid rafts, weak bands of both the

entire form and the cleaved form of TLR9 were detectable. Significantly, after BCR stimulation, a large portion of BCR was translocated within lipid rafts of fraction 11 in association with higher co-localization of both complete and cleaved TLR9, as shown by the densitometric analyses (Fig. 2B). Indirect immunofluorescence studies confirmed these observations. Prior to BCR stimulation, TLR9 was mostly found outside the lipid rafts. However, after BCR stimulation, BCR translocated within lipid rafts yielded to co-localization of TLR9 (Fig. 2C). These results suggest that cell surface TLR9 could act as a co-receptor of the BCR able to modulate the BCR activation response of B cells.

Effect of cell surface TLR9 stimulation

To evaluate the B cell response after stimulation of cell surface TLR9, we first evaluated its ability to bind CpG-B, the identified ligand of endosomal TLR9. By flow cytometry analysis, we found that the binding level of FITC-conjugated CpG-B on cell surface increased with concentration after incubation at 4°C to avoid internalization (Fig. 3A). But unexpectedly, we were unable to observe strong co-localization between membrane TLR9 and FITC-CpG-B through indirect immunofluorescence examination (Fig. 3B). This study indicates that cell surface TLR9 does not bind preferentially the well-known ligand of endosomal TLR9.

Therefore, we decided to activate the B cells using anti-TLR9 Ab without permeabilization to ensure specific stimulation of the plasma membrane TLR9. The early tyrosine phosphorylation response was firstly assessed. After 3 min of anti-BCR stimulation, the phosphorylation of the proteins had clearly increased, and was also up-regulated after 5 min of stimulation with CpG-B as well as anti-TLR9 stimulation (Fig. 4A, left). Since cell surface TLR9 co-localized with the BCR in lipid rafts after BCR stimulation, we wondered whether it could act as a co-receptor for the BCR. We observed that, like co-stimulation of BCR and endosomal TLR9, co-stimulation of the BCR and cell surface TLR9 enhanced the phosphorylation profile of the protein lysates (Fig. 4A, right) that lead to increased activation of the MAP kinase ERK pathway. Thereby, we estimated the activated status of B cells stimulated with anti-TLR9 Ab by measuring CD25 expression by flow cytometry. In comparison to

stimulation of the BCR in the presence of IL-2, CD25 was not up-regulated on B cells after 24 hours of stimulation of cell surface TLR9 with anti-TLR9 (Fig. 4B). Its putative role as a BCR co-receptor was then appraised. However, in contrast to the co-stimulatory effect of endosomal TLR9 activated by CpG-B, anti-TLR9 Ab did not upregulate the BCR-induced expression of CD25, suggesting that B cells can not be activated through stimulation of cell surface TLR9. To go further, we determined the proliferative response of B cells. After 5 days, B cells proliferated following anti-BCR and IL-2 stimulation, mainly in association with CpG-B stimulation, but not with anti-TLR9 stimulation (Fig. 4C). These results suggest that cell surface TLR9 differs from endosomal TLR9 and does not act synergistically with the BCR to promote the B cell response.

Because signalling cascades can be initiated from the cell surface TLR9 (Fig. 4A), we wondered whether TLR9 on the plasma membrane could restrain the B cell response induced by the stimulation of endosomal TLR9. Activation of B cells following CpG-B stimulation was thus inhibited in the presence of anti-TLR9 Ab leading to a significant down-regulation of CD25 expression (Fig. 5A). Furthermore, the weak proliferative response observed after CpG-B stimulation was dampened by anti-TLR9 Ab co-stimulation (Fig. 5B). Interestingly, we found that the synergistic effect of endosomal TLR9 and BCR-induced proliferation was also abrogated by the concomitant stimulation of cell surface TLR9 (Fig. 5B), indicating that TLR9 on the plasma membrane may play the role of a negative co-receptor. The same results were obtained with all anti-TLR9 Ab tested (not shown). Yet, proliferation of B cells was strikingly inhibited when anti-TLR9 Ab was added to CpG-B alone or to CpG-B associated with anti-BCR and IL-2 (Fig. 5C) supporting the notion that the cell surface TLR9 might be a potent negative regulator of the endosomal TLR9-induced B cell response.

Discussion

The unavailability of the TLR9 active form on cell surfaces has long been considered a key element to discriminate between microbial DNA and self DNA. However, our data demonstrate that the complete form and the potentially active cleaved form of TLR9 are expressed on the plasma membrane of B lymphocytes. Interestingly, we observed cell surface TLR9 co-localization with activated BCR in lipid rafts, suggesting that plasma membrane TLR9 could influence BCR-dependent activation of B cells. However, we were puzzlingly unable to demonstrate an interaction of cell surface TLR9 with CpG-B, its synthetic ligand. Yet, binding of ligands to TLR9 requires an acidic environment (Rutz, Metzger et al. 2004). Thus, it is likely that the pH of the extracellular milieu is not sufficiently lowered to allow interaction of the known ligands with cell surface TLR9.

It cannot be ruled out that cell surface TLR9 results from a novel TLR9 variant. For instance, it has been described that the P99L variant, though retaining its ability to bind normally CpG-B, displayed severely compromised functional response regarding NF-κB activation and cytokine production (Kubarenko, Ranjan et al. 2010). Another TLR9 allele, R892W, is also hyporesponsive to CpG-B. It is characterized by increased MyD88 adaptor binding but defective co-localization with CpG-B leading to impaired B cell response as seen by decrease IL-6 and IL-10 production by B cells after CpG-B stimulation (Knezevic, Pavlinic et al. 2012). This particular mutation appears to change the surface charge and hydrophilicity of TLR9. While the homotypic dimmer formation seems normal the heterotypic interaction of TLR9 with MyD88 is affected. A stronger association is detected that might be responsible for impaired downstream signalling, as previously observed with a truncated TLR9 form that strongly interacts with MyD88 and is defective in signalling on its own (Ewald, Lee et al. 2008). Future works are needed, to determine whether novel TLR9 variant might account for the TLR9 cell surface expression leading to downstream signalling independent of CpG-B binding.

Alternatively, the possibility exists that TLR9 expressed on the plasma membrane interacts with ligands different from those of the endosomal TLR9, leading to the

activation of alternative signallings. This hypothesis is reinforced by our functional experiments. Whilst endosomal TLR9 signals synergized with BCR signals to enhance B cell activation and proliferation as previously observed (Busconi, Bauer et al. 2007), stimulation of cell surface TLR9 did not cooperate with the BCR to increase the B cell responses. Moreover, cross-linking of cell surface TLR9 with anti-TLR9 Abs negatively regulates the endosomal TLR9-induced B cell response. These findings argue for a negative regulatory activity of plasma membrane TLR9 on B cell responses following endosomal TLR9 stimulation. Differential signalling pathways have been identified depending on receptor location. In intestinal epithelial cells, stimulation of basolateral-expressed TLR9 induces activation of NF-κB pathway after degradation of IκBα, whereas apical TLR9 signals induces accumulation of ubiquitinated IκB in the cytoplasm preventing NF-κB activation (Lee, Mo et al. 2006). This specific polarization of TLR9 might contribute to restrain inflammatory responses in a bacteria-enriched environment, with apical TLR9 stimulation delivering negative signals in balance with activation signals triggered by basolateral TLR9 stimulation (Lee, Gonzales-Navajas et al. 2008). The signalling pathway activated following cell surface TLR9 engagement remains to be identified, but might likely differ from endosomal TLR9. While cell surface TLR4 activated TIRAP-MyD88 signalling at the plasma membrane, endocytosed TLR4 induced TRAM-TRIF signalling from early endosomes (Kagan, Su et al. 2008). Similarly, plasma membrane and endosomal TLR9 might activate differential signalling pathways leading to a control of the B cell activation and proliferative response. Additional experiments are required to identify these cascades.

