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Development and characterization of a novel therapeutic bispecific antibody targeting BCMA and PDL1 for the treatment of multiple myeloma

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“Dinanzi alle difficoltà non ci arrendiamo,
ma proviamo a cambiare strategia”

A Marco,
grazie per aver colorato i miei giorni più grigi,
sei stato la mia forza e la mia più grande motivazione.

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COMMONLY USED ABBREVIATIONS

ADCC	Antibody-dependent cellular cytotoxicity
ADCP	Antibody-dependent cellular phagocytosis
ADCs	Antibody drug conjugates
APH1	Anterior pharynx 1
APP	Amyloid precursor protein
APRIL	Proliferation-inducing ligand
ASR	Age-standardized rate
Akt	Protein kinase B
BAFF	B-cell activator of the TNF-α family
BAFF-R	BAFF receptor
BCMA	B cell maturation antigen
BCR	B cell receptor
BM	Bone marrow
BM-ECs	Boone marrow endothelial cells
BM-MSC	BM mesenchymal stromal cells
bsAbs	Bispecific antibodies
CAR	Chimeric antigen receptor
CAR-Ts	Chimeric antigen receptor T-cell
CCL12	C-C Motif Chemokine Ligand 12
CCL2	C-C Motif Chemokine Ligand 2
CCL3	C-C Motif Chemokine Ligand 3
CCL5	C-C Motif Chemokine Ligand 5
CD3ζ	Type 1 TM protein of the CD3 complex
CDC	Complement-dependent cytotoxicity
CDRs	Complementarity determining regions
C_H	Constant domain of Ig heavy chain
C_L	Constant domain of Ig light chain
CRD	Cysteine-rich domain
CSR	Class-switch recombination
CTLs	Cytotoxic T lymphocytes
CXCL12	CXC motif chemokine 12
CXCR4	CXC chemokine receptor type 4
DCs	Dendritic cells
ECM	Extracellular matrix
EMD	Extramedullary disease
Fab	Fragment antigen-binding variable region
Fc	Crystallizable fragment
FRs	Frameworks
H	Ig heavy chain

HSCT	Hematopoietic stem cell transplantation
HSPG	Heparan sulfate proteoglycans
ICAM-1	Intercellular adhesion molecule-1
ICI	Immune checkpoint inhibitor
ICOS	Inducible T-cell co-stimulator, CD278
IFN-I	Type I interferon
Ig	Immunoglobulin
IgH	Immunoglobulin heavy locus
Igκ	Immunoglobulin kappa locus
Igλ	Immunoglobulin lambda locus
IKK	Inhibitory kappa B kinases complex
IL	Interleukin
IκB	I kappa B kinase inhibitor of NFkB
JNK	c-Jun N-terminal kinases
L	Ig light chain
M1 macrophages	Classically activated macrophages
M2 macrophages	Alternative activated macrophages
mAbs	Monoclonal antibodies
MAdCAM-1	Mucosal vascular addressin cell adhesion molecule 1
MDSCs	myeloid derived suppressor cells
MGUS	Monoclonal gammopathy of undetermined significance
MHC	Major histocompatibility complex
MICA	The MHC class I polypeptide-related sequence A
MIP-1a	Macrophage inflammatory protein-1a
MIP-1α	Macrophage inflammatory protein-1α
MM	Multiple myeloma
MMAF	Microtubule inhibitor monomethyl auristatin F
MM-PCs	Multiple myeloma plasma cells
mTOR	Mammalian target of rapamycin
MZ	Marginal zone of a lymph nodes
NCSTN	Nicastrin
NFkB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NK cells	Natural killer cells
NKG2D	Natural Killer group 2D receptor
NO	Nitric oxide
OS	Median overall survival

OX-40	TNF Receptor Superfamily Member 4 also known OX-40
PBMCs	peripheral blood mononuclear cells
PCL	Plasma cell leukemia
PCs	Plasma cells
PD-1	Programmed cell death 1
PDL1	Programmed cell death ligand 1
PDL2	Programmed cell death ligand 2
PI3K	Phosphatidyl Inositol 3-Kinase
PSEN1	Presenilin 1
PSEN2	Presenilin 2
PSENEN	Presenilin enhancer
PSGL-1	P-selectin glycoprotein ligand-1
PTPN11	Protein Tyrosine Phosphatase Non-Receptor Type 11
RAG 1/2	Recombination-activating gene 1/2
RANK	Receptor Activator of NFkB
RANKL	RANK ligand
ROS	Reactive oxygen species
RSS	Recombination signal sequences
sBCMA	Soluble B cell maturation antigen
scFv	Single-chain variable fragment
SHM	Somatic hypermutation
SMM	Smouldering myeloma
SPR	Surface plasmon resonance
TAA	Tumor-associated antigen
TAAxCD3	Bispecific T cell engagers
TACI	Transmembrane activator and CAML interactor
TCR	T-cell receptor
TGF-β	Transforming growth factor β
T_H	Helper T cells
TNFRSF	TNF receptor superfamily
TNFSF	Tumor necrosis factor superfamily
TRAF	Tumor necrosis factor receptor-related factor
Tregs	Regulatory T cells
VCAM-1	Vascular cell adhesion molecule 1
V_H	Variable domain of Ig heavy chain
V_L	Variable domain of Ig light chain

CHAPTER 1:
GENERAL INTRODUCTION

MULTIPLE MYELOMA: THE CLINICAL PROBLEM

EPIDEMIOLOGY

Multiple myeloma (MM) is a malignancy characterized by an accumulation of clonal plasma cells (PCs) in the bone marrow (BM) leading to specific end-organ damage: destructive bone lesions, anemia, kidney injury, hypercalcemia and recurrent infections.

MM is the third most common hematological malignancy after non-Hodgkins lymphoma and leukemia in 2020, contributing 14% of the incidence of overall cases of leukemia, lymphoma, and multiple myeloma worldwide[1]. It is predominantly a disease of older people, aged 50 years or older, with a median age of onset of 70 years[2]. In 2022 a study published on *The Lancet Haematology* [3] reported that the age-standardized rate (ASR) of MM incidence was 1,78 per 100,000 people globally in 2020.

DIAGNOSIS AND PROGNOSIS

Most cases of MM present *de novo*, but a proportion can be preceded by 2 sequential premalignant conditions termed monoclonal gammopathy of undetermined significance (MGUS) and smouldering myeloma (SMM; also known as asymptomatic myeloma).

MGUS is present in approximately 3% of white individuals aged 70 years or more, and its incidence increases with age[4]. By definition it is asymptomatic and usually discovered by accident with the discovery of a blood serum (M-protein <30g/L) or urinary paraprotein (monoclonal light chain excretion <500mg/24h)[5]. In

MGUS patients, a small population of clonal PCs is detectable in the BM (<10%), without evidence of end-organ damage, and an abnormal free light chain ratio[5]. The risk of progression to active MM is around 1% per year [6]

SMM is a condition characterized by a larger BM PCs clone compared to MGUS (10-60%), serum paraprotein >30g/l, monoclonal light chain excretion ≥500mg/24h and no evidence of end-organ impairment[5]. The risk of progression of SMM to active MM decreases with the time from diagnosis: 10% per year in the first 5 years, 3% per year in next 5 years and 1% annually at 10 years from the onset [7].

Symptomatic MM is a clinical condition characterized by a large clone of PCs in BM (≥10% or ≥60%) or by biopsy-proven plasmacytoma, detectable monoclonal protein in serum or urine and one or more symptoms indicative of MM related end-organ injury (known as 'CRAB criteria')[5]. Plasmacytoma is a rare PCs dyscrasia characterized by a localized accumulation of neoplastic monoclonal PC in bone, or in soft tissues. Unlike MM, plasmacytoma happens without systemic involvement[8]. The CRAB clinical features include hypercalcemia, renal failure, anemia and bone lesions (osteopenia, fractures and lytic lesions). Symptoms like fatigue, weight loss and recurrent bacterial infections are also detectable in active MM patients.

Serum biomarkers of disease burden like albumin and β-2-microglobulin levels as well as genetic risk factors have been

used by the *International Myeloma Working Group* to define an *International Staging System* [9] which allows to stratify disease severity and thereby estimate prognosis (Table 1)[9,10].

Table 1. Serum biomarkers levels defined by the International Staging System for Multiple Myeloma

Stage	*Criteria*		*5-y survival rate*
	Serum β2-microglobulin	*Serum albumin*	
I	*<3,5 mg/dl*	*≥3,5g/dL*	*77%*
II	*>3,5 and <5,5 mg/dl*	*<3,5g/dL*	*62%*
III	*>5,5 mg/dl*	*Any*	*47%*

Table adapted from Cowan et al. [10]*.*

Since 2000, median overall survival (OS) of MM has improved from 30 to 126.6 month thanks to new treatments; however, approximately 20% of people with newly diagnosed MM still have poor outcomes.

MOLECULAR PATHOGENESIS AND DISEASE EVOLUTION

The humoral immune response is driven by B cells (see chapter below) which produce immunoglobulins and is characterized by two phases: the recognition phase and the activation phase[11]. In the recognition phase, naïve B cells specifically bind antigens by their surface immunoglobulin (Ig) receptors. In the activation phase, antigen and other stimuli, including helper T cells (T_H), stimulate the proliferation and differentiation of specific B cell clones in the germinal center. Progeny of the clones may persist as memory cells or may undergo class-switch recombination (CSR) and somatic hypermutation (SHM) during the process of affinity maturation of the antigen binding site.

B cells, after antigen and T cell induced activation, proliferation and differentiation, leave the tissue and reach the BM, where they undergo the last step of maturation, differentiating into antibody-producing PCs (see chapter below). CSR and SHM are essential for antibody diversification and humoral immune responses. However, these events represent a risk of genomic errors, which are considered to be the triggering events of a pre-malignant stage[12].

Genetic modifications may also take place during Ig rearrangement in immature B cells. Pilarski et al. corroborate this premise reporting the presence of CD34+ B cells in peripheral blood mononuclear cells (PBMCs) of MM patients, holding hyperdiploidy and translocations. These genetic mutations were absent in the CD34- cells, assuming the

presence of a “MM stem-like cells” population[13,14]. In MGUS, these genetic errors can represent the primary and secondary genetic events that cause MGUS and then progression to symptomatic MM[15,16].

Primary genetic events, such as hyperdiploidy *and translocations,* represent the starting events triggering the evolution from MGUS to MM. Around 50% of all MM are hyperdiploid in the form of trisomies of chromosomes 3, 5, 7, 9, 11, 15, 19 and 21, either singly or in combination[4,17]. The other half of MGUS and MM patients show “non-hyperdiploid” karyotypes that can be further categorized into: hypodiploid (44/45 chromosomes), pseudodiploid (44/45 to 46/47), near-tetraploid (>74;), and hyperhaploid karyotypes (24–34)[4,18]. Translocations are mainly associated with the hypodiploid and pseudodiploid karyotypes and mainly involved the immunoglobulin heavy (IgH) locus (14q32)[4,19,20] during pre-B cells differentiation (see chapter below). IgH translocations cause the upregulation of the involved oncogenes by their juxtaposition with the strong IgH transcriptional promoters[4,20]. In Table 2 are reported the most common IgH translocations with affected gene, gene function, frequencies and prognostic factor. In MM patients other translocations may involve the immunoglobulin lambda (Igλ) locus (frequency is 10% at onset and 20% at relapse) [4,21] and Igκ locus (frequency <5% of newly diagnosed MM) [4,22].

Secondary genetic events are oncogenic point mutations that occur during the transition to symptomatic myeloma. These

events are summarized in Figure 1 and include: mutations in RAS oncogenes (NRAS, KRAS, BRAF, EGR1, and FGFR3) [4,23], mutation in Cyclin D genes, causing the loss of tumor suppressor functions, and mutation impacting on DNA repair mechanisms (TP53)[4,24,25]. Alteration of PI3K and NFkB pathways are also observed at rather later disease stages [4,26(p1),27]. Finally epigenetic changes such as DNA hypomethylation and hypermethylation can be observed that drive the progression from MGUS to symptomatic MM[4,28].

Table 2. Most common translocation of IgH locus (14q32) in multiple myeloma

IgH translocation	Affected gene	Frequency of mutation and Gene Function	Prognostic impact
t (4;14)	FGFR3*	11-15% Drives MM cells proliferation	High risk
	MMSET*	11-15% Involved in MM pathogenesis, has epigenetic effects	High risk
t (6;14)	CCND3*	1-2 % Drives MM cells proliferation	Standard risk
t (11;14)	CCND1*	15% Regulates cell cycle	Intermediate risk
t (14;16)	MAF*	3-5 % Promotes MM cells division, DNA synthesis and increases adhesion to bone marrow stromal cells	High risk
t (14;20)	MAFB*	1 % Induces MM cells proliferation and blocks drug-induced apoptosis	High

**FGFR3: fibroblast growth factor receptor 3. MMSET multiple myeloma SET-domain containing protein. CCND3: cyclin D3. CCND1: cyclin D1. MAF and MAFB: belong to the MAF family, are leucine zipper-containing transcription factors.*

A group of high-risk disease patients (20–30% of all cases) are associated with poor outcomes after standard therapy. Their condition arises from the interplay of several genetic lesions leading to high proliferation rates, evasion of apoptosis, and therapy resistance. Gain of chromosome 1q21[4,29] and biallelic inactivation of TP53 or concurrent mutation, are considered markers for high-risk diseases[4,25]. When MM clones lose their dependency on the bone marrow microenvironment, they can migrate to the bloodstream or infiltrate other organs. Plasma cell leukemia (PCL) is defined by the presence of $>2\times10^9$ (2.000/µl) clonal PCs in the peripheral bloodstream[30,31] while extramedullary disease (EMD) is marked by proliferation and infiltration of MM cells in various extramedullary organs[32]. Organs most commonly infiltrated by MM cells are skin/muscle (24%), pleura (12%), lymph nodes (10%), liver (9%), and central nervous system (6%). At diagnosis, the skin is the major affected site, while in relapsed/refractory MM, liver, kidneys, lymph nodes, central nervous system, breast, pleura, and pericardium are mostly involved[33]

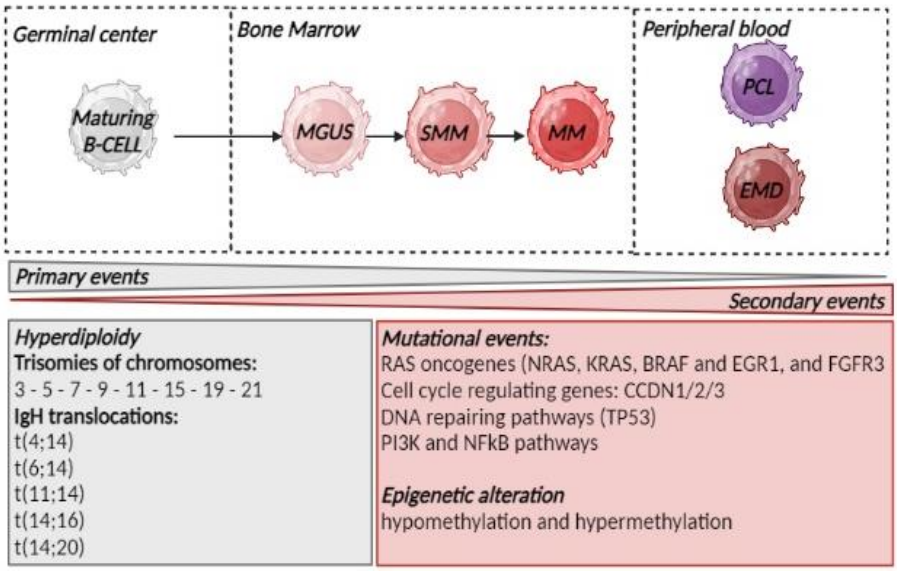

Figure 1. Pathophysiology of multiple myeloma.
This depicts primary and secondary genetic events causing the transition from MGUS to symptomatic myeloma. Created with Biorender.com.

