γ-secretase directly sheds the survival receptor BCMA from plasma cells

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ABSTRACT

The importance of B cells and plasma cell in autoimmune diseases such as multiple sclerosis (MS) has been widely established in the last decades. The role of antibody producing plasma cells is crucial, as those cells may persist for extended periods as long-lived plasma cells. The survival of these plasma cells is closely regulated by B cell maturation antigen (BCMA), a membrane-bound receptor for its agonist ligands BAFF and APRIL that belong to the tumor necrosis factor superfamily. While the release of soluble endogenous receptors as regulatory mechanism is already well known for some TNFR-SF members, the generation of soluble BCMA (sBCMA) has so far not been described.
Having identified by ELISA the presence of sBCMA in serum of healthy controls, the aim of this study was first to investigate the potential role of sBCMA as biomarker in B cell-mediated autoimmune diseases such as multiple sclerosis and systemic lupus erythematosus (SLE). Further we aimed to characterize the source of sBCMA, the biochemical mechanisms behind the generation of sBCMA and the functional consequences of BCMA shedding.

We observed that in MS, sBCMA levels in spinal fluid were elevated and associated with intracerebral IgG production; in systemic lupus erythematosus, sBCMA levels in serum were elevated and correlate with disease activity. Unexpectedly we found that sBCMA was directly shed from human Ig-secreting cells by γ-secretase. γ-secretase was previously found to cleave remaining fragments of membrane proteins only after their extracellular domain had been cleaved by another protease. It is also best known for processing of amyloid precursor protein (APP) and Notch. We thus demonstrated a novel function of γ-secretase, direct shedding of a membrane protein. By artificially extending the extracellular domain of human BCMA, we were able to show that its direct shedding is facilitated by the short length of BCMA's extracellular domain. Further *in vitro* analysis showed that BCMA shedding limits the surface expression and ligand-binding of BCMA on Ig-secreting cells, and reduces ligand-mediated NF-κB activation. Moreover sBCMA blocked the activity of the ligand APRIL, acting as a decoy. Finally, to get further insights into the *in vivo* relevance of BCMA shedding, we used two different animal models: Ova/LPS-immunized C57/BL6 mice and a murine SLE model with spontaneous formation of germinal centers and plasma cells. Inhibition of the γ-secretase and BCMA shedding in these mice resulted in enhanced BCMA surface expression in plasma cells and in an increased number of plasma cells in the bone marrow.

Together, shedding of BCMA constitutes a novel immunoregulatory feed-back mechanism that limits a critical survival receptor on Ig-secreting cells and thus regulates the survival of plasma cells in the bone marrow. Moreover direct shedding of BCMA constitutes a novel function of γ-secretase. Finally, sBCMA itself could serve as a potential biomarker for B cell involvement in human autoimmune diseases.

ABBREVIATIONS

ADAM	A disintegrin and metalloprotease
ADEM	Acute disseminated encephalomyelitis
APP	Amyloid precursor protein
APRIL	A proliferation-inducing ligand
AZA	Azathioprine
BAFF	B cell activating factor of the TNF-family
BAFF-R	BAFF-Receptor
BCMA	B cell maturation antigen
BM	Bone marrow
BrdU	Bromodeoxyuridine
CIS	Clinical isolated syndrome
CLL	Chronic lymphocytic leukemia
CNS	Central nervous system
CSF	Cerebrospinal fluid
CTF	C-terminal fragment
CYC	Cyclophosphamide
GA	Glatiramer Acetate
GC	Germinal center
GCS	Glucocorticosteroid
HC	Healthy control
HCQ	Hydroxychloroquine
ICD	Intracellular domain
Ig	Immunoglobulin
ISC	Ig secreting cells
mBCMA	Membrane bound BCMA
MMF	Mycophenolate Mofetil
MOG	Myelin oligodendrocyte glycoprotein
MS	Multiple sclerosis
NTF	N-terminal fragment
NTZ	Natalizumab
OCB	Oligoclonal band
OND	Other neurological disease
PBMC	Peripheral blood mononuclear cells
PC	Plasma cell
PCR	Polymerase chain reaction
PEN-2	Presenilin enhancer 2
PPMS	Primary progressive multiple sclerosis
Pred	Prednisolone
PS	Presenilin
RRMS	Relapsing remitting multiple sclerosis
sBCMA	Soluble BCMA
SLE	Systemic lupus erythematosus
SPMS	Secondary progressive multiple sclerosis

TACI	Transmembrane activator and CAML interactor
TNF	Tumor necrosis factor

1 INTRODUCTION

1.1 Multiple sclerosis

The immune system features extremely efficient effector mechanisms that can eliminate a broad variety of pathogens. It is able to distinguish between the components of a healthy individual (the self) and external antigens (the non-self) resulting in immunological self-tolerance. First described by Paul Ehrlich as “horror autotoxicus”, autoimmunity represents a breakdown or failure in the mechanisms of self-tolerance. In autoimmune diseases the immune response is activated by self-antigens or autoantigens and gives rise to autoreactive effector cells and to autoantibodies. This can lead to a variety of autoimmune diseases which are further classified into systemic autoimmune diseases, which affect multiple organs simultaneously such as SLE and in organ-specific specific autoimmune diseases such as MS.

MS is an autoimmune inflammatory disease of the central nervous system (CNS) and was first described in 1868 by Jean-Martin Charcot. The pathogenicity includes recurrent and acute focal inflammatory demyelinating lesions with axonal injury and astrocytic scar formation, resulting in intermittent neurological disturbance followed by progressive accumulation of disability (Compston and Coles 2008).

The overall worldwide estimated number of people diagnosed with MS was approximately 2.3 million in 2013, with the highest prevalence in North America and Europe (140 and 108 /100.000 respectively). MS is as many other autoimmune diseases twice more common among women than men and the average age of onset is 30 years. MS still being an incurable disease, patients live with this progressive neurological disease for many decades.

Depending of the lesion site, patients with MS can present with a variety of neurological deficits such as change in sensation, impaired balance and coordination, loss of vision and muscle weakness. The clinical course of MS is variable among patients and is classically divided in three subtypes, relapsing-remitting MS (RRMS), secondary progressive MS (SPMS) and primary progressive MS (PPMS). Approximately 85 % of MS patients begin with RRMS, a course of recurrent but mostly reversible neurological deficits where relapses can be followed by periods of month to years with no new signs of disease activity. SPMS is regarded as a common late stage of disease that follows RRMS after 8 – 20 years and is characterized by continuous and irreversible neurological disability unassociated with

relapses. PPMS is a less common form, only present in about 15 % of the patients, in which the disease progresses continuously from the beginning without any remission.

Despite being the object of intensive research in the last decades, the initial cause of MS remains elusive. Linkage studies and genome-wide association studies (GWAS) have identified several susceptibility loci associated with MS, which are located in gene regions involved in immune system functions, in particular within the major histocompatibility complex (MHC) (International Multiple Sclerosis Genetics, Wellcome Trust Case Control et al. 2011). The relationship between environmental factor including sun exposure, vitamin D levels and infection by Epstein-Barr virus and MS remains debated (Ramagopalan, Dobson et al. 2010).

1.2 Humoral immunity in MS

T-cells have classically been considered the primary immune drivers in MS (McFarland and Martin 2007). However, this notion has recently been reassessed and the roles of B cells as well as the importance of the innate immune system have increasingly been recognized (Krumbholz, Derfuss et al. 2012). Evidences for the involvement of B cells and for the B cell fostering CNS environment in the pathogenicity of MS include:

- Long-term persistence of intrathecal immunoglobulin (Ig) production with oligoclonal bands (OCB) present in more than 95% of MS patients (Freedman, Thompson et al. 2005).

- Presence of clonally expanded B cells located in the brain parenchyma and CSF responsible for the production of OCB (Owens, Ritchie et al. 2003; von Budingen, Gulati et al. 2010; Lovato, Willis et al. 2011; Obermeier, Lovato et al. 2011).

- Memory B cells and plasmablasts are the predominating B-lineage cells in the CSF of patients with MS; their numbers correlate with disease activity (Krumbholz, Derfuss et al. 2012).

- A detailed analysis of the white matter pathology in MS reveals four different lesion patterns (Lucchinetti, Bruck et al. 2000). Most interestingly, type II lesions show

immunoglobulin G (IgG) as well as active complement deposition present in the demyelinated areas of lesion type II.

- Antibodies against conformationally intact MOG (Myelin oligodendrocyte glycoprotein) are very rare in adult MS patients, but occur in about one-fourth of patients with childhood MS and acute disseminated encephalomyelitis (ADEM) (Mayer and Meinl 2012). While Anti-MOG antibodies rapidly and continuously declined in patients with ADEM, longitudinal study noted the persistence of anti-MOG IgG with fluctuations for a period of up to 5 years in children with MS (Probstel, Dornmair et al. 2011). More recently, the clinical and histopathological features of MOG-antibody-associated encephalomyelitis has been described (Spadaro, Gerdes et al. 2014).

- Therapeutic efficacy of plasmapheresis and B cell targeting therapies such as Rituximab, Ocrelizumab (Bar-Or, Calabresi et al. 2008; Hauser, Waubant et al. 2008; Kappos, Li et al. 2011).

Apart from their role as antibody producing cells, B cells can influence the inflammatory environment in general, as they produce both pro-inflammatory and anti-inflammatory cytokines and can serves as APC to T cells (**Figure 1**).

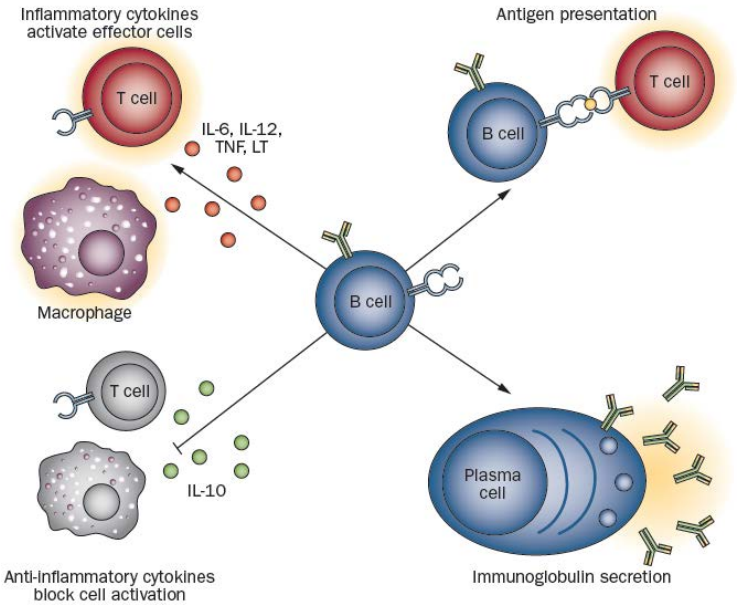

Figure 1. Proinflammatory and regulatory effects of B cells. *B cells can develop into immunoglobulin-secreting plasma cells, efficiently present their cognate antigen to T cells, and secrete inflammatory cytokines that activate T cells and macrophages. B cells can also secrete cytokines that block T cells and macrophage activation. Abbreviation: LT, Lymphotoxin. (Krumbholz, Derfuss et al. 2012).*

1.3 Systemic lupus erythematosus

Systemic lupus erythematosus (SLE) is an idiopathic, systemic autoimmune disease and is characterized by multisystem immune-mediated injury in the setting of autoimmunity to nuclear antigens. SLE most often harms the heart, joints, skin, lungs blood vessels, liver, kidney, and nervous system. The course of the disease is unpredictable, with period of illness (called flares) alternating with remissions.

Although the initial cause of the disease remains unclear, there has been accumulating evidence for a key role of B cells, as initiating, amplifying and effector cells. The BAFF/APRIL pathway, in particular, is over-activated in SLE and plays an important role in the pathogenesis of the disease (Vincent, Morand et al. 2014). This became particularly evident when, in 2011, belimumab, a monoclonal antibody targeting human BAFF, was shown in randomized clinical trials to be efficacious in a subset of patients with SLE and has now become the first approved targeted therapy for SLE (Furie, Petri et al. 2011; Navarra, Guzman et al. 2011).

1.4 The BAFF/APRIL-system

1.4.1 Structure, expression and signaling of BAFF, APRIL and their receptors

The cytokines B cell activating factor of the TNF-family (BAFF, also known as BLyS) and a proliferation-inducing ligand (APRIL) belong to the TNF ligand superfamily. BAFF and APRIL are initially synthesized as membrane-bound proteins and can be released as soluble forms by proteolytic processing at a furin consensus site (Moore, Belvedere et al. 1999; Schneider, MacKay et al. 1999). Both cytokines display the characteristic homotrimeric structure of the TNF family (Karpusas, Cachero et al. 2002; Wallweber, Compaan et al. 2004). Soluble BAFF can further assemble as virus-like particles, forming a particle

containing 20 trimers (BAFF 60-mer). Proteoglycans are highly glycosylated O-linked glycoproteins that can be found as a soluble or membrane bound form. Soluble APRIL can bind to these proteoglycans. Remarkably, the proteoglycan-binding site of APRIL is distinct from the binding site of BCMA and TACI. The binding of APRIL to proteoglycans can therefore serve to concentrate APRIL, creating APRIL-riche niches, i.e. for plasma cells (Huard, McKee et al. 2008). Furthermore, an intergenic splicing between *tweak* and *april* genes generating TWE–PRIL, a hybrid ligand containing the full receptor-binding domain of APRIL, has been described (Pradet-Balade, Medema et al. 2002).

BCMA, TACI and BAFF-R differ from the typical type I transmembrane TNF-receptors, which contain cysteine-rich domain (CRDs) in their extracellular domains. First, the three receptors are type III single pass transmembrane protein lacking a signal peptide. Further, while TACI contains two CRDs, BCMA has only one and BAFF-R has no CRDs. TACI can exist as two splice variants. While BAFF in its different forms can bind to all three receptors, APRIL binds to TACI and preferentially to BCMA (**Figure 2**).

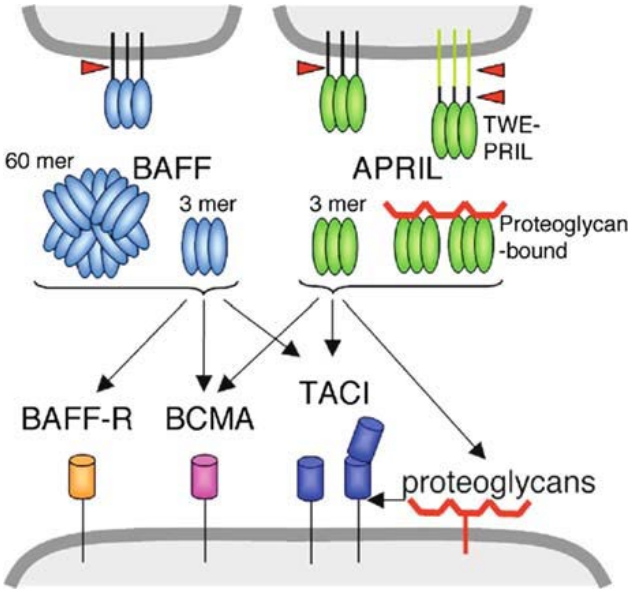

Figure 2. The BAFF and APRIL system. *Red arrowheads indicate furin consensus cleavage sites. Glycosaminoglycan side chains of proteoglycans are shown as thick wavy red lines. Two splicing isoforms of human TACI differing in the presence or absence of the first cysteine-rich domain are depicted (Schneider et al. 2010)*

BAFF and APRIL are mainly produced by innate immune cells (neutrophils, monocytes, dendritic cells and follicular dendritic cells). The receptors for BAFF and APRIL are mainly expressed by B cells (**Figure 3**). BAFF-R is largely expressed by all B cells except for bone marrow plasma cells. BAFF-R expression decreases and is eventually lost, as B cells differentiate into PC. TACI is found on memory B cells and on tonsillar and bone-marrow derived plasma cells.

BCMA is mainly expressed by plasma cells, in lymphatic tissue and bone marrow. It can also be detected on tonsillar memory B cells. BCMA expression is induced as memory B cells differentiate into plasma cells (Darce, Arendt et al. 2007). Interestingly, BCMA expression inversely correlates with the loss of BAFF-R expression. Moreover, BCMA and TACI are also expressed by keratinocytes (Alexaki, Pelekanou et al. 2012).

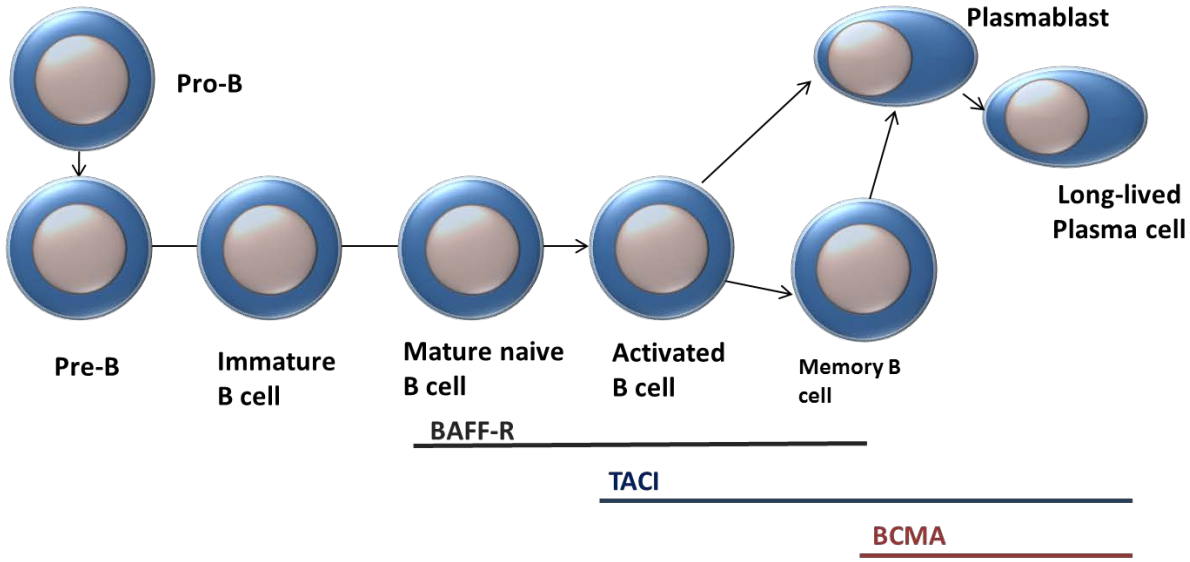

Figure 3. Surface expression of BAFF/APRIL-receptors during physiological B cell development modified from Krumbholz, Derfuss et al. 2012

While BAFF can bind to all three receptors (BAFF-R, BCMA, and TACI), APRIL binds to TACI only and especially to BCMA with high affinity. Stimulation of BCMA and TACI activate the classical NFκB pathway stimulation. BAFF-R on the other hand can activate the alternative NFκB pathway and in weakly manner also the classical NFκB pathway (Hatzoglou, Roussel et al. 2000).

1.4.2 Physiological relevance of the BAFF/APRIL pathway

1.4.2.1 B cell homeostasis and tolerance

The cytokines BAFF and APRIL regulate multiple aspects of B cell functions and homeostasis. The pro-survival activity of BAFF was clearly shown by an almost complete lack of follicular and marginal zone B cells and by an impaired B cell maturation beyond the T1 stage in BAFF deficient and in BAFF-R deficient mice (Schiemann, Gommerman et al. 2001; Shulga-Morskaya, Dobles et al. 2004). BAFF-R is considered a positive regulator of B cell homeostasis, because it transmits survival signals, whereas signaling through TACI decreases the size of the B cell pool (Mackay and Schneider 2008). It remains to be determined to what extent these findings in mice can be extrapolated to humans.
The function of APRIL is not as well established as for BAFF. While one group reported intact B cell and T-cell development and *in vitro* function in APRIL knock-out mice (Varfolomeev, Kischkel et al. 2004), a second research group showed that APRIL-deficient mice, despite having T and B cell development and *in vitro* proliferation, presented with enlarged germinal centers and had an increase IgG response to T-cell dependent antigen (Castigli, Scott et al. 2004). Nevertheless, in both studies antibody class switch recombination was found to be impaired.

1.4.2.2 Role of BCMA in plasma cell survival and antigen presentation

Antibody producing cells, generated during a germinal-center reaction may persist for extended periods as long-lived plasma cells in survival niches in the bone marrow. This is the basis for persisting high affinity IgG against pathogens. Importantly, not only the bone marrow, but also inflamed tissues can provide a survival niche for plasma cells and thus constitute an efficient way of providing maximal amounts of specific antibodies at the site of inflammation (Radbruch, Muehlinghaus et al. 2006). In MS the inflamed CNS might act as a survival niche and promote survival of long-lived plasma cell, which would explain the persistence of OCB in the CSF of MS patients (Meinl, Krumbholz et al. 2006). Within those survival niches, BCMA is essential for the maintenance of long-lived plasma cells (**Figure 4**). This essential role of BCMA became evident through studies with BCMA (-/-) mice which displayed impaired survival of long-lived bone marrow PCs compared with wild-type controls (O'Connor, Raman et al. 2004).

The BCMA dependent maintenance of long-lived plasma cells seems to be largely mediated via its main ligand APRIL (Belnoue, Pihlgren et al. 2008; Benson, Dillon et al. 2008; Huard, McKee et al. 2008). In addition to its role in the survival of long-lived plasma cells, BCMA is also implicated in the regulation of antigen presentation activity of activated B cells (Yang, Hase et al. 2005). The authors showed that ligation of BCMA by APRIL resulted in the up-regulation of surface molecules critical for Ag presentation such as CD40, CD80, CD86, MHC class II, and ICAM-1/CD54 and induced antigen presentation in splenic B cells.

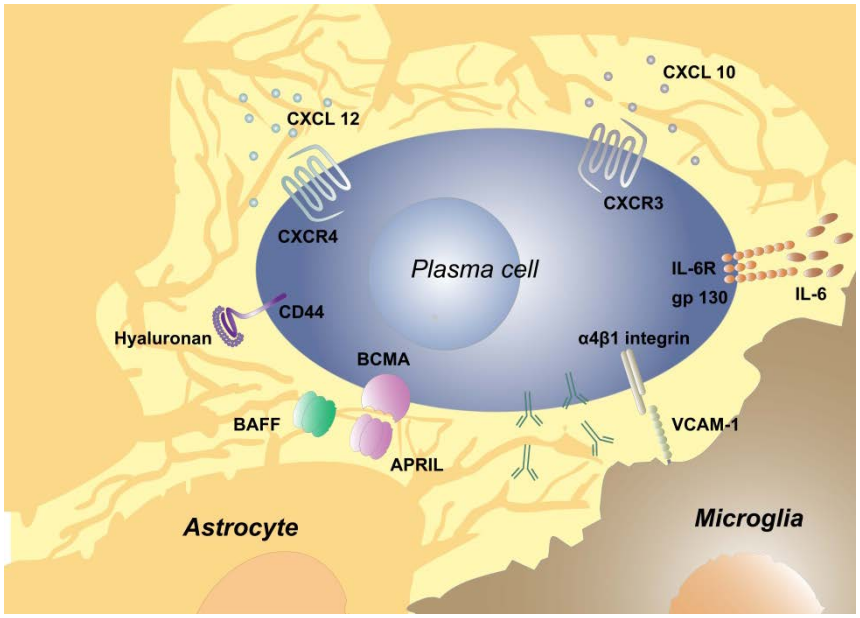

Figure 4. Plasma cell-surface phenotype and ligands in MS lesions. *Central nervous system (CNS) resident cells (mainly astrocytes and microglia) produce mediators that promote the local survival of plasma cells. All of the displayed mediators are elevated in MS lesions. The corresponding receptors on plasma cells are shown. The cytokines B cell–activating factor of the tumor necrosis factor family (BAFF), A proliferation-inducing ligand (APRIL) and interleukin-6 (IL-6) promote plasma cell survival (Meinl, Krumbholz et al. 2006).*

1.4.3 The BAFF/APRIL-system in autoimmunity

1.4.3.1 In mice

BAFF transgenic mice develop clinical and paraclinical features similar to SLE/Sjögren syndrome, with enlarged B cells compartments and lymphoid organs, high titers of dsDNA-antibodies and rheumatoid factors. Hypergammaglobulinemia, circulating immune complexes and glomerulonephritis with immunoglobulin deposits were also detected (Mackay, Woodcock et al. 1999; Fletcher, Sutherland et al. 2006). Further studies showed that serum BAFF levels were elevated in a collagen-induced arthritis (CIA) mice model (Zhang, Park et al. 2005). Remarkably, the silencing of BAFF gene in those CIA mice prevented the development of autoimmune arthritis (Lai Kwan Lam, King Hung Ko et al. 2008). Autoimmunity in BAFF-transgenic mice is T-cell independent and Myd88 dependent (Groom, Fletcher et al. 2007). A possible explanation to these findings is that upregulation of BAFF expression *in vivo* would result in the rescue of self-reactive B cells from peripheral elimination (Thien, Phan et al. 2004).

The role of APRIL remains certainly more unclear. Excess of APRIL leads to the development of B1 B cell lymphomas in APRIL-transgenic mice, but it does not cause apparent autoimmunity (Planelles, Carvalho-Pinto et al. 2004). However, increased production of APRIL was detected in lupus prone mice (NZB/W lupus mice) and selective antibody-mediated APRIL blockade in these mice delayed disease development (Huard, Tran et al. 2012).

Exacerbation of disease in lupus-prone BCMA-deficient mice, suggests that BCMA has a direct or indirect negative regulatory role. In these mouse models, exacerbation of disease might occur by abnormal signaling of BAFF or APRIL through TACI or BAFF-R in the absence of BCMA (Jiang, Loo et al. 2011).