Furthermore, our results raised several questions. To counteract the endosomal TLR9 pathways, cell surface TLR9 must be activated by interaction with a ligand that is still to be identified. Whether anti-TLR9 Ab mimics binding of soluble ligand or cell surface ligand expressed on the plasma membrane of B cells in cis or on the plasma membrane of other B cells or of other cell types in trans remains to be determined. This aspect appears important since identification of the natural ligand will help to understand this novel regulatory event that lead to control B cell responses. Another critical aspect of TLR9 activation is to decipher the way by which CpG-B reaches the

endosomal compartment. According to our observations, plasma membrane TLR9 does not supply the binding receptor for CpG-B endocytosis. DEC-205 has been recently identified as a cell surface receptor for CpG-B that contributes to its uptake and delivery to the endosomal TLR9. B cells express significant levels of DEC-205 which is important for CpG-B-dependent response (Kato, McDonald et al. 2006). In DEC-205-deficient mice, B cells are severely hampered in their ability to up-regulate CD40, CD86 and MHC class II molecules and showed a profound inability to produce IL-6 in response to CpG-B stimulation. DEC-205 is required for optimal CpG-B uptake to consequently facilitate B cell activation by promoting the delivery of captured CpG-B to endosomal TLR9 (Lahoud, Ahmet et al. 2012). However, in this model B cells can also acquire CpG-B uptake in a DEC-205-independent manner. This suggests that in addition to DEC-205, particular signalling platforms might be required for B cells to be fully responsive to CpG-B stimulation of endosomal TLR9 and possibly to cell surface TLR9 activation. In this respect, a novel molecular signalling platform has been characterized in macrophages to be essential for ligand activation of TLR9 and cellular signalling. This complex contains G-protein coupled receptor (GPCR), metallo-proteinase-9 (MMP9) and the lysosomal sialidase neuramimidase 1 (Neu1) (Abdulkhalek and Szewczuk 2013). It is likely that CpG-binding to endosomal TLR9 induces conformational changes (Latz, Verma et al. 2007) which potentiate GPCR-signalling through MMP9 activation inducing Neu1. Activated Neu1 then hydrolyzes α-2,3-sialyl residues linked to β-galactosides on TLR9. This structural modification would trigger the formation of homotypic dimmer that facilitate the recruitment of MyD88 adaptor and subsequent cellular response. On the cell surface, Neu1 appears also as an important intermediate of several TLR ligand-induced receptor activation and subsequent cellular function (Amith, Jayanth et al. 2009). Thus, TLR4 activation on macrophages is dependent on Neu1 in conjugation with GPCR and MMP9 signalling (Finlay, Abdulkhalek et al. 2010). Transactivation of cell surface receptors necessitates a molecular platform containing GPCR signalling in association with MMP9 and Neu1 cross-talk required for all identified cell surface TLRs (Abdulkhalek, Guo et al. 2012). Whether cell surface expressed TLR9 needs similar signalling platform to activate the B cell response after

binding of its specific ligand remains elusive. However, the detection of cleaved form and the up-regulation of tyrosine phosphorylated proteins subsequent to anti-TLR9 stimulation suggest that conformational changes also occurred for TLR9 on the B cell surface.

Moreover, additional proteins are required for cell surface as well as endosomal TLR activation by their respective ligands. As an example, CD14 is associated with MyD88-dependent cell surface TLR4 and constitutively interacts with MyD88-dependent TLR9 in macrophages (Baumann, Aspalter et al. 2010). Interestingly, the absence of CD14 reduces nucleic acid uptake and alters TLR-dependent cytokine production suggesting that CD14 promotes selective nucleic acid and acts as a co-receptor for endosomal TLR9 activation. CD14 could contribute to the engagement of nucleic acids by a holo-receptor in analogy to LPS-recognition by the TLR4-CD14 complex (Fitzgerald, Rowe et al. 2004). Alternatively, CD14 might act indirectly by providing a physical platform for the recruitment of factors required to assemble a fully functional receptor complex (Schmitz and Orso 2002). On the surface of B cells that lack CD14 expression, other molecules may play similar functions. For example, HMGB-1, although not membrane associated, binds and enhances nucleic acid uptake into the endosome, suggesting it could play a role in autoimmune responses (Tian, Avalos et al. 2007). Currently, HMGB-1 can form immune complex with nucleic acid and stimulate the BCR of autoreactive B cells (Avalos, Kiefer et al. 2010). However, TLR2, TLR4 as well as CD24-SiglecG/10 on the B cell surface have been identified as receptors for HMGB-1 (Li, Liang et al. 2013) that promote B cell reactivity. The possibility therefore exists that HMGB1 could also interact with cell surface TLR9 to influence the B cell activation. Finally, the BCR is an alternative receptor that provides efficacious endocytosis of the intracellular ligands by B cells (Lanzavecchia and Sallusto 2007). Consequently, activation of autoreactive B cells requires efficient regulatory mechanisms to avoid aberrant autoimmune reactions (Marshak-Rothstein 2006). Our data suggest that responses of B cells to endosomal TLR9 either alone or in association with the BCR can be curtailed by engagement of cell surface TLR9 which might provide a novel regulatory event of autoreactive B cells.

Whether cell surface TLR9 negative regulator is defective in autoimmune conditions is an open question that warrants further investigation. It has been recently demonstrated that TLR9 possesses a paradoxical role. Required for activation of anergic self reactive anti-DNA B cells, TLR9 also promotes tolerance by restricting their survival (Nickerson, Christensen et al. 2013). We may infer that endosomal and plasma membrane signals may offset each other in order to influence the final behavior of autoreactive B cells. Defect in TLR9 signalling from cell surface would then encourage the development of autoimmune reactions and could be a novel therapeutic target in autoimmune diseases.

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LEGEND OF FIGURES

Figure 1 **TLR9 is expressed on B cell surface. A.** Flow cytometry analysis of cell surface expression of TLR9 on B cells from cord blood, peripheral blood and tonsil. **B.** Proteins on the surface of B cells were biotinylated, solubilized and separated on gel electrophoresis before Western blot analyses, and compared to whole cell lysates. Anti-EEA1 and anti-β actin Abs were used as controls for the presence of endosomal and plasma membrane proteins, respectively. Anti-CD20 Ab was used to confirm the presence of a well-known cell surface expressed protein. Anti-TLR9 Ab revealed a 130kDa complete form and a 60kDa Nterminal cleaved form of TLR9. **C.** Ratio of cleaved TLR9/complete TLR9 obtained after densitometric analyses (mean±SD of 3 experiments). **D.** Schematic representation of the different forms of TLR9 expressed on the cell surface of B cells. The Nterminal cleaved fragment can interact with the Cterminal cleaved form and with the complete form. The black square indicates the epitope location recognized by the anti-TLR9 Ab used in flow cytometry and Western blot studies.