B-CELL DEVELOPMENT AND B-CELL SUBSET

B lymphocytes develop in the BM from hematopoietic precursor cells. Functional rearrangement processes, taking place in immunoglobulin gene loci, drive early stages of B cell development. In the following paragraphs, the B cell differentiation process as a function of the immunoglobulin gene segments recombination will be described. In the first part an overview regarding the immunoglobulins structure, isotypes, functions and genes loci organization as background will be given.

STRUCTURE AND FUNCTION OF IMMUNOGLOBULINS

Immunoglobulins (Ig_s) are glycoproteins belonging to the immunoglobulin superfamily, they are heavy molecules (~150 kDa) with a size of about 10 nm. Ig_s have a Y-shaped structure which consists of four polypeptides: two heavy (H) chains and two light (L) chains connected by disulfide bonds[34] (Figure 2). Each chain is composed of homologous structural domains called Ig domains, containing about 70–110 amino acids. Ig domains are classified into variable or constant depending to their size and sequence/function. Ig domains are made of a characteristic immunoglobulin fold, in which two beta sheets create a "sandwich" shaped globular structure, held together by interactions between conserved cysteines and other charged amino acids. These structures are quite stable and allow to build

molecules with different domains, each with its function. During antibody synthesis by B cells, 2 identical L chains bind the H chain homodimer, and the structure is held together and stabilized by several disulfide bridges (shown in yellow in Figure 2.). The final structure of immunoglobulins allows antibody molecules to perform dual functions: antigen binding, through the fragment antigen-binding variable region (Fab), and the mediation of the effector immune mechanisms by the crystallizable fragment (Fc)[34] (Figure 2).

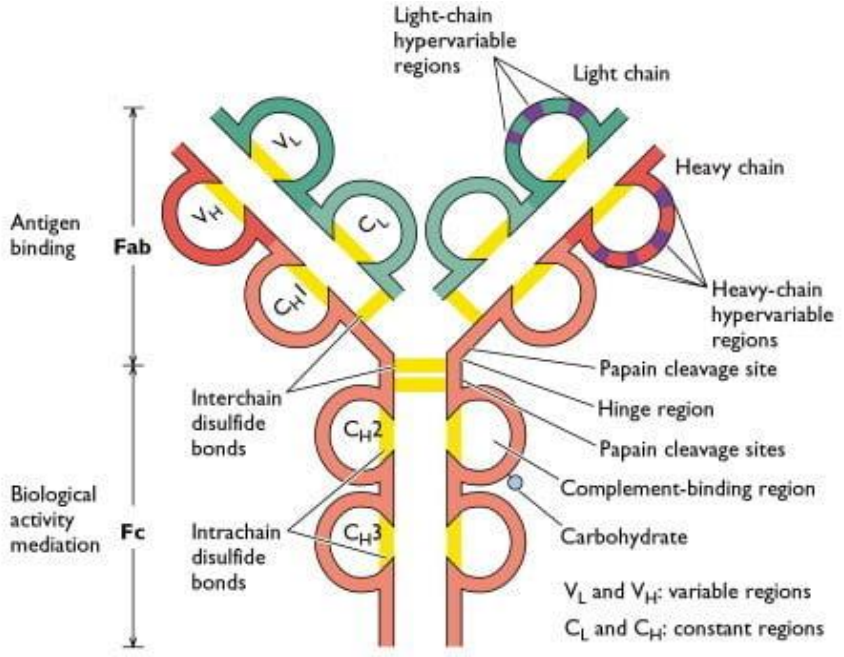

Figure 2. Immunoglobulin protein structure.
In this drawing is depicted a schematic diagram of an IgG molecule. Source: Sino Biological Inc.

There are five types of mammalian **Ig H-chain**[34]: α, δ, ε, ɣ, and μ. The type of the H-chain defines the class of an antibody: IgA, IgD, IgE, IgG, and IgM antibodies, respectively. Each H-chain is characterized by constant (C_H) and variable (V_H) immunoglobulin domains, with C_H domains identical in all antibodies of the same isotype. The constant region of γ, α and δ chains is composed of three tandem C_H, and a hinge region for added flexibility; while μ and ε chains have a constant region composed of four C_H. The C regions of different antibody isotypes differ in the effector functions they perform, due to the differences in the Fc region, i.e. the tail part of the antibody molecule (Table 3 and Figure 2). The variable region of the H chain is the same for all antibodies produced by a single B cell or B cell clone.

Table 3. Immunoglobulin isotype and effector function.

Isotype	***H-chain type***	***number of C_H Ig domains***	***Functions***
IgA	*α*	*3*	*Mucosal immunity, neonatal passive immunity*
IgD	*δ*	*3*	*Antigen receptor in naïve B-cells, only membrane form*
IgE	*ε*	*4*	*Immediate hypersensitivity*
IgG	*ɣ*	*3*	*Opsonization, CDC*, ADCC*, neonatal and passive immunity*
IgM	*μ*	*4*	*Antigen receptor in naïve B-cells, CDC*

**CDC: complement dependent cytotoxicity; ADCC antibody dependent cell-mediated cytotoxicity. Adapted from Cellular and molecular immunology book* [34]*.*

In mammals there are two types of Ig L-chain [34], lambda (λ) and kappa (κ), functionally identical and constituted by one constant (C_L) and one variable domain (V_L). Each antibody contains two identical L chains, κ or λ, and both can occur with any of the five types of H chains.

IMMUNOGLOBULIN GENES ORGANIZATION

Separate multigene families, on different chromosomes, encode for L and H chains of immunoglobulins (Figure 3):

- chromosome 14q32.2 contains the IgH chain locus [35];
- chromosome 2q1.2 contains the Igκ chain locus [36];
- chromosome 22q11.2 contains the Igλ chain locus [37].

As shown in Figure 3, all immunoglobulin gene loci contain three gene segments: V (variable), J (joining) and C (constant). The IgH locus contains also another gene segment called D (diversity) (Figure 3). The V regions of L chains are encoded by a random rearrangement and recombinations of VJ segments. And the V regions of H chains are derived by rearrangement and reorganization of VDJ segments. Individual exons (C gene segments) encode in contrast the different C Ig domains [38]. The human IgH locus contains 200 VH genes (of these, approximately 39 are functional), over 27 D segments, 6 J segments, and a C region of 9 functional genes and 2 pseudogenes[38,39]. Depending on the individual haplotype approximately 30 Vκ and 30-36 Vλ functional germline segments were described [38].

The nucleotide sequence of V gene segments of both heavy and light chains is composed of three hypervariable sequences (i.e. sequences that are most diverse between the different VH and VL segments), called complementarity determining regions (CDRs), that are situated between four less diversified sequence

termed frameworks (FRs). The CDR sequences come close together during folding of Ig molecules and form the antigen binding site, which is therefore hypervariable between different Ig molecules.

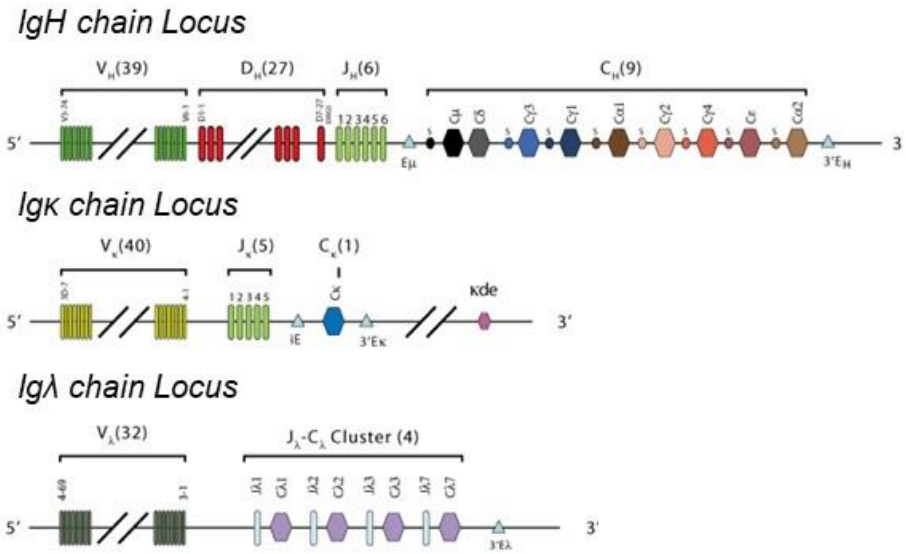

Figure 3. Chromosomal structure of immunoglobuline loci. *This figure depicts the Ig organization of Ig H,* κ and λ*. Adapted from Schroeder and Cavacini et al.* [38]

Recombination of V regions occurs during early B cell development and takes between specific sites on the DNA called recombination signal sequences (RSS) associated with each gene segment [38] (Figure 4). VDJ rearrangements of the H-chain and VJ rearrangement of the L-chains generate an enormous antibody repertoire, approximately 3×10^{11} combinations are possible, although some are not selected due to self-reactivity.

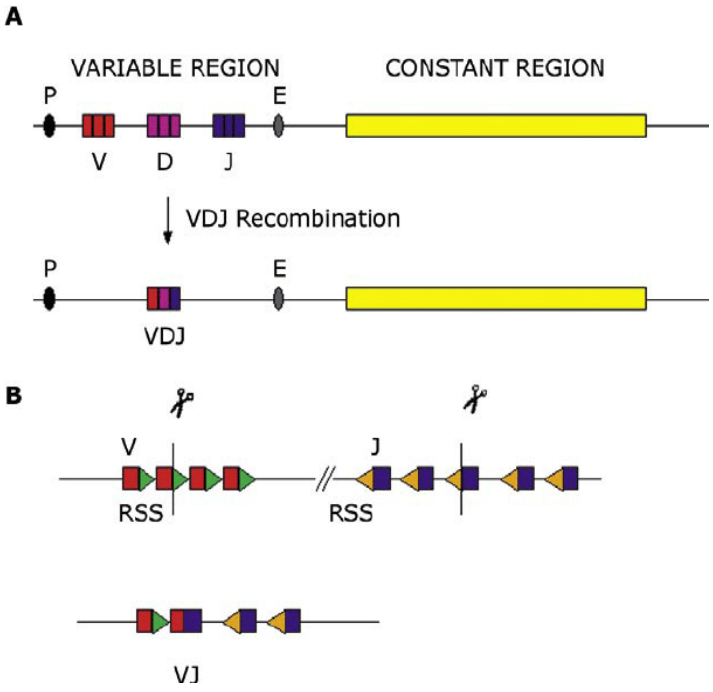

Figure 4. Recombination events in Ig gene loci.
A. In IgH locus, recombination events occur between one D and one J gene segment. Any DNA between these two gene segments is deleted. This D-J recombination is followed by the joining of one V gene segment, from a region upstream of the newly formed DJ complex, forming a rearranged VDJ gene segment. (B) In Igκ and Igλ loci recombination events occur between one V and one J gene segment. The portion of DNA between rearranged genes is shed. The cut takes place between rearranged DNA segments and the adjacent RSS motifs. V segments (red), RSSs (green and orange), J segments (purple) RSS (orange). P: promoter, E: enhancer. Adapted from Market and Papavasiliou[40].

B-CELL DIFFERENTIATION PROCESS AS A FUNCTION OF THE VDJ-VJ RECOMBINATION

According to the VJ and VDJ rearrangement, 3 stages of B cell development are defined: pro–B cells, pre–B-cells and immature B cells (Figure 5). The first stage, pro–B cells is characterized by a RAG 1/2–dependent (Recombination-activating gene) rearrangement of the D and J segments of the H-chain[41]. A second rearrangement that joins an upstream V region to the rearranged DJ segment opens the entry into the pre–B-cell stage[42]. Following rearrangement of the V-J fragments of L-chain genes, functional k or λ polypeptides are produced, combined with the functional H-chain and give rise to an IgM molecule. Cells expressing an IgM on the cell surface are termed immature B cells. These cells leave the BM, begin to express IgD and thus become mature naïve IgM^+ IgD^+ B cells. Naïve B cells migrate to the spleen, here they receive survival signals through BAFF-R and finalize early development by differentiating into marginal zone (MZ) or follicular B cells. MZ B cells after contact with cognate antigen may develop into short-lived plasma cells. The fate of naive B cells is dependent upon their encounter with the specific antigen. The contact with cognate antigen activates follicular B cells that, supported by T_H cells proliferate and may differentiate into memory B cells or PCs in the germinal center. Activation of B cells induces AID enzymes (activation-induced cytidine deaminase) and other components of the somatic hypermutation and class-switch system, thus allowing these

processes to take place. Somatic hypermutation and selection of hypermutated B cell clones results in increased affinity of the BCR/secreted antibody, whereas class-switching modifies the Fc of the antibody molecules produced and therefore changes the functional capability of these antibodies and the antibody class isotype.

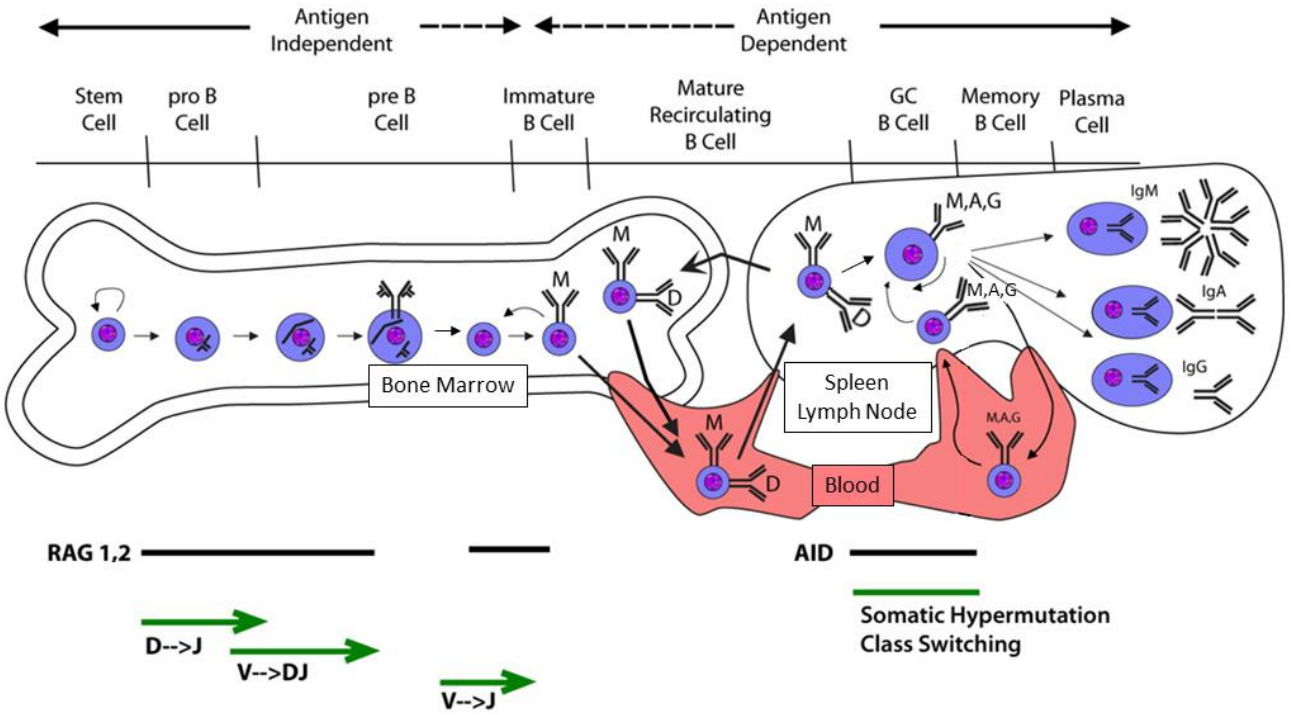

Figure 5. B-cell development and B-cell subsets.
In this drawing is depicted B-cell development as a function of immunoglobulins gene rearrangement. Illustration adapted from Schroeder and Cavacini et al.[38]

B CELL SURVIVAL AND MAINTENANCE

THE ROLE OF THE TNF CYTOKINE AND RECEPTOR SUPERFAMILIES.