1.4.3.2 In human

The BAFF/APRIL pathway has been found to be constantly involved in various autoimmune diseases.

In MS, BAFF expression was upregulated in CNS lesions; local production of BAFF by astrocytes in the inflamed CNS might promote survival of long-lived plasma cell, which

would explain the persistence of OCB in the CSF of MS patients (Krumbholz, Theil et al. 2005).

Serum concentrations of BAFF and APRIL are higher in patients with systemic lupus erythematosus (SLE) (Stohl 2003) and Wegener's granulomatosis (Krumbholz, Specks et al. 2005). The importance of BAFF in the pathogenesis of SLE became evident when in 2011, belimumab, a monoclonal antibody against human BAFF, became the first approved targeted therapy for SLE (Furie, Petri et al. 2011). As to the expression pattern of BAFF/APRIL receptors in SLE patients, BAFF-R expression on B cells was lower compared to healthy control B cells while BCMA expression was substantially increased on SLE B cells, especially on memory cells and plasmablasts (Kim, Gross et al. 2011).

1.5 Soluble receptors

1.5.1 Mechanisms of generations

The production of soluble receptors, by attenuating or promoting cytokine signaling, constitutes an essential and evolutionary highly conserved regulatory mechanism of immunity and inflammation. These soluble receptors can be generated by various underlying mechanisms such as proteolytic cleavage of the ectodomain, alternative splicing and exosome release. These three mechanisms are not mutually exclusive (Heaney and Golde 1998).

1.5.1.1 Shedding

The proteolytic cleavage of the extracellular domain (also known as shedding) is typically catalyzed by a metalloproteinase of the ADAM (a disintegrin and metalloprotease) family, in particularly ADAM10 and ADAM17 (TACE). Amongst the large number receptor undergoing proteolytic cleavage, notorious examples include TNFR1, TNFR2, CD30, CD40, and IL-6Rα. TNF-receptor superfamily. In the case of type I oriented proteins the γ-secretase complex may further cleave the remaining fragment within the plasma membrane (Fluhrer and Haass 2007).

1.5.1.2 Alternative splicing

The second important mechanism for the generation of soluble receptors is the alternative splicing of mRNA transcripts which deletes the transmembrane domain, thus giving rise to soluble receptors that contain signal peptides and lack transmembrane domains. The receptors are therefore secreted, rather than membrane-associated proteins. Soluble forms generated by alternative splicing of TNFR2, IL6-Rα, sFAS and most recently TNFR1 have been described (Gregory, Dendrou et al. 2012).

1.5.1.3 Exosomes

Exosomes are membranous vesicles of endocytic origin (diameter between 40–100 nm) that are released by different cell types into the extracellular space. The exosome-associated receptors can thus act as soluble receptors. For example, a full-length TNFR1 receptor associated to exosome-like vesicles and able to bind TNF-α has been reported to be constitutively generated by human vascular endothelial cells (Hawari, Rouhani et al. 2004).

1.5.2 Functional relevance

The conserved existence of soluble receptors and the complex regulation of their production and function suggest that they have an important role in normal physiology, with multi-facetted consequences.

Firstly, generation of soluble receptors by proteolytic cleavage leads to a down-modulation of the number of receptors on the cell surface, thus preventing signal generation by ligand. Second, provided that the shedded extracellular domain can still bind its ligand, the generated soluble receptor can become a decoy receptor. In this model, the soluble receptor acts as a non-signaling decoy competing with the membrane-bound receptors for ligand binding (**Figure 5**). In addition, the soluble receptor can serve as a binding protein that prevents its ligand from further degradation or clearance, as in the case of growth hormone receptor (GH-BP). More rarely, soluble receptors associate with membrane-bound subunits to influence signal transduction, as is seen among the IL6 family of receptor. In the case of IL-6R, the complex formed by sIL-6Rα and IL-6, can bind to a membrane-bound signal transducing subunit (gp130) located on cells which do not express IL-6R (and would thus be unresponsive to IL-6). This pathway is referred to as trans-signaling (Rose-John 2012).

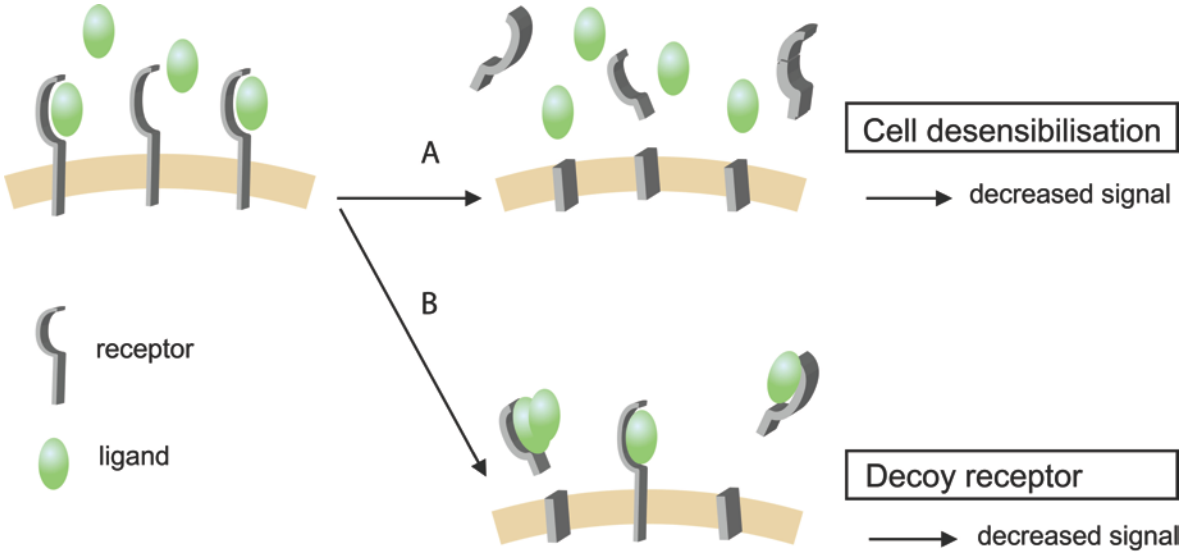

Figure 5. Mechanisms of actions of soluble receptors. *(A) Generation of soluble receptors by proteolytic cleavage downmodulates number of membrane-bound receptors and prevent signal generation by ligand. (B) Soluble receptors as decoy receptors compete with membrane-bound receptors for ligand binding resulting in decreased receptor mediated signal generation.*

1.6 The γ-secretase complex

The γ-secretase, a protease that cleaves within the membrane, has long been of particular interest to Alzheimer's disease research for its role in the production of amyloid beta (Aß). The γ-secretase has the ability to cleave various type I membrane protein after these have undergone ectodomain shedding. The remaining stub can then be further processed by the γ-secretase. This process is known as regulated intramembrane proteolysis (**Figure 6**), in which a membrane protein undergoes two consecutive cleavage by two distinct proteases controls the communication between cells and the extracellular environment (Lichtenthaler, Haass et al. 2011).

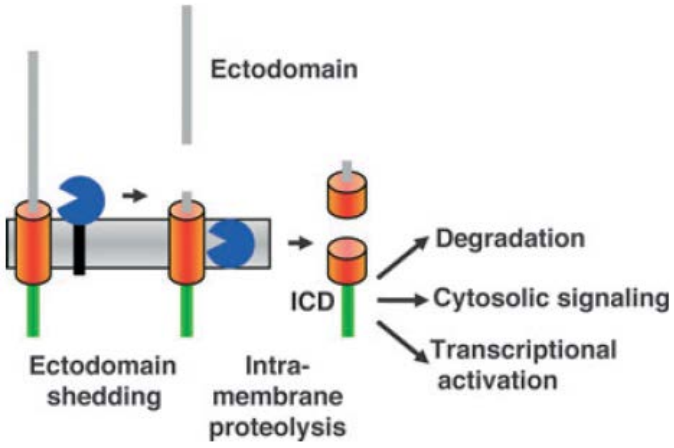

Figure 6. Schematic representation of regulated intramembrane proteolysis. *A membrane protein undergoes two consecutive proteolytic cleavages by two distinct proteases (shown in blue). First, a membrane-bound protease cleaves close to the transmembrane domain, resulting in shedding of the ectodomain. The remaining membrane-bound fragment is further cleaved by an intramembranous protease. A small peptide is secreted into the luminal/extracellular space and the intracellular domain (ICD) is released into the cytosol, where it may either be further degraded, or participate in cytosolic signaling reactions or translocate to the nucleus and stimulate transcriptional activation of target genes. Regulated intramembrane proteolysis substrates are mostly single span membrane proteins. The membrane is represented as a grey box (Lichtenthaler, Haass et al. 2011).*

1.6.1 Subunits and assembly

Over the years, biochemical and genetic approaches have identified four components of the γ-secretase complex, presenilin, nicastrin, anterior pharynx defective homolog 1 (aph-1), and presenilin enhancer 2 (pen-2) (**Figure 7**). Within the γ-secretase complex, presenilin is the catalytically active subunit and undergoes endoproteolysis upon maturation to form a stable NTF/CTF complex. Nicastrin undergoes a glycosylation/maturation process, which is crucial for the assembly and maturation of the γ-secretase complex and gamma-activity (Shirotani, Edbauer et al. 2003; Chavez-Gutierrez, Tolia et al. 2008).

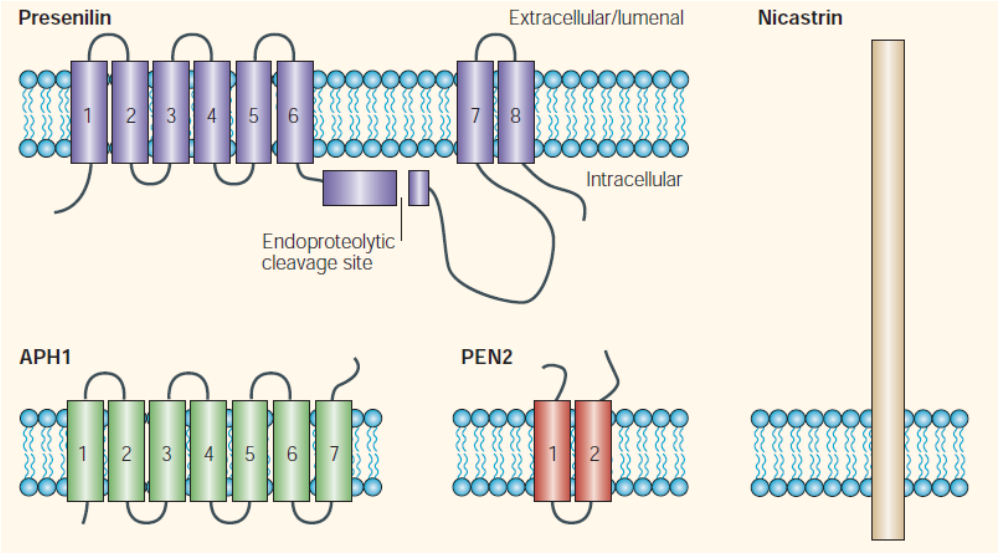

Figure 7. The components of the gamma-secretase complex. *Presenilin, which is endoproteolysed during its maturation, contains the catalytic aspartyl residues. Nicastrin, APH1 and PEN2 are important for the maturation and trafficking of the complex (Kopan and Ilagan 2004).*

1.6.2 The role of γ-secretase in biology and disease

Amyloid beta (Aβ) peptides are generated by the amyloidogenic pathway of amyloid precursor protein (APP) processing. APP, a transmembrane protein, is cleaved by α- or β-secretases, generating large, soluble, secreted fragments and membrane associated carboxy-terminal fragments (CTFs). Aβ peptides could vary in size, from 38 to 43 aminoacids. The predominant isoforms are Aβ40 (90%) and the more fibrilogenic Aβ42 (10%). They are generated after β-secretase (also known as BACE1, β-site APP cleaving enzyme) cleavage, followed by γ-secretase cleavage. Aβ peptide has the ability to auto-aggregate, so it can exist as monomers, dimers or oligomers; which in turn can generate fibrils that have β-sheet structure, and can deposit to form extracellular plaques (neuritic plaques). These processes are believed to be implicated in the pathogenesis of Alzheimer and in particular in the pathogenesis of familial Alzheimer's disease cases.

In addition, the role of the γ-secretase in the Notch signaling pathway is especially crucial and conserved. In the Notch pathway, binding of the Notch ligand to its receptor induces Notch signaling by releasing the intracellular domain of the Notch receptor through a cascade of proteolytic cleavages by TACE and γ-secretase. The released intracellular domain of Notch then translocates into the nucleus where it can further modulate gene expression (Schroeter,

Kisslinger et al. 1998; De Strooper, Annaert et al. 1999). In cancers, molecular genetic alterations are responsible for constitutive activation of Notch pathway, which all result in increased levels of intracellular Notch-IC. Since the Notch signaling pathway is a critical component in the molecular circuits that control cell fate during development, aberrant activation of this pathway can contribute to tumorogenesis.

Over the past decades, inhibitors for γ-secretase have been actively investigated for their potential to block the generation of Aβ peptide in Alzheimer disease as well as for their antitumor effects (i.e. hematological malignancies, melanoma, breast cancer) with limited benefit so far.

2 OBJECTIVES

We aimed at identifying features of humoral immunity that might be altered in autoimmune diseases. Maturation and survival of B cells and plasma cells is largely regulated via the ligands BAFF and APRIL with their receptors BCMA, BAFF-R and TACI. This dissertation focuses on BCMA, which regulates the survival of plasma cells. Having identified the presence of a soluble form of BCMA in serum of healthy controls, we aimed to characterize five major aspect of this novel soluble receptor.

1) Is sBCMA a potential biomarker in autoimmune diseases with a B cell involvement (MS and SLE)?

2) Which cells release sBCMA and how is this release regulated?

3) What is the biochemical mechanism behind the generation of sBCMA?

4) What is the functional consequence of BCMA shedding for the BCMA expressing cell?

5) What is the function of sBCMA?

3 MATERIALS AND METHODS

3.1 Materials

3.1.1 Clinical samples

Plasma and the corresponding CSF from 37 untreated patients diagnosed with either clinical isolated syndrome (CIS, n=10) or MS (n=27) and from 20 untreated patients with other neurological diseases (OND) as well as CSF pairs from 25 additional MS patients before and about one year after continuous natalizumab therapy were provided by Prof. Dr. Tomas Olsson and Dr. Mohsen Khademi. 5 CSF samples from neuroborreliosis patients were given by Prof. Dr. W. Pfister (Department of Neurology, Universitätsklinikum Großhadern, Munich, Germany). Longitudinally sera samples of 10 patients with MS who were treated with high doses of steroids (1000 mg/d methylprednisolone i.v. for 3-5 days) due to a relapse were provided by Prof. Dr. Frank Weber. Samples were obtained directly before treatment, 3 days and 4 weeks later. Serum samples from 17 untreated and 22 treated SLE patients came from Prof. Dr. Falk Hiepe. The treated group included patients treated with glucocorticosteroid, hydroxychloroquine, azathioprine, cyclophosphamide and mycophenolate Mofetil. Our study included 26 plasma samples and 29 serum samples of 34 healthy control donors. The IgG production within the brain compartment (intrathecal) was calculated as IgG-index ((CSF IgG / CSF albumin) / (serum IgG / serum albumin)). This study was approved by the Ethics Committee of the Ludwig Maximilian University of Munich. Informed consent was obtained according to the Declaration of Helsinki.

Table 1. Clinical data of patients included in this study

Age (year)	sex	Diagnosis	Treatment	Related Figure
52	F	RRMS	GA + GCS	Figure 9
41	F	RRMS	GA + GCS	Figure 9
50	M	RRMS	GCS	Figure 9
40	M	RRMS	GCS	Figure 9
39	M	RRMS	AZA + GCS	Figure 9
29	F	RRMS	GCS	Figure 9
51	F	RRMS	GCS	Figure 9
29	M	RRMS	IFN-beta 1a + GCS	Figure 9
32	F	CIS	GCS	Figure 9
33	F	RRMS	GA + GCS	Figure 9

				Figure 9
50	F	RRMS	NTZ	Figure 9
33	F	RRMS	NTZ	Figure 9
34	M	RRMS	NTZ	Figure 9
28	M	RRMS	NTZ	Figure 9
50	F	RRMS	NTZ	Figure 9
56	M	RRMS	NTZ	Figure 9
37	F	RRMS	NTZ	Figure 9
38	F	RRMS	NTZ	Figure 9
31	F	RRMS	NTZ	Figure 9
56	M	RRMS	NTZ	Figure 9
24	F	RRMS	no treatment	Figure 8
26	F	CIS	no treatment	Figure 8
28	M	RRMS	no treatment	Figure 8
34	F	RRMS	no treatment	Figure 8
24	F	CIS	no treatment	Figure 8
43	F	CIS	no treatment	Figure 8
33	F	RRMS	no treatment	Figure 8
38	F	RRMS	no treatment	Figure 8
32	F	RRMS	no treatment	Figure 8
44	F	RRMS	no treatment	Figure 8
37	F	CIS	no treatment	Figure 8
45	F	RRMS	no treatment	Figure 8
62	F	RRMS	no treatment	Figure 8
51	M	RRMS	no treatment	Figure 8
21	F	RRMS	no treatment	Figure 8
59	F	RRMS	no treatment	Figure 8
42	F	RRMS	no treatment	Figure 8
40	F	CIS	no treatment	Figure 8
21	M	CIS	no treatment	Figure 8
33	F	CIS	no treatment	Figure 8
53	M	RRMS	no treatment	Figure 8
32	M	RRMS	no treatment	Figure 8
24	F	CIS	no treatment	Figure 8
37	F	RRMS	no treatment	Figure 8
38	F	RRMS	no treatment	Figure 8
45	F	RRMS	no treatment	Figure 8
24	F	RRMS	no treatment	Figure 8
27	F	RRMS	no treatment	Figure 8
22	F	RRMS	no treatment	Figure 8
26	F	RRMS	no treatment	Figure 8
62	F	SPMS	no treatment	Figure 8
46	F	SPMS	no treatment	Figure 8
59	M	SPMS	no treatment	Figure 8
62	M	SPMS	no treatment	Figure 8

39	M	SPMS	no treatment	Figure 8
45	F	SPMS	no treatment	Figure 8
54	F	SPMS	no treatment	Figure 8
46	F	Neuroborreliosis	no treatment	Figure 8
69	F	Neuroborreliosis	no treatment	Figure 8
66	M	Neuroborreliosis	no treatment	Figure 8
55	M	Neuroborreliosis	no treatment	Figure 8
61	M	Neuroborreliosis	no treatment	Figure 8
45	f	SLE	no treatment	Figure 10
47	f	SLE	no treatment	Figure 10
33	f	SLE	no treatment	Figure 10
27	f	SLE	no treatment	Figure 10
62	f	SLE	no treatment	Figure 10
38	f	SLE	no treatment	Figure 10
40	f	SLE	no treatment	Figure 10
40	f	SLE	no treatment	Figure 10
31	f	SLE	no treatment	Figure 10
39	f	SLE	no treatment	Figure 10
26	f	SLE	no treatment	Figure 10
22	f	SLE	no treatment	Figure 10
20	f	SLE	no treatment	Figure 10
22	f	SLE	no treatment	Figure 10
43	f	SLE	no treatment	Figure 10
55	f	SLE	no treatment	Figure 10
21	f	SLE	no treatment	Figure 10
29	f	SLE	Pred, HCQ	Figure 10
38	f	SLE	Pred, HCQ, AZA	Figure 10
39	f	SLE	Pred, HCQ, CYC	Figure 10
54	f	SLE	Pred, AZA	Figure 10
47	f	SLE	Pred, HCQ	Figure 10
63	f	SLE	HCQ, AZA	Figure 10
28	f	SLE	HCQ	Figure 10
38	f	SLE	Pred	Figure 10
36	f	SLE	Pred, MMF	Figure 10
41	f	SLE	Pred, HCQ	Figure 10
30	f	SLE	AZA	Figure 10
60	f	SLE	HCQ, AZA	Figure 10
25	f	SLE	HCQ	Figure 10
31	f	SLE	Pred, HCQ, MMF	Figure 10
27	f	SLE	Pred, CYC	Figure 10
45	f	SLE	Pred, HCQ, CYC	Figure 10

22	f	SLE	Pred, HCQ, CYC	Figure 10
28	f	SLE	Pred, HCQ, AZA	Figure 10
23	f	SLE	Pred	Figure 10
25	f	SLE	Pred, CYC	Figure 10
31	f	SLE	Pred, AZA	Figure 10
32	f	SLE	Pred	Figure 10
38	F	Sensineuronal hearing deficit		Figure 8
68	F	Cerebrovascular disease		Figure 8
29	M	Sensory symptoms		Figure 8
34	F	Sensory symptoms		Figure 8
45	F	Migraine		Figure 8
26	M	Sensory symptoms		Figure 8
41	F	Sensory symptoms		Figure 8
19	F	Vertigo		Figure 8
31	M	Sensory symptoms		Figure 8
35	F	Syringomyelia		Figure 8
51	M	Spinal stenosis		Figure 8
30	F	Neurastenia		Figure 8
65	M	Alcohol-related spastic paraparesis		Figure 8
37	F	Sensory symptoms		Figure 8
49	F	Hearing deficit		Figure 8
53	F	Depression		Figure 8
52	F	OND		Figure 8
33	F	Fatigue		Figure 8
40	M	Bipolar disorder		Figure 8
32	M	Schizophrenia		Figure 8

Abbreviations used in this table: GA: Glatiramer Acetate; GCS: Glucocorticosteroid, NTZ: Natalizumab, PRED: Prednisolon, HCQ: Hydroxychloroquine, AZA: Azathioprine, MMF: Mycophenolate mofetil, CYC: Cyclophosphamide.