Figure 2 TLR9 and BCR co-localized within lipid rafts on B cell surface after BCR activation. B cells were stimulated or not with 10µg/ml anti-IgM. A. Co-localization of TLR9 and BCR was evaluated on lipid raft-enriched protein fraction with anti-TLR9 and anti-µ chain Abs. Lipid rafts were enriched in fraction 11, as depicted by staining with cholera toxin B (CTB). B. Densitometric analyses (mean±SD of 3 experiments). C. Co-localization (yellow color) of TLR9 (green color) and BCR or CTB (red color) before and after BCR stimulation. Confocal microscopy analysis was performed after staining of B cells with anti-TLR9 Ab revealed with FITC-conjugated anti-Ig and with anti-IgM Ab revealed with TRITC-conjugated anti-Ig or with TRITC-conjugated CTB.

Figure 3 **TLR9-independent binding of CpG-B on the B cell surface. A.** Flow cytometry analysis of CpG-B binding on the surface of B cells after incubation at 4°C of different concentrations (0.3µM, 1µM and 3µM) of FITC-conjugated CpG-B. **B.** Confocal microscopy analysis of B cells stained at 4°C with FITC-conjugated CpG-B and anti-TLR9 Ab revealed with TRITC-conjugated anti-Ig.

Figure 4 Differential responses of B cells after cell surface TLR9 or endosomal TLR9 stimulation. B cells were stimulated or not with 10µg/ml anti-IgM Ab with 100U/ml IL-2 in the presence or absence of 10µg/ml anti-TLR9 Ab or 0.25µM CpG-B. A. After stimulation of 3 and 5 min, total B cell lysates were prepared for Western blot analysis using anti-phosphotyrosine or anti-phospho ERK Abs. Detection of β-actin served as control. A representative experiment is shown. B. After 24 hours of stimulation, induction of CD25 expression on B cells was evaluated by flow cytometry using FITC-conjugated anti-CD25 Ab, * $P<0.05$, (mean±SD of 6 experiments). C. After 4 days of stimulation, the proliferative response was determined on flow cytometer by the dilution of CFSE expression from cells stained with CFSE before stimulation. Representative experiments are shown where dotted histograms correspond to the staining of unstimulated B cells.

Figure 5 **Cell surface TLR9 stimulation inhibits endosomal TLR9 responses. B cells were stimulated with 0.25µM CpG-B with or without 10µg/ml anti-TLR9 Ab in the presence or absence of 10µg/ml anti-IgM Ab with 100U/ml IL-2. A.** CD25-induced expression was determined by flow cytometry using FITC-conjugated anti-CD25 Ab after 24 hours, *$P<0.05$, (mean±SD of 6 experiments). **B.** Proliferation was evaluated after 4 days on flow cytometer by the dilution of CFSE expression in B cells stained before stimulation. A representative experiment is shown where dotted histograms correspond to CFSE staining of unstimulated cells. **C.** Cell surface

TLR9 inhibitory effect on proliferation was expressed as the percentage of inhibition relative to B cells cultured without anti-TLR9 Ab.

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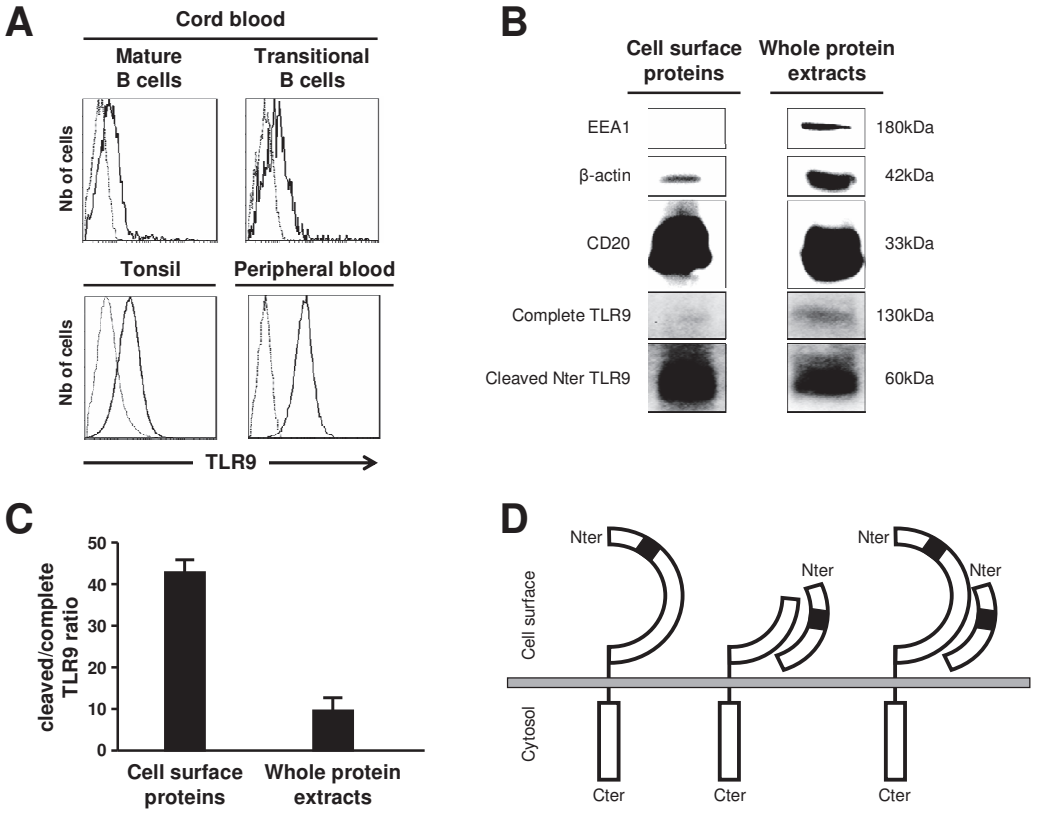
Figure 1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Mature B cells
Transitional B cells
Nb of cells
Tonsil
Peripheral blood
Nb of cells
TLR9
B
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180kDa
β-actin
42kDa
CD20
33kDa
Complete TLR9
130kDa
Cleaved Nter TLR9
60kDa
C
cleaved/complete TLR9 ratio
50
40
30
20
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Cell surface proteins
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D
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Cell surface
Cytosol
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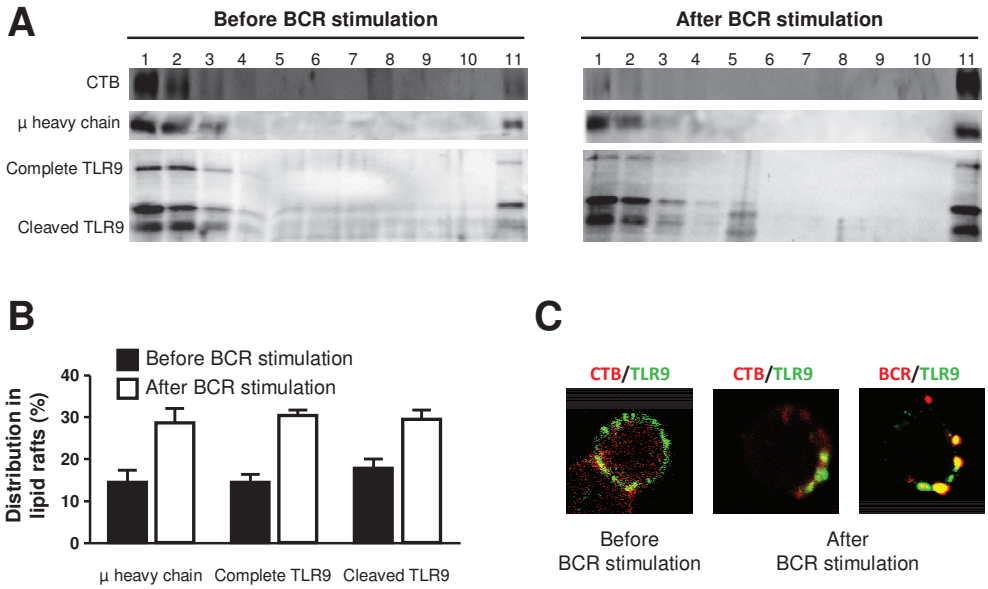
Cter