The survival of B cells in the periphery is balanced by the signaling induced by two B-lymphocyte stimulators: *BAFF*[43] (B-cell activator of the TNF-α family) and *APRIL*[44] (a proliferation-inducing ligand). *BAFF* and *APRIL* belong to the tumor necrosis factor superfamily (TNFSF) and are trimeric molecules[45,46]. *BAFF* and *APRIL* bind to *TACI* (transmembrane activator and CAML interactor) and *BCMA* (B cell maturation antigen). In addition *BAFF* binds to a third receptor BAFF-R[41]. APRIL can also bind to heparan sulfate proteoglycans (HSPG) present in the extracellular matrix or on the surface of plasma cells [47]. BAFF-R, TACI and BCMA belong to the TNF receptor superfamily (TNFRSF) and promote B-cell survival at different stages of B cell development[48] (Figure 6). *BAFF-R* is expressed on immature B cells in the bone marrow and is highly expressed until the mature B cell stage, on naïve and memory B cells [49]. BAFF-R and TACI are co-expressed on memory B cells and on marginal zone B cells [41,50]. BCMA expression is exclusive for the B-cell lineage, in particular it is found on differentiated PCs[51]; it is selectively induced during PC differentiation in parallel with the loss of BAFF-R [52].

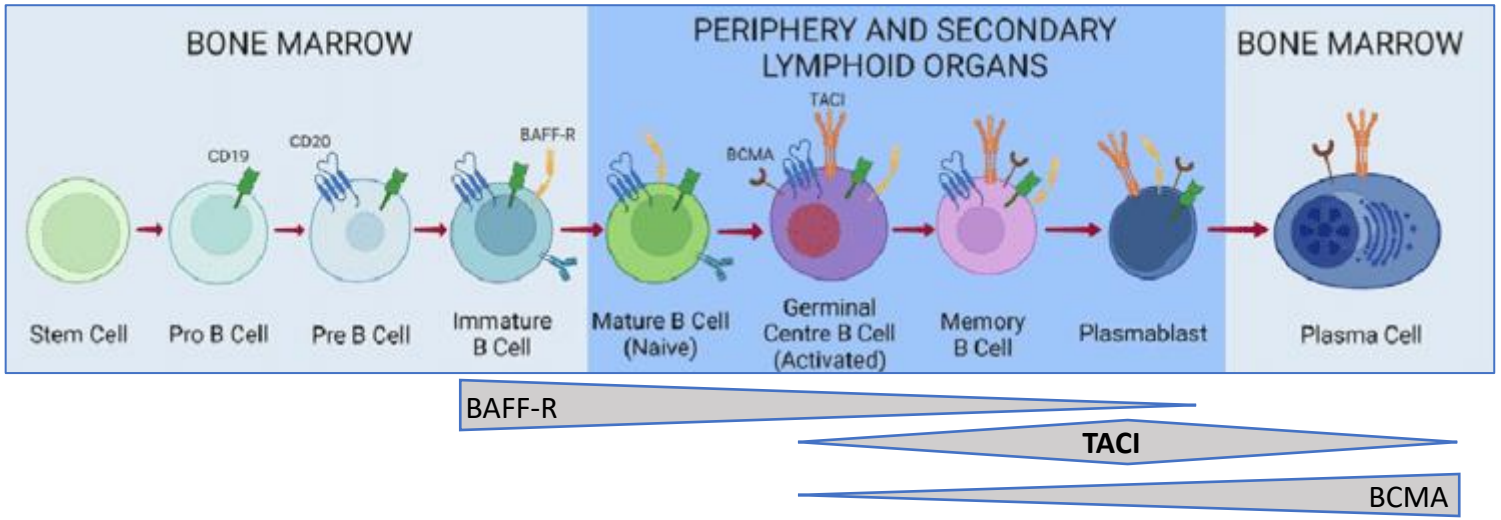

Figure 6. Expression of BAFF-R, TACI and BCMA throughout B-cell maturation.
Adapted from Kanatas et al [53]

The main role of BAFF-R is to promote immature B-cell differentiation while BCMA is essential for survival and proliferation of long-lived PCs in the BM[51]. TACI promotes survival of PCs and activated B cells [54]. It also has a role in antibody production, somatic hypermutation and class switch recombination[55,56].

The ligands BAFF and APRIL are first synthetized as type II transmembrane proteins by myeloid or stromal cells [57,58]. BAFF can exists either in membrane-expressed or soluble cytokine forms, while APRIL is processed intracellularly in the Golgi apparatus prior to secretion[59] (Figure 7). BAFF binds with higher affinity BAFF-R, followed by TACI, and binds weakly BCMA. APRIL binds most strongly BCMA and TACI, but not to BAFF-R [60]. Soluble BAFF can be processed to a trimeric molecule, which may associate to form 60-mers (20 trimers) (Figure 7). The role of the BAFF 60-mer is unclear, but it is a biologically active entity that can bind to receptors[60]. When APRIL binds to HSPG both in the extracellular matrix or on a cell's surface, it may oligomerize in 3-mers [47] (Figure 7). TACI is better activated by the oligomeric forms of BAFF and APRIL[49]. APRIL and BAFF can also assemble as mixed aggregates containing two APRIL and one BAFF (BAA), or two BAFF and one APRIL protomers (ABB) having distinct receptor binding specificities [61]. Both ABB and BAA bind to BCMA and TACI, but only ABB binds to BAFF-R (with a lower affinity compared to monomeric BAFF)[61]. APRIL binding to receptors promotes the survival of mature B cells and

long-lived PCs[60]. Both APRIL and BAFF cytokines stimulate class-switch recombination[49].

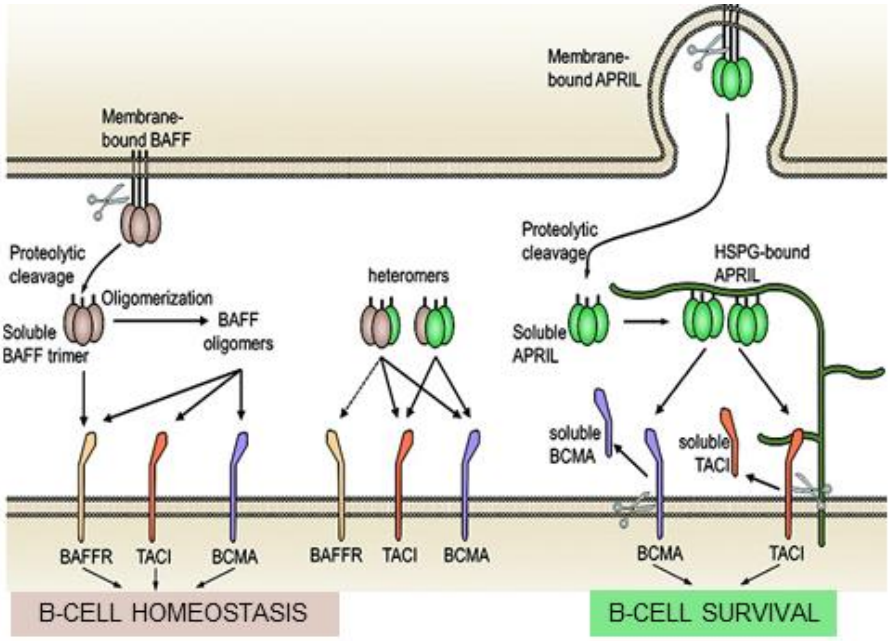

Figure 7. BAFF and APRIL production, processing and interaction with their receptor. *Scissors indicate proteolytic shedding of cytokines and receptor (discussed in follow chapters). From Samy et al.*[49]

B CELL SURVIVAL: THE SIGNALING EVENTS

Survival of B cells in the periphery depends on the expression of a functional BCR and is balanced by BAFF/APRIL-induced signaling. As shown in Figure 8, after binding to their receptors, APRIL and BAFF stimulate the activation of two main survival pathways such as: the protein kinase B/mammalian target of rapamycin (Akt/mTOR) and most importantly the induction of NF-κB, signaling pathways [41,62].

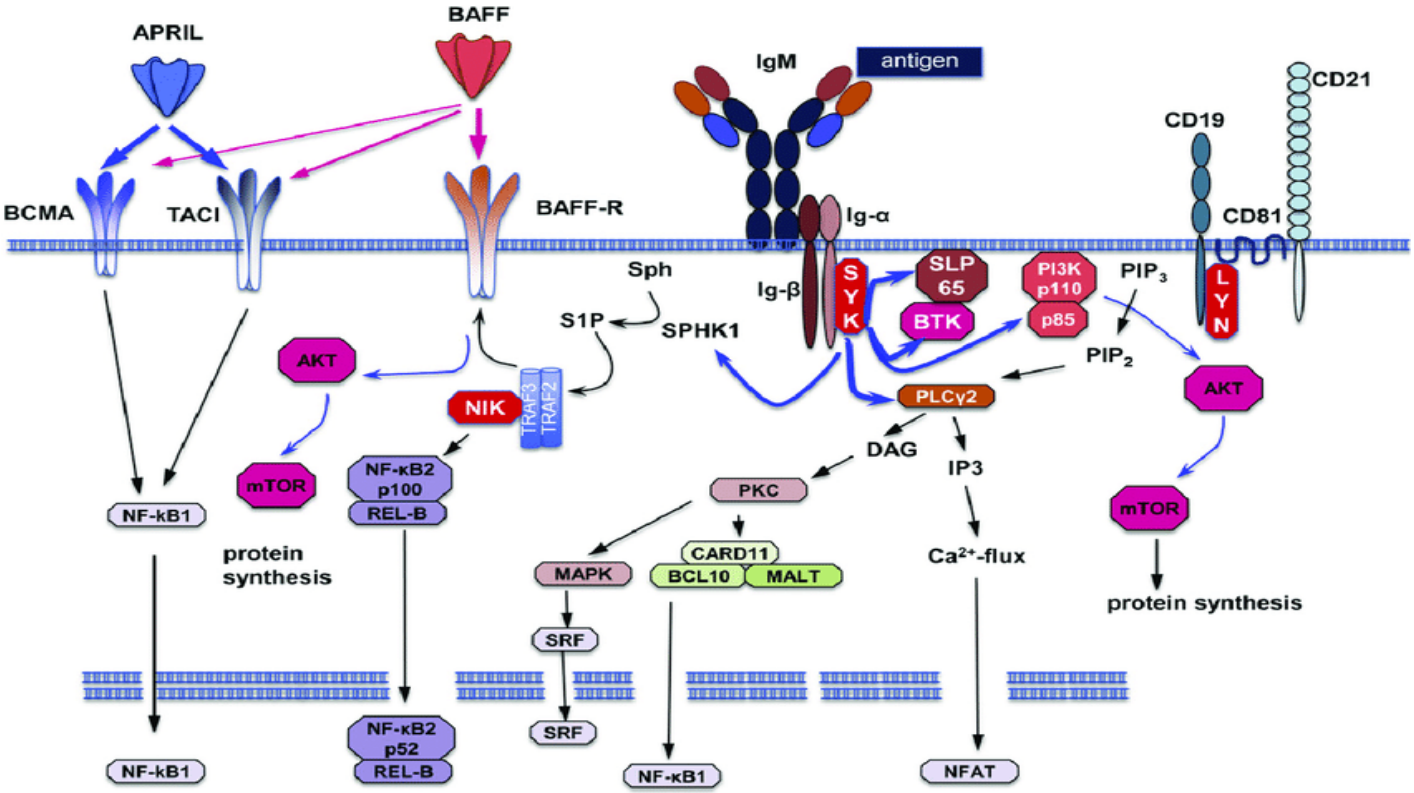

Figure 8. BCR, BAFF-R, TACI and BCMA signaling cascades events. *In this cartoon is depicted a diagram of molecular pathways regulating B cells survival. Source Pieper et al.*[41]

BCMA-RELATED SIGNALING PATHWAY

Prevalent BCMA-related signaling pathways are the NF-κB (Figure 9) and JNK signaling pathways.

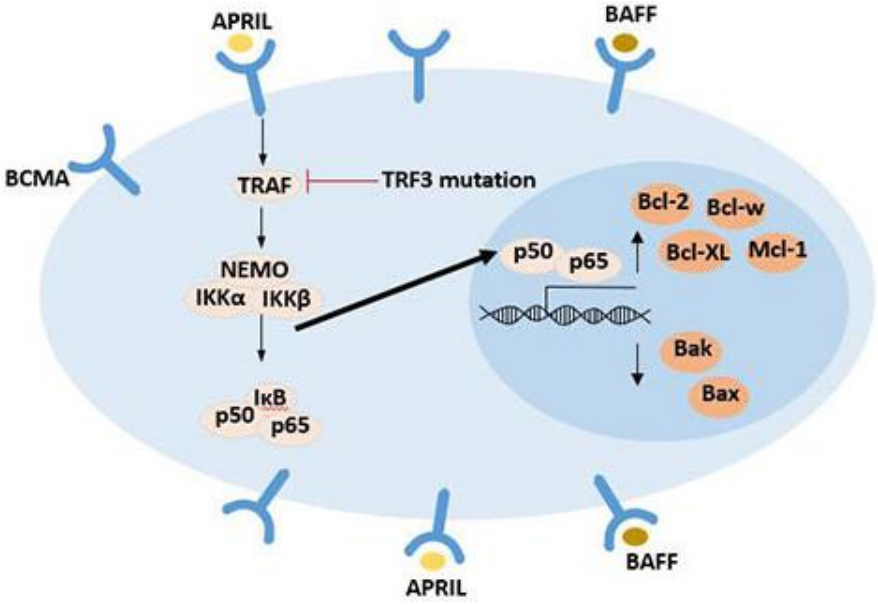

Figure 9. BCMA-related NF-κB signaling pathway in PC cells. *Source Cusabio web site (https://www.cusabio.com/c-20947.html)*

Proliferation and survival rate of normal and neoplastic PCs are mainly regulated by the NF-κB signaling pathway[63,64] (Figure 9). Inactive NF-κB exists in the cytoplasm bound with its inhibitor IκB (I kappa B kinase). The binding of BCMA with its ligands activates the classical NF-κB signaling pathway. In this signaling cascade TRAF (tumor necrosis factor receptor-related factor) activates the IKK complex (IKKα, IKKβ, IKKγ/NEMO) and phosphorylates IκB, which induces the p50 and p65 nuclear factors. The translocation of p50 and p65 to the nucleus results in the up-regulation of anti-apoptotic proteins (as Bcl-2, Bcl-x

L, Mcl-1 and Bcl-w) and down-regulation of pro-apoptotic proteins (as Bax and Bak) (Figure 9).

PROTEOLYTIC SHEDDING OF BAFF/APRIL RECEPTORS CONTROLS SURVIVAL OF B CELL SUBSETS

In view of the signaling capacity of BAFF/APRIL at various stages of B cell development, through interaction with their receptors BAFF-R, TACI and BCMA, a precise balance of the BAFF–APRIL system is essential to guarantee immune competence. Many authors, have recently described a new level of regulation of this system: BCMA, TACI and BAFF-R are all shed by ɣ-secretase and in some cases by metalloproteases which reduces their cell-surface expression and ligand-mediated B-cell survival [54,65–67]. Only ɣ-secretase substrates with short ectodomains are directly shed by the enzyme, while substrate proteins with long extracellular domains need to be first truncated by other proteases (e.g. metalloproteases) before the recognition and cleavage by ɣ-secretase[68]. ɣ-secretase is a multi-subunit intramembrane protease (Figure 10), that cleaves single-pass transmembrane proteins at or within their transmembrane domains[68]. The major catalytic unit of ɣ-secretase is presenilin 1 (PSEN1) and alternative catalytic protein is presenilin 2 (PSEN2). Other components of the complex are nicastrin (NCSTN), anterior pharynx 1 (APH1a and APH1b) which stabilizes, and presenilin enhancer (PSENEN), which activates PSEN1/2[69] (Figure 10). The most well-known substrate of ɣ-

secretase is amyloid precursor protein (APP) in the central nervous system (CNS), a protein that is thought to play an important role in the pathogenesis of Alzheimer's disease and may be responsible for the familial form of the disease, in part due to mutations of ɣ-secretase or of APP itself.