3.1.2 Antibodies

Table 2. Primary and secondary antibodies

Antibody	Source	Concentration/Dilution
anti-hBCMA (C12A3.2)	Biogen Idec	FC: 1 µg/ml WB: 1 µg/ml
anti-hBCMA (A7D12.2)	Biogen Idec	FC: 5 µg/ml
anti-mBCMA, biotin (BAF593)	R&D systems	FC: 5 µg/ml
anti-presenilin (PSEN1)	Epitomics	WB: 1:2500
anti-nicastrin (N1660)	Sigma-Aldrich	WB: 1:5000
anti-CD40L, FITC (TRAP1)	BD PharMingen	FC: 1:40

anti-CD138, FITC (B-A38)	Diaclone	FC: 1:10
anti-CD19, PE (HIB19)	BD PharMingen	FC: 1:40
anti-CD19, Cy7 (HIB19)	Ebioscience	FC: 1:40
anti-CD27, Cy7 (O323)	Ebioscience	FC: 1:20
anti-CD38, eFluor® 450 (HB7)	Ebioscience	FC: 1:20
anti-CD138, PE (281-2)	BD Biosciences	FC: 1:100
anti-kappa, Pacific Orange (187.1)	DRFZ	FC: 1:50
anti-CD21 (7E9)	BioLegend	FC: 1:100
anti-CD23 (B3B4)	BioLegend	FC: 1:100
anti-CD24 (clone M1/69)	BD Biosciences	FC: 1:200
anti-CD93 (AA4.1)	BioLegend	FC: 1:50
anti-CD117 (2B8)	BD Biosciences	FC: 1:200
anti-IgM (RMM-1)	BioLegend	FC: 1:20
anti-B220 (RA3-6B2)	DRFZ	FC: 1:400
anti-IgD (11-26c)	DRFZ	FC: 1:200
anti-GL-7 (GL-7)	DRFZ	FC: 1:200
anti-CD4 (GK1.5)	DRFZ	FC: 1:200
anti-CD8 (53-6.7)	BioLegend	FC: 1:50
anti-FLAG™ (M2)	Sigma-Aldrich	FC: 5 µg/ml ELISA: 5 µg/ml
goat-anti-mouse IgG2b, Alexa Fluor® 647	Invitrogen	FC: 1:500
goat-anti-mouse Ig, PE	Dako	FC: 1:50
goat-anti-mouse IgG, HRP (True blot®)	Ebioscience	WB: 1:1000
anti-rabbit IgG, HRP	Promega	WB: 1:2500-5000
mouse anti-C99 (4G8)	Covance	FC: 2 µg/ml

3.1.3 Cytokines

Table 3. Cytokines, TLR7/TLR8- agonist

Protein	Description	Product number	Company
Recombinant Mega APRIL H98	Extracellular domain of mouse APRIL (aa 98-232) fused at the N-terminus to mouse ACRP30*headless* (aa 18-110) and a FLAG®-tag. Binds to human and mouse BCMA and TACI. Does not bind to proteoglycans.	AG-40B-0035-C010	Adipogen
Recombinant human BAFF-FLAG	Extracellular domain of human BAFF (aa 83-285) fused at the N-terminus to a linker peptide (6 aa) and a FLAG®-tag	ALX-522-025-C010	Enzo Life Sciences
Recombinant human BAFF		2149-BF-010	R&D
Recombinant		202-IL	R&D

human IL-2			
R848	Resiquimod; TLR7/TLR8- agonist	SML0196-10MG	Sigma Aldrich
Recombinant human IL-21		14-8219-62	eBioscience

3.1.4 Protease inhibitors

Table 4. Protease inhibitors

Target protein	Target protease	Product number	Company
DAPT	γ-secretase	565770	Merck Millipore
LY-411575-I (stereoisomer SRR)	γ-secretase	SML0506	Sigma Aldrich
L-685,458	γ-secretase	2627/1	R&D systems
RO49290	γ-secretase	S1575	Selleckchem
TAPI-1	MMPs and TACE	579053	Calbiochem Merck

3.1.5 ELISA

Table 5. ELISA assays

ELISA	Description	Product number	Source
Human BCMA ELISA DuoSet	Sandwich ELISA, consisting of 2 polyclonal antibodies against human BCMA	DY193	R&D
Quantokine human BAFF ELISA	Solid Phase Sandwich ELISA consisting of a monoclonal capture and a polyclonal detection against human BAFF	DBLYS0B	R&D
LEGEND MAX™ Human APRIL/TNFSF13 ELISA	Solid Phase Sandwich ELISA consisting of a monoclonal capture and a polyclonal detection against human APRIL	439307	BioLegend
Human APRIL Platinum ELISA	Sandwich ELISA, consisting of 2 polyclonal antibodies against human APRIL	BMS2008	eBioscience
Human IgG ELISA assay	Sandwich ELISA	3850-1AD-6	Mabtech

3.1.6 Reagents and buffers

Table 6. Reagents and buffers

PBS (phosphate-buffered saline)	137 mM 2.7 mM 10 mM 2 mM	NaCl KCl, Na_2HPO_4 KH_2PO_4 pH 7,4
Cell isolation buffer	5 g/L 2 mM	Bovine Serum Albumin EDTA
RBC lysis buffer	150 mM 1 mM 0,1 mM	NH_4Cl $KHCO_3$ Na_2EDTA
NP-40 lysis buffer	150 mM 50 mM 1 %	NaCl, Tris pH 7.5 Nonidet P-40
BS^3 crosslinking conjugation buffer	20 mM NaCl 0.5 M	Sodium phosphate, pH 8,6
BS^3 quenching buffer	1 M	Tris HCl pH 7.5
Silver stain- Fixation buffer	50% Vol. 12% Vol.	Methanol Acetic acid
Silver stain- Sensitizing buffer	0.2 g/L	$Na_2S_2O_3$
Silver stain- Silver nitrate solution	2 g/L 0.075% Vol.	$AgNO_3$ Formaldehyde
Silver stain- Developer	60 g/L 5 mg/L 0.05% Vol.	Na_2CO_3 $Na_2S_2O_3$ Formaldehyde
Flow cytometry buffer	1% BSA 0.1 %	FCS Sodium azide
MES Running buffer (20x)	1 M 1 M 2 % (g/V) 20 mM	MES TRIS-base SDS EDTA H_2O, pH 7,3
Laemmli Loading buffer 3x	6 % 30 % 6 M 0.006 % 7.5 % ß-mercaptoethanol 0.1875 M	SDS Glycerol Urea bromophenol blue Tris-Cl pH 6,8
Lower Tris Buffer	1.5 M 0.4 %	Tris Base SDS pH 8.8
Upper Tris buffer	0.5 M 0.4 %	Tris Base SDS pH 6.8

SDS running buffer (10x)	250 mM 1.92 M 1% (g/V)	Tris Glycine SDS
Transfer buffer (10x)	250 mM 2.4 M	Tris Glycine
I-Blocking buffer	PBS 0.1 % 1 g/L	 Tween 20 I-Block™ (Tropix)
TE Buffer (10x)	108 g/L 55 g/L 4.4 % Vol.	Tris Boric acid 0,5 M EDTA pH 8.0, H_2O

3.1.7 Gels

Table 7. Gels

Tris-Glycine 8% polyacrilamide (1 gel)	4.4 ml 1.6 ml 2 ml 15 µl 15 µl <u>Stacking gel:</u> 1.7 ml 0.25 ml 0.65 ml 7.5 µl 7.5 µl	H_2O Acrylamide 40 % Lower Tris Buffer TEMED APS H_2O Acrylamide 40 % Upper Tris Buffer TEMED APS
Tris-Glycine 12% Urea (1 gel)	1.1 ml 2.6 ml 2.25 ml 2 ml 15 µl 15 µl <u>Stacking gel:</u> 0,45 ml 1.25 ml 0.25 ml 0,65 ml 7.5 µl 7.5 µl	H_2O Urea 8M Acrylamide 40 % Lower Tris Buffer TEMED APS H_2O Urea 8M Acrylamide 40 % Upper Tris Buffer TEMED APS

3.2 Cell culture

3.2.1 General cell culture method

Suspension cell lines, JLK-6 and J558L, were maintained by medium renewal after 3 days. Therefore the cell suspensions were centrifuged at 300 g for 5 min and subsequently resuspended at a concentration of $1x10^5$ -$1x10^6$ cells/ml medium.

Adherent cell lines, HEK293, HeLa, L-cells were passaged once they reached 80-90% confluency by detachment with Trypsin-EDTA (PAA Laboratories, Coelbe, Germany), rescue from the protease trypsin with 5 volumes of DMEM media, centrifugation at 1000 rpm for 5 min at 4°C, and suspension in media for plating.

BCMA-transfected MEF-cells were detached with EDTA 0,5mM for 2-5 min at 37°C buffer to avoid tryptic digestion of BCMA.

Cell viability was assessed by mixing the cell suspension 1:1 with trypan blue solution (0.4%, 1:4 in PBS, pH 7.4; Invitrogen) and quantifying on a hemocytometer under the microscope. The cells were typically grown in T25, T75, or T175 cell culture flasks (BD Biosciences).

3.2.2 Cell lines

The plasmacytoma suspension cell line JK-6L (Ref. Burger et. al) was cultured in Roswell Park Memorial Institute medium (RPMI; Gibco, Darmstadt, Germany) media, supplemented with 10% fetal calf serum (FCS; Gibco) and 1% penicillin/streptomycin. After sequential adaptation, the plasmacytoma cells were grown in serum-free condition (Hybridoma 6 direkt from Bio&SELL, Nürnberg, Germany) with 1% Penicillin/Streptomycin (Invitrogen, Darmstadt, Germany). They were incubated in 37°C with 10% CO_2.

HEK293 (human embryonic kidney), and HeLa (human cervical adenocarcinoma) cells were cultured in DMEM (Gibco®, Karlsruhe, Germany) supplemented with 10% FCS (Biochrom AG, Berlin Germany) and 1% penicillin/streptomycin (Gibco).

L-cells stably transfected with CD40L were cultured in DMEM supplemented with 10% FCS and 4.8 mg/ml Geneticin® G418.

Mouse embryonic fibroblast (MEF) cells derived from $PS1/2^{-/-}$ mice (kindly provided by Bart de Strooper) were cultured in Dulbecco's modified eagle medium (DMEM, Invitrogen) supplemented with 10%FCS and 1% Penicillin/Streptomycin. They were incubated in 37°C with 5% CO_2.

Plasmacytoma mouse cells J558L were cultured in DMEM high Glucose supplemented with 10% FCS, 1% penicillin/streptomycin (Gibco) and L-glutamine in a final concentration of 2mM (PAN Biotech, Aidenbach, Germany) and incubated at 37°C with 5% CO_2.

3.2.3 Isolation of human peripheral blood mononuclear cells by density gradient centrifugation

Blood samples from healthy donors were obtained using the S-Monovette® EDTA K2-Gel system. The blood was then diluted 1:1 with PBS, and carefully poured into 50ml falcons containing 15ml of Pancoll (human Pancoll, density 1.077 g/l PanBiotech). After 20min centrifugation at 2000rpmi (Room temperature, without brakes), the enriched lymphocyte fraction was collected and washed twice in PBS (300g, 10min, 4°C) and then resuspended in complete RPMI consisting in consisted of RPMI supplemented with 10% fetal calf serum (FCS; Gibco), 1% Penicillin/Streptomycin solution (Gibco), and 1% sodium pyruvate (Gibco). Approximatively 1-2 10^6 lymphocytes /ml blood were obtained. Cells were then directly stimulated or further isolated as described below.

3.2.4 Human B cell isolation

Human B cells were positively selected from Peripheral blood mononuclear cells (PBMC) obtained from healthy donors as described above using CD19 Microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany). First PBMC were resuspended in isolation buffer (0.5 % BSA, 2 mM EDTA, pH 7.2). After determining cell number, the PBMCs were centrifuges (300 g, 10 min, room temperature) and then resuspended into 80µl buffer /10^7 cells. 20µl of CD19 microbeads, magnetic beads labeled with anti-human CD19 antibodies (Miltenyi Biotec) were added per 10^7 cells for 15min at 4°C. Cells were then washed and then resuspended in 500µl isolation buffer. The magnetic separation was performed using MS or LS columns and a MACS® Separator (Miltenyi Biotec). The appropriate column was placed in the magnetic field and the cell suspension was applied to the column. The column was then washed three times with the isolation buffer. The column was then removed from the separator and the CD19 positive, magnetically labeled cells were then flushed out of the columns by pushing a plunger into the column. The collected CD19 positive cells were culture in complete RPMI supplemented with 10% FCS, 1% sodium pyruvate and 1% Penicillin/Streptomycin.

3.2.5 Human B cell stimulation and differentiation

In order to differentiate human native B cells into Ig-secreting cells, two experimental approached were used:

3.2.5.1 CD40L/IL-21 stimulation

CD40L transfected L-cells were suspended in culture medium without genetecin®. The cells were then irradiated (7500 rad) and plated in a 96-well flat bottom plate at 5000 cells /well. Isolated human B cells (see above) were added to the irradiated CD40L-L cells in a concentration of 80,000 cells /well and cultured in RPMI complete for 5 days in the presence of IL-21 at a final concentration 50 ng/ml (Ettinger, Sims et al. 2005).

3.2.5.2 Resiquimod/IL-2 stimulation

PBMC were obtained from healthy donors by density gradient centrifugation and resuspended in RPMI complete (300,000 cells /ml). Resiquimod at a final concentration of 2.5 µg/ml and IL-2 at a final concentration of 1000 UI/ml were added for 7 days (Pinna, Corti et al. 2009).

3.2.6 Mouse B cell isolation and survival assay

Spleens from C57/BL6 mice were homogenized. A single cell suspension was obtained using a 100 µM nylon mesh (Merck Millipore). Erythrocyte lysis was done by suspending the cells in 2ml RBC lysis buffer for 5 min at room temperature. The splenocytes were then washed with PBS, centrifuged (300 g, 10 min, 4°C) and suspended into RPMI complete at a concentration of $1x10^8$ cells/ml in a 5 ml polystyrene tube. B cells were magnetically isolated (negative selection) using the EasySep™ Mouse B Cell Isolation Kit (Stemcell Technologie, Vancouver, Canada) using the protocol from the manufacturer. Normal rat serum (Stemcell) and EasySep™ Mouse B cell isolation cocktail (Stemcell) were added. The latter is a combination of biotinilated monoclonal antibodies directed against CD4, CD8a, CD11b, CD43, CD49b, CD90.2, Ly-6C, TER119 and an FcR blocker. After 10 min incubation at

room temperature, streptavidin coated magnetic particles (EasySep™ Streptavidin Rapidspheres ™) were added. The magnetic separation was performed using the EasySep™ Magnet.

Flatt bottom, 96-well microtiter plates (Nunc) were precoated overnight at 37°C with anti-IgM (5 µg/ml) in RPMI. The plates were washed with sterile PBS and the B cells were plated at $3x10^6$ cells /well. APRIL-FLAG (100 ng/ml, Adipogen) was added for 48h and cross-linked with anti-FLAG mAb antibody at a concentration of 1 µg/ml (Sigma-Aldrich). Cells were stained for flow cytometry against CD19 and cell survival was quantified by flow cytometry using TO-PRO®-3 Iodide viability dye (Invitrogen Life Technologies) and APRIL-induced survival was calculated as followed: 100 x [(cell survival in presence of APRIL – cell survival without APRIL) / cell survival without APRIL].

3.3 General protein analytics

3.3.1 Cell lysates

Adherent MEF Cells were washed twice with cold PBS. Complete protease inhibitor cocktail (Roche Applied Science, Penzberg, Germany) was added to cold NP-40 lysis buffer (150 mM NaCl, 50 mM Tris pH 7.5, 1% Nonidet P-40). Cells were suspended by scraping and pipetting in the complete NP-40 lysis buffer. After 1h incubation on ice, cell suspensions were transferred to precooled aliquots, and centrifuged (20,000 g, 15 min, 4°C). Supernatant were collected and stored at -80°C.

3.3.2 Immunoprecipitation

3.3.2.1 Samples

Blood samples from healthy donors were obtained using the S-Monovette® Serum system. The tubes were then centrifuged (3000 g, 15 min, RT) and the upper serum fraction was collected. Plasmacytoma cells (JLK6) were centrifuged (300 g, 10 min), resuspended in serum-free hybridoma media (1,000,000 cells/ml) and cultured for 3 days. Cells were then centrifuged and the supernatant was collected. Human B cells were isolated and stimulated

with CD40/L and IL21 as described above. After 5 days, the cells were centrifuged and the supernatant was collected. To avoid contamination by dead cells and cell debris, all samples were centrifuged twice at 300 g (10 min, 4°C) followed by 20,000 g centrifugation (30 min, 4°C). Supernatant were collected and transferred to a new tube between each centrifugation step. Sample were then stored at -20°C.

3.3.2.2 Magnetic beads preparation, antibody coupling and crosslinking

Magnetic beads with recombinant protein G (Dynabeads Protein G, Invitrogen) were used for all immunoprecipitation experiments. 35 µg of the monoclonal antibody anti human BCMA C12, 35 µg of the monoclonal antibody anti human BCMA A7, and 15 µg of the polyclonal antibody anti-human BCMA (R&D), 35 µg of isotype control IgG and 35 µg of isotype control IgG2b diluted in 200 µl of PBS-Tween (0,02 %) were incubated with 1.5 mg magnetic beads. Magnetic beads were washed twice with PBS-Tween using MagCellet™Magnet (R&D). Antibodies couple to the magnetic beads were cross-linked using following protocol: Beads were washed twice in crosslinking conjugation buffer. The beads were then incubated at room temperature with tilting/rotation twice for 30 min in 250 µl of a freshly prepared solution of 5 mM BS3 (bis[sulfosuccinimidyl] suberate). The reaction was quenched by adding 12.5 µl of BS3 Quenching for 15 min at room temperature. The beads were washed 3 times with PBS-Tween (0.02%).

3.3.2.3 Immunoprecipitation

Immunoprecipitation for further Western blot analysis was performed by incubation of 1 ml serum, 1 ml plasmacytoma cell supernatant or 5 ml human B cell supernatant with the antibody-coupled magnetic beads (overnight with tilting and rotating at 4°C). In order to immunoprecipitate a larger amount of sBCMA for latter mass spectrometry analysis, successive (10x2ml) incubation of plasmacytoma supernatant were performed using anti-human BCMA A7 and IgG2b coupled magnetic beads.

3.3.2.4 Elution

The magnetic beads-antibody-antigen complex was washed twice with PBS-tween (0.02%) and three times with PBS alone. The bead suspension was then transferred to a clean tube before the elution.

For further immunoblotting analysis, denaturing elution was performed by adding 20µl premixed NuPage LDS Sample buffer and NuPage Reducing Agent (mixed as per manufacturer's instructions) and heated for 10min at 90°C. The tubes with the beads were then placed on the magnet and the supernatant was collected.
For further mass spectrometry analysis, acidic elution was done by incubating the beads 3 times with a 1M Glycine solution, pH 3 for 20 min each at 56°C. The eluate was neutralized by adding 1M TRIS, pH 9.

3.3.3 Gel electrophoresis, Coomassie staining and Western blot

3.3.3.1 Western blot detection of soluble BCMA upon immunoprecipitation

Samples were separated in 4 - 12 % polyacrylamide gels (Invitrogen, Darmstadt, Germany) using the NuPAGE SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) system with MES buffer as running buffer. Recombinant human BCMA-Fc was used as a positive control. Markers used were the Novex R Sharp Pre-stained protein standard (Invitrogen, Darmstadt,Germany) and the Spectra™ Multicolor Low Range protein ladder (Thermo Scientific).
For Coomassie staining, gels were incubated for 20 min in the Coomassie staining solution (0.1 % Coomassie Brilliant-Blue, 40 methanol, 10 % acetic acid) under constant shaking at room temperature. The background staining was afterwards eliminated by at least three subsequent 15 min incubations with the destaining solution (50 % methanol, 7 % acetic acid). Gels were eventually stored in 7 % acetic acid.
For Western blotting, gels were electrically transferred to a polyvinylidene difluoride (PVDF) membrane at 50 mA for 80 min using NuPAGE transfer buffer (Invitrogen) by semi-dry blotting (Bio-Rad). After blocking in 5 % milk at room temperature for at least 2 h, membranes were incubated overnight at 4°C with anti-human BCMA C12 diluted in blocking buffer. To avoid interference with residual heavy and light chains resent in the

immunoprecipitation eluate, in addition to crosslinking of the ip antibodies, the Mouse TrueBlot® ULTRA system was used (1:1000, 2h, room temperature). This HRP-conjugated rat-anti-mouse Ig preferentially detects the non-reduced form of mouse IgG.

3.3.3.2 Western blot detection of presenilin 1 and nicastrin

Total protein concentration of cell lysate was quantified using Pierce™ BCA Protein Assay kit (Thermo Scientific). 3x Laemmli Loading buffer with ß-mercaptol and 6M Urea was used as loading buffer.

For presenilin and nicastrin immunoblotting, samples were loaded on a 12% Urea Tris-Glycine gel and on an 8% polyacrylamide Tris-Glycine gel respectively. Samples were separated by electrophoresis (80 V, 90 min). Gels were transferred to a PVDF membrane by wet electroblotting (400 mA, 65 min, Bio-Rad). PVDF membranes were blocked with 5% milk and incubated overnight at 4°C with either a monoclonal rabbit anti-presenilin antibody or a polyclonal rabbit anti-Nicastrin antibody. HRP-conjugated goat-anti rabbit Ig was used as a secondary antibody. Primary and secondary antibodies for presenilin and nicastrin immunoblotting were diluted in PBS-Tween 0.05 %, BSA 0.5 % and in I blocking buffer respectively. Detection and imaging was realized with ECL (GE healthcare) using the Gel Doc™ system (Bio-Rad).

3.3.4 Mass spectrometric analysis and sample preparation

3.3.4.1 In solution digestion by trypsin followed by mass spectrometry

Immunoprecipitated soluble BCMA from supernatant of plasmacytoma cells obtained by acidic elution were desalted and concentrated using C18 StageTips™ (Thermo Scientific) following manufacturer's protocol. Samples were eluted in 80% acetonitrile (ACN) 5% formic acid. In-solution tryptic digestion, the LC-MSMS analysis and the subsequent mass spectrometric analysis (TQ OrbitrapXL mass spectrometer, Thermo Fischer Scientific) were performed by Stephanie Hauck (Research Unit Protein Science, Helmholtz Zentrum München).

3.3.4.2 Silver staining, in gel digestion by trypsin and chymotrypsin followed by mass spectrometry

Immunoprecipitated soluble BCMA from supernatant of plasmacytoma cells were eluted in denaturing conditions and loaded on a NuPAGE® Novex® 4-12% Bis-Tris Gel (MES running Buffer, 50 V 30min then 100 V 30 min). The gel was fixed with a fixation solution twice for 15 min and then washed three times with 50% ethanol. The gel was then sensitized with 0.02 % $Na_2S_2O_3$ for 1-2 min. After the gel was washed with H_2O for 5 min, it was stained with silver nitrate for 20min. The gel was the washed for 1 min with H_2O and developed over a period of 5 min. After stopping the staining with 0.5 % glycine for 15 min, the obtained band was cut. In gel tryptic and chymotryptic digestion, followed by the LC-MSMS analysis and the subsequent mass spectrometric analysis (TQ OrbitrapXL mass spectrometer, Thermo Fischer Scientific) were performed by Stephanie Hauck (Research Unit Protein Science, Helmholtz Zentrum München).

3.4 Molecular cloning

3.4.1 General molecular cloning methods

3.4.1.1 Transformation of bacterial cells

Plasmids were transformed into *Escherichia coli* (*E. coli*) cells for amplification. Typically, 10 ng of plasmid preparation or 1 µL of ligation reaction was added into 50 µL of electro-competent DH5-cells. After incubation on ice for 30 min, the cells were heated-shocked at 42 °C for 90 sec in a thermomixer, and then returned to ice. For recovery, 450 µL of LB-media was added and then the cells were placed in a 37 °C shaker at 150 rpm for 30 min. Afterwards, the cells were plated on LB- agar plates supplemented with the appropriate selection antibiotic and incubated at 37°C.

3.4.1.2 Plasmid purification from bacterial cells

Bacterial colonies of interest were picked and inoculated to LB-media supplemented with the appropriation selection antibiotic. Plasmid DNA was purified from *E. coli* cells by using miniprep and maxiprep kits (Qiagen, Hilden, Germany), following protocol from the manufacturer. The resulting plasmid DNA was used for sequence verification, further cloning procedures, or cell transfection.

3.4.1.3 Agarose gel electrophoresis

To cast an agarose gel, agarose powder was dissolved in TBE buffer by heating and poured into a self-assembled mold with an appropriate comb. The percentage of agarose varied from 1-1.5% depending on the interested DNA size. SYBR@Safe DNA Gel Stain (1:10,000, Invitrogen) was added into the gel mixture at to visualize DNA under UV light. The gel was then placed in TBE buffer in a horizontal electrophoresis chamber. Samples were mixed with 6x gel loading dye (Thermo scientific) and loaded into the wells. DNA ladders (100 bp or 1 kb; New England Biolabs) were included for size recognition. The electrophoresis conditions were 100 mV for 30 min to 1 h at room temperature. Afterwards, the gels were visualized in a UV light box.

3.4.1.4 DNA extraction from agarose gel

DNA molecules were eluted from agarose gel pieces cut from the whole gel with a scalpel in the UV light box. The elution was done using QIAquick Gel Extraction Kit following manufacturer's protocol.

3.4.1.5 Cloning primers

Table 8. Cloning primers

Name	Description	Sequence 5'-3'
BCMA-SgfI-fw	*Sgf*I restriction site	GAGGCGATCGCCATGTTGCAGAT
BCMA-MluI-rev	*Mlu*I restriction site	GCGACGCGTTTACCTAGCAGAAATTGATT

BCMA-EcoRI-fw	*Eco*RI restriction site	GATCGAATTCCGGATGTTGCAGATGGCTGG
BCMA-NotI-rv	*Not*I restriction site	GATCGCGGCCGCATTCTTACCTAGCAGAAATTGATTTCTC
BCMA-EcoRI-fw	*Eco*RI restriction site	ATCCGGAATTCATGTTGCAGATGGCTGGGC
BCMA-SgfI-Rv	*Sgf*I restriction site	GCGGCGATCGCTCCTTTCACTGAATTGGTCACACTTG

3.4.1.6 Plasmids

Table 9. Plasmids

Name	Backbone	description
BCMA WT	pCMV-XL5	Human BCMA
pCMV6-AN-DDK	pCMV6	Destination vector, DDK tag N-terminal
pcDNA3.1 Zeo(+)	pcDNA3.1	
psPAX2	psPAX2	
pCDNA3.1 (-)-VSV-G	pCDNA3.1 (-)	
pCR8GWTOPO	pCR8/GW/TOPO	Entry vector
pLV FU-ΔZeo		Destination vector
peak12-SP-C99	pEAK	Expresses the C-terminal 99 amino acids of APP together with the APP signal peptide

3.4.1.7 Polymerase chain reaction

PCR was performed using a thermocycler (Biometra, Goettingen, Germany). All primers were designed to have an annealing temperature between 58-64 °C and were synthesized by Metabion (Martinsried, Germany). A typical amplification reaction involved a sample of template DNA (ex. cDNA, PCR product, plasmid, bacteria cells), two oligonucleotide primers, deoxynucleotide triphosphates (dNTPs), reaction buffer, magnesium, and a thermostable DNA polymerase. Taq DNA polymerase (Roche, Penzberg, Germany) was used unless otherwise specified. The reaction involved an initial denaturing step at 95 °C for 3 min. This was followed by typically 25-30cycles consisting of: (1) Denaturation step at 95 °C for 30sec, (2) Annealing step at between 55-60 °C for 30 sec, and (3) Elongation step at 72 °C.

The elongation time of the final cycle was set to 10 min to ensure that all products were fully extended. The PCR product was stored at 4°C.

3.4.1.8 DNA digestion with restriction endonuclease

Restriction digestion using endonucleases was performed on PCR products and plasmids using conventional restriction. For each enzyme, the digest reaction was carried out according to the manufacturer's recommendation of buffer usage and temperature for incubation. Double digest reactions were carried out if a compatible buffer existed and if the two recognition sequences were sufficiently far apart from each other to avoid steric hindrance.

Table 10. Restriction enzymes

Enzyme	Restriction site 5'-3'	Manufacturer
SgfI	GCGAT*CGC	Promega
*Mlu*I	A*CGCGT	New England BioLabs
EcoRI-HF	G*AATC	New England BioLabs
NotI-HF	GC*GGCCGC	New England BioLabs

3.4.1.9 DNA ligation

DNA fragments generated by PCR or by restriction were ligated to the vector to produce a plasmid. After determining the DNA concentration of each fragment or the linearized vector, a ratio of 1 (vector) to 4 (insert) was used to form a complete plasmid. T4 DNA ligase (Invitrogen) was used here and reactions were set up according to the protocol provided by the manufacturer, and incubated at room temperature for 60 min or at 16 ℃ overnight. After the ligation reaction, 10 µL of the ligation product was used to transform *E. coli* cells (Section 3.1.2.1), typically DH5*α*.

3.4.1.10 DNA quantification

DNA concentration was measured on a Nanodrop spectrophotometer (Peqlab, Erlangen, Germany). After a blank measurement was done with the DNA buffer, 2 µL of DNA sample was loaded onto the stage for photometric measurement at 260 nm, and the concentration and purity of the DNA sample were measured.