Figure 2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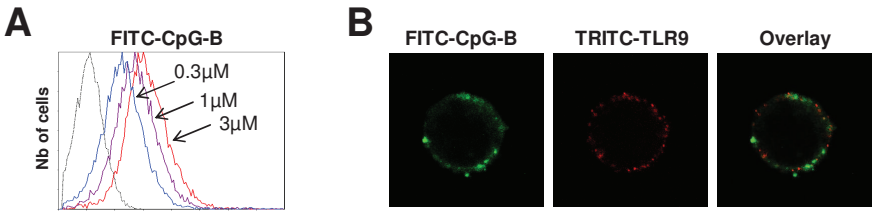
Before BCR stimulation
After BCR stimulation
1 2 3 4 5 6 7 8 9 10 11
CTB
μ heavy chain
Complete TLR9
Cleaved TLR9
B
Before BCR stimulation
After BCR stimulation
Distribution in lipid rafts (%)
40
30
20
10
0
μ heavy chain
Complete TLR9
Cleaved TLR9
C
CTB/TLR9
CTB/TLR9
BCR/TLR9
Before BCR stimulation
After BCR stimulation

Figure 3


A
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0.3µM
1µM
3µM
B
FITC-CpG-B
TRITC-TLR9
Overlay

Figure 4

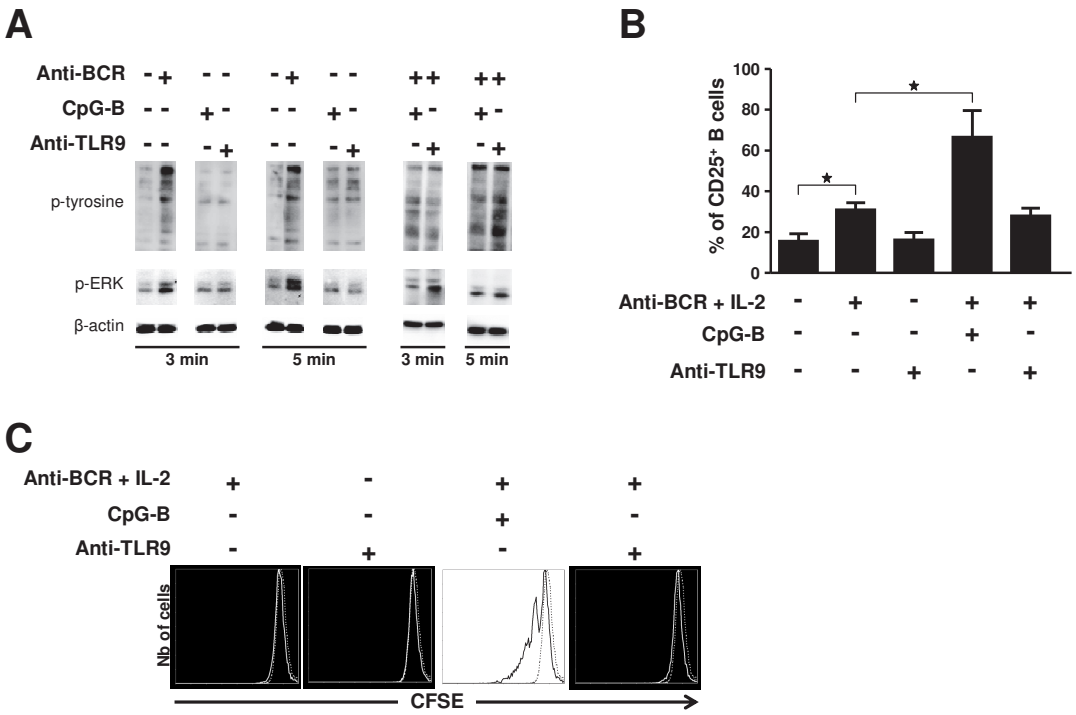
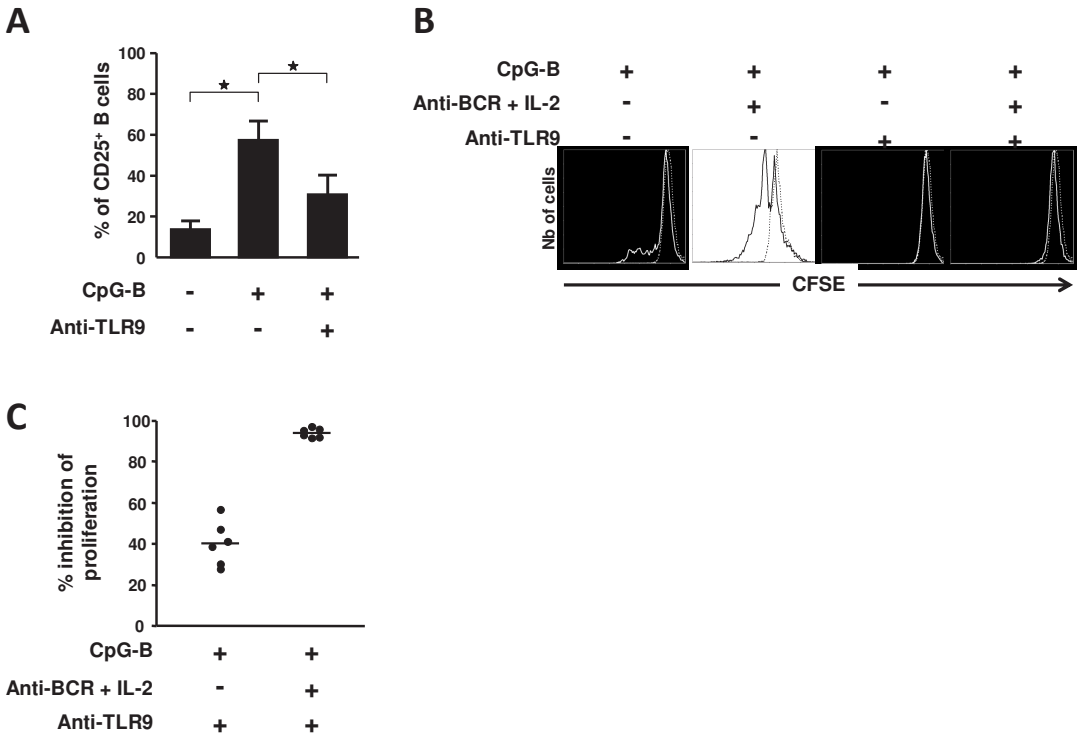

A
Anti-BCR
CpG-B
Anti-TLR9
p-tyrosine
p-ERK
β-actin
3 min
5 min
3 min
5 min
B
% of CD25+ B cells
100
80
60
40
20
0
Anti-BCR + IL-2
CpG-B
Anti-TLR9
C
Anti-BCR + IL-2
CpG-B
Anti-TLR9
Nb of cells
CFSE

Figure 5


A
% of CD25+ B cells
100
80
60
40
20
0
*
*
CpG-B - + +
Anti-TLR9 - - +
B
CpG-B + + + +
Anti-BCR + IL-2 - + - +
Anti-TLR9 - - + +
Nb of cells
CFSE
C
% inhibition of proliferation
100
80
60
40
20
0
CpG-B + +
Anti-BCR + IL-2 - +
Anti-TLR9 + +

III. GENERAL DISCUSSION

Our study deals with various aspects of immunology and autoimmunity starting from the implication of the B cell survival factor BAFF, and its receptor BR3 in EC survival to the identification of a new splice variant of BAFF. We also focused on B cell biology and the functions of TLRs and showed the presence of TLR9 on the plasma membrane of B cells and its effect on the TLR9-induced B cell response.