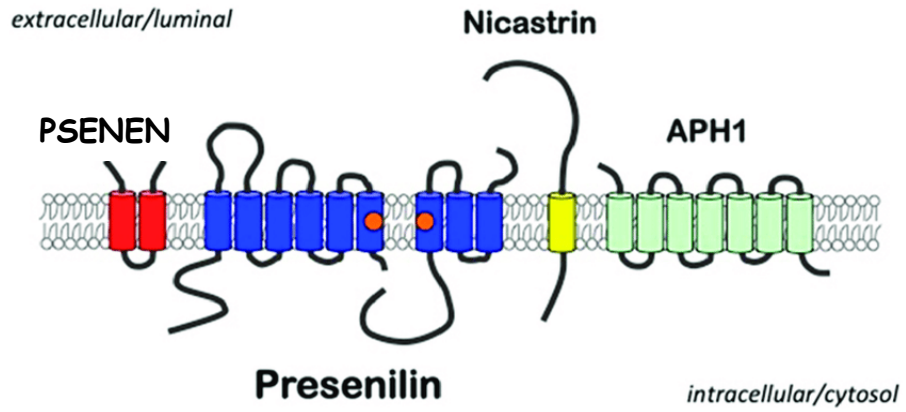

Figure 10. Structure of ɣ-secretase enzyme.
In this picture is depicted a schematic overview of the ɣ-secretase complex consisting of: PSENEN, presenilins, nicastrin and APH proteins. Catalytic sites in presenilins are indicated by orange circles. Adapted from Oikawa et al [70].

SHEDDING MECHANISMS OF BAFF AND APRIL RECEPTORS

BCMA has a small extracellular domain, compared to other surface receptors that are targeted by ɣ-secretase and is also one of the few molecules that is directly shed by the enzyme, without requiring a first shortening of the extracellular domain by another protease (Figure 11). Indeed, BCMA extracellular domain is composed of a single cysteine-rich domain (CRD) of fifty-four amino acids. In contrast, TACI, like many other targets of ɣ-secretase has a long extracellular ectodomain and needs to be cleaved by ADAM10 or ADAM17 (metalloproteases) before being available for ɣ-secretase shedding [66] (Figure 11). The property of BCMA makes it a unique molecule, directly shed by the enzyme, within the wide family of ɣ-secretase targets [71]. The shed soluble extracellular domains of BCMA (sBCMA) and TACI (sTACI) act as decoy receptors, neutralizing APRIL and BAFF [54] (Figure 11). sBCMA is also elevated in the serum of MM patients with progressive disease and correlates with disease severity [72]. The elevated level may reflect the fact that MM patients have considerable amounts of BCMA positive cells that shed the protein in the plasma.

BAFF-R is shed only by metalloproteases and only in cells that express also TACI, and after the binding of BAFF to both receptors[65]. BAFF trimer induces cleaving by ADAM10, and BAFF 60-mer induces shedding by ADAM17 [54,65].

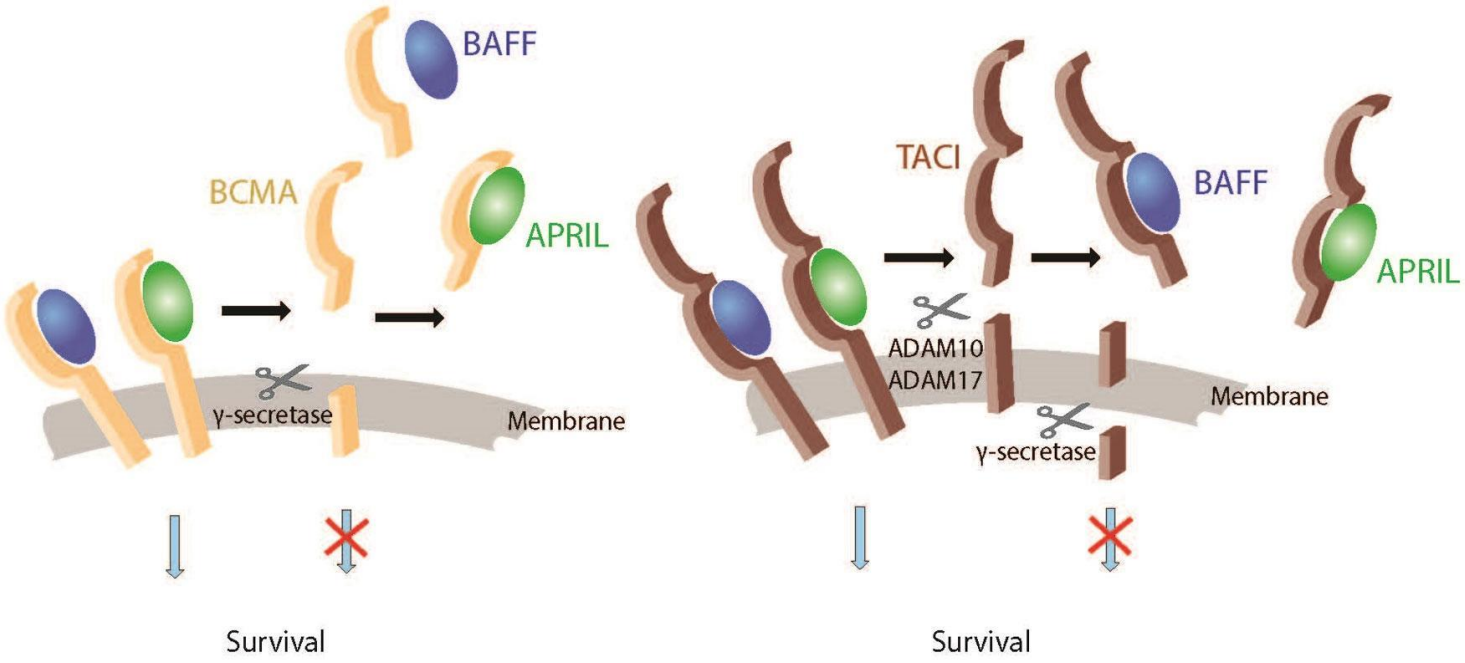

Figure 11. Shedding mechanisms of BCMA and TACI.
BCMA is directly shed by ɣ-secretase, TACI is cut by ADAM10 or ADAM17 before being available for ɣ-secretase shedding. Source Meinl et al [54].

THE BONE MARROW MICROENVIRONMENT IN MULTIPLE MYELOMA

Disease evolution of MM from the premalignant conditions, as MGUS and SMM, to symptomatic MM and eventually to PCL or EMD is importantly affected by the BM microenvironment. The interaction between the non-cellular and cellular compartment in the BM niche contributes to create an immunosuppressive tumor microenvironment which promotes MM-PCs proliferation and survival.

THE BONE MARROW MICROENVIRONMENT: NON-CELLULAR COMPARTMENT AND PLASMA CELLS TRAFFIKING.

The BM niche non-cellular compartment consists of extracellular matrix (ECM) proteins and soluble factors that govern MM-PCs recirculation and homing to the BM niche. Normal and neoplastic PC reach the BM niche via sinusoids, here they interact with different adhesive molecules mediating cell homing, retention and recirculation in the BM[73]. Normal and neoplastic PC trafficking are regulated by the adhesive mechanisms. The main protagonists of adhesive events in BM niche are the chemokine receptor CXCR4, expressed on normal and MM PCs, and the α4β7 integrin receptor[73] (Figure 12). The interaction of CXCR4 with its ligand CXCL12, a chemokine expressed in the BM, induces the activation α4β1 integrin on MM-PCs and its binding to the ligand VCAM-1 expressed on BM endothelial cells (BM-ECs)[73–75]. The α4β1 integrin also interacts with fibronectin in the

BM sustaining MM-PCs proliferation through IL-6[73,76]. The homing of MM cells is also regulated by the interaction of α4β7 integrin (Figure 12), expressed on MM-PCs, with its ligands MAdCAM-1, on BM-ECs, and fibronectin[77]. Neoplastic PCs express on their surface the P-selectin glycoprotein ligand-1 (PSGL-1) receptor whose binding with P-selectin on BM-ECs enhances cell rolling in the early phase of entry of MM-PCs in BM microenvironment[75,78]. All together these events cause the lodging and retention of neoplastic PCs in the BM microenvironment. During the late stage of MM disease, MM-PCs lose their dependency from the retention signals generated by the BM niche and become able to migrate to extramedullary organs by the blood stream[73].

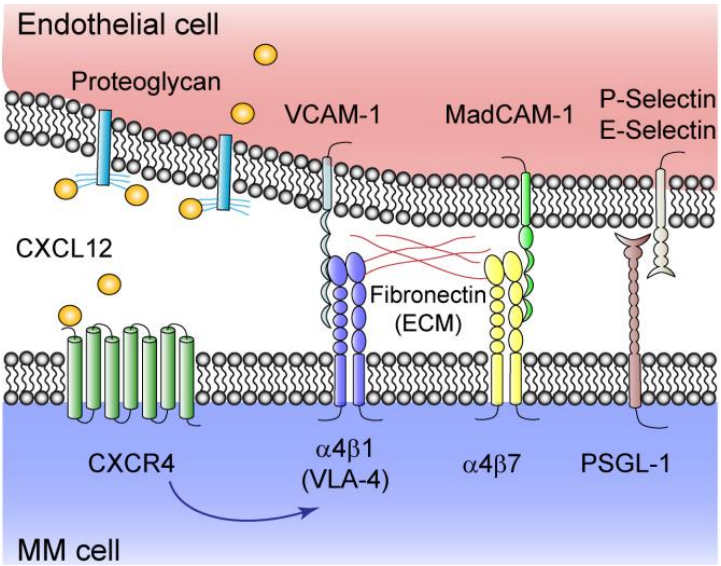

Figure 12. Multiple myeloma PCs homing and trafficking in BM microenvironment. *In this figure are depicted the ECM proteins and the soluble factors that govern MM-PCs recirculation and homing in the BM niche. Adapted from García-Ortiz et al [73].*

THE BONE MARROW MICROENVIRONMENT: THE CELLULAR COMPARTMENT.

The BM microenvironment is composed of hematopoietic and non-hematopoietic cells. The hematopoietic compartment includes myeloid cells, T and B lymphocytes, NK cells and osteoclasts, whereas the non-hematopoietic compartment includes BM mesenchymal stromal cells (BM-MSC) and their descendant stromal cells as endothelial cells, pericytes, adipocytes, osteolineage cells, osteoclasts and fibroblasts[73,79]. BM-MSC exert a key role in MM-PCs homing; they secrete high levels of CXCL12 and thus along with α4β1 and α4β7 integrins regulate neoplastic PCs lodging and retention [73] . In addition, BM-MSC contribute to the establishment of a premetastatic niche. Several studies have reported that the presence of genetic mutations on BM-MSC fosters the growth of neoplastic PCs by generating an inflammatory microenvironment. Dong et al.[80] in their study report that BM-MSC mutated in the PTPN11 gene secrete high levels of CCL3, a chemokine promoting the recruitment of inflammatory monocytes which predisposes to myelomonocytic leukemia development. Infiltrated monocytes, by IL-1β activity, contribute to generate an inflammation state characterized by the proliferation of osteoblasts, fibroblasts and BM-MSCs themselves.

The cellular compartment of the BM niche has also a fundamental role in MM-PCs survival and proliferation. Several cell types secrete IL-6, whose receptor is expressed on

neoplastic PCs. In addition, the cellular compartment supplies APRIL and BAFF which bind BCMA on MM-PCs surface. In BM niche, IL-6 is mainly produced by perivascular cells[81] and other BM-MSC after contact with PC[82,83]. Both IL-6 and APRIL are secreted by eosinophils[84] and megakaryocytes[85].

THE ROLE OF BONE MARROW MICROENVIRONMENT IN MM DEVELOPMENT AND PROGRESSION

MM disease progression correlates with the establishment of an immunosuppressive BM tumor microenvironment. Once neoplastic PCs reach the BM niche, they abnormally secrete cytokines and growth factors which promote immune escape and the survival and growth of neoplastic cells [79]. In addition, lodged MM-PCs, by secretion of cytokines, induce extracellular matrix and cell-cell contact remodeling, facilitating the eventual egress of neoplastic cells from the BM [73].

Tumor escape from the host immune system combined with its suppression are key points in MM progression[79,86]. Under healthy conditions, NK cells and cytotoxic T lymphocytes (CTLs) are the main populations which drive an efficient anti-tumor response. In MM, the induction of an immunosuppressive tumor microenvironment sustains the expansion of immunosuppressive cell populations, such as myeloid derived suppressor cells (MDSCs) and regulatory T cells (Tregs)[79]. In the tumor microenvironment other cell subsets, such as dendritic cells (DCs), macrophages and NK cells actively participate to disease progression[79].

MULTIPLE MYELOMA PROGRESSION: THE ROLE OF MYELOID DERIVED SUPPRESSOR CELLS

The MDSCs are a heterogeneous pool of immature myeloid cells that differentiate into macrophages, granulocytes, or dendritic cells under normal conditions. Differentiation of immature myeloid cells is inhibited in cancer, resulting in an accumulation of MDSCs[79,87,88]. This cell subset exerts its immunosuppressive role by inhibiting T-cell proliferation via secretion of arginase, ROS and NO[89]. In MM, MDSCs participate in disease progression by stimulating MM-PCs to secrete CCL5, MIP-1α and IL-6 cytokines; furthermore MDSCs suppress T_H cells functions and promote the induction of Treg [73]. In addition, previous studies have described a high concentration of MDSCs in peripheral blood and BM of MM patients when compared to healthy donors[90,91].

MULTIPLE MYELOMA PROGRESSION: THE ROLE OF REGULATORY T CELLS

Tregs are a subset of CD4+ T lymphocytes that are anergic and suppressive. These cells mediate their suppressive functions on antigen-presenting cells or on T_H cells, by expressing anti-inflammatory cytokines (IL-10 and TGF-β) or by cell-to-cell contact [73,79]. MM-PCs, by producing IFN-I and indoleamine 2, 3-dioxygenase, promote Treg cell proliferation and inhibit T_H cells. Finally, the cytokines produced by Tregs, together with other MM-PCs derived signals have a synergistic effect on T_H cells activity[73]. Previous studies have described an increase in

number of functional Tregs, expressing high levels of IL-10 and TGF-β[92], in peripheral blood of MM patients[93]. High expression of Treg correlates with the presence of paraprotein, a shorter time to progression[93] and shorter overall survival[94].

MULTIPLE MYELOMA PROGRESSION: THE ROLE OF DENDRITIC CELLS

DCs are BM-derived antigen-presenting cells which promote immunity or tolerance. DCs present self and non-self antigens to naive T cells with consequent differentiation into T_H and memory T cells [95]. Furthermore, DCs can trigger other T-cell mediated responses according to their functional conditions. Banerjee et al described the possible in vitro expansion of Tregs $FOXP3^+$ by DCs derived from MM patients[96]. In addition, García-Ortiz et al described the role of DCs in supporting MM-PCs proliferation by cell-to-cell contact through the CD80/CD86-CD28 pathway and secretion of IL-3[73].

MULTIPLE MYELOMA PROGRESSION: THE ROLE OF MACROPHAGES

The monocyte-macrophage lineage is a key element of leukocyte infiltration in the MM-BM microenvironment[97]. Macrophages can be divided into “classically activated” or M1 and “alternatively activated” or M2 macrophages[98]. In the MM BM niche, M1 macrophages trigger an immune response against MM-PCs by producing NO, ROS and by acting as antigen-presenting cells. This anti-tumor activity is inhibited by MM-PCs which unbalance the M1/M2 ratio towards the M2 population in the MM-BM niche. M2 macrophages polarization and migration into the BM is

induced by the CXCL12, CCL2, CCL3, and CCL14 chemokines, that are secreted by MM-PCs[99–101]. M2 macrophages promote MM-PCs survival by repressing drug-induced caspase activation. Myeloma cells express PSGL-1 and intercellular adhesion molecule-1 (ICAM-1) that interact with E/P selectins and CD18 on M2 cells. These contact-mediated mechanisms enable macrophages to protect MM-PCs from apoptosis via stimulation of SRC, ERK1/2 kinases, and c-MYC[102]. In addition, MM associated macrophages play a pro-tumoral role by non-contact-mediated mechanisms. They supply immunosuppressive agents in the BM niche (IL-10, TGF-β1 and Arginase-1)[79], proangiogenic factors and cytokines (VEGF, FGF-2 and IL-8) and angiogenesis modulating enzymes (matrix metalloproteinases, cycloxygenase-2, and colony-stimulating factor-1)[103].