3.4.1.11 Transfection protocol

HEK293T oder Hela cells were plated to be 70-90% confluent after 24h. Lipofectamin 2000 (Invitrogen) was diluted in Opti-MEM™ (Invitrogen) medium (1:20-1:100, 5 min, RT). DNA (0,5 µg-10 µg) was diluted in the same volume of Opti-MEM™. The diluted DNA was then added to the lipofectamin 2000 and the mixture was incubated for 20 min at RT. The DNA lipofectamin mixture was then added directly to the cells.

3.4.2 Soluble BCMA generation

To produce soluble BCMA and soluble BCMA with N-terminal-FLAG tag, HEK293 cells were transfected within 10 cm cell culture dishes with expression plasmids of full-length human BCMA and full-length human BCMA with an N-terminal-FLAG (BCMA-FLAG) using lipofectamine 2000 (Invitrogen Life Technologies), as described above. Supernatant was harvest after 48h. Control supernatant was produced by parallel transfection of HEK293 cells with the corresponding empty vector. sBCMA concentrations were determined by ELISA.

3.4.3 NF-κB assay

To measure NF-κB activation, HEK293T cells were transiently transfected with a plasmid containing a firefly luciferase reporter gene under the control of an NF-κB transcriptional response element, a plasmid with a Renilla reniformis luciferase reporter gene for normalization and the indicated amounts of expression or control plasmids using Lipofectamine 2000 (Invitrogen Life Technologies). To determine the effect of γ-secretase inhibition on NF-κB activation, BCMA-transfected cells were treated with DAPT or a solvent control and 6 hours later stimulated with APRIL-FLAG or BAFF. To analyze a possible decoy function, DAPT along with BAFF or APRIL-FLAG were added to BCMA-Fc or supernatants generated by HEK293T cells that had been transfected with full-length BCMA or an empty control vector as described above. These supernatants were incubated at 37°C for 30 minutes and then added to BCMA or TACI-transfected and DAPT-treated cells used for the reporter assay. 16 hours after stimulation cells were harvested and cell lysates were prepared using passive lysis buffer (Promega) and the reporter gene activity was measured

using firefly luciferase substrate (Biozym) and renilla luciferase substrate (Promega) respectively (cooperation with Franziska Hoffmann).

3.4.4 Wild-type human BCMA, N-terminal-FLAG-BCMA and extended hBCMA-BCMA

A human BCMA cDNA clone (SC125656) was purchased from Origene. The plasmid DNA was sequenced following the manufacterer's protocol and using the provided sequencing primers. To obtain BCMA with an N-terminal FLAG-tag, full-length human BCMA (h184) from a human BCMA cDNA clone (OriGene Technologies) was first amplified by PCR. Primers were designed to append *Sgf*I and *Mlu*I cloning sites at the 5' and 3' respectively end of the BCMA sequence. The restriction of the obtained PCR fragment and the digestion of the destination vector pCMV6-AN-DDK were performed by conventional double digestion with *Sgf*I and *Mlu*I (NEB buffer 3). Both restriction products were loaded on an agarose gel. The bands of interests were cut and eluted using (QIAquick Gel extraction Kit). Ligation was performed using a 1 (PCR fragment) to 4 (vector) ratio as described above. 10µl of the ligation product was used to transformed DH5α *E. coli* cells. Afterwards, the cells were plated on LB- agar plates supplemented with Ampicillin and incubated at 37°C. Colonies of interest were picked and inoculated to LB-media with Ampicillin. Bacterial DNA was extracted as described previously. Control digestion using *Sgf*I and *Mlu*I as well as vector sequencing using the provided sequencing primers was done.

To obtain a BCMA variant with a prolonged extracellular part, the extracellular part of hBCMA was amplified by PCR using human BCMA cDNA clone (SC125656) as a template and primers designed to append *Eco*RI and *Sgf*I cleavage sites at the 5' and 3' site respectively of the extracellular domain. The obtained PCR product and the N-terminal-FLAG-BCMA plasmid (see above) were digested using conventional double digestion by *Eco*RI and *Sgf*I. The ligation resulted in a human BCMA construct that contains a full length human BCMA and an additional extracellular domain. The original N-terminal Flag is removed during the process.

The plasmid peak12-SP-C99 expresses the C-terminal 99 amino acids of APP together with the APP signal peptide. C99 is a direct substrate for γ-secretase. Peak12-SP-C99 was obtained by cloning SP-C99 from the pCEP4 vector into the peak12 plasmid (Mitterreiter, Page et al. 2010).

3.4.5 BCMA transduction and rescue of MEF PS-/- deficient cells

3.4.5.1 Generation of hBCMA expressing ® Gateway lentiviral plasmid

Full-length human BCMA (h184) from a human BCMA cDNA clone (OriGene Technologies) was first amplified by PCR. Primers were designed to append *Eco*RI and *Not*I cloning sites at the 5' and 3' respectively end of the BCMA sequence. The restriction of the obtained PCR product and the digestion of the vector pcDNA3.1/Zeo(+) were performed by conventional double digestion with *Eco*RI-HF and *Not*I-HF (NEB buffer 4). After gel purification of the restriction products, ligation was performed as described above. The BCMA insert was released from the obtained vector pcDNA3.1/Zeo(+)-hBCMA by double digestion with BamH1 and NotI (NEB buffer 3) and subcloned into the GATEWAY® entry vector pCR8GWTOPO, resulting in a plasmid containing the human BCMA gene with 2 flanking recombination sequences called "att L 1" and "att L 2". The transfer of BCMA or LR reaction into the destination lentiviral vector pLV FU-ΔZeo (containing the "att R" recombination sites) was performed following the manufacture's protocol (Invitrogen, Life technologies, Carlsbad CA). To summarize, the LR recombination reaction was performed with a 1:1 ratio (entry vector: destination vector). And TE buffer was added to reach an end volume of 8µl. LR Clonase™II was added. After 1 hour incubation, the reaction was terminated using 1µl Proteinase K (collaboration with Peer-Hendrik Kuhn, Neuroproteomics, Klinikum rechts der Isar, Technische Universität München, Munich).

3.4.5.2 Generation of a stable BCMA-expressing MEF PS (-/-) cell line

Lentiviruses were generated by transient cotransfection of HEK293T cells with the plasmids psPAX2, pCDNA3.1 (-)-VSV-G as previously described (Kuhn et al. 2010). For generation of MEF PS1/2 (-/-) cells stable expressing BCMA, transfer vectors FKP-5XUAS-BCMA and FUΔZeo-Gal4-VP16 were separately packed into lentiviral particles. For the rescue of Presenilin-1 expression in MEF PS1/2 (-/-) cells, lentiviral particles were generated with the transfer vector FU-Valentin-PS1wt or as a control the catalytically inactive Presenilin-1 mutant D385. For transduction, medium was replaced by fresh antibiotic-free DMEM High Glucose supplemented with Pyruvate and 10% fetal calf serum medium 1 day after transfection. Overnight conditioned medium containing both FUΔZeo-Gal4-VP16 and FKP-

5XUAS-BCMA lentiviral particles was filtered through 0.45 µm sterile filters and directly added to MEF PS1/2 (-/-) cells. After 6 h, medium was exchanged against cell type-specific growth medium of the target cells. After determining the adequate concentration of the selection antibiotic by performing a killing curve with different puromycine concentrations, BCMA expressing MEF PS (-/-) cells were cultured in the presence of 2µg/ml puromycine to eliminate non-transduced cells. Afterwards, Presenilin expression was rescued with FU-Valentin-PS1 wt particles or FU-Valentin-PS1-D385A particles as a control.
MEF PS(-/-) cells, MEF PS(-/-) BCMA, MEF PS(-/-) BCMA-D385A and MEF PS(-/-) BCMA-PS1 were plated in a 6-well plate (250,000cells/well). After 24h, BCMA surface expression was measured by flow cytometry as described in section 3.6. Supernatant was collected to measure sBCMA levels. Detection of Nicastrin, Presenilin and BCMA on cell lysate was performed by immunoblotting as described earlier (collaboration with Peer-Hendrik Kuhn, Neuroproteomics, Klinikum rechts der Isar, Technische Universität München, Munich).

3.5 Enzyme-linked immunosorbent assay (ELISA)

Soluble BCMA was detected in serum, plasma, CSF and cell culture supernatant using a commercially available human BCMA ELISA kit (R&D, DY193), following manufacturer's instructions. To avoid contamination with dead cells or cell debris, samples were sequentially centrifuged, 2x 10 min at 300 g, 4 °C and 30 min at 20,000 g, 4 °C. Between each centrifugation step, supernatants were transferred to a clean tube. Samples were incubated overnight at 4 °C. Absorption was measured with a Wallac Victor2 1420 multilabel counter (PerkinElmer) at 450 nm.

Soluble BAFF was measured using precoated ELISA plate (DBLYS0B, R&D). Samples were prepared and incubate as described above.

To quantify human IgG levels in cell culture supernatants, ELISA assay for human IgG (Mabtech) was used. Absorption was measured with a Wallac Victor2 1420 multilabel counter (PerkinElmer) at 405. Day-to day controls were used to allow comparison within value of different ELISA plates.

3.6 Flow cytometry

Cells were transferred into 96-well V-bottom plates (Nunc), centrifuged at 300 g for 5 min at 4°C and washed twice with 200 µl flow cytometry buffer (Table 6). Cell pellet was resuspended in 50 µl/well flow cytometry buffer containing directly labeled antibodies (Table 2) and incubated for 30 min at 4°C in the dark. Cells were washed three times and resuspended in 200 µl flow cytometry buffer.

Human BCMA cell surface expression was measured by staining with anti-human BCMA C12 at 5µg/ml. RPE-conjugated goat anti-human IgG was used as a secondary antibody. Mouse BCMA expression was detected by staining with a biotin-conjugated goat anti-mouse BCMA polyclonal antibody followed by staining with RPE-conjugated streptavidin.

For multi-staining of BCMA with CD19, CD27 and CD38, anti-human BCMA A7 was used followed by staining with mouse anti-IgG2b-647 secondary antibody.

APRIL-binding to human plasmacytoma cells was detected as followed. After overnight treatment with or without DAPT, cells were washed and incubated for 4 hours (37°C) with APRIL-FLAG. The cells were then washed with FACS buffer and stained with anti-FLAG M2 antibody (Sigma-Aldrich). RPE-conjugated goat anti-human IgG was used as a secondary antibody. Fluorescence was measured using a FACSverse™ flow cytometer (BD) and data were analyzed with FlowJo 7.6 software (TreeStar, Ashland, USA).Cell survival was quantified by flow cytometry using TO-PRO®-3 Iodide viability dye (1:4000, Invitrogen)

In mice, single cell suspensions were prepared from bone marrow and spleen. The detection of plasma cells was carried out with anti-CD138-PE (clone 281-2; BD Biosciences) for surface staining and anti-kappa Pacific Orange (clone 187.1; DRFZ) for intracellular staining. B and T cells were identified with following anti-mouse antibodies: CD21 (clone 7E9, BioLegend), CD23 (clone B3B4, BioLegend), CD24 (clone M1/69, BD Biosciences), CD93 (clone AA4.1, BioLegend, CA, USA), CD95 (clone Jo2, BD Biosciences), CD117 (clone 2B8, BD Biosciences), IgM (clone RMM-1, BioLegend), B220 (clone RA3-6B2, DRFZ), IgD (clone 11-26c, DRFZ), GL-7 (clone GL-7, DRFZ), CD4 (clone GK1.5, DRFZ) and CD8 (clone 53-6.7, BioLegend). Identification of B cell subsets in the spleen: B1: $IgM^{high}CD21^{low/-}CD23^{-}CD93^{-}$. Follicular B cells: $IgM^{+}CD21^{+}CD23^{+}CD93^{-}$.Marginal zone B cells: $IgM^{high}CD21^{+}CD23^{-}CD93^{-}$. GL-$7^{+}$: GL-$7^{+}IgD^{-}$. Identification of B cell subsets in the bone

marrow: pro-B cells: $B220^{+}CD93^{+}CD117^{+}$. pre-B cells: $B220^{+}CD24^{+}IgM^{-}IgD^{-}$. Immature B cells: $B220^{+}CD24^{+}IgM^{+}IgD^{-}$. Mature B cells: $B220^{+}CD24^{low/-}IgM^{+}IgD^{+}$. Cytometric analysis was performed using a FACSCanto II cytometer (BD Biosciences) and data were analysed with FlowJo software (Tree Star Inc.). Source and working concentration of antibodies used are listed in Table 6. Flow cytometric analyses in animal experiments were performed by our collaborators (see below).

3.7 Immunization and γ-secretase inhibitor treatment *in vivo.*

Two different mouse models were applied. First, two months old C57/BL6 mice were immunized intraperitoneally with 100 µg OVA and 10 µg LPS in alum (Pasare and Medzhitov 2005) and sacrificed 10 days later. Immunized mice received an intraperitoneal dose (10 mg/kg) of LY-411575-I on day 9, followed by another dose 6 hours before sacrifice. These animal experiments were performed in collaboration with Dr. Yuan-Yuan Chu and Prof. Dr. Marc Schmidt-Supprian at the Department of Internal Medicine III, Klinikum Rechts der Isar, Technische Universität München, Munich.

Second, female 16-18 weeks NZB/W F1 mice were bred at the animal facility of the German Rheumatism Research Center Berlin (DRFZ) under defined, pathogen-free conditions. To distinguish between short-lived and long-lived plasma cells, we fed the mice bromodeoxyuridine (BrdU, Sigma-Aldrich, 1mg/ml) with 1 % glucose in drinking water for two weeks. One week after start of BrdU feeding, mice were treated daily with the γ-secretase inhibitor LY-411575-I intraperitoneally at a dose of 10 mg/kg for 7 days. These animal experiments were performed in collaboration with Dr. Qingyu Cheng and Prof. Dr. Falk Hiepe at the Deutsches Rheuma-Forschungszentrum in Berlin.

4 RESULTS

4.1 Contribution statement

NF-κB assays were performed in collaboration with **Dr. Franziska Hoffmann** (Institute of Clinical Neuroimmunology, LMU Munich).

BCMA transduction and rescue of MEF PS-/- deficient cells were performed in collaboration with **Dr. Peer-Hendrik Kuhn** (Neuroproteomics, Klinikum rechts der Isar, Technische Universität München, Munich).

Immunization of C57/BL6 mice, treatment with γ-secretase inhibitor and flow cytometric analysis of bone marrow cells were performed by **Dr. Yuan-Yuan Chu and Prof. Dr. Marc Schmidt-Supprian** (Department of Internal Medicine III, Klinikum Rechts der Isar, Technische Universität München, Munich).

γ-secretase treatment of NZB/W mice and flow cytometric analysis were performed by **Dr. Qingyu Cheng and Prof. Dr. Falk Hiepe** at the Deutsches Rheuma-Forschungszentrum in Berlin.

Mass spectrometric analyses were performed by **Dr. Stephanie Hauck** (Research Unit Protein Science, Helmholtz Zentrum München).

4.2 Detection of soluble BCMA

4.2.1 sBCMA in healthy subjects and patients with neuroinflammatory diseases

We first addressed the question whether soluble forms of BCMA could be detected in serum and plasma samples of healthy controls and patients with neuroinflammatory diseases.
Indeed, sBCMA was regularly detected by ELISA in the plasma of each of 83 analyzed donors. sBCMA levels were similar in healthy controls (mean=17.95 ng/ml), in patients with CIS or MS (mean=20.93 ng/ml) and in patients with other neurological diseases (mean=20.30 ng/ml) (**Figure 8A**). We compared sBCMA levels in 14 matched serum/plasma pairs and found that the levels in serum and plasma were very similar (difference for each pair less <10%, **Figure 8B**). Moreover, sBCMA was detected in the cerebrospinal fluid (CSF) of patients with other non-inflammatory neurological diseases (OND, mean= 0.091 ng/ml) and at enhanced levels in the CSF samples of patients with CIS and MS (mean= 0.348 ng/ml, $p< 0.001$), and neuroborreliosis (mean= 0.754 ng/ml, $p< 0.001$, Kruskal-Wallis test followed by Dunn's Multiple Comparison Test) (**Figure 8C**). We did not observe significant differences between the sBCMA levels in the different clinical forms of multiple sclerosis (**Figure 8D**; clinical isolated syndrome, CIS, mean= 0.277 ng/ml; relapsing remitting multiple sclerosis, RRMS, mean=0.370 ng/ml; Secondary progressive MS, SPMS, mean= 0.386 ng/ml).

A hallmark of MS is the persisting Ig production, which is regularly quantified in the neurological clinical routine by the IgG-index, calculated as follows, IgG-Index = (CSF IgG / CSF albumin) / (Serum IgG / serum albumin)). We found a strong correlation between sBCMA levels in the CSF and the intrathecal IgG production (**Figure 8E**). This correlation was evident when all CSF samples were considered ($p <0.0001$, $r= 0.85$, Spearman rank correlation) and also within the MS group ($p< 0.0001$, $r= 0.77$, Spearman rank correlation).

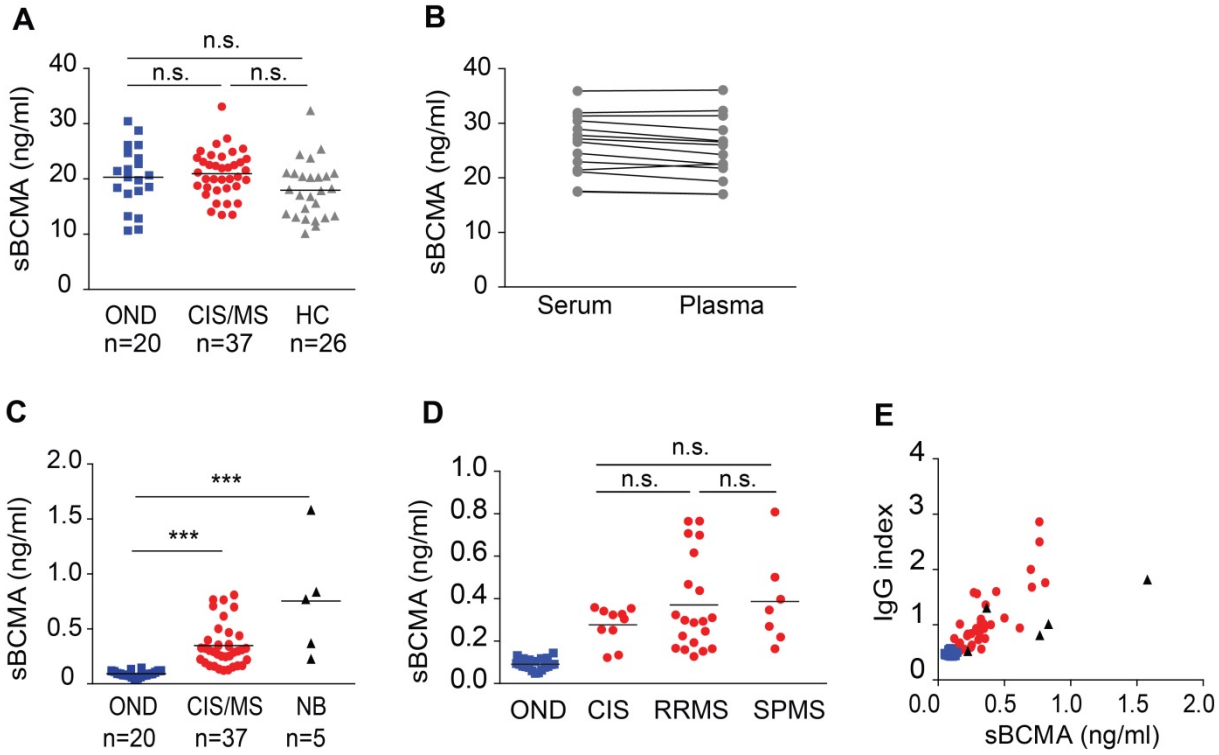

Figure 8. sBCMA is elevated in the spinal fluid of patients with neuroinflammatory diseases and correlates to local IgG production. *(**A**) sBCMA plasma concentrations were determined by ELISA in healthy controls (grey symbols, HC), CIS/MS (red symbols) and OND patients (blue symbols). (**B**) sBCMA levels were measured by ELISA in plasma/serum pairs of 14 healthy controls.* Bars represent means. *(**C**) sBCMA in the CSF was determined by ELISA in patients with a clinically isolated syndrome (CIS) or multiple sclerosis (MS), neuroborreliosis (black symbols, NB), and other neurological diseases (OND) ($p<0.001$, Kruskal-Wallis test followed by Dunn's Multiple Comparison Test). (**D**) sBCMA levels in different subset of multiple sclerosis (measured in a) were determined. (**E**) sBCMA in the CSF correlated strongly with the intrathecal IgG production represented by the IgG-Index. This correlation was evident when all analyzed CSF samples were considered ($p <0.0001$, $r= 0.85$) and also within the MS/CIS group ($p< 0.0001$, $r= 0.77$, Spearman rank correlation). Modified from Laurent, Hoffmann et al. 2015.*

4.2.2 Effects of immunosuppressive treatments on sBCMA levels in patients with multiple sclerosis

We aimed to examine whether immunosuppressive treatments in MS such as steroids or natalizumab would lead to alterations of the levels of sBCMA.

Natalizumab, a blocking antibody to α4-integrin, which prevents the entry of lymphocytes into the CNS and the IgG production (Ransohoff and Engelhardt 2012), reduces relapses in MS patients and is given as a monthly infusion. To further investigate the effect of

natalizumab on sBCMA levels, we analyzed longitudinally the CSF of 25 patients before initiation of natalizumab treatment and 12 months after continuous treatment. This treatment reduced the level of sBCMA in 24/25 patients in the CSF after 12 months (mean=1.10 ng/ml before, and mean=0.686 ng/ml after natalizumab treatment; p<0.0001, Wilcoxon matched-pairs ranked-test), the strongest decrease of sBCMA was observed in those MS patients with a particularly high level of sBCMA **(Figure 9A)**.

We have analyzed longitudinally 10 MS patients, who received high-dose (1000 mg/d) methylprednisone (Urbason®) IV over 3 to 5 days, a common treatment of relapses in MS. Serum samples were obtained before the first steroid application, on the 3rd day before the 3rd infusion and 4 weeks later. We noted a significant decline of sBCMA 3 days after begin of methylprednisone treatment (mean= 24,55 ng/ml at baseline; mean=18.58 ng/ml at 3 days, $p < 0.05$, Friedman test), while four weeks later an increase of the sBCMA levels compared to the 3 days after treatment was observed (mean=21.70 ng/ml). **(Figure 9B)**

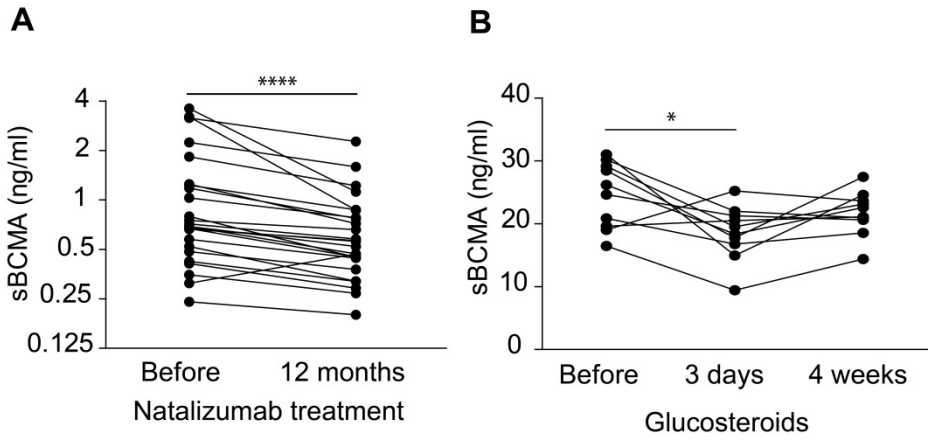

Figure 9. sBCMA levels under immunosuppressive treatments in blood and CSF. *(**A**) sBCMA in the CSF of 25 MS patients (other patients than those in Figure 1) were determined before and 12 months after natalizumab treatment. Natalizumab treatment reduced sBCMA levels in the CSF in 24/25 patients (p<0.0001, Wilcoxon matched-pairs ranked-test). (**B**) sBCMA levels in the serum of 10 MS patients were determined before, 3 days and 4 weeks after the beginning of high dose glucocorticoid treatment, which reduced sBCMA levels within 3 days (p<0.05, Friedman test). Modified from Laurent, Hoffmann et al. 2015.*

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4.2.3 sBCMA in patients with systemic lupus erythematosus

Since the BAFF/APRIL pathway plays an important role in the pathogeny of SLE, we went on analyzing the presence of sBCMA in SLE patients. We found that in these patients, serum levels of sBCMA and BAFF were elevated as seen in an untreated and in a treated cohort (**Figure 10A, 10B**). APRIL levels in healthy controls (mean= 62.7 ng/ml) were found elevated when compared to the treated SLE patients (mean= 43.2 ng/ml, p= 0.0033, Kruskal-Wallis test followed by Dunn's Multiple Comparison Test), and when compared to all SLE patients. Immunosuppressive treatment of SLE patients reduced sBCMA levels (**Figure 10A**). We noted a strong correlation of serum sBCMA and disease activity as measured by the SLE disease activity index (SLEDAI), which reflects the severity of systemic organ involvement (**Figure 10D**). We observed an inverse correlation with the paraclinical marker complement factor 3 (**Figure 10E**). There was a trend (p= 0.0767, r= 0.23, spearman correlation) for correlation between anti-dsDNA titer and sBCMA in these SLE patients. Serum BAFF levels were elevated in our SLE cohort (**Figure 10B**; mean= 0.61 ng/ml in healthy controls; mean= 2.11 ng/ml in SLE patients, p= 0.0004, Kruskal-Wallis test followed by Dunn's Multiple Comparison Test), and correlated with sBCMA levels (p= 0.0013, r= 0.47, spearman correlation).