In the first part, we showed that ECs can express BAFF and its receptor BR3. BR3 modulates the survival of ECs and this phenomenon is PKCδ dependent. We also showed that different forms of BAFF are implicated in the survival of EC mediated by BR3, suggesting that differently glycosylated or non-glycosylated forms are present and act differently.

The second study deals with the functions of a newly identified transcriptional variant of BAFF. This variant lacks the exon 4 in humans. The exon 4 of the *BAFF* gene in humans shares a similarity with the exon 5 in mice. We also observed this transcriptional variant in mice in which the exon 5 had been spliced out. The mechanism of splicing regarding the formation of this new splice variant was also studied in details. We reported that Δ4BAFF acts, in association with p50 from the NF-κB pathway, as a transcription factor for its own parent gene and that Δ4BAFF expression is required for regulatory B cell functions.

The last part of our study shows the presence of TLR9 on the plasma membrane of B cells. Both the full form and the active cleaved form of TLR9 were found on the plasma membrane. A study identified that the cell surface TLR9 could act as a co-receptor of BCR and modulate the BCR-activated B cell response. The cell surface TLR9 plays the role of a negative regulator of the endosomal TLR9-induced human B cell response. However, the ligand of TLR9 at the cell surface is still not identified as CpG-B, the well-known ligand for endosomal TLR9, and does not bind to the cell surface TLR9.

In the first part of our study we investigated the role of BR3 and BAFF on EC survival. This is the first time that BR3 is implicated in the survival of epithelial cells. BAFF interacts with three receptors of the TNF family: BAFFR/BR3 (Thompson, Bixler et al. 2001; Yan, Brady et al. 2001), BCMA (Gras, Laabi et al. 1995) and TACI (von Bulow and Bram 1997). However, additional evidence suggests that BR3 is the main receptor used by BAFF to maintain its survival function. Indeed, BR3 knock-out mice display far less peripheral B cells

and a decreased production of circulating immunoglobulins which is also the case in BAFF knock-out mice (Sasaki, Casola et al. 2004). Mice lacking both TACI and BCMA do not exhibit a significant loss of B cells (Shulga-Morskaya, Dobles et al. 2004). In addition, the interaction between BR3 and BAFF is also important for the survival of other cell types. Neuronal cells express both BAFF and its receptor BR3. In neuronal cells, BR3 deficiency reduces the survival of neuronal cells (Tada, Yasui et al. 2013). Thus, it is clear that different cells express BAFF and its receptor BR3 and that the interaction of BAFF with this receptor modifies the survival properties of these different cells. Indeed, we also observed that the SGEC from SS patients and the EC lines express BAFF and its receptor BR3. However, the expression of other BAFF receptors, such as BCMA and TACI, was not observed in both SS patients and HSG cells, suggesting that BR3 is the only BAFF receptor expressed by ECs. However, BR3 expression by the EC is not related to SS because we also observed BR3 expression in healthy controls. Inhibiting the function of BR3 by BR3-specific siRNA results in increase of apoptosis and a reduced proliferation in ECs.

The protein kinase C (PKC) is a family of proteins that acts as a major regulator of cell death, tumor progression and cell proliferation. PKCδ has long been implicated in the apoptosis process of different cells (Basu 2003; Griner and Kazanietz 2007). PKCδ needs to be cleaved catalytically to become active and to induce apoptosis (LaGory, Sitailo et al. 2010). The intracellular localization of PKCδ is essential because it enables its ability to induce apoptosis through distinct signalling pathways. Depending on the cell types, and apoptotic stimuli PKCδ translocates to different subcellular organelles (Brodie and Blumberg 2003). PKCδ translocated to the mitochondria or the nucleus induces apoptosis. On the contrary, PKCδ localization in the endoplasmic reticulum protects against apoptosis (Gomel, Xiang et al. 2007). The tyrosine phosphorylation of PKCδ is crucial for regulating its nuclear localization and its proteolytic cleavage. Two tyrosine phosphorylation sites in the regulatory domain and two near the caspase cleavage sites have been shown to facilitate PKCδ nuclear translocation and proteolytic cleavage respectively (Kaul, Anantharam et al. 2005; DeVries-Seimon, Ohm et al. 2007). PKCδ possesses a nuclear localization sequence and the nuclear localization of PKCδ from the cytoplasm is required to induce apoptosis (DeVries, Neville et al. 2002). The translocation of PKCδ to the nucleus induces the apoptosis of cells in different ways; the interaction of the DNA-dependent protein kinase (DNA-PK) with PKCδ being one of them. Apoptosis can also occur when activated PKCδ phosphorylates DNA-PK that gets disassociated from DNA. This inhibits the repair of the

DNA and induces DNA fragmentation (Bharti, Kraeft et al. 1998). PKCδ has been shown to act as a lamin kinase acting on lamin B, thus contributing to the disassociation of nuclear lamina during cell apoptosis (Cross, Griffiths et al. 2000). Different studies show that PKCδ regulates p53 levels by increasing the transcription of the p53 gene and by phosphorylating p53, which is important for p53-mediated apoptosis (Abbas, White et al. 2004; Yoshida, Liu et al. 2006).

As in other cell types, PKCδ also induces apoptosis in ECs. ECs from the parotid glands of PKCδ$^{-/-}$ mice show significantly decreased apoptosis after stimulation with genotoxins. Moreover, reintroducing wild-type PKCδ restores the apoptosis procedure. The parotid gland ECs from PKCδ$^{-/-}$ mice showed a decrease in cytochrome c release along with poly (ADP-ribose) polymerase (PARP) cleavage and caspase 3 activation (Humphries, Limesand et al. 2006).

In our report, we observed an increase of EC apoptosis when BR3 was blocked with anti-BR3 antibody. The same phenomenon was observed when siRNA specific to BR3 was used to inhibit the expression of BR3. We also observed increased translocations of PKCδ to the nucleus of EC in BR3-blocking conditions which was associated with increased apoptosis in epithelial cells. In line with previous results, we also observed an increased translocation of PKCδ into the nucleus in BR3-blocking conditions. The effect of BAFF on PKCδ translocation has been previously described in B cells, where BAFF was reported to prevent translocation and accumulation of PKCδ to the nucleus (Mecklenbrauker, Kalled et al. 2004). Our result is in complete agreement with this BAFF induced inhibition of the translocation of PKCδ into the nucleus because in EC, we observed that the interaction between BAFF and BR3 maintains PKCδ outside the nucleus.