MULTIPLE MYELOMA PROGRESSION: THE ROLE OF NK CELLS

NK cells exert their anti-tumor capacity through the Natural Killer group 2D (NKG2D) receptor which efficiently promote tumor cell killing by binding ligands on their surface. The MHC class I polypeptide-related sequence A (MICA) molecule is a ligand for the NKG2D receptor, whose high levels were described in the blood of MM patients. These data suggested that in MM the proliferation of NK cells was not combined with their activation, presumably due to a MICA downregulatory effect [104–106]. Another mechanism which could explain a loss of function of NK cells in MM patients, may be their expression of programmed cell death 1 (PD-1). The interaction between PD-1, on NK cells, with the

ligand PD-L1, on MM cells, may lead to a downmodulation of NK cells function versus MM-PCs[107].

MULTIPLE MYELOMA PROGRESSION THE ROLE OF OSTEOLINEAGE CELLS

In MM patients an osteolineage imbalance has been described, with loss of osteoblasts in favor of osteoclasts, that triggers osteolytic process and progression of tumor burden[73,108]. Neoplastic PCs secretions such as IL-6 and IL-3 with the macrophage inflammatory protein-1a (MIP-1a) are factors that promote osteoclasts activation. However, the main protagonist of enhanced osteoclastogenesis in MM is the transmembrane signaling receptor RANK, expressed on osteoclasts. The binding of MM-PCs to neighboring BM-MSC, within the BM, causes a rise of the expression of the RANK ligand (RANKL). These events promote osteoclastogenesis by the induction of RANK/RANKL/NF-κB and JNK pathways on osteoclast precursor cells[109]. RANKL is also involved in inhibition of osteoclast apoptosis [110].

THE PD1/PDL1 PATHWAY INBALANCE IN THE MULTIPLE MYELOMA BM MICROENVIRONMENT

PD1 is a type I transmembrane protein of the CD28 family[111], it is expressed on activated and exhausted T and B cells and binds the PDL1 and PDL2 ligands. The interaction between PD1 and PDL1 causes the suppression of T cells function and cytokine production, supporting the generation of an immunosuppressive microenvironment. PDL1 is not expressed on normal epithelial tissues and PC but it is highly expressed on MM-PCs and solid tumors[112].

In MM, the BM microenvironment plays a pivotal role in the activation of the PD1/PDL1 pathway. Previous studies reported increased levels of PDL1 on MM-PCs after inflammatory cytokines incubation[112] and BM-MSCs co-culture in vitro[113]. In addition, higher expression of PD1 on T and NK cells was observed in MM patients than in healthy donors[79]. These data suggest that by PDL1 expression MM-PCs escape the host immune response and increase the tumor burden. Promising data are reported in the literature regarding the efficacy of a PD1/PDL1 monotherapy in preclinical studies of MM. Rosenblatt et al.[114] reported the ability of an anti-PD1 mAb, named CT-011, to activate T-cell responses in an *ex vivo* model of MM BM microenvironment. Kearl et al. demonstrated increased survival of MM-bearing mice treated with an PDL1 mAb after lymphodepletion[115]. Due to high expression of PD1 and PDL1 in MM BM microenvironment, modulation of PD1/PDL1 signaling pathways may have therapeutic potential in

microenvironment targeted therapy.

The rationale to target the PD1/PDL1 pathway in MM is also reinforced by clinical evidence. PD1 and PDL1 are known to be expressed in MM and are prognostic factors for worse outcomes in this disease[112,116] . Patients whose myeloma cells expressed high levels of PDL1 had higher MM cell infiltrating their BM compared with other MM patients. In addition to the T cell inhibitory effect, PDL1 molecules on myeloma cells are associated with aggressive cell behavior, including increased proliferative potential and resistance to chemotherapeutic drugs[113]. Therefore, PD1/PDL1-mediated signals are involved in the pathophysiology of myeloma, and modulating this pathway may have therapeutic potential in myeloma patients.

MULTIPLE MYELOMA: THE CURRENT STATUS IN THERAPY

The therapeutic landscape of MM has evolved over the past decade with the discovery and validation of proteasome inhibitors and immunomodulatory agents[117,118]. Current therapies for MM often cause remissions, but most patients eventually relapse and die[119,120]. There is substantial evidence for an immune-mediated elimination of MM cells in patients after allogeneic hematopoietic stem cell transplantation (alloHSCT); however, the toxicity of allo-HSCT is high, and few patients are cured so that alternative treatments are needed[120,121]. In addition, MM is most prevalent in elderly patients, the majority of whom are ineligible for HSCT (allo or auto). In the spectrum of new agents in development for the treatment of MM, monoclonal antibodies (mAbs), bispecific antibodies (bsAbs), antibody drug conjugates (ADCs) and chimeric antigen receptor T-cell therapy (CAR-T) have emerged as a potential strategy, taking advantage of specific surface antigens highly expressed on malignant cells and mostly absent from other tissues[119]. Approved drugs used for the treatment of MM are summarized in Table 4.

Table 4. Approved drugs for MM treatment.

Drug Class	***Generic Drug Name***	***Mechanism of Action***
Immunomodulatory drugs	*Thalidomide, Lenalidomide, Pomalidomide, Iberdomide, Mezigdomide*	*Bind to an E3 ubiquitin ligase complex, which ubiquitinates disease-related proteins resulting in their proteasome degradation.*
Proteasome Inhibitors	*Bortezomib, Carfilzomib, Ixazomib*	*Inhibition of the 20S proteasome. Drug effect is reversible for bortezomib and ixazomib, irreversible for carfilzomib.*
Unconjugated monoclonal Antibodies	*Daratumumab, Isatuximab*	*CD38 mAbs. Induce of *ADCC, apoptosis and *CDC. CD38 is expressed MM-PCs.*
	Elotuzumab	**SLAMF7 mAbs*
Antibody-drug conjugate	*Belantamab mafodotin*	*BCMA mAb conjugated with a* *MMAF, an antimitotic agent that blocks the polymerization of tubulin.
Bispecific antibody	*Teclistamab*	*BCMAxCD3 IgG4*
Selective inhibitors of nuclear export	*Selenixor*	*Induce apoptosis of MM-PCs. Suppress the export of tumor suppressor proteins and growth factors from the nucleus by blocking exportin 1.*
Chimeric antigen receptor T cells	*Decabtagene Vicleucel*	*Autologous BCMA *CAR-T cells, infuse in patient after lymphodepleting chemotherapy. CAR-T cells product in the patient recognizes and kills tumor cells, leading to expansion of the CAR T cells in a patient.*
Alkylators	*Melphalan +cyclophosphamide*	*Nitrogen mustard compounds alkylators induce cellular death by crosslinking cancer cells DNA.*
Glucocorticoids	*Dexamethasone*	*Indirectly repress target genes via interaction with *NF-κB and *AP-1*

**ADCC antibody dependent cell-mediated cytotoxicity; CDC: complement dependent cytotoxicity; SLAM7, surface antigen CD319; MMAF, monomethyl auristatin F; CAR-T cells, Chimeric antigen receptor T cell; NF-κB, nuclear factor kappa B; AP-1, activator protein 1. Adapted from Cowan et al.*[10]

BCMA-BASED CURRENT THERAPIES

RATIONALE TO TARGET BCMA IN MULTIPLE MYELOMA

BCMA is highly expressed on MM-PCs and the serum levels of its ligands, APRIL and BAFF, are a mean 5-fold higher in MM patients than in healthy donors[122–124]. A study has shown that combining anti-BCMA immunotherapy with inhibition of APRIL, allows to reinforce T-cell cytotoxicity against MM-PCs and counteract the immunosuppressive status of MM-BM microenvironment[125].

In addition, high levels of serum sBCMA correlate with the levels of PCs infiltration in the BM and sBCMA is considered a marker of prognosis and treatment response. Previous studies reported that patients with favorable response to therapy show lower levels of sBCMA compared to patients with progressive disease. In addition, patients with high levels of sBCMA in the early stage of the disease, have a major risk of progression to symptomatic MM[126,127]. Since sBCMA is derived from the shedding of membrane BCMA by ɣ-secretase, its high serum levels may reflect a high tumor burden. In addition the reduced surface density of BCMA on MM-PCs may result in reduced efficacy of BCMA-targeted therapies[128]. Finally, sBCMA may inhibit anti-BCMA based therapies. To overcome this challenge the combination of a ɣ-secretase inhibitor with a BCMA-targeting therapy in MM is now under investigation in clinical trials[129,130]. Taking together these data make BCMA an ideal target for the

treatment of MM, with the caveat that temporary ɣ-secretase inhibition during treatment may help increase efficacy.

Current novel BCMA-based therapies for MM are represented by: antibody-drug conjugates (ADCs), bispecific T cell engagers (TAAxCD3), and chimeric antigen receptor T cells (CAR-Ts).

BCMA ANTIBODY DRUG CONJUGATES

ADCs are mAbs targeting a tumor antigen covalently linked to a cytotoxic agent capable of mediating cancer cell death. Among ADCs, the FDA has currently approved for MM Belantamab Mafodotin (Bel, GSK2857916, anti-BCMA-MMAF ADC)[131]. Bel is an afucosylated humanized IgG1 directed against BCMA and covalently linked to MMAF (microtubule inhibitor monomethyl auristatin F)[132]. Bel binds the BCMA on MM-PCs membrane and after its internalization within cells, releases MMAF which causes cell-cycle arrest and apoptosis[133]. The presence of a defucosylated Fc region, on Bel's structure, allows the binding of effector cells and consequent induction of antibody-dependent cellular cytotoxicity and phagocytosis [134].

BISPECIFIC T CELL ENGAGERS

Bispecific T cell engagers (TAAxCD3) for MM are engineered to have a dual specificity for a MM-PCs antigen and patient's T cells [134]. An approved TAAxCD3 for the treatment of relapsed and refractory MM is Teclistamab (Tec, JNJ-64007957). Tec is an IgG4 bispecific antibody binding the CD3 receptor on T-cells and BCMA on neoplastic PCs[133].

Due to its dual binding sites, Tec engages $CD3^+$ T cells close to $BCMA^+$ cells, resulting in T cell activation and T cell-mediated cytotoxicity. This effect does not depend on T cell receptor specificity or reliance on major histocompatibility complex (MHC) Class 1 molecules on the surface of antigen presenting cells and is therefore very effective in activating all T cells[133]. Other TAAxCD3 presently under investigation are Elranatamab (PF-06863135, BCMAxCD3)[135],TNB-383B (BCMAxCD3)[136], and Alnuctamab (CC-93269)[137]. Recently, novel tri-specific molecules targeting BCMA are under investigation in preclinical studies. Vrohlin et al described the anti-myeloma potency of a trispecific molecule CD3xBCMAxPDL1. Engagement of T cells against BCMA positive neoplastic cells, combined with the blocking of the PD1/PDL1 pathway, improves MM-PCs killing, when compared to a BCMAxCD3 bispecific antibody treatment, in a human MM xenograft mouse model[138]

BCMA CAR-T CELLS

CAR-Ts are genetically modified T cells that express a CAR directed against a specific tumor antigen. A CAR molecule is generally constructed with an extracellular scFv (single-chain variable fragment), targeting the tumor antigen, joined to a CD3ζ intracellular signaling domain, in sequence with co-stimulatory molecules (CD28 or 4-1BB)[139]. This structure allows to efficiently redirect the modified T cells cytotoxicity specifically towards cancer cells. To obtain a CAR-Ts product, usually autologous T cells, derived from a patient's leukapheresis, are transfected with

the CAR by using a viral vector and are expanded ex vivo. Autologous CAR-Ts are infused in patient after lymphodepleting chemotherapy in order to favor CAR-Ts expansion and activity[133.]

BCMA represents an ideal target for CAR-Ts therapy. Indeed the FDA has currently approved two autologous BCMA-targeting CAR-Ts: Idecabtagene Vicleucel (Ide-Cel, bb2121)[140] and Cilta-cel ciltacabtagene autoleucel LCAR-B38M/JNJ-4528 (Carvykti)[141]. Ide-Cel is approved for the treatment of MM patients relapsed after four lines of therapy (including a proteasome inhibitor, an Immunomodulatory drug, and an anti-CD38 mAb)[142] and is now under investigation in clinical trials to assess its use in in first-line therapy[143] or at early relapse[144]. Ide-Cel is a CAR-T that carries a scFv targeting BCMA, a CD3ζ signaling domain and a costimulatory domain. Its efficacy appears to be independent of BCMA and sBCMA expression levels[140].

Cilta-cel is a CAR-T that carries two scFvs in tandem targeting BCMA, a CD3-ζ signaling domain and a 4-1BB costimulatory domain[141]. Cilta-cel was authorized by the FDA in February 2022, as therapy for MM patients relapsed and refractory after more than 4 lines of therapy (including a proteasome inhibitor, an Immunomodulatory drug, and an anti-CD38 mAb[145]). Other CAR-Ts products are now being evaluated in clinical trials with promising results. In particular encouraging data are reported in an early-phase clinical trial with bispecific CAR-Ts targeting BCMA and CD38[141,146] .

BISPECIFIC ANTIBODIES FOR CANCER THERAPY

GENERAL ASPECTS OF BISPECIFIC ANTIBODIES STRUCTURE AND MECHANISM OF ACTION

The 2 Fab arms of a natural antibody are usually monospecific and bear two identical antigen-binding sites. Engineered bsAbs are bivalent and harbor two different antigen-binding sites. BsAbs can be produced by a variety of methods, including fused hybridomas and genetic engineering methods[147 148].

The design and manufacturing technologies of bsAbs is complex, because natural IgGs are made of pairs of heavy and light chains, which are bound together non-covalently and assemble during IgG synthesis in the cells. Thus, forcing the correct and stable association of potentially two different heavy and light chains in order to obtain bispecific molecules is complex. One particular means to induce correct pairing is to make use of scFv technology (Figure 13). Using this technology, it is possible to combine the variable sequence of H chain and L chain in a single polypeptide chain, introducing a linker sequence between them, in order to produce a single chain molecule with all the properties of the Fv fragment of a natural antibody (Figure 13). The $scFv_s$ are widely used units to make CARs as well as bispecific antibodies, since they can be linked in tandem in multiple units or can be attached to one end of a natural IgG heavy chain or to other elements derived from different proteins.

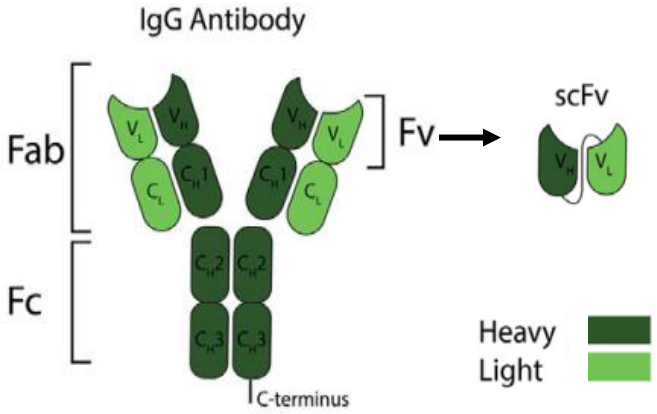

Figure 13. Schematic overview of an IgG antibody and of a scFv. *An IgG antibody consists of Fab and Fc regions. The binding part of the Fab region is called the single chain variable fragment (scFv). Adapted from Suurs et al* [149]*.*

In order to achieve the correct pairing of H and/or L chains to produce bsAbs carrying or not scFv fragments, a number of technologies have been devised during the last decades [149], some of which are shown in Figure 14 and 15.