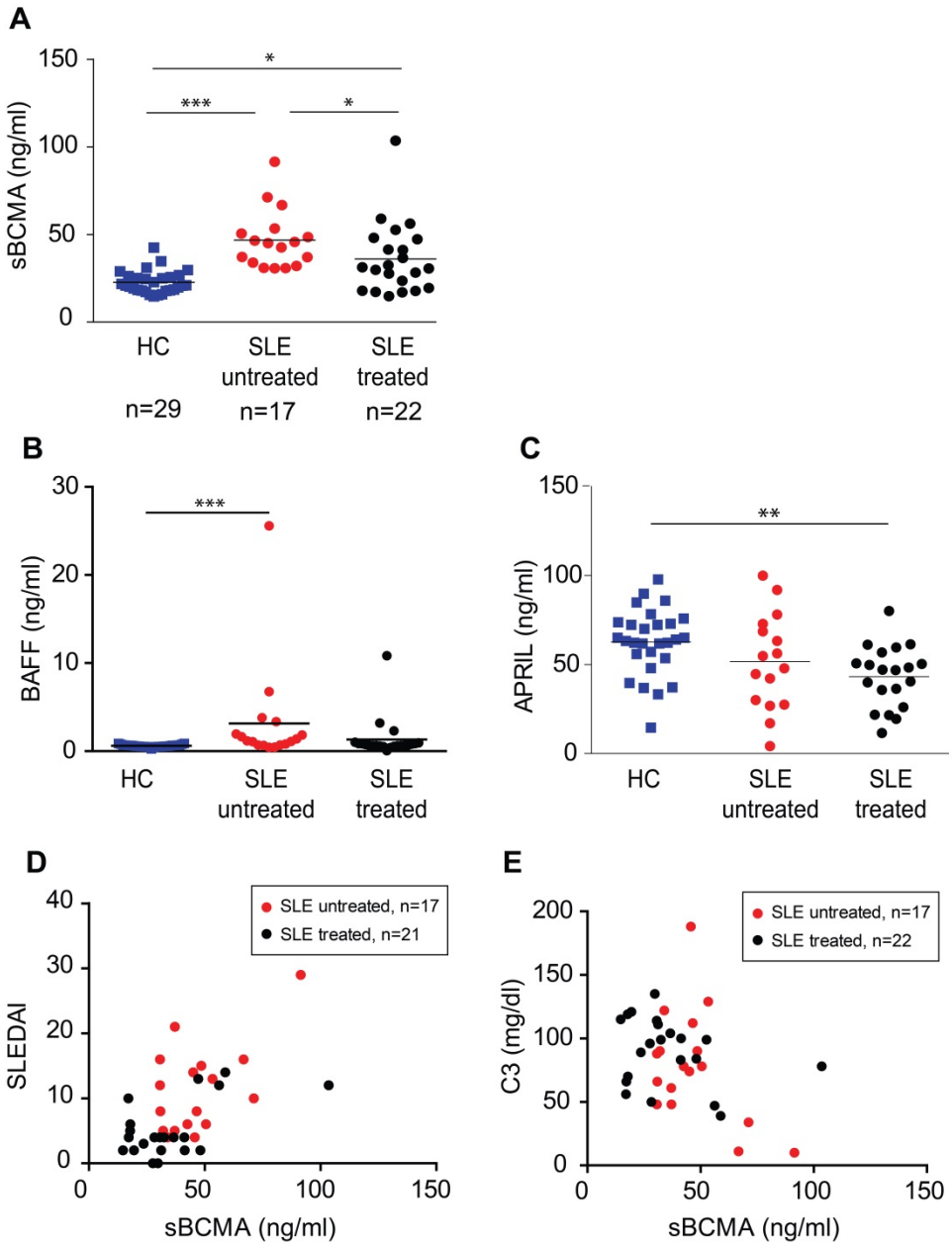


Figure 10. sBCMA is elevated in the serum of patients with systemic lupus erythematosus and correlates to paraclinical and clinical disease activity. *(**A**) sBCMA determined by ELISA is elevated in treated and untreated patients with SLE in comparison to healthy controls (HC) (***, p<0.001 and *, p<0.05, Kruskal-Wallis test followed by Dunn's Multiple Comparison Test). (**B**) BAFF levels in the serum was determined by ELISA and was found elevated in untreated SLE patients (***, p<0.001 Kruskal-Wallis test followed by Dunn's Multiple Comparison Test) (**C**) APRIL levels in the serum was determined by ELISA (**, p<0.001 Kruskal-Wallis test followed by Dunn's Multiple Comparison Test) and was found reduced in the serum of SLE patients (**D**) sBCMA in serum of SLE patients correlated strongly with disease activity quantified with SLE disease activity index (SLEDAI) (p<0.001; r= 0.54, Spearman correlation). (**E**) sBCMA in serum of SLE patients inversely correlated with the level of the complement factor C3 (p<0.0374, r= -0.29, Spearman correlation). Bars represent means. Modified from Laurent, Hoffmann et al. 2015.*

4.2.4 sBCMA is released by human B cells upon differentiation into Ig-secreting cells

Previous studies have determined that BCMA upregulation is a consequence of B cell differentiation into Ig-secreting cells (ISC) (Darce, Arendt et al. 2007). To assess whether this was also applicable for sBCMA production we applied two different protocols. On one hand, human B cell were isolated from blood of healthy donors and differentiated in a T-cell dependent manner, into ISCs trough stimulation over 5 days with CD40L and IL-21. Alternatively, total peripheral blood mononuclear cells were obtained from healthy donors and activated by stimulation with the TRL7/8 agonist resiquimod (R848) and IL-2 as represented below (**Figure 11**).

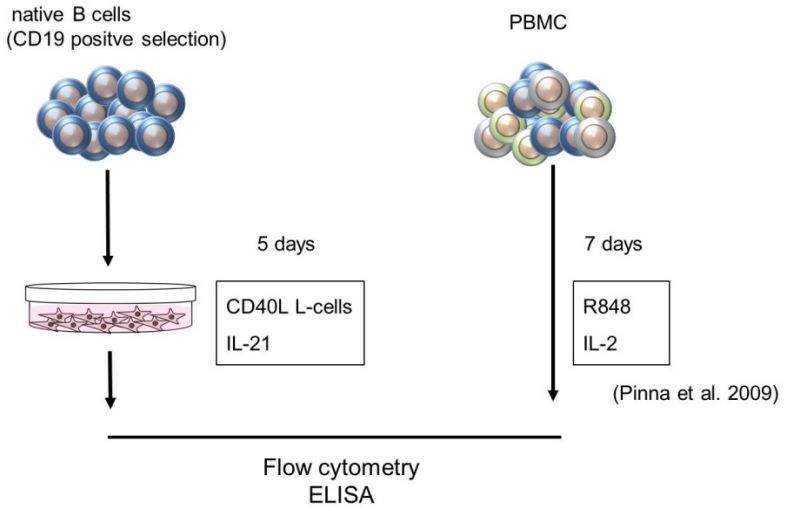


Figure 11. In vitro differentiation of native B cells into Ig-secreting cells with either CD40/IL-21 or Resiquimod (R848)/IL-2 stimulation. PBMC contain native B cells

While human B cells activated via CD40L alone released low, but detectable levels of sBCMA, this was strongly enhanced by addition of IL-21 (**Figure 12A**). After, stimulation of PBMCs with R848/IL-2 for 7 days (**Figure 12B**), release of sBCMA was observed as well. The appearance of sBCMA was concomitant to the occurrence of Ig-secreting cells. Upon activation with CD40L+IL-21, we could distinguish two cell B cell populations, $CD19^{+}CD38^{-}$ and $CD19^{+}CD38^{+}$ cells (**Figure 12C**). While mBCMA was absent on unstimulated B cells, we saw a low mBCMA expression on $CD19^{+}CD38^{-}$ cells and a strong mBCMA expression on $CD19^{+}CD38^{+}$ cells (**Figure 12D**). After sorting $CD38^{+}$ and $CD38^{-}$ cells and culturing them for another 24 h without further stimulation, we determined the amount of shed BCMA

(**Figure 12E**). This revealed a close correlation between released sBCMA and surface expression of mBCMA (**Figure 12F**).

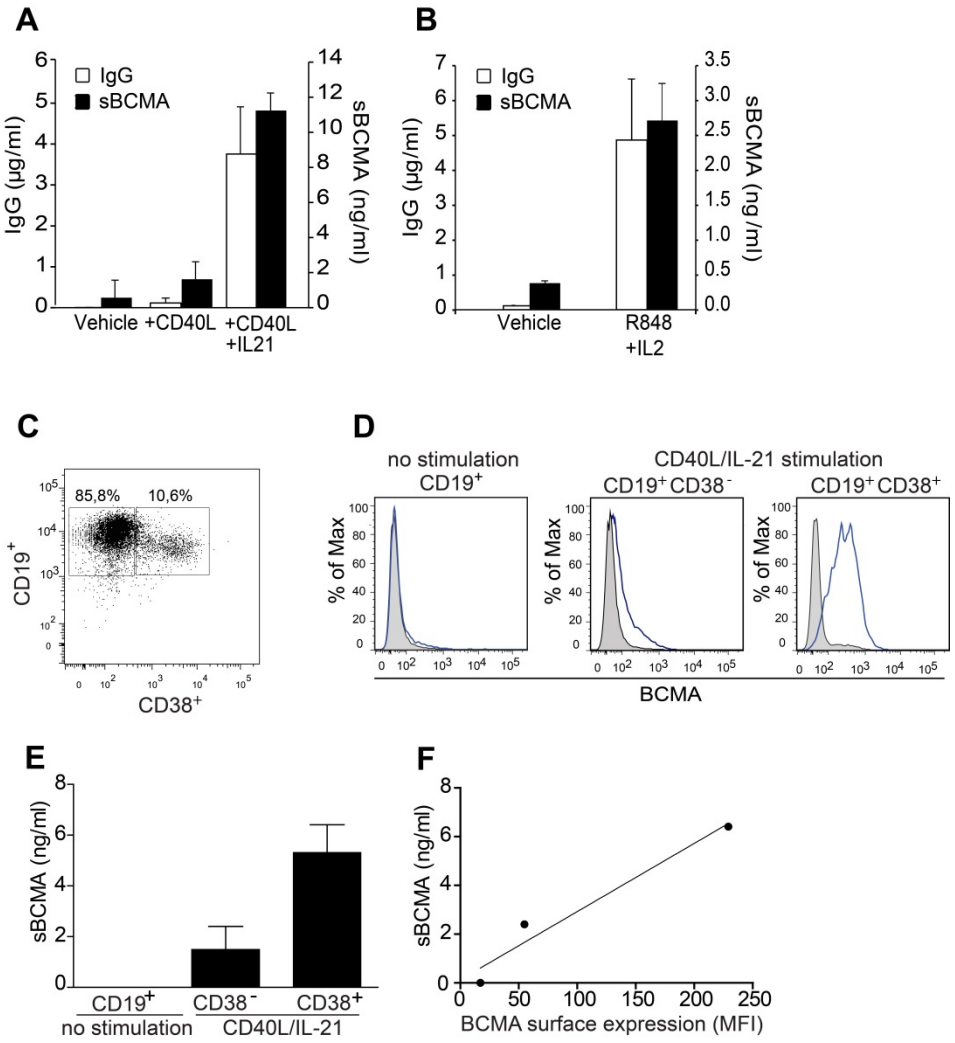


Figure* 12. *sBCMA is released when B cells differentiate towards plasma cells. *(**A**) Human purified B cells were activated for 5 days as indicated; IgG and sBCMA in the supernatant were quantified by ELISA. Combined data of 3 independent experiments (mean±SEM, p=0.0073, paired t-test). (**B**) PBMC were stimulated with R848+IL-2 for 7 days. IgG and sBCMA in the supernatant were quantified by ELISA. Combined data of 3 independent experiments (mean±SEM, p= 0.0227, paired t-test). (**C-E**) Human purified B cells were stimulated with CD40L + IL-21. (**C**) After CD40L/IL-21 stimulation 2 cell populations $CD19^+CD38^-$ and $CD19^+CD38^+$ could be distinguish. (**D**) Surface BCMA was measured by flow cytometry on unstimulated B cells, $CD19^+CD38^-$ cells and $CD19^+CD38^+$ cells. (**E**) Sorted $CD38^+$ and $CD38^-$ cells, were cultured for another 24 h and the amount of shed sBCMA was measured by ELISA, combined data of 2 independent experiments. (**F**) Correlation between sBCMA release and surface expression of BCMA for a single replicate. Modified from Laurent, Hoffmann et al. 2015.*

4.3 Characterization of sBCMA

4.3.1 Immunoprecipitation of sBCMA and Western blot analysis

We set out to investigate the biological biochemical features and mechanisms underlying the release of sBCMA. We found that BCMA and the plasma cell marker CD138 were uniformly expressed by the cell line JKL-6 (**Figure 13**). Moreover sBCMA was detected by ELISA in the supernatant.

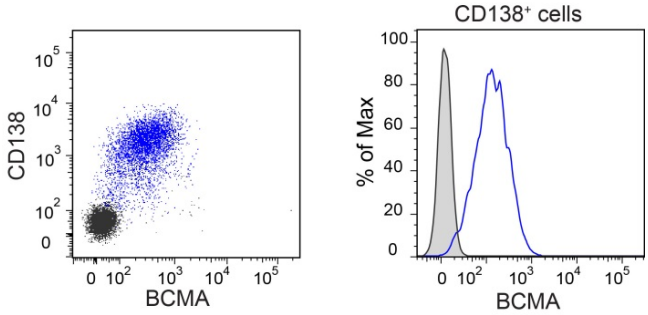


Figure 13. BCMA and CD138 expression in human plasmacytoma cells. *BCMA and CD138 expression was measured by flow cytometry in JKL-6 cells (closed histogram: isotype control; open histogram: BCMA staining).*

In order to gain further insights into the biochemical features of sBCMA we immunoprecipitated sBCMA from supernatant of the plasmacytoma cell line JKL-6 using either anti-hBCMA mAb A7D12.2 or C12A3.2. (**Figure 14A, B**).

Immunoprecipitation of sBCMA from serum and supernatant of native B cells cultured with CD40L and IL-21 was performed using respectively either a goat anti-hBCMA polyclonal Ab or the mAb A7D12.2. (**Figure 14A**). These antibodies were coupled to Dynabeads® Protein G and cross-linked with bis-sulfosuccinimidyl-suberate (BS^3). Before immunoprecipitation supernatants and serum samples were centrifuged twice at 500 g for 10 minutes and once at 20.000 g for 30 minutes to avoid sample contamination with cell debris. After successive incubation with either supernatant of plasmacytoma cells or serum, we eluted with glycine or SDS loading buffer, and then performed SDS gel separation. BCMA was then detected either by Western blot (**Figure 14A**) using the mAb C12A3.2 which is directed against the extracellular domain of BCMA or by silver staining (**Figure 14B**).

The molecular weight of sBCMA detected by immunoblot and silver staining was about 6 kDa. This roughly corresponded to the size of the extracellular domain BCMA (54 amino acids, calculated MW 5.8 kDa). The full-length receptor is constituted of 184 amino acids with an expected calculated MW of 20 kDa)

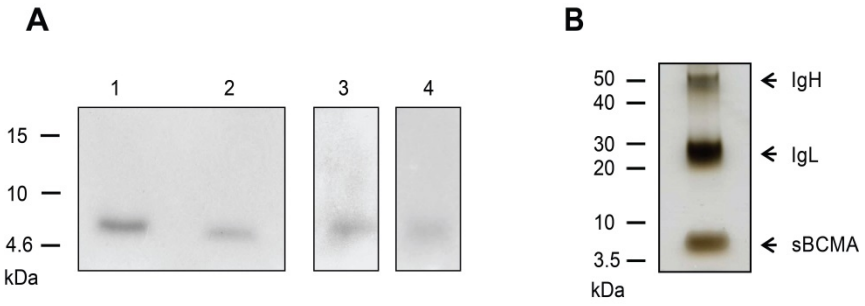
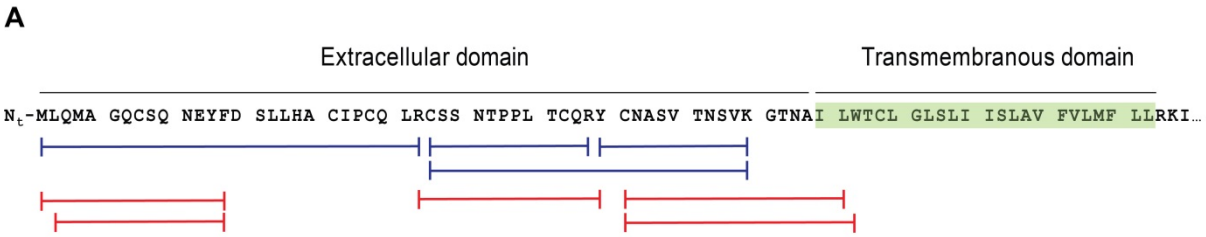


Figure 14. Immunoprecipitation of sBCMA reveals M.W. around 6kDa. *sBCMA was immunoprecipitated from supernatant of plasmacytoma cells (A, lanes 1, 2), serum (A, lane 3), and from supernatant of native B cells cultured with CD40L plus IL-21 (A, lane 4) with anti-BCMA mAb A7D12.2 (A, lanes 1 and 4), the mAb C12A3.2 (A, lane 2), or goat-anti-BCMA (A, lane 3). Western blot for BCMA (**A**) and silver staining of sBCMA immunoprecipitated from plasmacytoma supernatant (**B**) was performed. IgH: Immunoglobulin heavy chain; IgL: Immunoglobulin light chain. Modified from Laurent, Hoffmann et al. 2015.*

4.3.2 Mass spectrometry analysis of sBCMA

sBCMA was concentrated by immunoprecipitation and obtained by acidic elution. The eluate was then desalted and concentrated using C18 microcolumns. Then two approaches were followed. A) The material was digested in solution by trypsin or chymotrypsin and analyzed by mass spectrometry (LTQ Orbitrap XL). B) The i.p. material was separated via an SDS gel, silver stained (Chevchenko protocol) and the band corresponding to BCMA as detected by Western blot was excised and analyzed by mass spectrometry (LTQ Orbitrap XL).

Mass spectrometry revealed that sBCMA comprised not only the complete extracellular domain, but also part of the transmembrane region, indicating that it is released or shed by an intramembranous protease (**Figure 15A**). The peptides identified are listed in the table below (**Figure 15B**).



B

	Trypsin digestion (in solution)	Trypsin digestion (in gel)	Chymotrypsin digestion (in gel)
peptide sequence	MLQMAGQCSQNEYFDSL LHACIPCQLR	CSSNTPPLTCQR	MLQMAGQCSQNEY
		CSSNTPPLTCQRYCNA SVTNSVK	LQMAGQCSQNEY
	CSSNTPPLTCQR		CNASVTNSVKGTNAIL
	YCNASVTNSVK	YCNASVTNSVK	CNASVTNSVKGTNAILW
			RCSSNTPPLTCQRY

Figure 15. sBCMA comprises the extracellular domain and part of the transmembranous region of BCMA. (A) *The band at 6 kDa (from Fig. 2.1.b) and sBCMA obtained by immunoprecipitation were analyzed by mass spectrometry. Amino acid sequences of BCMA and peptides identified after tryptic (blue) or chymotryptic (red) digestion are shown. No peptide was detected with a C-terminal amino acid that was not a site for either tryptic or chymotryptic cleavage, indicating that the precise cleavage site of γ-secretase within the membrane needs to be identified.(**B**) Identified peptides of mass spectrometry analysis of sBCMA. Modified from Laurent, Hoffmann et al. 2015.*

4.4 Direct shedding of sBCMA by γ-secretase

4.4.1 γ-secretase inhibitor DAPT inhibits sBCMA generation and increases BCMA cell surface expression

BCMA receptor is a single pass type-III oriented transmembrane protein, thus featuring an extracellular N-terminus while lacking a signal peptide. Since sBCMA contains part of the transmembrane domain, the intramembranous γ-secretase appeared to be a prime protease candidate. We therefore tested the γ-secretase inhibitor DAPT (N-[N-(3,5-Difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester) on sBCMA generation and BCMA surface expression on our plasmacytoma cell line and on activated human B cells. In addition, we analyzed in parallel the effects of the metalloprotease inhibitor TAPI-1.

We observed that overnight DAPT treatment almost completely abrogated even at low concentrations the release of sBCMA in a plasmacytoma cell line in a dose-dependent manner (**Figure 16B**), while simultaneously increasing surface expression of BCMA (**Figure 16A).** In contrast, TAPI-1 had little or no effect on sBCMA release and surface expression (**Figure 16C, D**). Toxicity-induced, unspecific reduction of sBCMA shedding and BCMA surface expression was observed at a vehicle concentration of 0.5 % DMSO, which corresponds to 50 µM TAPI-1.

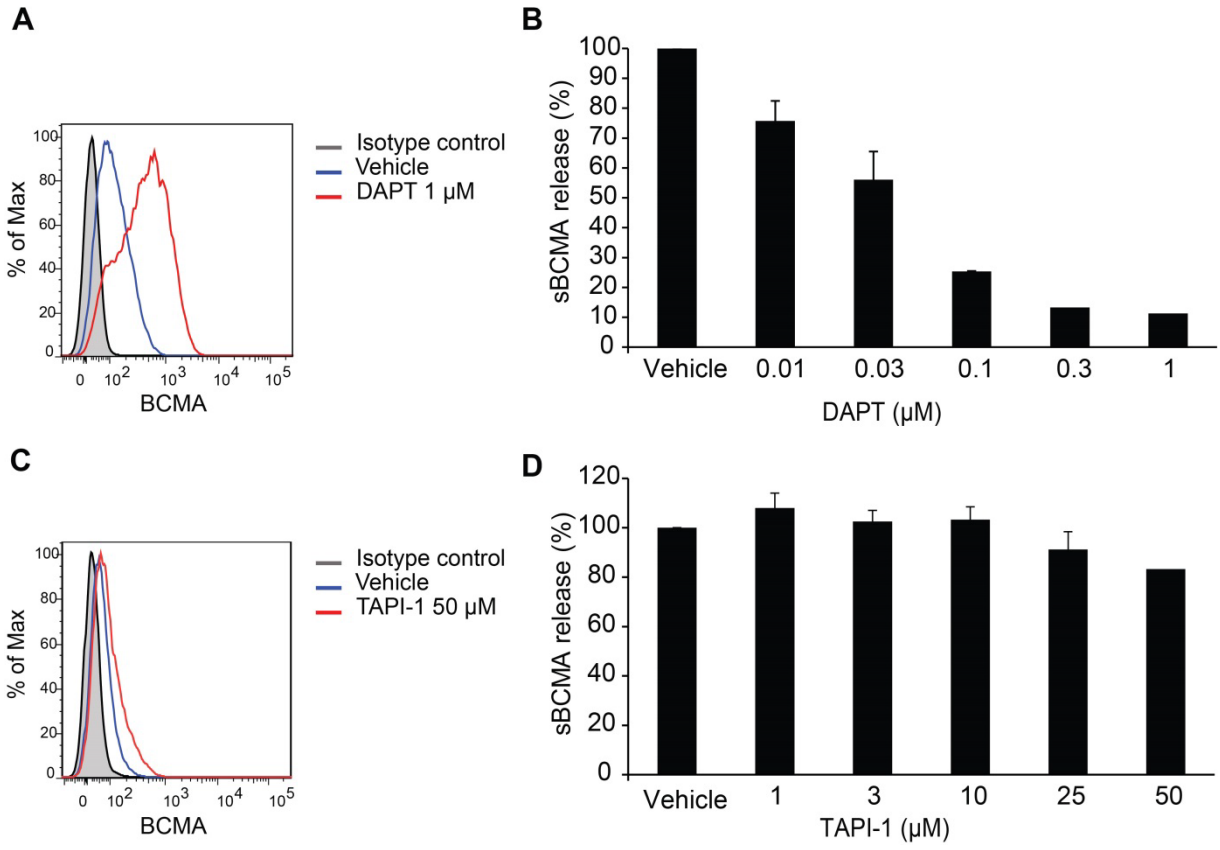


Figure 16. γ-secretase inhibitor DAPT blocks BCMA shedding in plasmacytoma cells. *The human plasmacytoma cell line JK-6L was incubated overnight with DAPT or TAPI-1. Surface expression of BCMA was measured by flow cytometry (**A, C**) and sBCMA in the supernatant was determined by ELISA (**B, D**). Error bars indicate the SEM of technical replicates, representative of 3 experiments. Modified from Laurent, Hoffmann et al. 2015.*