Previous results from our laboratory showed that B cells can induce the apoptosis of ECs in B cell- EC co-culture model. This EC apoptosis requires the interaction between B cells and ECs. This interaction induces the caspase 3 activation, translocates PKCδ to the nucleus, promotes the phosphorylation of Histone H2B and subsequently results in EC apoptosis (Varin, Guerrier et al. 2011). In SS, the lesions of exocrine glands consist of lymphocytic infiltration including B cell infiltrations. Thus, it is possible that infiltrating B cells expressing BAFF compete with EC expressing BR3 to interact with BAFF. Moreover it might also be possible that, in a co-culture model, the EC apoptosis induced by B cells is caused by a defective signal received by the BR3 expressed on ECs, leading to PKCδ

activation and to its nuclear localization.

Earlier, it has been shown that BAFF performs most of its activity *when in soluble form/through its soluble forms* and, in this way, regulate the survival of B cells (Stadanlick and Cancro 2008). However, the glycosylation of BAFF makes it harder to detect in serum which could explain why, some autoimmune disease patients, BAFF levels in serum remain within or below normal range (Pers, Devauchelle et al. 2007). Indeed, reports suggests that BAFF could assume unpredictable glycoforms (Bossen and Schneider 2006), an alternative variant such as Δ3 (Gavin, Ait-Azzouzene et al. 2003) and intergenic splice variants isoforms (Pradet-Balade, Medema et al. 2002). In a report from our group, Le Pottier et al. showed that polyclonal antibodies are more efficient than monoclonal antibodies in capturing the various forms of BAFF in serum. Namely, polyclonal antibodies recognize both the glycosylated and the non-glycosylated forms of BAFF. These reports are further strengthened by our observations.

In addition, we observed that some but not all forms of BAFF are involved in the survival of ECs. The rate of survival of the ECs decreases when BAFF is neutralized through the use of a polyclonal rabbit anti-BAFF antibody. However, the neutralization of BAFF by a mouse anti-BAFF monoclonal antibody does not have any effect on BAFF-mediated EC survival in our model. This observation supports the theory that different forms of BAFF have different effects. In our model, the BAFF form involved in EC survival was not neutralized by the mouse anti-BAFF monoclonal. Hence, we did not observe any change in EC proliferation. Rather, when BAFF was neutralized by a polyclonal rabbit anti-BAFF antibody we observed a decrease in the survival of epithelial cells.

These differential effects of the various forms of BAFF were further reinforced with the observation that in EC supernatant, the mouse anti-BAFF monoclonal antibody detects only one form of BAFF present at 21 kDa whereas the polyclonal rabbit anti-BAFF antibody detects an additional form of BAFF at 17 kDa along with the 21 kDa form. It seems that the BAFF form observed at 17 kDa is responsible for the BR3 mediated survival of ECs. However, a detailed analysis of this form and its glycosylation status is required / this form and its glycosylation status need to be further evaluated.

BAFF production by SGECs was also shown previously. Moreover, this production of BAFF by ECs can induce the activation of B cells and the secretion of auto-

antibody. The local production of BAFF by ECs also influences the disease by altering B cell differentiation and the formation of ectopic germinal-center-like structures (Groom, Kalled et al. 2002; Jonsson, Szodoray et al. 2005). In SS, it has been already established that viral infection is associated with the disease. Indeed, ECs display different TLRs, and stimulation with ds RNA and poly (I: C) increases BAFF mRNA and protein in SGECs. Still, the specific signaling pathways leading to BAFF production by epithelial cells remain unidentified. Nevertheless, BAFF production has been shown to be partially dependent on signalling through the TLR and IFN pathways (Ittah, Miceli-Richard et al. 2008). Our report also shows the production of BAFF by SGECs, and the expression of the BAFF-receptor. This is also the first time that different forms of BAFF have been shown to act differently after interacting with BR3.

In our report we observed the sheer importance of the interaction between BR3 and the specific forms of BAFF in the survival of ECs. This is particularly true in SS. Indeed, EC play an important role in the pathogenesis. Increased EC apoptosis and various apoptosis-related molecules such as FAS-FASL, perforin, granzymes have already been detected within minor SG lesions of SS patients (Polihronis, Tapinos et al. 1998). This increased apoptosis generates an autoimmune response in SS. Because of the apoptosis, nuclear antigens such as autoantigenic Ro(SSA) and La(SSB) ribonucleoproteins are exposed to the surface leading to autoantibody responses (Ohlsson, Jonsson et al. 2002).

Rituximab treatment leads to the depletion of B cells in the peripheral blood and SGs of SS patients (Pijpe, van Imhoff et al. 2005; Devauchelle-Pensec, Pennec et al. 2007). This treatment was shown to increase BAFF levels (Lavie, Miceli-Richard et al. 2007). The increased BAFF levels may be attributed to B cell depletion in the peripheral blood and the ensuing lack of receptors for binding. Since rituximab treatment also causes B cell depletion in SGs, (Daridon, Devauchelle et al. 2007) it could occasion an absence of competition between B cells and ECs for BAFF. In that situation, BAFF could act as an anti-apoptotic factor for ECs by interacting with the BR3 present on ECs which would result in EC survival.

Another important aspect in our study involves the treatment of SS patients by anti-BAFF immunotherapy such as Belimumab. Belimumab is a recombinant fully human IgG1-λ mAb targeting only soluble BAFF (Fairfax, Mackay et al. 2012). This drug has completed phase III trials and has been approved for SLE treatment (Navarra, Ishimori et al.

2011). It is currently under phase II clinical trials in SS (clinicaltrials. gov identifier NCT01160666). In our report, we observed that different forms of BAFF play different roles. Consequently, it would be important to evaluate the forms of BAFF that are recognized by Belimumab prior to its use in anti-BAFF immunotherapy. Depending on the required forms, anti-BAFF treatment could also deprive ECs in BAFF and favors apoptosis.

The BAFF gene encodes 4 different mRNAs: the well-characterized full-length *BAFF*, a longer variant called *φBAFF*, a shorter variant designated *Δ3BAFF* and a novel transcriptional variant that was recently identified by our group: Δ4BAFF, in which exon 4 is excised. (Gavin, Ait-Azzouzene et al. 2003). The larger transcript *φBAFF* was identified in the human cell lines HL-60 and U937, but sequencing proved this transcript to be non-functional because of the incomplete splicing of the intronic sequences that lead to the formation of a premature stop codon (Gavin, Ait-Azzouzene et al. 2003). 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 the full-length BAFF (Gavin, Duong et al. 2005). It has been shown that ΔBAFF in mice was indeed able to oppose endogenous BAFF functions in an *in vivo* setting and when ΔBAFF is overexpressed under the control of a human CD68 promoter, a significant phenotype is obtained in independent transgenic lines, with reduced follicular and MZ B cell numbers. We demonstrated that Δ4BAFF was induced by IFN-γ, modifying the function of SC35 (a member of the SR protein family) and favoring the increase of hnRNP C1/C2 that in turn regulates SC35. Our work aimed to understand the functional role of Δ4BAFF. We demonstrated that the glycosylated form of Δ4BAFF acts as a transcription factor, upregulating a large number of genes.