Two classes of bsAbs can be distinguished according to the presence or absence of the Fc region: the IgG-like and non-IgG-like bsAbs, respectively[150]. IgG-like bsAbs (Figure 14) bear an Fc fragment, which facilitates the purification procedure and enhances the stability of the bsAbs in vivo. Indeed, IgG-like bsAbs have a longer half-life in vivo, up to several weeks, as compared with non-IgG-like bsAbs, which have a half-life of hours or at maximum days. The Fc fragment of IgGs, in particular of human IgG1, interacts with effector molecules and receptors on immune cells and is necessary for the IgGs to mediate ADCC,

CDC and ADCP. In addition, IgG Fcs interact with the neonatal FcRn which favors recycling of IgGs through the cells rather than degradation, thus prolonging significantly IgG half-life in vivo [150].

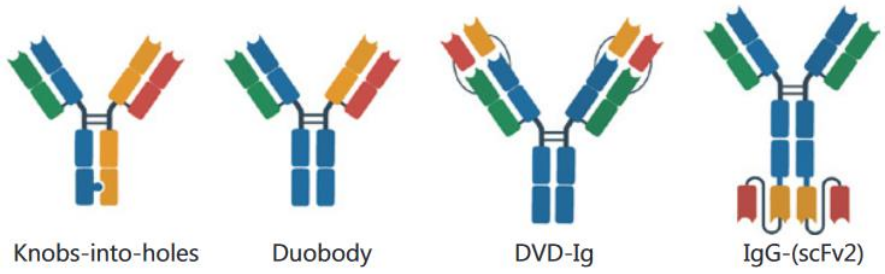

Figure 14. Schematic diagram of representative IgG-like bsAbs. *In this cartoon are depicted some IgG-like BsAb constructs, obtained with different platform technologies developed to prevent random association of H and L chains. DVD-Ig, dual-variable-domain immunoglobulin; scFv, single-chain variable fragment. Adapted from Zhang et al [150].*

Non-IgG-like bsAbs class (Figure 15), in particular are often based on $scFv_s$, are smaller molecules (they lack Fc) and can therefore be simply manufactured, for example also in prokaryotic cells. They rely on genetic engineering techniques only, allowing to customize the number and specificities of antigen binding sites. Examples of non-IgG-like bsAbs are shown in Figure 15.

Figure 15. Schematic diagram of representative Non-IgG-like bsAbs. *In this cartoon are depicted some Non-IgG-like bsAb constructs, obtained with different platform technologies developed to prevent random association of H and L chains. BiTE, bispecific T-cell engager; DART, dual-affinity retargeting molecule; TandAb, tandem diabody. Adapted from Zhang et al* [150]*.*

SPECIFICITIES OF BISPECIFIC ANTIBODIES

BsAbs have a dual specificity, thus depending on the nature of targeted antigens they can trigger different mechanisms of action *Zhang et al.* [150]:

1. bispecific T cell engagers (TAAxCD3) direct cytotoxic effector T cells against neoplastic cells;
2. bsAbs may act as agonist of co-stimulatory receptors of effector T cells, such as ICOS and OX-40. Hence, this type of bsAbs amplify the initial activating signals provided to effector T cells from TCR;
3. bsAbs directed against two inhibitory immune checkpoints induce the activation and revitalization of effector T cells toward neoplastic cells more efficiently than antibodies directed against a single checkpoint inhibitor;
4. bsAbs targeting two tumor antigens allow to more effectively and specifically target neoplastic rather than normal cells; this is especially true if the TAA are

expressed at low levels on tumor cells or are also expressed to some extent on normal cells;

5. bsAbs may inhibit other signaling pathways that favor tumor growth or metastasis, such as angiogenic factors or TGF-β signaling; this targeting will therefore add a function compared to a standard mAb

BISPECIFIC ANTIBODIES TARGETING IMMUNE CHECKPOINTS

BsAbs targeting immune checkpoints can be classified in three categories: targeting two inhibitory checkpoints, targeting co-stimulatory and inhibitory checkpoints and targeting immunomodulatory checkpoints and non-checkpoint targets [150].

BISPECIFIC ANTIBODIES TARGETING DUAL INHIBITORY IMMUNE CHECKPOINTS

Checkpoint inhibitors have recently been developed as important regulators of cancer, in both solid and hematopoietic tumors[151,152]. In patients with advanced squamous-cell non-small-cell lung cancer, Nivolumab (an anti-PD1 mAb), significantly increases survival, response rate, and progression-free survival compared to the standard chemotherapy treatment with docetaxel[151]. In addition, Nivolumab has been shown to have substantial therapeutic activity and an acceptable safety profile in patients with relapsed or refractory Hodgkin's lymphoma [152]. The checkpoint inhibitors Nivolumab and Ipilimumab (an anti- CTLA-4 mAb) have been shown to have

complementary activity in metastatic melanoma [153]. Unfortunately, the use of a combined immune checkpoint blockade therapy frequently causes acute immune-related adverse effects and an MHC restriction of the TCR, exacerbating immune escape[154,155]. In addition, the use of mAbs like Ipilimumab and Nivolumab, is frequently associated with the onset of severe autoimmune disease especially when a systemic strategy is designed to deplete Tregs[156]. In order to trigger potent anti-tumor immune responses, while reducing toxicity and avoiding Treg depletion in normal tissues, the use of bsAbs, targeting two inhibitory checkpoints, is being currently investigated, for example CTLA-4 and PD-1[157]. Farhangnia et al.[157] have recently published a review summarizing the clinical trials currently investigating the efficacy of bsAbs targeting CTLA-4 in combination with other inhibitory checkpoints in various types of cancer. These trials report positive clinical outcomes in terms of prolonged anti-tumoral response and reduced immune-related toxicities. However, further investigations are needed to clarify questions related to the safety, effectiveness, and the range of tumors that could be treated[157].

BISPECIFIC ANTIBODIES TARGETING CO-STIMULATORY AND INHIBITORY CHECKPOINTS

Targeting co-stimulatory and inhibitory checkpoints can facilitate bridging of effector T cells to cancer cells with consequent proliferation and reinvigoration of T cells, hence maximizing the

therapeutic response. Kuang et al [158] in their report described the effect of an OX40xPD-L1 bsAb in different tumor mouse models. OX40 is an inducible co-stimulatory checkpoint, transiently expressed after TCR engagement on CD4+ and CD8+ T cells and constitutively expressed on Treg in the tumor microenvironment. They described the ability of OX40xPD-L1 bsAb to boost T cell functions and to induce a dose-dependent anti-tumor response.

Another example of co-stimulatory x inhibitory checkpoints bsAb is reported by Sainson et al[159]. The authors studied the effects of KY105, an ICOSxPDL1 IgG1 bsAb that activates ICOS in a PD-L1-dependent manner. ICOS is a co-stimulatory checkpoint constitutively expressed after activation of TCR. They demonstrated the capability of KY105 to deplete ICOS+ Treg cells and increase IFN-γ secretion.

BISPECIFIC ANTIBODIES TARGETING INHIBITORY CHECKPOINTS AND NON-CHECKPOINT TARGETS

A therapeutic approach targeting tumor antigens/growth factors with immunomodulatory checkpoints can increase the tumor-killing effects, specifically in the tumor microenvironment expressing the targeted molecules, rather than in normal tissues. BsAbs that target cytokines activate T cells and reduce drug resistance[150]. Recently encouraging data have been reported on bsAbs targeting immunomodulatory checkpoints with tumor antigens (such as EGFR[160]) and growth factors (such as TGFβ[161]).

Encouraging preclinical and clinical data are available on molecules targeting simultaneously TGF-β and PDL1[161]. TGF-β prevents tumor expansion in the early stages, but enhances metastasis and drug resistance in late stages [150]. The literature reports that the activation of TGF-β pathways reduces the effects of anti-PD-1/PD-L1 monotherapy [162], reinforcing the rationale to simultaneously block TGF-β and PDL1. Currently, at least 2 bsAbs targeting PD-L1 and TGF-β (M7824 YM101 and BiTP), are being developed[161]. Preclinical and clinical studies report potent anti-tumor activities of these bsAbs with manageable side effects[163–166]. Indeed, with the dual blocking of PDL1 and TGF-β, it may be possible to switch tumors from an immune-exclusion into immune-inflamed state[161].

SCOPE OF THE THESIS

The aim of my PhD thesis was to identify and characterize useful antigens to be used as targets for a novel therapeutic bispecific antibody (bsAb) for multiple myeloma (MM), as well as to design, produce and characterize functionally in vitro this novel bsAb.

The antigens identified are a tumor associated antigen (TAA), BCMA (CD269), expressed specifically in normal and neoplastic plasma cells, as well as checkpoint inhibitor, PDL1, which may be expressed by MM cells and immune cells of the tumor microenvironment.

The project planned the following investigations:

1. To analyze the expression of BCMA in B cell neoplasias and its regulation and shedding by the ubiquitous, membrane-associated enzyme ɣ-secretase. As part of this study, we identified a single MM patient with high and dysregulated BCMA expression and characterized in more detail genetically this single patient MM cells, in a case analysis and report.
2. To analyze the expression of PD1/PDL1 in B cell neoplasias that are BCMA positive, i.e. MM and more differentiated B-NHL.
3. To design, clone, produce and purify a novel IgG1-like, tetravalent bsAb directed against BCMA and PDL1 and bearing a fully functional human Fc.

4. To measure accurately the antigen binding affinities of the bsAb in comparison with the parent mAbs, towards the natural, full-length and cell-associated antigens, as well as towards the recombinant extracellular fragments thereof, in isolation or bound simultaneously. This was planned in order to measure accurately the relative binding affinities of the 2 Fabs, measure their simultaneous binding to the 2 antigen targets and the possible structural interaction between the two antigen binding sites of the bsAb.
5. To demonstrate the functionality of the 2 Fabs of the BCMAxPDL1 bsAb, i.e. their capacity to inhibit the function of the BCMA ligand APRIL and of the PDL1-PD1 axis in T cell activation assays.
6. To investigate the functionality of the bsAb Fc moiety, i.e. the capacity of the molecule to induce in vitro either complement dependent cytotoxicity (CDC) and/or antibody dependent cellular cytotoxicity (ADCC) mediated by NK cells.
7. To measure the functionality of the molecule altogether, and its capacity to mediate the killing of BCMA-positive MM target cells in vitro via both its Fabs and Fc moieties, in presence of a cocktail of immune effector cells in long term assays that may, in part, mimic the in vivo microenvironment.

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CHAPTER 2

DEVELOPMENT OF A BISPECIFIC IgG1 ANTIBODY TARGETING BCMA AND PDL1

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Submitted to Antibodies

ABSTRACT

We designed, produced and purified a novel IgG1-like, bispecific (bsAb) antibody directed against BCMA, expressed by multiple myeloma (MM) cells and an immune checkpoint inhibitor (ICI), PDL1, expressed in the MM microenvironment. The BCMAxPDL1 bsAb was fully characterized in vitro in comparison with the parent mAbs. BCMAxPDL1 bsAb bound specifically and simultaneously, with nM affinity, to both native, and cell-associated membrane-bound antigens and to the recombinant soluble antigen fragments, as shown by immunophenotyping analyses and surface plasmon resonance (SPR), respectively. Binding affinity to PDL1 was about 10-fold lower in the bsAb compared to equivalent mAb, in part due to steric hindrance associated with the more internal anti-PDL1 Fab. Affinity for PDL1 in the bsAb format was however similar to that for BCMA, even during simultaneous binding of the 2 antigens. The bsAb was able to functionally block both antigen targets with IC_{50} in the nM range, i.e. to inhibit binding of APRIL to BCMA and to block PD1-PDL1 interaction, thus inducing TCR-dependent T cell activation in a bioassay. The bsAb Fc was functional, inducing human complement dependent cytotoxicity as well as ADCC by NK cells in 24 hours killing assays. Finally, BCMAxPDL1 was effective in 7 days killing assays with peripheral blood mononuclear cells as effectors, inducing up to 75% target MM cell line killing at a physiologically attainable, 8 nM, concentration. The fine analysis of binding and function of the

BCMAxPDL1 bsAb *in vitro* provides the necessary data to perform future *in vivo* studies of this novel bsAb in mouse models.

INTRODUCTION

Although a number of non-conjugated monoclonal antibodies (mAbs) have demonstrated significant therapeutic activity against cancer, many others have proved not to be sufficiently effective[1–3]. Bispecific antibodies (bsAbs) allow the targeting of two different antigens, thus adding another function to the drug compared to mAbs. BsAbs can also be trifunctional, for example if the chosen Fc domain is that of a human IgG1, capable of binding FcγRs and activating immune cells. Fc also binds to FcRn and prolongs antibody half-life *in vivo* through recycling[4]. Thus, the choice of specificities and overall structure of novel bsAbs has to consider the final biological activities that are desired from the drug. Relative affinities of the two arms of a bsAb are also important elements to consider during development, according to the desired function of the molecule and properties of the antigens that are targeted[5].

We have recently developed a novel platform for bsAb engineering, that allows us to produce IgG-like, Fc-bearing bsAbs with bivalent binding to each chosen antigen[6]. Using this platform, we have set out to design a novel bispecific molecule to target multiple myeloma (MM). We have focused our attention on BCMA as primary specificity, because this molecule is expressed by mature normal and neoplastic B cells, including

MM, plasma cell leukemia (PCL), as well as some differentiated diffuse large cell lymphomas (DLBCL), but mostly absent from other tissues[7,8]. However, BCMA is expressed at relatively low levels on MM and DLBCL cells, in part due to shedding by membrane-associated ɣ-secretase[9,10]. Different approaches have been applied to develop anti-BCMA therapeutics, in particular T cell engaging bispecifics, antibody-drug conjugates and CAR-T cells, each of which may have advantages and limitations[11–13].

Immune checkpoint inhibitors (ICI) may be expressed by tumor cells or their microenvironment and diminish the activity of IgG1 therapeutic mAbs. MM and DLBCL cells or tissues often express PD1 and/or its ligand PDL1. We reasoned that adding an anti-PDL1 moiety to an anti-BCMA antibody in a bispecific format may thus allow blocking the PD1/PDL1 axis within the tumor, since the anti-BCMA moiety would concentrate the antibody mostly in the tumor bed, potentially increasing efficacy of the anti-BCMA and reducing the toxicity of the anti-PDL1 moiety. A BCMAxPDL1 bsAb was thus designed and produced, based on the IgG1 format developed and patented in our laboratory[6]. We here describe the construction of this novel tetravalent and trifunctional bsAb and characterize its antigen binding and functional activities *in vitro*.

MATERIALS AND METHODS

Therapeutic antibodies

The construction of the transfer vectors carrying light chains and fused or standard chimeric IgG1 heavy chains for bsAb and mAb was performed by genetic engineering, essentially as described previously (patent WO/2013/005194)[6,14]. The antibodies were based on the VH/VL sequences of anti-BCMA J22.9 antibody (patent WO 2014/068079)[15] and anti-PDL1 atezolizumab (MPDL3280A, US8217149B2). The generation and cloning of recombinant baculoviruses expressing 2 chains (mAbs) or 3 chains (bsAb), antibody expression in insect Sf9 cells and purification were performed as described[6,14,16]. The following antibodies were investigated: anti-BCMA, anti-PDL1, anti-BCMAxPDL1 produced in Sf9 cells and purified at CNRS. In some experiments, commercially available anti-PDL1 atezolizumab (Tecentriq®, Roche, Basel, Switzerland) was used as control. IgG mix from human serum (Sigma-Aldrich, St Louis, MO, USA), was used as reference for SPR analysis. Anti-CD38 daratumomab, anti-CD20 rituximab, anti-EGFR cetuximab and commercial atezolizumab were obtained from the local Pharmacy.