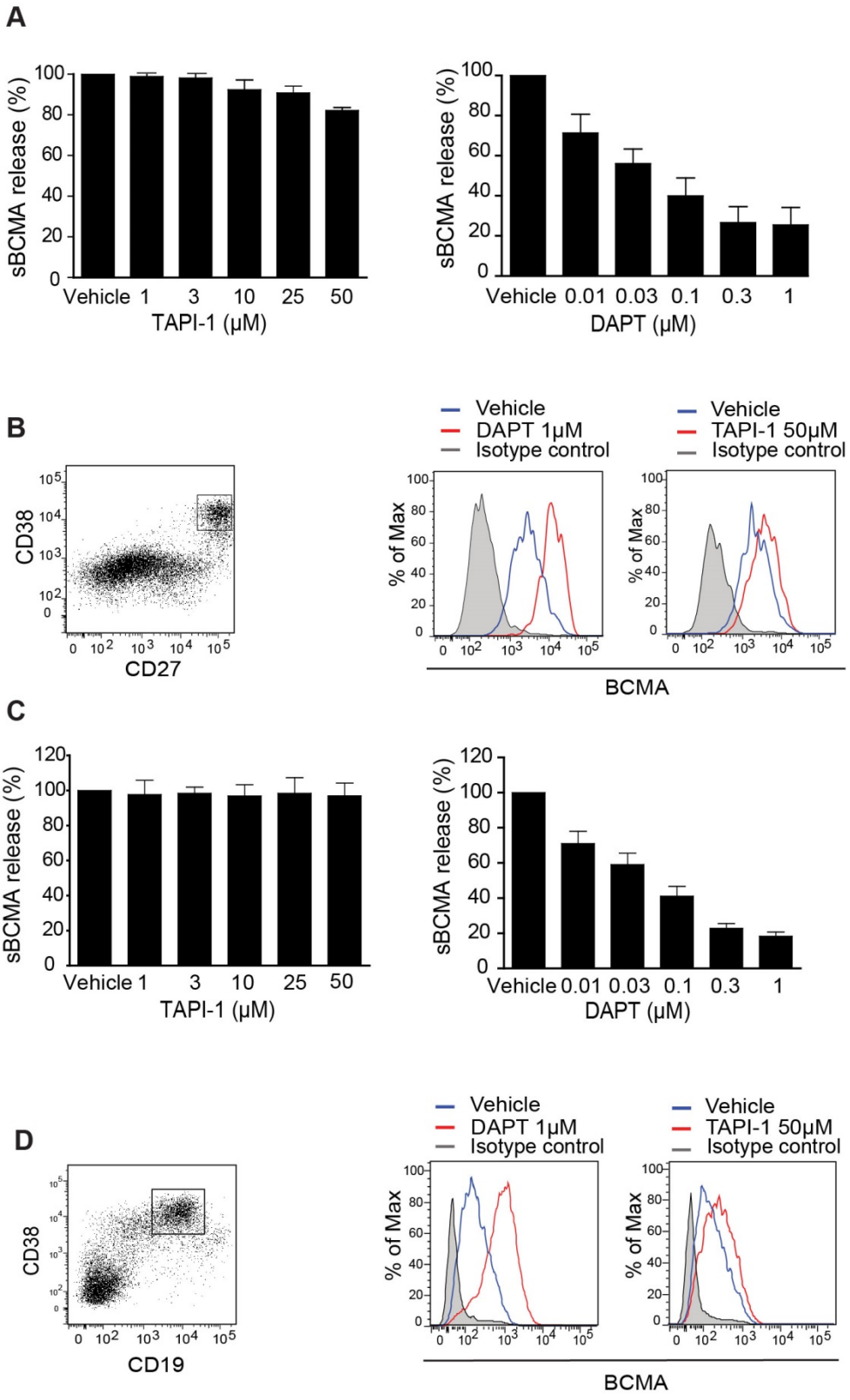

A
sBCMA release (%)
Vehicle 1 3 10 25 50
TAPI-1 (μM)
Vehicle 0.01 0.03 0.1 0.3 1
DAPT (μM)
B
CD38
CD27
Vehicle
DAPT 1μM
Isotype control
TAPI-1 50μM
% of Max
BCMA
C
D
CD19

Figure 17

Figure 17. γ-secretase inhibitor DAPT reduces release of sBCMA and enhances surface expression of BCMA on activated human B cell. *(**A, B**) Human B cells were differentiated to Ig-secreting cells via CD40L+IL-21. (**A**) Release of sBCMA upon treatment with DAPT or TAPI-1 was measured by ELISA. sBCMA release was normalized to the amount of sBCMA shed under vehicle conditions, which was set as 100%. Combined data of 3 independent experiments (mean±SEM). (**B**) These activated primary human B cells were sub grouped based on expression of CD27 and CD38. A high surface expression of BCMA was seen on the $CD27^{++}CD38^{+}$ subset. Surface expression of BCMA was enhanced by DAPT treatment, while TAPI-I had little effect. (**C, D**) Human PBMC were stimulated with R848+IL-2 for 7 days. (**C**) Release of sBCMA upon treatment with DAPT or TAPI-1 was measured by ELISA. sBCMA release was normalized to the amount of sBCMA shed under vehicle conditions, which was set as 100%. Combined data of 3 independent experiments (mean±SEM). (**D**) High surface expression of BCMA was seen on the $CD19^{+}CD38^{+}$ subset; this was further enhanced by DAPT, while TAPI-I had little effect (Laurent, Hoffmann et al. 2015).*

To determine the effect of γ-secretase on human primary B cells, PBMCs were obtained from healthy donors. Native B cells were isolated and differentiated into ISCs with CD40L and IL-21 as described earlier. In a second approach total PBMCs were also activated by stimulation with the TRL7/8 agonist resiquimod (R848) and IL2, which resulted in the apparition of IgG secreting cells. Cells were then washed and stimulated overnight with DAPT and TAPI-1. Similar as in the plasmacytoma cell line, we found a dose-dependent decrease of the sBCMA release **(Figure 17A)** and an increased BCMA surface expression **(Figure 17B),** upon DAPT treatment, while only minimal effect was seen upon TAPI-1 treatment (**Figure 17 C-D)**. The DAPT effect was already observed at the low working concentration of 0.1 µM.

Moreover, we compared the effect of transition (LY-411575-I, LY685,458) and non-transition state (DAPT, RO4929097) inhibitors of the γ-secretase on BCMA shedding from human B cells. Human PBMCs were first stimulated with R848+IL2 for 7 days, then $CD19^{+}$ B cells were positively selected and cultured overnight in the presence of these γ-secretase inhibitors. We found that RO4929097, LY-411575-I, and LY685,458 had similar effects as DAPT on the shedding of mBCMA as seen in FACS and ELISA (**Figure 18A, B**).

Further, to formally verify that the stereoisomer SSR of LY-411575, referred as LY-411575-I, was also a γ-secretase inhibitor, we transfected HEK293T cells with human BCMA or with the established γ-secretase substrate C99 and treated them overnight with either DAPT (1 µM), with LY-411575-I (100 nM) or with the appropriate vehicle. As expected LY-411575-I inhibited both C99 and BCMA shedding by the γ-secretase (**Figure 18C, D).**

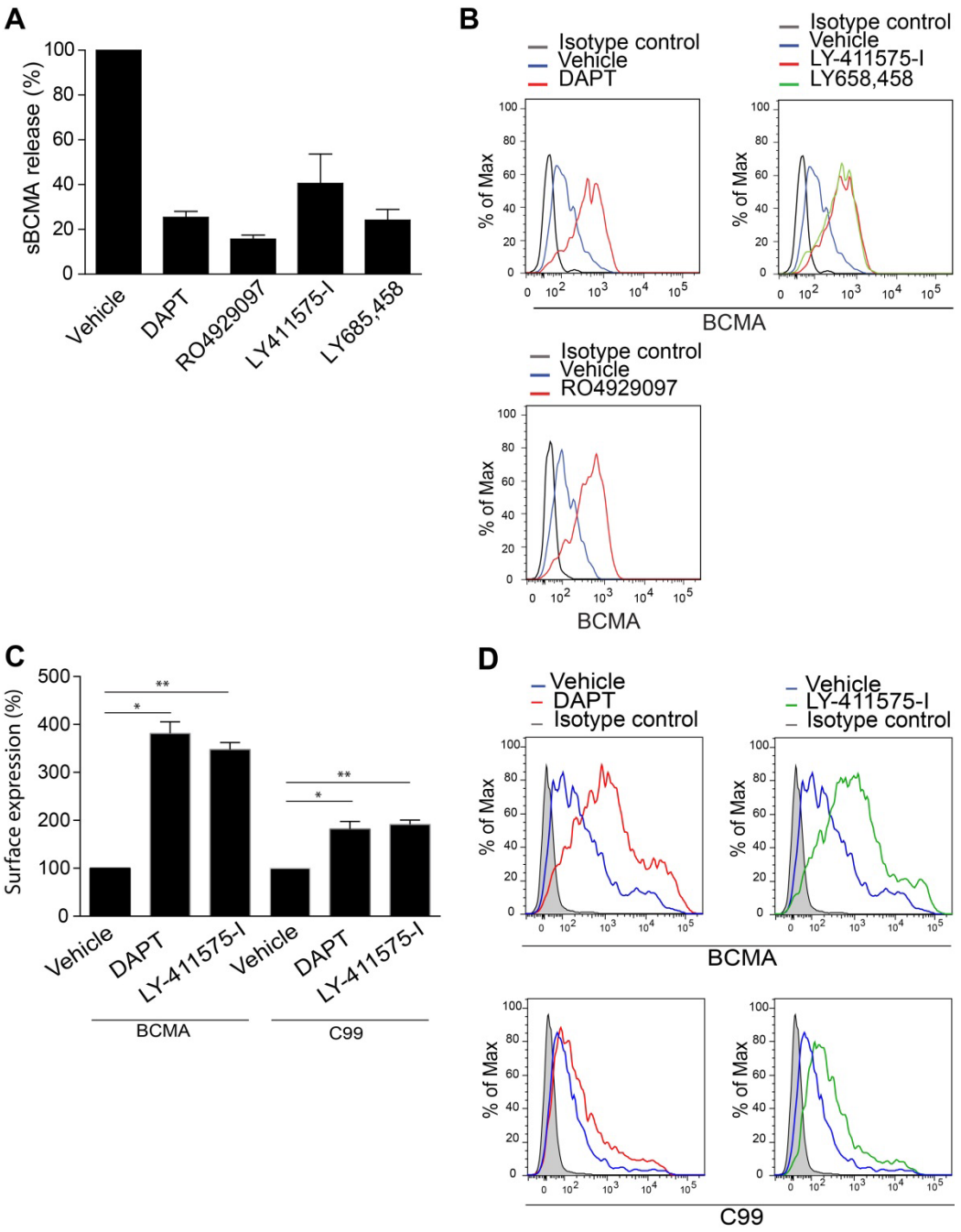


Figure 18. Different types of γ-secretase inhibitors block the release of sBCMA. *Human PBMCs were stimulated with R848 and IL-2 for 7 days and then CD19$^+$ B cells were positively selected. (**A**) After an overnight culture with the indicated γ-secretase inhibitors the released sBCMA in the supernatant was measured by ELISA, (mean±s.e.m. of three experiments) (**B**) After an overnight culture with the indicated γ-secretase inhibitors, BCMA surface expression was determined after gating on CD19$^+$CD38$^+$ Ig-secreting cells, which represented in this experiment about 50% of the CD19$^+$ cell population.. The concentrations of the γ-secretase inhibitors were DAPT 1 µM, RO4929097 10 µM, LY-411575-I 100 nM and LY685,458 1 µM. Representative of 3 independent experiments, mean±s.e.m.. (**C-D**) HEK293T cells were transfected with human BCMA or with the established γ-secretase substrate C99 and treated overnight with either DAPT (1 µM), with LY-411575-I (100 nM) or*

*with the appropriate vehicle. Surface expression of BCMA or C99 was measured in by flow cytometry. (**A**) Surface expression of human BCMA or C99 upon DAPT and LY-411575-I treatment. Mean±s.e.m. of 6 replicates, *, p <0,05; **, p<0,01, unpaired t-test. (**B**) Representative histograms of BCMA and C99 surface expression upon DAPT (1 µM) and LY-411575-I (100 nM) treatment. Modified from Laurent, Hoffmann et al. 2015.*

4.4.2 sBCMA cleavage is mediated by Presenilin-1

To formally prove that sBCMA cleavage depends on the γ-secretase complex, we applied mouse embryonic fibroblast (MEF) cells derived from PS1/2 (PS-/-) double knockout mice. These PS1/2 deficient cells were first stably transduced with full length human BCMA and then stably transduced with either wt PS1 or a catalytically inactive D385A mutant PS1. Expression of wt PS1 strongly reduced surface expression of BCMA and resulted in about 15 fold higher level of sBCMA in the supernatant, while the enzymatically inactive D385 had no effect (**Figure 19A-D**). Expression and endoproteolysis of PS1 in these transfectants was formally shown by immunoblotting of cell lysates with antibody PSEN1 (and maturation of nicastrin (NCT) by immunoblotting with antibody N1660 (**Figure 19C**).

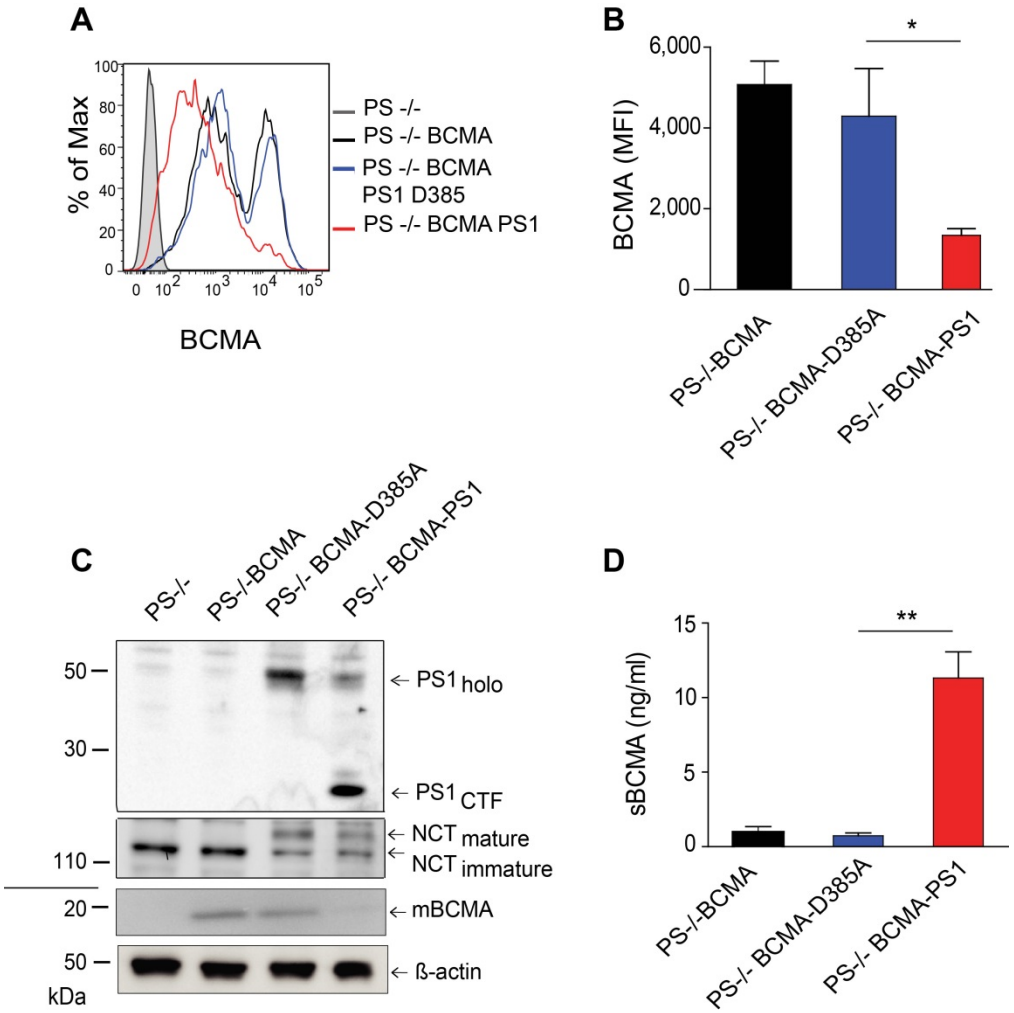


Figure 19. Release of sBCMA requires active presenilin. *(**A-D**) Presenilin-deficient MEF cells (PS-/-) were transduced with BCMA (PS-/- BCMA) and then with wild-type PS1 (PS-/- BCMA PS1) or with a catalytically inactive mutant (PS-/- BCMA PS1-D385A). BCMA surface expression (**A,B**) and sBCMA release (**D**) was determined. In (**A**) a representative experiment is shown, in **B,D** mean±SEM of four independent experiments is given (respectively p=0.0313 and p=0.0033, *, p <0,05; **, p<0,01 paired t-test). (**C**) Cells used in a, b, d were analyzed by immunoblotting for expression of full-length PS1 ($PS1_{holo}$), for autoendoproteolysis of PS1 generating a C-terminal fragment ($PS1_{CTF}$) reflecting an active state of the γ-secretase, for maturation of nicastrin (NCT) and for full-length BCMA (BCMA).* (Laurent, Hoffmann et al. 2015)

4.4.3 Shedding of BCMA by γ-secretase occurs without prior N-terminal trimming and is length-dependent

So far γ-secretase has been known to cleave the remaining c-terminal fragment of a membrane protein after shedding of its extracellular domain, but not to directly shed any membrane protein. Therefore we used an additional experimental approach to confirm that the γ-secretase directly cleaved BCMA without prior N-terminal trimming by another protease.
A human BCMA clone with an N-terminal FLAG-tag was constructed and transiently transfected into HeLa cells. sBCMA was detected in the supernatant of cells transfected with either BCMA-FLAG or BCMA alone to a similar extent as seen by ELISA using anti-BCMA for coating and anti-BCMA for detection (**Figure 20**). When we used anti-FLAG for coating and anti-BCMA for detection, we found sBCMA-FLAG in the supernatant of the BCMA-FLAG transfectants (**Figure 20B**). The concentration of sBCMA-FLAG decreased when increasing levels of DAPT were added to the cells. The extent of DAPT mediated down-regulation of sBCMA and sBCMA-FLAG was comparable (**Figure 20A,B**). These results strongly suggest that γ-secretase directly cleaves BCMA-FLAG without prior N-terminal trimming by another protease.

BCMA has an unusual short extracellular domain (54 aa). To test whether BCMA shedding might be length-dependent, we doubled its extracellular part artificially obtaining a variant called BCMA-BCMA with an extracellular part of 108aa. We transfected wildtype BCMA and the BCMA-BCMA construct into HEK cells. While the γ-secretase inhibitor DAPT strongly enhanced the surface expression of mBCMA and blocked the release of sBCMA after transfection of wild-type BCMA, DAPT had little or no effect on surface expression or release of hBCMA-BCMA (**Fig. 20 C, D and E**). Thus, the short extracellular domain of mBCMA facilitates its direct cleavage by γ-secretase

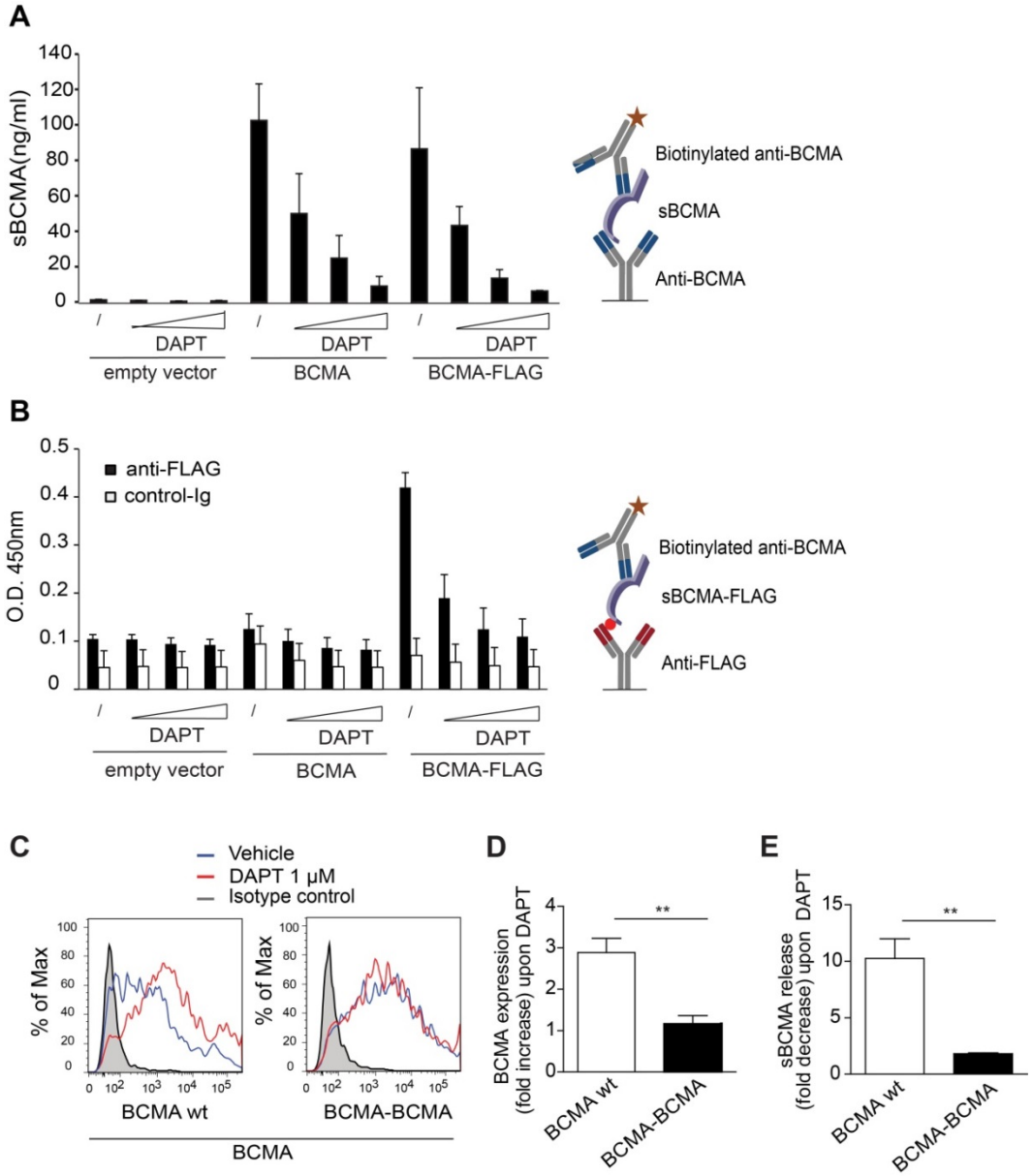


Figure 20. Release of sBCMA occurs without prior N-terminal trimming and is length-dependent. *(**A-B**). HeLa cells were transfected with plasmids coding for full-length BCMA or BCMA with an N-terminal-FLAG and then cultured with increasing amounts of the γ-secretase inhibitor DAPT (0.02 µM, 0.1 µM, 0.5 µM). 24h after transfection supernatants were harvested and the released sBCMA was analyzed by ELISA. In (**A**) ELISA wells were coated with anti-BCMA and in (**B**) with anti-FLAG or a control IgG (anti-MOG 8.18 C5), both were developed with anti-BCMA. Schemes of the ELISAs are shown on the right. Combined data of 2 independent experiments (mean±SEM). (**C-E**) Human BCMA wild type (wt) or BCMA-BCMA with a doubled extracellular domain of BCMA were transfected into HEK293T cells. (**C,D**) Surface expression of BCMA was determined in the absence or presence of the γ-secretase inhibitor DAPT (1 µM). (**D**) shows the combined data of 3 experiments (p=0.0049,unpaired t-test. (**E**) shows the effect of DAPT on the released sBCMA after transfection with BCMA wt or BCMA-BCMA (mean±SEM. of 3 experiments), p=0.0081,unpaired t-test. Modified from Laurent, Hoffmann et al. 2015.*

4.5 Functional consequences of sBCMA

4.5.1 Inhibition of sBCMA shedding increases APRIL-binding to cell surface and increases APRIL-mediated induction of HLA-DR and NF-κB activation *in vitro*

In order to assess the consequences of sBCMA shedding to the binding of APRIL to the cell bound BCMA we added APRIL-FLAG to plasmacytoma cells, which had been preincubated with or without DAPT overnight. Cell bound APRIL-FLAG was then detected by flow cytometry using an anti-FLAG antibody. We observed that the enhanced BCMA expression after DAPT was accompanied with an increased binding of its ligand APRIL-FLAG **(Figure 21A).**

To analyse whether DAPT-mediated increased BCMA expression was accompanied by enhanced cellular responsiveness to APRIL we used two read-out systems, induction of HLA-DR on human B cells or NF-κB activation in BCMA-transfected HEK cells, and obtained similar results. In BCMA transfected HEK293T cells, DAPT increased NF-κB activation induced via APRIL and also via BAFF (**Figure 21B**). As APRIL stimulation via BCMA has been described to lead to higher MCH-II expression (Yang, Hase et al. 2005) in mice, we looked at HLA-DR expression upon APRIL stimulation in R848/IL2 primary activated human B. We noted that stimulation of these primary activated human B cells with APRIL led to higher surface expression of HLA-DR, which was further enhanced by the γ-secretase inhibitor DAPT, in particular at suboptimal concentrations of APRIL. This effect was slight, but significant, as seen in 6-8 replicates with different concentrations (**Figure 21C**).