We also demonstrated for the first time that Δ4BAFF, a splice variant of *BAFF* lacking exon 4, can function as a transcription factor for its own parent gene. Δ4BAFF also induces the expression of many genes involved in the immune response. Different TLRs (-2, -6, -9 and -10) are increased. *AICDA* (AID gene) and miR-155 are also shown to be increased after Δ4BAFF transfection. These genes have critical roles in the establishment and control of tolerance (Isnardi, Ng et al. 2008; Tili, Croce et al. 2009; Meyers, Ng et al. 2011). Indeed, TLR ligation results in the production of pro-inflammatory cytokines, increased antigen expression, antibody production, proliferation and differentiation in B cells (Huggins, Pellegrin et al. 2007; Jiang, Lederman et al. 2007). As Δ4BAFF increases the expression of

BAFF, the effect of Δ4BAFF in various autoimmune diseases becomes even more significant because BAFF acts as one of the determinants in the development of autoimmune disorders (Mackay, Silveira et al. 2007).

BAFF possesses two potential *N*-glycosylation sites but only the residue N^{124} is glycosylated (Schneider, MacKay et al. 1999). The importance of the N-glycosylation status for BAFF has been already described, particularly for the ΔBAFF variant (Gavin, Ait-Azzouzene et al. 2003), but also for the full-length form of BAFF in the serum of patients with autoimmune diseases (Le Pottier, Bendaoud et al. 2009). We also previously observed that Δ4BAFF was located in the endoplasmic reticulum and the nucleus and that the nonglycosylated form of Δ4BAFF was not found in the nucleus. This observation suggests that the glycosylation status of Δ4BAFF is an important factor that contributes to its nuclear entry. It is also worth mentioning that the glycosylation is an important factor for the activation of transcription factors as shown by Chan et al. In their work, they observed that unglycosylated CREB-H (cyclic AMP-responsive element binding protein H) is maintained in an inactive form in the endoplasmic reticulum (Chan, Mak et al. 2010).

The splicing efficiency of individual exons is determined by multiple mechanisms including cell-specific patterns or in response to acute stimulation (Black 2003). IFN-γ stimulation for 24h was found to induce Δ4BAFF expression. The cytokine-mediated alternative splicing now clearly emerges as a potential regulatory mechanism, one that can operate on different time scales depending on mRNA and protein stability. The effects of IFN-γ on the alternative splicing phenomena have been described in other genes. For example, the isoforms of human tryptophanyl-tRNA synthetase (TrpRS) are regulated *in vivo* by IFN-γ through alternative mRNA splicing (Liu, Wang et al. 2004). Furthermore, IFN-γ can modify the global expression pattern of genes, including spliced variants. Ortis et al. (Ortis, Naamane et al. 2010) showed that, in purified rat pancreatic cells, IFN-γ, in association with other cytokines (IL-1β and TNF-α), modified the expression of more than 20 genes involved in RNA splicing. It also induced changes in the alternative splicing of more than half of the cytokine-mediated genes, showing that these cytokines could affect the alternative splicing in a tissue-specific manner.

In our report, we observed that the transfection of Δ4BAFF increases the expression levels of full-length BAFF. We also observed the presence of the protein in the nucleus of B cells, which led to the working hypothesis that it could act as a transcription

factor. Inorder to operate properly, in eukaryotic cells, the genetic material and the transcriptional system in the nucleus are separated from the translational and metabolic system of the cytoplasm by the nuclear membrane. If a protein is to act as a transcriptional regulator it must be able to penetrate the nuclear membrane to reach the nucleus. Nuclear pore complexes present on the nuclear membrane allow the passive transport of ions and small proteins (Lange, Mills et al. 2007). The transport of proteins to the nucleus needs the help of specific soluble career proteins called importeins (Gorlich, Prehn et al. 1994). These soluble proteins bind to the nuclear localization sequence (NLS) that is exposed on the protein surface and subsequently help the transport of proteins to the nucleus (Lange, Mills et al. 2007). However, the Δ4BAFF protein does not express any NLS sequence, suggesting that it cannot bind to importeins and reach the nucleus. However, in our study, we found that Δ4BAFF can bind to the consensus NF-κB binding site (-1040-840) of the BAFF promoter. Furthermore, our results show that the NF-κB component p50 helps the translocation of Δ4BAFF to the NF-κB binding site on the BAFF promoter and acts as a transcription factor. This interaction probably enables the localization of Δ4BAFF into the nucleus.

The interactions between the NF-κB dimers or monomers and the heterologous transcription factors through direct binding have been already described and markedly influence the transcriptional responses (Oeckinghaus, Hayden et al. 2011). NF-κB p50 lacks a transactivation domain and therefore usually forms a heterodimer to be transcriptionally active (Ghosh, May et al. 1998). We discovered that the Δ4BAFF sequence contains a perfect match between aa145 and aa153, corresponding to the 9aa transactivation domain, and is therefore able to form a heterodimer transcriptionally active with p50. *In vitro* studies have shown that p50 can associate with other transcriptional activators such as Bcl-3 (Fujita, Nolan et al. 1993) or p300 (Deng and Wu 2003) to activate transcription. NF-kB p50 can also form a complex with the transcriptional co-activator CREB to activate IL-10 transcription in macrophages (Cao, Zhang et al. 2006). Interestingly, among the genes that are upregulated after Δ4BAFF transfection, the promoter of the miR-155-encoding BIC gene contains two putative NF-κB sites able to bind *in vitro* the NF-κB proteins p50 and p65 in nuclear extract from MC3 cells (Gatto, Rossi et al. 2008). Furthermore, p50 plays a critical role in the induction of the AID gene expression because AID induction in B cells is impaired in p50-/- mice (Snapper, Zelazowski et al. 1996). Finally, by blocking the ubiquitinination of p50, Bcl-3 stabilizes a p50 complex that inhibits the TLR gene transcription and limits the strength of the TLR responses (Carmody, Ruan et al. 2007).

Studies on murine colitis and experimental autoimmune encephalomyelitis (EAE) have demonstrated that regulatory B cells leads to the generation of Treg cells which in turn regulate T cell-dependent immune responses. However, B cells have also been shown to directly inhibit T cell proliferation through cell to cell contact, even leading to anergy, or apoptosis of T cells, and the modulation of the inflammatory response. In this regard, CD40 engagement on B cell appears to be a requisite for the induction of functional Breg cells in mice.

In human, stimulation of CD40 brings about the development of B cells with suppressive properties. Furthermore, signalling in the absence of CD40 makes B cells unable to regulate inflammatory response (Mizoguchi, Mizoguchi et al. 1997; Fillatreau, Sweenie et al. 2002). TLR9 was also described as a prerequisite to induce B cell regulation of inflammatory responses (Lenert, Brummel et al. 2005). We have recently observed that combination of CD40 and TLR9 associated stimulation can successfully induce functional regulatory B cells (Lemoine, Morva et al. 2011). In our current finding, we observe that TLR9 and CD40 stimulation could induce Δ4BAFF expression. This observation led us to hypothesize that there must be a direct connection between Δ4BAFF expression and the regulatory properties of B cell.

In accordance to our hypothesis, reducing the expression of Δ4BAFF by siΔ4BAFF resulted in a marked decrease of TGF-β production and regulatory T cell induction. Neutralizing Δ4BAFF in B cells also resulted in a significant decrease on the inhibition of T cell proliferation. However, IL-10 production was not downregulated after Δ4BAFF inhibition. This result is in accordance with our previous results showing that human regulatory B cells can inhibit the proliferation of T cells through a mechanisms independent of IL-10 but through the induction of Foxp3 regulatory T cells, while the Th1 differentiation is controlled by IL-10-dependent signalling (Lemoine, Morva et al. 2011). It has been observed that human tonsil B cells constimulated with CpG ODNs and CD40L have a sigh proliferative response and produce large quantities of IL-10 and show IL-10-dependent regulatory properties (Jamin, Morva et al. 2008). Although regulatory B cell efficiency was mainly associated to their production of IL-10 (Mizoguchi, Mizoguchi et al. 2002), there is also strong evidence that part of the immunosuppressive function of regulatory B cells are mediated through the induction of regulatory T cells (Wei, Velazquez et al. 2005).