Cells

Peripheral blood (PB) or bone marrow (BM) samples were collected in EDTA from healthy donors or patients diagnosed with MM or B-NHL, after informed consent and local ethical committee

study approval. Samples were collected in accordance with the Declaration of Helsinki of 1975, as amended in 2013. Cells (MNCs) were purified by standard Ficoll-Hypaque gradient centrifugation (Seromed, Biochrom AG, Berlin, Germany). In some cases, MM cells from BM were first purified on anti-CD138 immunoaffinity columns (AutoMACS, Miltenyi Biotec, Bergisch Gladbach, Germany). Cells were either used immediately or cryopreserved in 10% DMSO (Li StarFish, Milano, Italy) for later use.

The human cell lines used in the present study were: CEM (acute lymphoblastic leukemia, ALL); HDLM2 (Hodgkin lymphoma, HL); MEC-1 (chronic B leukemia, B-CLL); REH, TOM-1 and 697 (precursor B cell acute lymphoblastic leukemia, pre-B ALL); GRANTA 519 and JEKO (mantle cell lymphoma, MCL); KARPAS 422, DOHH2, SU-DHL4, SU-DHL16 and RCK8 (Diffuse large B cell lymphoma, DLBCL); ALBANES, BJAB, RAMOS, RAJI and NAMALWA (Burkitt lymphoma, BL); PA698 and AS238 (AIDS derived non-Hodgkin B-cell lymphoma, AIDS-NHL); EBV-LCL, IM9 and SKW6.4 (Epstein-Barr virus immortalized lymphoblastoid B-cell lines, EBV-LCL); KMS11, KMS12, KMS18, KMS20, H929, OPM2, RPMI 8226, and U266 (multiple myeloma, MM). All cell lines were maintained in RPMI-1640 medium (Life Technologies, Carlsbad, CA, USA), supplemented with 10% fetal bovine serum (FBS, Euroclone, Milan, Italy), and 2 mmol/L L-glutamine (Euroclone), except HDLM2, TOM-1, DHL4 and RAJI which were supplemented with 20% FBS. Cell lines were routinely tested for the absence of mycoplasma.

Production of stable BCMA transfectants

BJAB cells were stably transfected with GFP-tagged full-length BCMA cDNA by infection with the BCMA-Lenti ORF-Myc/DDK/GFP tagged lentivirus (Origene, Herford, Germany), following the manufacturer's instructions. The BCMA-Lenti ORF encodes a puromycin antibiotic resistance gene for selection in mammalian cells. Stably infected cells were selected by supplementing the complete medium with 0.5 µg/ml of puromycin antibiotic, until >95% of BJAB-BCMA$^+$ expressing cells was reached. BCMA expression was verified at various times and prior to experiments, by assessing the percentage of GFP and/or BCMA positive cells by flow cytometry (see below).

CEM cells ($1.5x10^6$) were stably transfected with an expression plasmid bearing the full-length human BCMA cDNA (3 µg pUNO1-TNFRS17, InvivoGen, San Diego, CA, USA) using the nucleofector kit V and Amaxa IIb nucleofector device (Lonza, Basel, Switzerland). After 15 days of expansion, transfected CEM-BCMA$^+$ cells were purified by staining with a mouse anti-human BCMA antibody (Biolegend, San Diego, CA, USA) followed by FITC-labelled goat anti-mouse IgG secondary antibody (BD Biosciences, San Jose, CA, USA), and immunoselection with anti-FITC magnetic beads (Miltenyi Biotec). After two rounds of immunoselection, a purity >94% of CEM cells stably expressing BCMA was obtained. Expression was stable over time.

Flow cytometry

mBCMA expression on cell lines was analyzed after overnight cell culture in the presence or in the absence of 1 µM N-[N-(3,5-Difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester (DAPT, Selleckchem, Cologne, Germany). Detection was done by direct immunofluorescence and flow cytometry, using an anti-human BCMA-PE (clone 19F2, Biolegend) antibody.

Binding affinity of home-produced mAbs and bsAbs was measured by indirect immunofluorescence. The KMS11 ($BCMA^+$) or HDLM2 ($PDL1^+$) cell lines were incubated with increasing and equimolar concentrations of bsAb or mAbs (from 1.2 nM to 160 nM), washed and labelled with excess ($25x10^6$ nM) FITC-labelled anti-human Fc, mAb (clone HP-6017, Sigma-Aldrich). Binding was measured by flow cytometry on a FACSCanto II instrument (BD Biosciences).

Either BM-derived mononuclear cells (MNC-BM) or $CD138^+$ purified cells were used for immunophenotypic analysis of primary MM. Peripheral mononuclear cells (PBMCs) were used in the case of B-NHL. Primary cells were cultured overnight in StemSpan SFEM medium (Stemcell Technologies, Vancouver, Canada), supplemented with 10% FBS in presence or absence of 1 µM DAPT. After incubation, cells were stained with a mouse anti-human BCMA-PE-CY7 (clone 19F2, Biolegend) and mouse anti-human CD138-FITC, CD38-PerCp and CD19-APCH7 (BD Biosciences) (for MM cells) or mouse anti-human CD19-APCH7 and CD20-V450 (BD Biosciences) (for B-NHL). In some cases,

MNC-BM and PBMCs were also characterized for the expression of PD1 and PDL1 on neoplastic cells, T-lymphocytes and monocytes by direct staining with mouse anti-human PD1-APC (clone MIH4), PDL1-PE (clone MIH1), CD3-APCH7 and CD14-PerCp antibodies (BD Biosciences). For all antibodies, respective isotype controls were used. Stained cells were analyzed on a FACSCantoII cytometer.

Surface Plasmon Resonance (SPR)

SPR analysis was carried out with the ProteOn XPR36 Protein Interaction Array system (BioRad, Milan, Italy). The system allows for immobilization of up to six ligands on parallel lanes of the same sensor surface (including a reference channel). The flow channels can be rotated 90° so that up to six analyte solutions can be flowed in parallel on all the immobilized ligands[17].

MAbs and bsAb were immobilized through an amine-coupling process on the surface of a GLH CMD 700L sensor chip (XanTec bioanalytics, Düsseldorf, Germany), as previously described[18]. Briefly, the chip surface was activated by flowing a solution made with 50 mM N-hydroxysuccinimide and 400 mM 1-ethyl-3 -(3-dimethylaminopropyl)-carbodiimide (NHS/EDC), for 5 min at 30 µl/min. Then, the antibodies were flowed for 5 min at 30 µl/min, at the concentration of 30 µg/ml in sodium-acetate pH 5.0 (NaOAc). The remaining activated carboxyl groups were deactivated by flowing 1 M ethanolamine for 5 min at 30 µl/min. Immobilization levels were 1359, 2400 and 4120 resonance units

(RU, where 1000 RU = 1 ng protein/mm^2), for anti-BCMA, anti-PDL1 and BCMAxPDL-1 bsAb, respectively. A reference surface was prepared in a parallel channel of the same chip flowing commercial, unrelated IgGs, which immobilized at a level of 2352 RU. After chip rotation, the following analytes were injected simultaneously on all the immobilized antibodies: recombinant human BCMA extracellular domain Fc chimera (R&D system, Minneapolis, Minnesota, USA; indicated hereinafter as recBCMA) and recombinant human PDL1 extracellular domain-Fc chimera (R&D system, indicated hereinafter as recPDL1), diluted in SPR running buffer (Dulbecco's Phosphate Buffered Saline with 0,005 % Tween-20) at different concentrations, as indicated. Analytes flowed over immobilized ligands for 3 min at a rate of 30 µL/min. Dissociation was measured in the following 5-25 min. The SPR signals on the sensorgrams, expressed as RU, were corrected by subtracting the nonspecific response in the reference channel. The kinetic parameters, association and dissociation rate constants (k_a and k_d in table 1) and the equilibrium dissociation constant (K_D) were obtained using the Langmuir model globally fitting to entire sensorgrams (association and dissociation phases).

April binding inhibition

CEM-$BCMA^+$ cells ($2x10^5$) were incubated with equimolar doses of BCMAxPDL1 bsAb or BCMA mAb (2.2 nM to 60 nM) for 10 minutes at 4°C. After washing, cells were incubated for 20 minutes at 4°C with 90nM of recombinant multimeric human

Flag-tagged April protein (Adipogen Life Sciences, San Diego, CA, USA) followed by FITC-labeled mouse anti-Flag antibody (Sigma-Aldrich). April binding was then analyzed by flow cytometry on a FACSCanto II instrument.

Inhibition of PD1- PDL1 signaling

To assess PDL1 functional blocking by anti-PDL1 antibodies, the cell-based PD1/PDL1 Blockade Bioassay (Promega, Madison, Wisconsin, USA) was used, according to the manufacturers' instructions. Briefly, one day before performing the assay, the $PDL1^+$ aAPC/CHO-K1 cells were seeded in a 96-well plate. The next day, $PDL1^+$ aAPC/CHO-K target cells were incubated with serial 2.5-fold equimolar dilutions of PDL1 mAbs and BCMAxPDL1 bsAb or negative control cetuximab (from 66 nM to 0.1 nM). The $PD1^+$ Jurkat T effector cells were then added and the plate incubated for 6 hours in a 37°C, 5% CO_2 incubator. The bioluminescent signal was then activated with the Bio-Glo™ Luciferase Reagent and quantified on a FLUOstar OPTIMA plate reader (BMG Labtech GmbH, Ortenberg, Germany). In some experiments, the recBCMA (33 nM) was added to the blocking antibodies. A recPDL1 was used as control.

Complement dependent cytotoxicity (CDC)

CDC was performed as previously described[14]. Briefly, BJAB-$BCMA^+$ cells were incubated overnight with 1 µM of DAPT, then plated at $2x10^5$ cells/well in a 96-well plate and exposed to equimolar concentrations of BCMAxPDL1 bsAb or mAbs, in presence of 50% pooled human serum (HS), as source of

complement. Rituximab (RTX) was used as positive control. After 4 hours of incubation at 37°C, the lysis was quantified by flow cytometry (FACSCanto II instrument) as percentage of 7-aminoactinomycin D (7AAD, BD Bioscience) positive cells.

NK mediated ADCC (24 hours killing assays)

The KMS11, OPM2 and KMS12 cell lines were used as targets, after overnight culture with 1 µM DAPT. The target cells were first stained with the 0.5 µM 5(6)-Carboxyfluorescein diacetate N-succinimidyl ester (CFSE; Sigma-Aldrich, Merck KGaA) before plating at $1x10^5$ /well in round bottom 96-well plates. Healthy donor PBMCs were then added as effector cells at a 5:1 effector (E): target (T) ratio, in presence or absence of equimolar concentration of BCMAxPDL1 bsAb or mAbs. Daratumomab was used as a positive control. After 24 hours[19], cells were harvested and stained using the GFP-certified TM Apoptosis/Necrosis detection kit (Enzo Life Science, Farmingdale, NY, USA), according to the manufacturer's instructions. Samples were analyzed by flow cytometry (FACSCanto II instrument).

Cell mortality was expressed as % cytotoxicity calculated with the formula:

$$\frac{\%\ \text{of death CFSE}^+\text{cells} \ - \ \%\ \text{spontaneous death of CFSE}^+\text{cells}}{100 \ - \ \%\ \text{spontaneous death of CFSE}^+\text{cells}}\ x\ 100$$

In some cases, NK cell activation was measured after 4 hours of co-culture of PBMC and KMS11 cells at 5:1 E:T ratio, by staining with anti-human CD56-APC (BD Biosciences) and anti-human

CD107a-PE (BD Biosciences) mAbs[14]. The degranulation of NK cells was quantified by flow cytometry (FACSCanto II instrument) as an increase in percentage of $CD107a^+$ cells in the $CD56^+$ population.

Long term cell mediated cytotoxicity (7-days killing assays)

$2.5x10^5$ KMS11 target cells, collected after overnight culture with 1 µM DAPT, were plated in 96-well dishes with healthy donor PBMC as effector cells, at a 5:1 E:T ratio. Cells were cultured in StemSpan SFEM medium supplemented with 10% FBS. 6 nM bsAb or mAbs were added and plates were put in a 37°C, 5%CO_2 incubator. After 3 days of culture cells were split at 1:2 ratio in fresh complete culture medium containing the same bsAb or mAbs. After 7 days[19,20], cells were collected and stained with 7-AAD and anti-human CD138-APCH-7 mAb (BD Biosciences). CountBright™absolute counting beads (Thermo Fisher, Waltham, MA) were added to the staining tube. Absolute count of live $CD138^+$ KMS11 target cells were analysed by flow cytometry (FACSCanto II instrument) and calculated with the formula:

$$\frac{Cells}{\mu l} = \frac{Cells\ count\ x\ Counting\ beads\ absolute\ number}{Counting\ beads\ count\ x\ sample\ volume}$$

Statistical analyses

Statistical significance was calculated using Student's t-test or Linear Regression t-test.

RESULTS

BCMA and PD1/PDL1 expression in neoplastic B cells

BCMA is known to be expressed on the surface of most MM cells. In 2015, plasma membrane BCMA (mBCMA) was shown to be actively cleaved and released from the cell surface by γ-secretase, a ubiquitous membrane protein complex[9]. This shedding results in relatively weak basal expression of mBCMA on most MM cell lines and primary samples. Blocking γ-secretase with inhibitors results in 3- to 4-fold increase in mBCMA mean fluorescence intensity (MFI) on MM and plasma cells[7,9,10]. Some reports have detected BCMA expression also on mature lymphoma cells, but this has been less systematically studied[8,21,22]. We therefore investigated by flow cytometry mBCMA expression in presence or absence of γ-secretase inhibitor DAPT in a panel of B cell lines, from immature pre-B to fully differentiated MM cells. The results are shown in Figure 1. The data confirm weak basal expression of mBCMA in all eight MM cell lines tested and strong upregulation in presence of DAPT. Among the other 21 B cell lines analyzed we observed that, in absence of DAPT, mBCMA was low or negative in most cell lines, except for one AIDS-derived B-NHL (PA682, 60% positive, Figure 1A). In contrast DAPT treatment led to >20% BCMA surface expression in 12 B-NHL and EBV-LCL lines (ranging from 20 to 90% positivity). All $BCMA^+$ positive lines were more mature lymphoma cells: 2/2 mantle cell lymphomas (MCL), 1 chronic lymphocytic leukemia (CLL), 2/5 diffuse large B cell

lymphomas (DLBCL), 2/5 Burkitt's lymphomas (BL), 2/2 AIDS-NHL and 3/3 EBV-LBL (Figure 1A). MFI analysis showed that 3 B-NHL cell lines, in presence of DAPT, expressed mBCMA at an intensity similar to that of MM cell lines (Figure 1B).

We then analyzed by flow cytometry primary samples from MM and B-NHL patients. As already described by others and by our group[23] most MM cells were positive for mBCMA but at a relatively low density, which was significantly and consistently increased by DAPT treatment (Suppl. Figure 1). The two primary Mantle Zone Lymphoma (MZL, patients 22 and 23) samples that could be analyzed also revealed low density of mBCMA expression, which was increased by ɣ-secretase inhibitor (Suppl. Figure 1). These data confirm that MM and a proportion of more differentiated B-NHL cells may be targeted by anti-BCMA drugs, at least in conditions that block ɣ-secretase activity[8,21,22].