Figure 21. Inhibition of sBCMA shedding increases APRIL-binding to cell surface and limits APRIL-mediated induction of HLA-DR and NF-κB activation in vitro. *(**A**) BCMA ligand APRIL-FLAG was added to plasmacytoma cells after pretreatment with DAPT and its binding was analyzed with anti-FLAG mAb. (**B**) HEK293T cells were transfected with full-length BCMA or an empty vector and a luciferase-based NF-κB-reporter. DAPT, APRIL and BAFF were added as indicated and NF-κB activation was determined. (**C**) PBMCs were activated with R848+IL-2 for 7 days. The expression of HLA-DR on $CD19^+$ cells was elevated by APRIL and this effect was further enhanced by DAPT. The respective isotype control stainings were not modified by the different stimuli (data not shown). A representative example is shown on the left. The effects might appear small, but the significance was elaborated in 6-8 independent replicates from 5 different donors and compiled data are shown on the right. (**, p<0.01; ****, p<0.0001 and ***, p<0.001, paired t-test). Modified from Laurent, Hoffmann et al. 2015.*

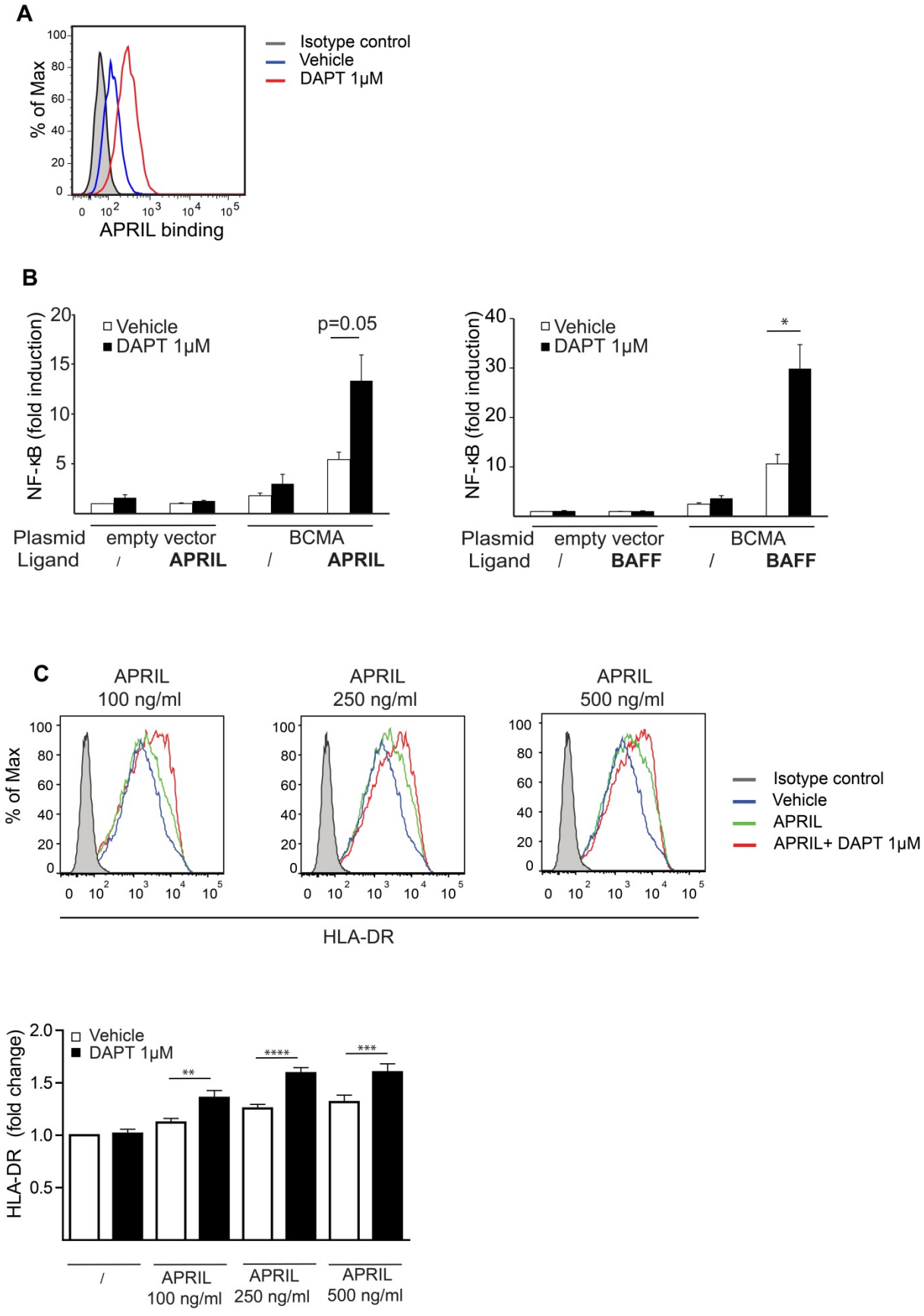

A
% of Max
APRIL binding
Isotype control
Vehicle
DAPT 1µM
B
NF-κB (fold induction)
Vehicle
DAPT 1µM
p=0.05
*
Plasmid
Ligand
empty vector / APRIL
BCMA / APRIL
empty vector / BAFF
BCMA / BAFF
C
APRIL 100 ng/ml
APRIL 250 ng/ml
APRIL 500 ng/ml
% of Max
HLA-DR
Isotype control
Vehicle
APRIL
APRIL+ DAPT 1µM
HLA-DR (fold change)
**
****
***
/
APRIL 100 ng/ml
APRIL 250 ng/ml
APRIL 500 ng/ml

Figure 21

4.5.2 sBCMA acts as a decoy receptor for APRIL and limits APRIL mediated B cell survival *in vitro*.

To further investigate the biological implications of sBCMA we set out to determine whether the shed receptor remained able to bind APRIL or BAFF thus functioning as a decoy receptor.

We established an ELISA, in which the anti-FLAG M2 mAb was used for coating in order to immobilize APRIL-FLAG or BAFF-FLAG on the ELISA plate. Then sBCMA from supernatant of HEK cells transfected with BCMA was added and incubated for 2 hours at room temperature. BCMA was detected as described above. We found that sBCMA bound APRIL, but not BAFF (**Figure 22**). This was different to membrane BCMA, which binds both APRIL and BAFF (Mackay and Schneider 2009). We found that the naturally occurring sBCMA also differed from recombinant BCMA-Fc, which binds both APRIL and BAFF (**Figure 22**).

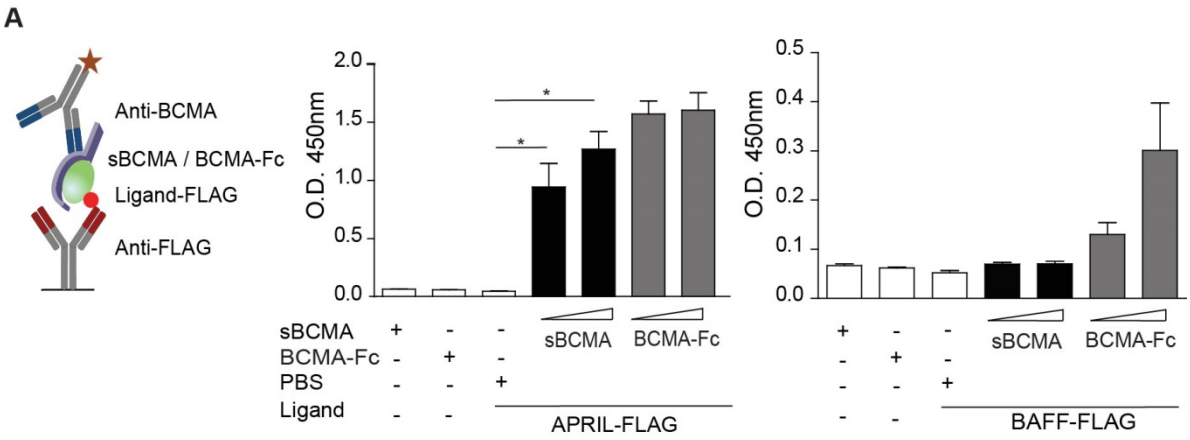


Figure 22. sBCMA binds APRIL. *A scheme of the ELISA is shown on the left; it detects BCMA-APRIL-FLAG (left panel) or BCMA-BAFF-FLAG (right panel) complexes, but neither BCMA nor APRIL-FLAG nor BAFF-FLAG alone (*, $p<0.05$, paired t-test). sBCMA was derived from the supernatant of plasmacytoma cultured under serum-free conditions. Combined data of 3 independent experiments (mean±SEM). Modified from Laurent, Hoffmann et al. 2015.*

These differences between sBCMA and BCMA-Fc were also seen in NF-κB reporter assays: in HEK cells transfected with BCMA, APRIL-mediated NF-κB activation was blocked by both sBCMA and BCMA-Fc, while BAFF-mediated activation via membrane bound BCMA was suppressed only by BCMA-Fc, not by sBCMA (**Figure 23A**). Since APRIL can signal

via membrane bound BCMA and TACI we transfected HEK cells also with TACI. These experiments showed that sBCMA inhibited APRIL, but not BAFF-mediated signaling via TACI (**Figure 23B**). sBCMA thus acts as APRIL-specific decoy.

We aimed to analyse whether sBCMA could limit APRIL-mediated survival of activated B cells. To measure APRIL-induced survival, mouse B cells, magnetically isolated from spleen, were cultured in 96-well microtiter plates, which were precoated with anti-IgM. APRIL-FLAG was added for 48h and cross-linked with anti-FLAG mAb antibody. Supernatants generated by HEK293T cells that had been transfected with either full-length BCMA or an empty control vector and therefore either contained sBCMA or did not, were added at a final sBCMA concentration of 200 ng/ml and 400 ng/ml. Cell survival was quantified by flow cytometry and APRIL-induced survival was calculated as followed: 100 x [(cell survival in presence of APRIL – cell survival without APRIL) / cell survival without APRIL]. We observed a dose-dependent inhibition of APRIL-induced survival by sBCMA. (**Figure 23C**).

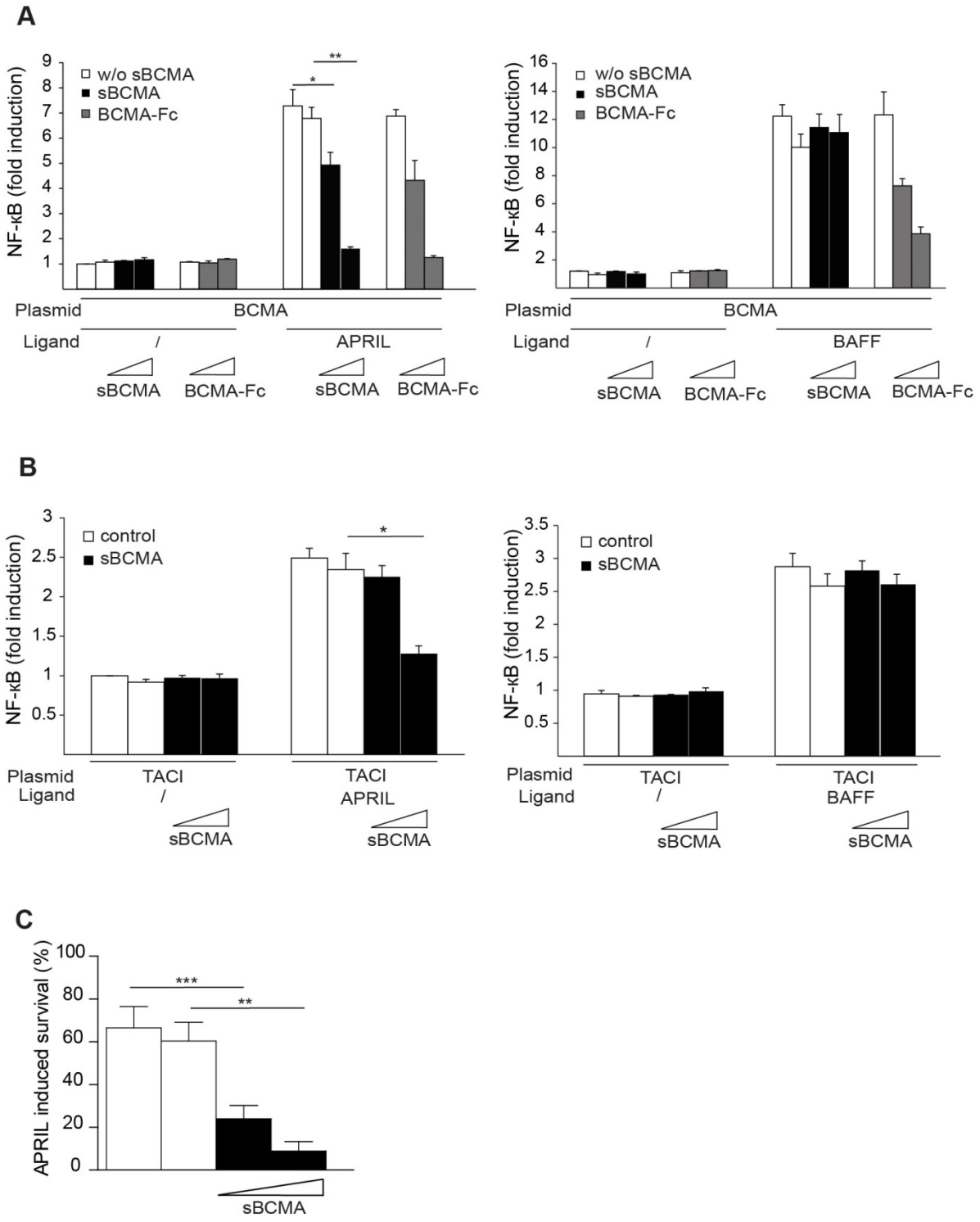


Figure 23. sBCMA is an APRIL-specific decoy. *(**A**) HEK293T cells were transfected with BCMA and activated with APRIL (left panel) or BAFF (right panel). sBCMA (50 and 200 ng/ml) was applied as indicated. sBCMA and control supernatant were obtained as mentioned above. BCMA-Fc (50 and 200 ng/ml) was used as a positive control. Combined data of 3 independent experiments (mean±SEM, *, p<0.05; **, p<0.01 paired test). (**B**) HEK293T cells were transfected with TACI and activated with APRIL or BAFF as indicated. sBCMA (50 and 200 ng/ml) was applied as indicated. sBCMA and control supernatant were obtained from HEK293T cells that had been transfected with full-length BCMA or an empty*

*control vector. BCMA-Fc (50 and 200 ng/ml) was used as a positive control. Combined data of 3 independent experiments (mean±SEM, *, p<0.05, paired t-test). (**C**) Murine B cells were activated via anti-IgM and cultured for 2 days with APRIL in the presence or absence of sBCMA (200 and 400 ng/ml). APRIL-induced survival was calculated as described in material and methods. sBCMA was obtained from supernatant from HEK293T cells transfected with full-length BCMA (black bars). Control supernatant was obtained after transfection with an empty vector (white bars). sBCMA significantly inhibited APRIL mediated survival (***, p<0.001 and **, p <0.01, paired t-test). Combined data of 6 independent experiments (mean± SEM). Modified from Laurent, Hoffmann et al. 2015.*

4.6 γ-secretase regulates BCMA shedding *in vivo*

4.6.1 γ-secretase inhibitors enhance BCMA expression of mouse cells *in vitro*.

To address the question whether sBCMA cleavage occurs in mice B cells, murine plasmacytoma cells J558L were treated with γ-secretase inhibitors DAPT, LY-411575-I and the respective vehicle controls overnight. Treatment with both inhibitors resulted in a similar strong increase in BCMA cell surface expression (**Figure 24)**.

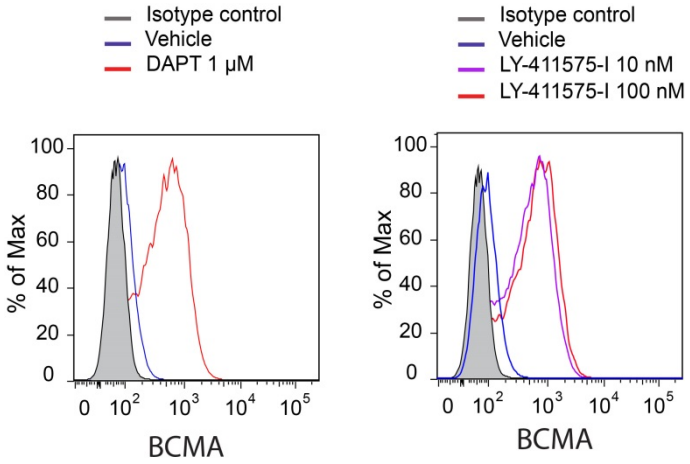


Figure 24. Increased BCMA expression in a murine plasmacytoma cell line upon treatment with γ-secretase inhibitors DAPT and LY-411575-I. *J588L plasmacytoma cells were treated over night with either DAPT (left panel) or the γ-secretase inhibitor LY-411575-I (right panel). BCMA surface expression was measured by flow cytometry.*

4.6.2 γ-secretase regulates BCMA and plasma cells *in vivo*

To address the question whether cleavage of mBCMA by γ-secretase occurred also *in vivo*, we used two different mouse models. First, we immunized mice with ovalbumin (OVA) and LPS to induce a T-dependent immune response and plasma cell differentiation (**Figure 25**). Treatment 9 days later with the γ-secretase inhibitor LY-411575-I for one day resulted in an enhanced surface expression of mBCMA in $CD138^{+}$ plasma cells in spleen and bone marrow (**Figure 26**). In the absence of a γ-secretase inhibitor, mBCMA was barely detectable on the surface of murine plasma cells, in line with previous observations (Peperzak, Vikstrom et al. 2013).

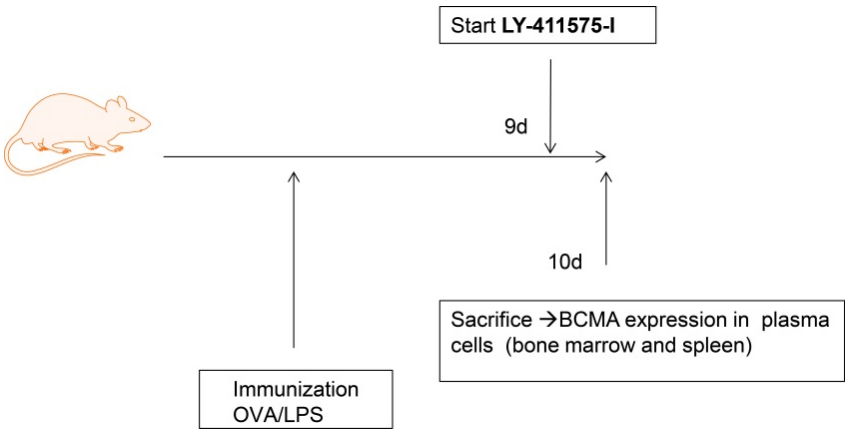


Figure 25. Immunization and treatment protocol. *Two months old C57/BL6 mice were immunized intraperitoneally with 100 µg ovalbumin and 10 µg LPS in alum and sacrificed 10 days later. Immunized mice received an intraperitoneal dose (10 mg/kg) of the γ-secretase inhibitor LY-411575-I on day 9, followed by another dose 6 hours before sacrifice.*

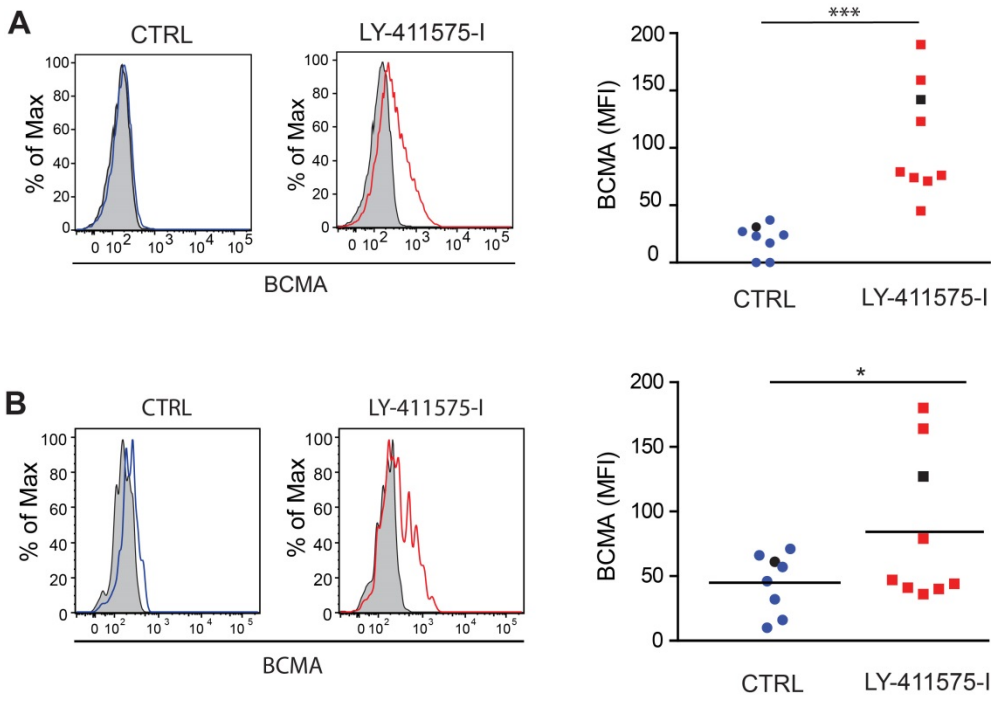


Figure 26. γ-secretase regulates surface display of BCMA in vivo. *Immunized mice were treated with the γ-secretase inhibitor LY-411575-I and the surface display of BCMA in splenocytes (A) or bone marrow (B) was measured by flow cytometry. BCMA expression on gated B220+CD138+ cells is shown, a representative example (left) and compiled data from all 17 analyzed animals (mean, ***, p<0.001, unpaired t-test) (right). The black symbols on the right indicate the samples shown on the left. Closed histograms indicate isotype controls. Modified from Laurent, Hoffmann et al. 2015.*

As a second model, we used NZB/W mice, a widely used murine SLE model, in which germinal centers can develop spontaneously (Luzina, Atamas et al. 2001). We noticed that also in this model, one day of treatment with the γ-secretase-inhibitor LY-411575-I was sufficient to induce an increased surface expression of BCMA on plasma cells in the spleen and bone marrow (**Figure 27A**). In order to distinguish between short-lived and long-lived plasma cells, mice were fed bromodeoxyuridine (BrdU). We found that inhibition of γ-secretase for one day enhanced mBCMA both on $BrdU^+$ plasma cells, or short-lived plasmablasts, and on $BrdU^-$ long-lived plasma cells in bone marrow and spleen (**Figure 27A)**. To analyse the effect on long-term treatment with γ-secretase inhibitors on the mBCMA and plasma cells survival, we treated NZB/W mice with LY-411575-I for 7 days. As expected, we observed an enhanced expression of mBCMA on $BrdU^+$ and $BrdU^-$ plasma cells in bone marrow and spleen (**Figure 27B**). Moreover, this prolonged treatment period enhanced the

number of $BrdU^+$ and $BrdU^-$ plasma cells in the bone marrow, but not in the spleen (**Figure 27C**). In the spleen, the absolute number of $BrdU^+$ plasma cells per organ decreased, but not their relative number (**Figure 27D**). In the bone marrow treatment with the γ-secretase inhibitor increased both the absolute and also the relative number of plasma cells (**Figure 27D**).