The pivotal role of Δ4BAFF as a transcription factor that controls BAFF expression and the control of immune response hold immense promise for the clinic. The potential for Δ4BAFF to be used as a therapeutic target will need 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.

Hence, it is our hypothesis that Δ4BAFF may very well play a pivotal role in the control of BAFF expression. Further studies regarding the function of Δ4BAFF and its role in regulating disease outcomes will be of immense interest to the autoimmune field. Attempting to discover the exact ways to control the ratio of BAFF to Δ4BAFF will be a fruitful avenue of future research.

In the third part of our study, we focused on B cells and TLR9. We showed that B cells express both the complete and the potentially active forms of TLR9 on their plasma membrane, though the specific ligand for the membrane-bound TLR9 has yet to be discovered. Indeed, we observed that TLR9 expression on the plasma membrane of B cells does not interact with CpG-B, a known synthetic ligand for TLR9 (Bauer, Kirschning et al. 2001). TLR9 recognizes microbial CpG DNA. The stimulatory effect of bacterial CpG-DNA is due to the presence of unmethylated CpG dinucleotides in a particular base context named CpG-motif (Krieg, Yi et al. 1995; Hemmi, Takeuchi et al. 2000). The interaction between TLR9 and its ligand needs endocytosis of the ligand to the endosomal or lysosomal vesicles formation. Moreover, it has been proven that CpG-DNA signalling through TLR9 depends on the acidification and the maturation of endosomes (Yi, Tuetken et al. 1998);(Ahmad-Nejad, Hacker et al. 2002). Thus, the reduced pH conditions (6.5-4.5) in the endosomes and lysosomes are actually perfect for this interaction (Mellman, Fuchs et al. 1986). There might be different theories that could account for the non-binding of CpG-DNA with the cell surface TLR9. It is likely that the pH of the extracellular environment is not sufficiently acid to initiate the interaction between Cpg-ODN ligands and the cell surface TLR9. Moreover, it is also possible that the cell surface TLR9 interacts with different ligands and initiates signalling mechanisms that differ from the endosomal ones. This second possibility is strengthened by two facts. Earlier we showed that, in intestinal ECs, the stimulation of basolateral TLR9 induces the activation of the NF-κB pathway, though the apical TLR9 signalling induces the accumulation of ubiquitinated IκBα in the cytoplasm, thereby

inhibiting the activation of NF-κB (Lee, Mo et al. 2006). Later, it was identified that this polarization of TLR9 in turn contributes to limit the inflammatory responses in bacterial-enriched condition, where apical TLT9 stimulation delivers a negative signal in balance with the activation signals triggered by basolateral TLR9 stimulation; thus contributing to homeostasis (Lee, Gonzales-Navajas et al. 2008). Signalling through TLR4 also varies according to its localization. Cell-surface TLR4 activates the TIRAP-MYD88 signalling at the plasma membrane, whereas endocytosed TLR4 activates the TRAM-TRIF signalling cascade (Kagan, Su et al. 2008). Thus, in our observation, it is possible that cell surface TLR9 interacts with different ligands and induces signalling through other pathways than the endosomal TLR9 pathways.

The possibility that a cell surface TLR9 signal through different pathways is further strengthened by our observation that B cell receptor (BCR) and surface TLR stimulation do not activate B cells. The proliferative response of B cells also differs according to the localization of TLR9. The stimulation of cell surface TLR9 with anti-TLR9 antibody and the co-stimulation with BCR and IL-2 do not induce proliferation, suggesting that the cell surface TLR9 differs from the endosomal TLR9 and does not act synergistically with the BCR to promote the B cell response. In contrast, reports indicate that endosomal TLR9 signals synergize with BCR signals and increase B cell activation, and proliferation (Busconi, Bauer et al. 2007).

TLR 9 has been shown to play a contradictory role. An *in vivo* study showed that TLR9 is responsible for the production of specific Abs in lupus prone mice and is correlated with increased disease severity (Christensen, Kashgarian et al. 2005; Wu and Peng 2006). The production of anti-DNA antibody is ascribed with the expression of TLR9 by B cells (Nickerson, Christensen et al. 2013). Moreover, in lupus, the tolerance-breaking of autoreactive B cells was observed because nucleic acids containing self-antigens, activate TLR9 help from the BCR (Christensen and Shlomchik 2007). Although TLR9 has been shown to increase anti-DNA-Ab generation in mice model of lupus, other studies have shown that TLR9 has no effect in elevated diseased conditions (Yu, Wellmann et al. 2006; Nickerson, Christensen et al. 2010) This tend to suggest a dual function of TLR9. Indeed, recent findings have shown that, on one hand, TLR9 is required to activate anergic self-reactive anti-DNA B cells but, on the other hand, controls self-tolerance by restricting their survival (Nickerson, Christensen et al. 2013). These reports indicate that there might be some

kind of regulating mechanism that controls the function of TLR9 in different autoimmune reactions. Indeed, in our report we observed that the engagement of the cell surface TLR9 inhibits the activation of B cells resulting from the endosomal TLR9 signalling. Moreover, the synergistic effect of the endosomal TLR9 and of the BCR-induced proliferation was also inhibited when the cell surface TLR9 was stimulated; indicating that the cell surface TLR9 acts as a negative regulator of the endosomal TLR9-induced human B cell response. This might be important in the context of autoimmunity where the endosomal and plasma membrane TLR9 functions could be regulate the final shaping of the autoreactive B cell pool in so doing, influence the ultimate behavior of these autoreactive B cells.

To conclude, our results can open new avenues in biology. The presence of both the full and the active form of TLR9 along with the various ways they operate from endosomal TLR9 can open new directions in TLR9 signalling. Our preliminary data suggest that the cell surface TLR9 negatively regulates the function of the endosomal TLR9. Recently, the dual self-contradictory role of TLR9 has been shown. Not only is TLR9 is required for the activation of self-reactive anti-DNA B cells, it also elevates tolerance by restricting their survival (Nickerson, Christensen et al. 2013). It might also possible that cell surface and endosomal TLR9 signalling counterbalance one another to determine the final behavior of the autoreactive B cells. However, further investigations are needed to identify the detailed functions and signalling of cell surface- expressed TLR9 and their impact. It is also surprising that CpG B, the known ligand of endosomal TLR9 does not bind to the cell surface TLR9. It will be of great interest to find out the exact ligand for membrane-bound TLR9 to facilitate different *in vitro* studies. TLR9 has long been implicated in various autoimmune diseases. TLR9 knock-out mice are severely affected by nephritis and skin diseases (Christensen, Shupe et al. 2006). So, it will be rewarding to investigate the role of the membrane-expressed TLR9 on different autoimmune diseases. Since the membrane-expressed TLR9 negatively regulate the role of the endosomal TLR9, it is also possible that defaults in the cell surface TLR9 signaling could promote autoimmune reactions and thus could be used as a therapeutic target.

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V - APPENDIX

Appendix 1

The complexity of the BAFF TNF-family members: Implications for autoimmunity

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Appendix 2

Interferon-gamma and SC35 protein regulate the alternative splicing of BAFF gene