Figure 27. γ-secretase regulates plasma cells in mice. *(**A**) NZB/W mice pretreated with BrdU received the γ-secretase inhibitor LY-411575-I (red) or vehicle (blue) for one day. Surface expression of BCMA on all $CD138^+$ plasma cells (PC) and the $BrdU^+$ and $BrdU^-$ PC subgroups in spleen and bone marrow (BM) was determined by flow cytometry (mean, ***, p<0.001; **, p <0.01, unpaired t-test) . (**B-D**) 7 days treatment of NZB/W mice with LY-411575-I (red) or vehicle (blue). (**B**) BCMA surface expression in the spleen and bone marrow (BM) on B220, and $BrdU^+$ and $BrdU^-$ plasma cells was determined. (**C**) Absolute number of plasma cells, $BrdU^+$ and $BrdU^-$ plasma cells in spleen and bone marrow. (**D**) Frequency (% of all cells in the organ) of plasma cells in spleen and bone marrow. Compiled data from 10 analysed animals per group (mean,*, p<0.05; **, p<0.01; ***, p<0.001; ****, p<0.0001, unpaired t-test). Modified from Laurent, Hoffmann et al. 2015.*

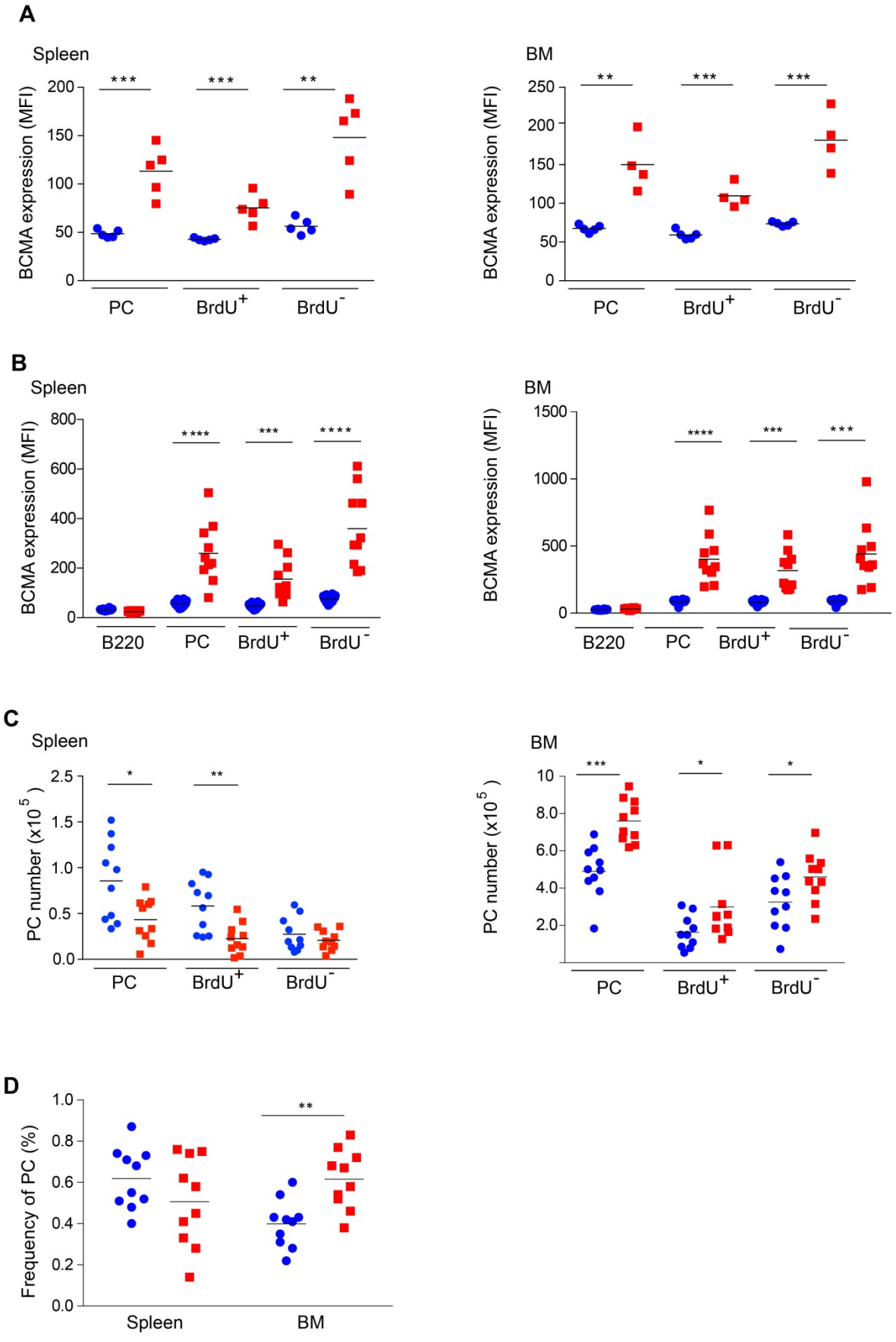

A
Spleen
BCMA expression (MFI)
PC
BrdU+
BrdU-
BM
B
Spleen
B220
BM
C
Spleen
PC number (x10^5)
BM
D
Frequency of PC (%)
Spleen
BM

Figure 27

5 DISCUSSION

5.1 sBCMA as a novel biomarker for B cell driven autoimmunity

A hallmark in MS is the persistence of plasma cells inside the CNS compartment, which is accompanied by local Ig production (Meinl, Krumbholz et al. 2006; von Budingen, Bar-Or et al. 2011). A possible explanation for this would be the local production of BAFF and APRIL by astrocytes leading to B cell fostering niches in the inflamed CNS inside the brain compartment (Krumbholz, Theil et al. 2005). BCMA being mainly expressed by plasma cells, we aimed to address the question whether this plasma cell fostering milieu in the CNS of MS patients would translate into altered sBCMA levels.

We therefore measured sBCMA in CSF of patients with multiple sclerosis, neuroborreliosis and other non-inflammatory neurological diseases and found that sBCMA was indeed elevated in the CSF of MS patients and that the increased levels closely correlated to the local Ig production. Increased sBCMA levels were also detected in CSF of patients with neuroborreliosis indicating that far from being a disease specific marker, increased levels of sBCMA rather indicates an intrathecal B cell driven immune response. In order to assess if these findings could be extrapolated to systemic B cell driven autoimmune diseases, we went on analysing sBCMA in serum of patients with SLE. In this disease, activation of the B cell compartment typically goes along with elevated levels of BAFF. We observed that serum sBCMA levels were elevated in SLE patients and that these levels closely correlated to the disease activity. Further *in vitro* experiments showed that native human B cells produce sBCMA upon differentiation into Ig-secreting cells thus pointing towards plasma cells in the CNS as a possible source for sBCMA in the CSF of MS patients.

The significance of soluble receptors as prognostic or therapeutic marker has been already well documented for several receptors, in particular for soluble TNF-receptors I and II. For example, in acute and systemic inflammatory responses, including bacterial sepsis (Froon, Bemelmans et al. 1994) and falciparum malaria (Kern, Hemmer et al. 1992), high serum sTNFR usually correlate with disease severity and mortality. A recent study from our group has shown that TACI receptor, which can bind both APRIL and BAFF, was shed by the metalloproteinase ADAM10 and resulted in an endogenous soluble TACI receptor which

acted as a decoy receptor for BAFF and APRIL. Similarly to BCMA, elevated sTACI levels were found in MS and SLE (Hoffmann, Kuhn et al. 2015).

Histological studies have shown that there is a clear heterogeneity between individual MS patients regarding the immunopathological patterns of disease seen in MS lesions. While some MS patients show lesions with T-cell/macrophage-associated patterns, other patient's lesions display antibody/complement-associated demyelination. The development of biomarkers, which would allow us to distinguish between the underlying immunopathological mechanisms, would be of tremendous clinical importance as it would enable to predict therapeutic response. Based on our results, sBCMA reflects activation of the BAFF/APRIL pathway and thus shows the promising features of a biomarker for B cell driven autoimmunity in MS and SLE and could serve as a further paraclinical marker for disease activity in SLE. Further studies will be needed to assess if sBCMA in MS patients can serve as a prognostic biomarker for disease activity and therapeutic response, in particular to B cell targeting therapies.

5.2 Direct cleavage of BCMA by γ-secretase

The generation of soluble receptors by proteolytic cleavage of the extracellular domain constitutes a highly conserved and common regulatory mechanism of immunity and inflammation with notorious examples including TNFR1, TNFR2, CD30, CD40, and IL-6Rα. So far, generation of soluble receptors by shedding required the proteolytic cleavage of the extracellular domain by a metalloproteinase. This first cleavage was believed to be a prerequisite for further cleavage by the intramembranous γ-secretase (Kopan and Ilagan 2004; Lichtenthaler, Haass et al. 2011).

In our work we found that BCMA however, is directly cleaved by the γ-secretase without any previous cleavage by a metalloproteinase. First, treatment of sBCMA producing cells with different γ-secretase specific inhibitors almost completely abrogates BCMA shedding and highly increases the cell surface expression of BCMA. Moreover, using MEF cells deficient for both PS1 and PS2 (PS-/-) transduced with human BCMA and rescued with either wt PS1 or a deficient form of PS1 (D385), we showed that a full-functioning PS1 unit is necessary for BCMA shedding. The direct shedding of BCMA without previous cleavage was confirmed by ELISA using HELA-cells transfected with human BCMA containing a N-terminal-FLAG tag.

This constitutes a novel proteolytic feature of the γ-secretase, as until now this enzyme complex has not been known to act on intact membrane proteins. γ-secretase does not cleave at a specific aa sequence, but its activity seems to be influenced by the length of the extracellular domain: little processing occurred when the extracellular domain exceeded 100 aa, while extracellular parts of about 50 aa permitted effective γ-secretase cleavage (Struhl and Adachi 2000). In line with these previous results we found that the artificial increase of the extracellular domain of BCMA resulted in a striking reduction of shedding. Direct cleavage of BCMA by γ-secretase is thus presumably facilitated by the unusually short extracellular domain of native BCMA (54 aa). BCMA is so far the only known direct substrate of γ-secretase. Screening for substrates of the γ-secretase, focussing on proteins with short extracellular domains, could help identify other direct γ-secretase substrates in the future.

5.3 Functional consequences of BCMA shedding

Soluble receptors can prevent binding of a cytokine to its membrane-bound receptors, thus inhibiting signal transduction and biological activity. They are thus generally regarded as antagonists for their ligands. The fact that both the membrane–bound and soluble receptors bind the same cytokine implies that competition takes place between the two receptor forms. It is essential to stress that the concentration of a soluble receptor required to block biological activity is dependent on a complex set of variables, including the relative affinities and binding profiles of membrane-bound receptors versus soluble receptors, the number of membrane-bound receptors on the target cells, cytokine and soluble concentration and the fraction of membrane-receptors that need to be occupied to result in a biological response. Beside the decoy function of the shed receptors, the release through proteolytic cleavage constitutes a common mechanism in which the cell evades the effects of a specific cytokine by reducing the number of membrane-bound receptors available. In this regard the overall physiological effect of soluble receptors is likely to be the one of a negative feed-back mechanism.

In addition to this regulatory aspect, which can be found enhanced in pathological states, mutations that affect the shedding mechanism in itself can lead to disease. A famous example is TNFR1. In humans, different mutations on the TNFR1 gene, that associate with impaired receptor shedding, are linked to a group of dominantly inherited autoinflammatory syndromes

named TRAPS (McDermott, Aksentijevich et al. 1999). The physiological role of TNFR1 shedding was explored *in vivo* with a knock-in mouse expressing a mutated non-sheddable TNFR1. These mice presented spontaneous autoinflammatory reactions as well as an enhanced susceptibility to experimental autoimmune encephalitis (Xanthoulea, Pasparakis et al. 2004). More recently, functional studies, regarding a single nucleotide polymorphism in the TNFRSF1A gene that encodes TNFR1 and constitutes a genetic risk factor for MS, showed that this MS risk allele directs, by modified alternative splicing, expression of a new soluble TNFR1 (Gregory, Dendrou et al. 2012).

5.3.1 BCMA shedding modulates APRIL-induced signaling

Being confronted to this novel soluble form of BCMA, we aimed to get further insights into the functional relevance of BCMA shedding. Soluble cytokine receptors, which either reduce or promote cytokine signaling, are important regulators of inflammation and immunity. Our findings show that inhibition of BCMA shedding by the γ-secretase leads to an increased surface expression of BCMA, which in turn leads to an enhanced APRIL- and BAFF-binding to the cell surface and increased NF-κB activation. Taken together these results show that shedding of BCMA can regulate APRIL and BAFF-induced signaling. Similar to other soluble receptors, the decrease of the number of BCMA on the cell surface through shedding acts as a down-modulation mechanism, thus preventing signal generation by APRIL and BAFF.

5.3.2 sBCMA is a new decoy receptor for APRIL

If a soluble receptor retains its capacity to bind it(s) ligand(s), it may act as a decoy receptor by preventing further binding of the ligands to membrane bound receptors and thus reducing the pool of biological active ligand in the system. In our work we found evidence for such a decoy function of sBCMA. Indeed, *in vitro* a NF-κB assay and a B cell proliferation assay demonstrated that sBCMA was able to bind and inhibit APRIL-mediated effects, while no binding and no inhibition of BAFF was observed. These findings differ from the binding

profile of membrane-bound BCMA and the soluble fusion protein BCMA-Fc, which can bind both.

One possible explanation for this difference in binding profile between sBCMA and BCMA-Fc would be a strong gain of avidity of the dimerization state of BCMA in BCMA-Fc. Binding assays showed an avidity contribution of dimeric versus monomeric recombinant BCMA, which resulted in a more then 1000-fold increase in apparent affinity of BCMA binding to BAFF (Day, Cachero et al. 2005).

It might appear at first sight paradoxical that sBCMA, which shows features of a decoy as we showed in this study, is elevated in autoimmune diseases involving B cells and antibodies. However increased levels of sBCMA, produced by locally or systemically accumulating Ig-secreting cells, should be considered as a feed-back inhibitory mechanism that is not sufficient to completely down-regulate the immune response. Similar observations have already been made with sTNFR in SLE. In spite of being a decoy, sTNFR1, is elevated in SLE where it correlates with disease activity and is considered as a disease activity marker (Aderka, Engelmann et al. 1992; Heaney and Golde 1998; Xanthoulea, Pasparakis et al. 2004).

Other possible functions of sBCMA, beyond the inhibitory aspects of sBCMA, include additional agonistic effect and are yet to be explored. Indeed, some soluble receptor such as IL6-receptor are involved in trans-signaling mechanisms, by which the complex formed by sIL-6Rα and IL-6, can bind to a membrane-bound signal transducing subunit (gp130) on cells which do not express IL-6R and are unresponsive to IL-6 (Rose-John 2012). Furthermore, it has been reported that some soluble receptors including sTNF-R and sIL-4R potentiate the activity of their own cytokines *in vivo* under certain condition (Ma, Hurst et al. 1996). The underlying mechanisms involve the formation of cytokine/soluble receptor complexes that can result in increased molecular stability and prolonged half-life.

Finally, the remaining intracellular part of the receptor, the C-terminal fragment after γ-secretase cleavage, can be involved in further intracellular signaling by translocating into the nucleus where it can modulate gene expression as it has been described for NOTCH- pathway (Schroeter, Kisslinger et al. 1998; De Strooper, Annaert et al. 1999).

The existence of these additional functions in case of BCMA shedding is still unknown and should be investigated in future.

5.3.3 *In vivo* relevance of BCMA shedding

Our *in vitro* findings support the existence of a new regulatory feature within the BAFF/APRIL pathway. In order to investigate the physiological relevance of BCMA shedding, we treated wild type mice with a γ-secretase inhibitor, and observed, similarly to our *in vitro* results, an increase of BCMA expression on the cell surface. These short-term applications of a γ-secretase inhibitor revealed that γ-secretase regulates the amount of BCMA on the surface *in vivo*. To get further insights into the *in vivo* consequences of BCMA shedding, NZB/W mice, a widely used murine SLE model, were treated with a γ-secretase inhibitor. This revealed that γ-secretase inhibition increased the number of plasma cells in the bone marrow. Interestingly, in the spleen, we observed no increase in plasma cell number upon γ-secretase inhibitor treatment, even though surface BCMA was upregulated on plasma cells, as seen in the bone marrow.

Based on our previous studies, the increased number of plasma cells in the bone marrow after γ-secretase inhibition is at least partially explained by the enhanced presence of mBCMA, since mBCMA mediates the induction of the survival protein Mcl-1 in bone marrow plasma cells (O'Connor, Raman et al. 2004; Peperzak, Vikstrom et al. 2013). Regarding, the differential effect of γ-secretase inhibition on the plasma cell number in spleen and bone marrow, a possible explanation can be given by the previous observation that BCMA induced high expression of Mcl-1 in bone marrow but not in spleen plasma cells. Also, BCMA-/- mice had reduced plasma cell numbers in the bone marrow, but not in the spleen (Peperzak, Vikstrom et al. 2013).

Together our result show that *in vivo* inhibition of the BCMA shedding by γ-secretase enhances BCMA surface expression in plasma cells and leads to an increase number of plasma cells in the bone marrow. This increased survival of plasma cells is likely mediated by APRIL and due to the enhanced surface expression of BCMA (**Figure 28**).

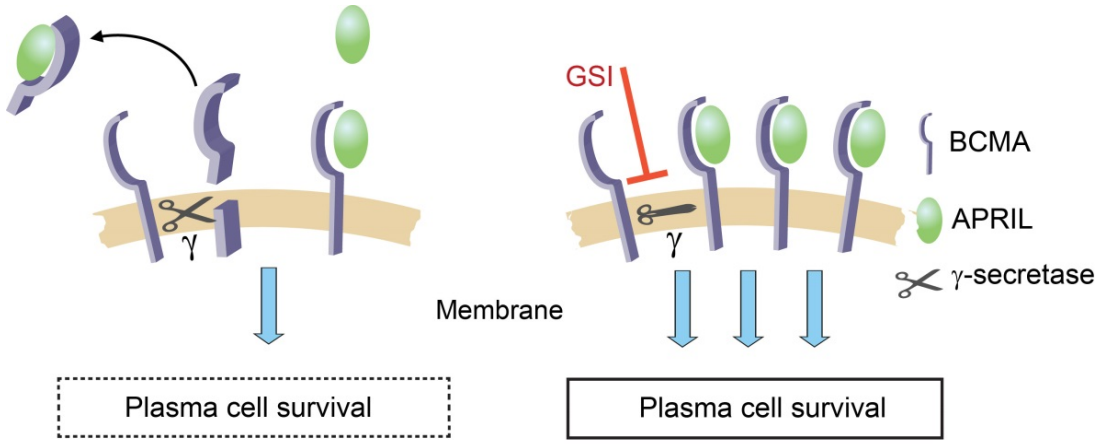


Figure 28. Functional consequences of BCMA shedding by γ-secretase. *Left: An active γ-secretase cleaves BCMA. This reduces the number of membrane-bound BCMA molecules and releases sBCMA which binds its ligand APRIL functioning as a decoy. Right: γ-secretase inhibitors (GSI) result in elevated BCMA on the surface and increased APRIL-mediated activation and survival. Modified from Laurent, Hoffmann et al. 2015.*

Long-term application of γ-secretase inhibitors *in vivo* would be required to analyse the effect of inhibition of BCMA shedding on disease course. Unfortunately, this would not allow specific conclusions about the long-term consequences of sBCMA inhibition, because the γ-secretase, through its effect on a broad range of substrate including NOTCH-receptors, regulates multiple effects in the immune system such as Th1 differentiation (Minter, Turley et al. 2005), T cell lineage commitment (Radtke, MacDonald et al. 2013), maintenance of marginal zone B cells (Simonetti, Carette et al. 2013), B cell activation (Yagi, Giallourakis et al. 2008), and survival of GC B cells (Yoon, Zhang et al. 2009). A further limitation of long-term treatment with γ-secretase inhibitors is the associated gastrointestinal toxicity, likely associated with the inhibition of NOTCH-cleavage.

The generation of a transgenic mouse displaying a non-sheddable BCMA receptor would avoid any NOTCH-associated toxicity and allow a most extensive study of the *in vivo* relevance of BCMA shedding. An important limitation is the lack of consensus cleavage patterns of γ-secretase as well as the expected multiple cleavage sites, which would likely necessitate a complete transmembrane swapping.

5.3.4 Regulation of BCMA shedding

The direct cleavage of BCMA by the γ-secretase poses the problem of the regulation of sBCMA generation. While ADAM proteases are subject to multiple regulatory mechanisms such as ligand binding, the limiting event of the intramembranous proteolysis by the γ-secretase has so far been the first extracellular cut by a distinct protease. Several non-mutual exclusive regulatory mechanisms are likely to be involved in the regulation of BCMA shedding.

This study shows that sBCMA release is a direct consequence of BCMA surface expression by human B cells upon differentiation into Ig-secreting cells. These results are consistent with the data of previous studies, which showed that BCMA expression is induced as memory B cells differentiate into plasma cells (Darce, Arendt et al. 2007). Membrane-bound BCMA is then shed by the γ-secretase. This shedding doesn't require any additional activation or ligand binding. The γ-secretase being ubiquitously expressed and at state of knowledge constitutively active without any known cytokine stimulation, the main regulatory event is thus likely to be the expression of BCMA upon differentiation of B cells, i.e. within a chronic autoimmune process, into plasma cells. Naturally, we cannot exclude that this shedding can be further enhanced by yet unknown mechanisms.

A second potential mechanism which could generate increased sBCMA levels would be the upregulation of BCMA expression on plasma cells. Previous studies have reported that plasma cells in SLE patients display enhanced surface BCMA expression in comparison to healthy controls (Kim, Gross et al. 2011). Accordingly we indeed found higher sBCMA levels in serum of SLE patients. As to which stimuli would lead to an increase BCMA expression on the cell surface by plasma cells, little is yet known. BCMA being mainly found in intracellular golgi-like structures, stimuli which would lead either to an upregulated transcription or to a translocation of intracellular BCMA to the cell surface can results in an enhanced surface BCMA expression and thus result in increased sBCMA levels.

Finally, consideration should be given to the possibility of a specific regulation of the γ-secretase activity. So far the limiting or regulating event to the intramembranous proteolysis by the γ-secretase has been the first extracellular cut by a distinct protease. These proteases, i.e. ADAM proteases, are subject to multiple regulation mechanisms such as ligand binding and inflammation. Since BCMA is directed cleaved by the γ-secretase, these usual regulation

mechanisms cannot apply and therefore other regulation mechanisms of the γ-secretase remain to be investigate.

5.3.5 sBCMA in biological therapy

The involvement of BAFF and APRIL in the pathogenesis of many autoimmune and oncological diseases is well established. These cytokines thus represent valid key target for biotherapeutical approaches. Soluble receptors such as sBCMA with the ability to inhibit specific cytokines may have the potential to significantly contribute to the treatment of such disease. In addition, they display lower immunogenicity, in contrast to murine anti-cytokine antibodies, that normally induce anti-mouse immunoglobulin responses in patients.

Nevertheless, cytokine and their soluble receptors are involved in a complex regulated pathway with many targets. Therefore extensive knowledge of the target pathway is still required to avoid unexpected consequences, such as seen in the Atacicept in multiple sclerosis (ATAMS) trial. In this placebo-controlled, double-blind, 36-week, phase 2 trial, a recombinant humanized fusion protein containing the extracellular part of TACI, a receptor binding both BAFF and APRIL, was administrated to MS patients. Unexpectedly the therapy led to increased clinical disease activity, suggesting that the role of BAFF and APRIL in multiple sclerosis is more complex than previously expected. (Kappos, Hartung et al. 2014). These findings stand in contrast to the beneficial effect B cell depleting therapies in multiple sclerosis (Bar-Or, Calabresi et al. 2008; Hauser, Waubant et al. 2008; Kappos, Li et al. 2011) and reveal a yet not fully understood BAFF/APRIL pathway.

In this regard, the generation of a recombinant BCMA receptor, displaying a similar APRIL specific binding profile to the endogenous sBCMA could offer several advantages. Previous studies showed that the BCMA dependent maintenance of long-lived PC is largely mediated via its main ligand APRIL (Belnoue, Pihlgren et al. 2008). Since those cells are believed to be the source of pathogenic auto-antibodies in several autoimmune diseases, the specific inhibition of APRIL could in its turn target long-lived plasma cells. The beneficial effect of an APRIL-specific antibody-mediated blockade was described in a murine SLE model (Huard, McKee et al. 2008). sBCMA could therefore serve as model for a future pharmacological agent blocking specifically APRIL.

Together, we found that BCMA is directly shed from plasma cells by γ-secretase and suggest that this shedding constitute a regulatory mechanism for plasma cell survival in the bone marrow. Furthermore shedding of BCMA could serve as a potential biomarker for B cell driven autoimmunity and could open ways for new therapeutic strategies.

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7 LIST OF FIGURES

8 LIST OF OWN PUBLICATIONS

Laurent SA, Hoffmann FS, Kuhn P-H, Cheng Q, Chu Y, Schmidt-Supprian M, Hauck SM, Schuh E, Krumbholz M, Rübsamen, H, Wanngren J, Khademi M, Olsson T, Alexander T, Hiepe F, Pfister HW, Weber F, Jenne D, Wekerle H, Hohlfeld R, Lichtenthaler SF, Meinl E

γ-secretase directly sheds the survival receptor BCMA from plasma cells.

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Hoffmann FS, Kuhn P-H, Laurent SA, Hauck SM, Berer K, Krumbholz M, Khademi M, Olsson T, Dreyling M, Pfister H-W, Alexander T, Hiepe F, Kümpfel T, Wekerle H, Hohlfeld R, Lichtenthaler SF, Meinl E.

The Immunoregulator Soluble TACI Is Released by ADAM10 and Reflects B Cell Activation in Autoimmunity.

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Spadaro M., Gerdes LA, Mayer MC, Ertl-Wagner B, Laurent SA, Krumbholz M, Breithaupt C, Högen T, Straube A, Giese A, Hohlfeld R, Lassmann H, Meinl E, Kümpfel T

Histopathology and clinical course of MOG-antibody-associated encephalomyelitis

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Rosche B, Laurent SA, Conradi S, Hofmann J, Ruprecht K, Harms L.

Measles IgG antibody index correlates with T2 lesion load on MRI in patients with early multiple sclerosis.

PLoS one, 2012; 7(1)

9 AFFIDAVIT/ EIDESSTATTLICHE VERSICHERUNG

Hiermit versichere ich an Eides statt, dass ich die vorliegende Dissertation „γ-secretase directly sheds the survival receptor BCMA from plasma cells“ selbstständig angefertigt habe, mich außer der angegebenen keiner weiteren Hilfsmittel bedient und alle Erkenntnisse, die aus dem Schrifttum ganz oder annähernd übernommen sind, als solche kenntlich gemacht und nach ihrer Herkunft unter Bezeichnung der Fundstelle einzeln nachgewiesen habe.

I hereby confirm that the dissertation „γ-secretase directly sheds the survival receptor BCMA from plasma cells “ is the result of my own work and that I have only used sources or materials listed and specified in the dissertation.

München, den Munich, date	Unterschrift Signature

LIST OF CONTRIBUTIONS

NF-κB assays were performed in collaboration with **Dr. Franziska Hoffmann** (Institute of Clinical Neuroimmunology, LMU Munich).

BCMA transduction and rescue of MEF PS-/- deficient cells war performed in collaboration with Peer-Hendrik Kuhn (Neuroproteomics, Klinikum rechts der Isar, Technische Universität München, Munich).

Immunization of C57/BL6 mice, treatment with γ-secretase inhibitor and flow cytometry analsys of bone marrow cells were performed by **Dr. Yuan-Yuan Chu and Prof. Dr. Marc Schmidt-Supprian** (Department of Internal Medicine III, Klinikum Rechts der Isar, Technische Universität München, Munich).

γ-secretase treatment of NZB/W mice and flow cytometric analysis were performed by **Dr. Qingyu Cheng and Prof. Dr. Falk Hiepe** at the Deutsches Rheuma-Forschungszentrum in Berlin.

Mass spectrometric analyses were performed by **Dr. Stephanie Hauck** (Research Unit Protein Science, Helmholtz Zentrum München).

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