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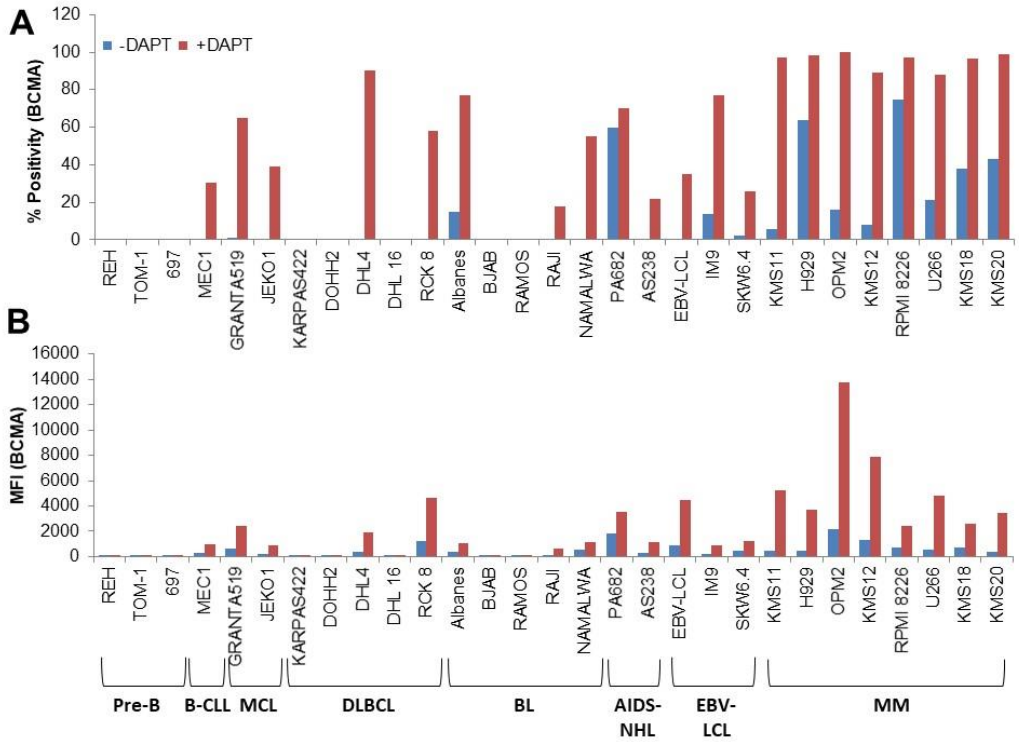
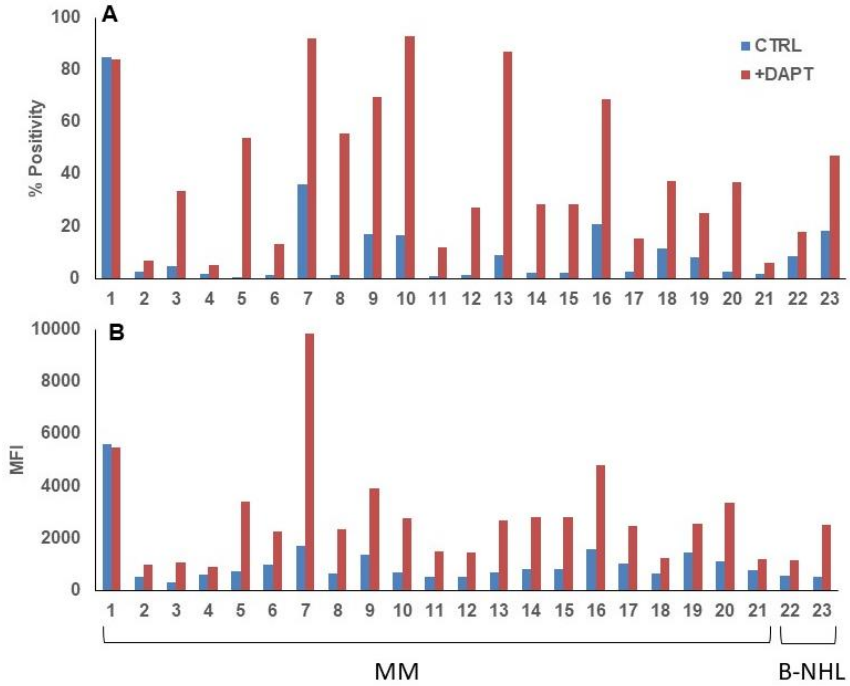


Fig. 1. Expression of mBCMA in B cell lines.

Membrane BCMA expression was assessed by flow cytometry in B cell lines cultured in presence or absence of γ-secretase inhibitor DAPT. (A) Percentage of BCMA+ cells. (B) Mean fluorescence intensity, MFI. Pre-B: precursor B cell acute lymphoblastic leukemia; B-CLL: chronic B leukemia; MCL: mantle cell lymphoma; DLBCL: diffuse large B cell lymphoma; BL: Burkitt lymphoma; AIDS-NHL: AIDS derived non-Hodgkin B-cell lymphoma; EBV-LCL: Epstein-Barr virus immortalized lymphoblastoid B-cell lines; MM: multiple myeloma.



Supplementary Fig.1. Expression of mBCMA in MM and B-NHL patients.
Membrane BCMA expression in presence or absence of γ-secretase inhibitor DAPT in MM and B-NHL neoplastic cells from 23 patients. The expression was assessed by flow cytometry. (A) Percentage of BCMA+ cells. (B) Mean fluorescence intensity, MFI. On the x-axis patients are identified with arabic numerals from 1 to 23. Patients 22 and 23 were diagnosed with marginal zone lymphoma (MZL)

We next analyzed PD1 and PDL1 expression in MM and other cells present in the MM BM microenvironment. We could access 10 BM samples from MM patients (Figure 2). The data show that PD1/PDL1 expression was very variable between samples, with only 4/10 MM samples expressing either PD1 or PDL1 on>5% of cells (Figure 2A and B). However, T-lymphocytes and/or monocytes/macrophages present in the BM of MM patients did express either PD1 or PDL1 to a significant degree in 70% of cases (Figure 2A and B). A similar heterogeneity in PD1/PDL1 expression was observed in the 3 B-NHL samples analyzed (data not shown).

These data suggest that a BCMAxPDL1 bsAb may be useful to target BCMA positive MM or B-NHL cells and stimulates the immune system within the tumor microenvironment.

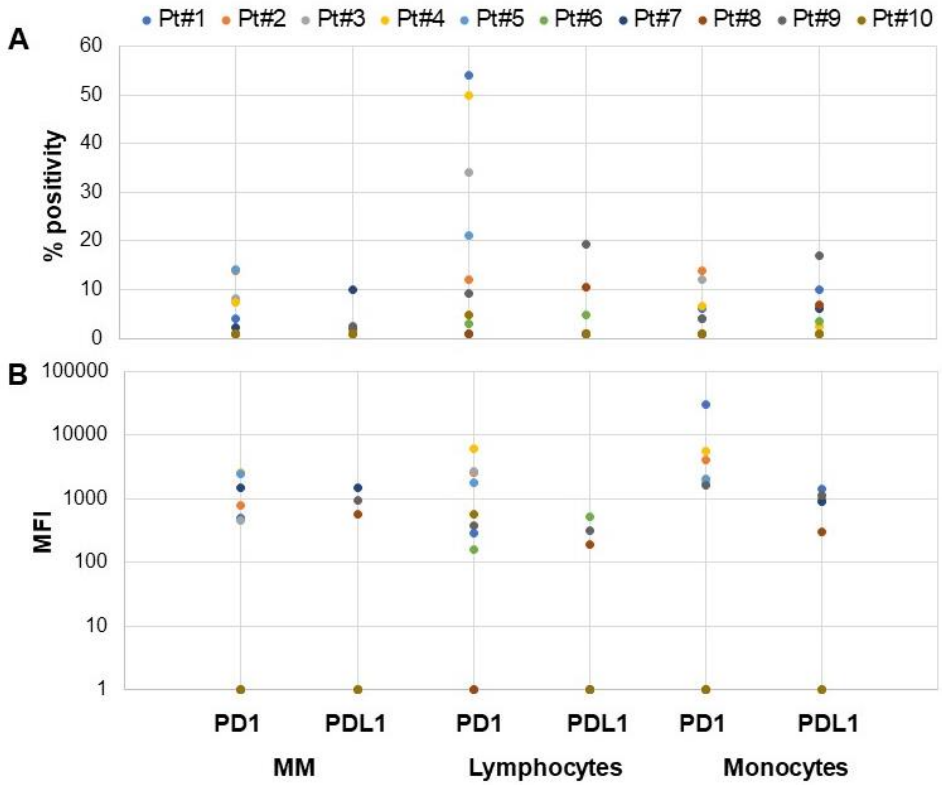


Fig. 2. Expression of PD1 and PDL1 in BM samples from MM patients.
Expression of PD1 and PDL1 in MM cells, T-lymphocytes and monocytes were analyzed in 10 MM BM samples. (A) Percentage of positive cells. (B) Mean fluorescence intensity, MFI.

Structure of the BCMAxPDL1 bsAb and affinity measurements

We designed and constructed a bsAb targeting both BCMA and PDL1, based on our patented IgG1 structure, which is bivalent for each antigen and has a fully functional IgG1 Fc[6] (Figure 3A). As expected, the bsAb was able to specifically bind to both BCMA and PDL1, as shown by staining of cell lines expressing either BCMA (KMS11) or PDL1 only (HDLM2) (Figure 3B). The binding affinity to the native antigens was also measured using the same cell lines stained with increasing amounts of the BCMAxPDL1 bsAb or parent mAbs. Binding affinity of bsAb was calculated to be about 15 nM for BCMA, similar to the affinity of parent anti-BCMA mAb (Figures 4A and C). The affinity of bsAb for PDL1 was 13 nM, whereas it was about 1 nM for the parent anti-PDL1 mAb (Figures 4B and C). This is expected since the anti-PDL1 moiety is more internal to the molecule and may experience partial steric hindrance induced by the anti-BCMA moiety, reducing affinity ([6,14] and see below).

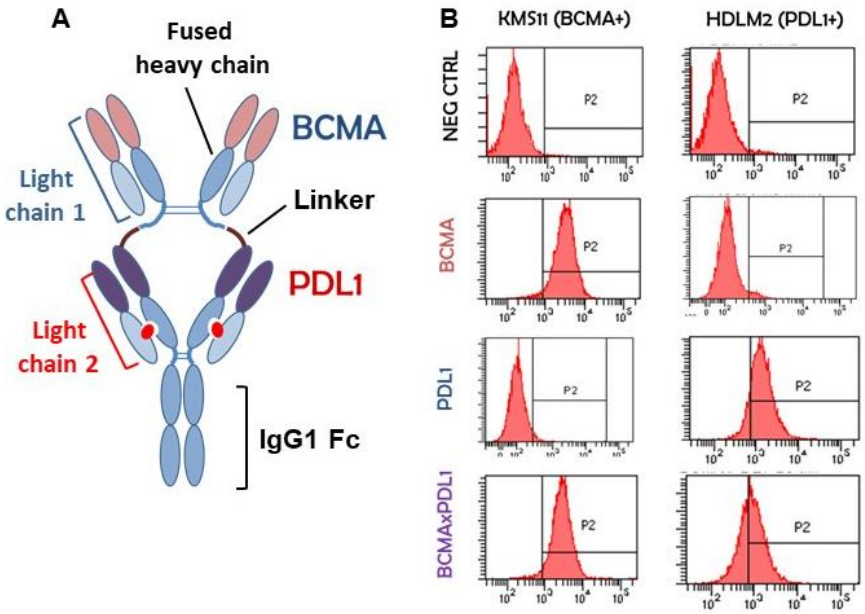
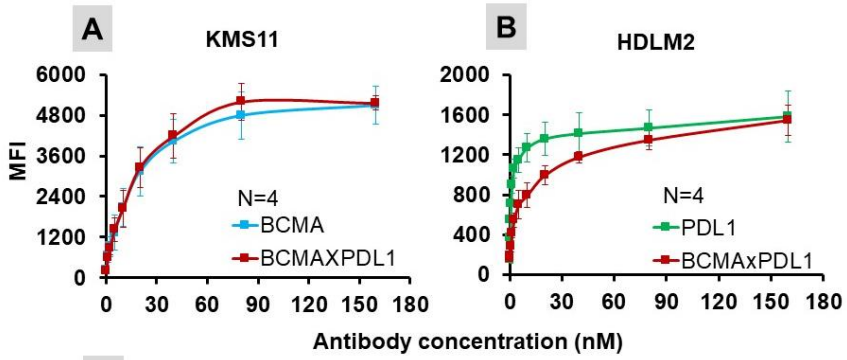


Fig. 3. Structure and specificity of binding of chimeric BCMAxPDL1 bsAb. *(A) Starting from the N-terminus, the fused heavy chain is composed of the anti-BCMA J22.9 antibody VH sequence-CH1 hinge1-linker-anti-PDL1 atezolizumab VH sequence-CH1 hinge2-CH2-CH3. The CH1 hinge-CH2 and CH3 sequences are from human IgG1. The two light chains (both k) are anti-BCMA J22.9 VL-CL and anti-PDL1 atezolizumab VL-CL. The red dot indicates the paired complementary mutations on CH1 and CL of anti-PDL1 moiety to drive correct light chain pairing. (B) Specificity of binding of the purified bsAb and respective mAbs was tested by flow cytometry on BCMA$^+$ and PDL1$^+$ single-positive cell lines, KMS11 and HDLM2 respectively.*



C

	Relative binding affinity for the antigens (nM)	
ANTIBODY	**BCMA**	**PDL1**
BCMA mAb	15	NA
BCMAxPDL1 bsAb	15	13
PDL1 mAb	NA	1

Fig. 4. Relative binding affinity of BCMAxPDL1 bsAb and respective mAbs for target antigens.
The relative affinity of the BCMAxPDL1 bsAb and anti-BCMA and anti-PDL1 mAbs was tested by flow cytometry, using increasing concentrations of primary antibodies and detection with anti-human Fc-FITC secondary antibody. (A) Binding of bsAb and mAbs to BCMA⁺ KMS11 cell line (B) Binding of bsAb and mAbs to PDL1⁺ HDM2 cell line. (C) relative binding affinities (IC_{50}) for each antigen; NA: not applicable.

In order to further characterize the antigen binding properties of the bsAb and parent mAbs, we performed SPR experiments, which allow to study in real-time the interaction between the antibodies and their targets and estimate the binding constants (k_a, k_d, K_D). All the antibodies were immobilized in parallel chambers of the same chip and the soluble recombinant extracellular domains of BCMA and PDL1 (recBCMA and recPDL1) were then injected either alone or in succession.

Representative sensorgrams with the binding profiles are presented in Figure 5 and the binding constants are summarized

in Table 1. No binding of recPDL1 was observed on immobilized anti-BCMA mAb and no binding of recBCMA was observed on immobilized anti-PDL1 mAb (Fig 5A and B). In all the other cases, we observed very high affinity interactions (sub-nanomolar KD values) characterized by very high association rate constants (ka) and very low dissociation rate constants (kd) (Fig 5A-C and Table 1).

The main findings were that:

1) recBCMA bound the bsAb with an affinity (mean K_D 19.5 pM) 5-fold higher than its affinity for the anti-BCMA mAb (K_D 106 pM);

2) recPDL1 bound the bsAb with an affinity (mean K_D 203 pM) 3-fold lower than its affinity for the anti-PDL1 mAb (K_D 71 pM);

3) the Rmax value (i.e. the maximum binding possible on the immobilized ligand) observed on the bsAb with recBCMA was higher than with recPDL1 (691 vs 241 RU on average). Taking into account the MW of the proteins (52 kDa for recPDL1 and 32 kDa for recBCMA), this means that the immobilized bsAb allows about 5-fold more binding of BCMA than PDL1. This is likely associated to the steric hindrance due to the disposition of the epitopes in the bsAb and already identified as apparent decreased affinity to native PDL1 protein in the bsAb compared to mAb (Figure 3 and 4).

4) We also observed that if recPDL1 was injected after recBCMA (Fig 5D), its binding to bsAb decreased by 15-25% (n=2 independent experiments). This decrease can be due either to a decrease of the number of available sites (decrease of Rmax by 20% on average) or a 2-fold decrease of affinity, or both. This

again is possibly due to the disposition of the epitopes in the bsAb, and/or to conformational change induced by BCMA on the more internal PDL1 epitope (Figure 3 and 4).

5) When recBCMA was injected after recPDL1 (Fig 5D), a much smaller decrease of binding (7-12%, n=2 independent analysis) was found. Similar SPR results were obtained with a variant BCMAxPDL1 bsAb carrying a slightly different linker sequence between the two moieties (data not shown).

We conclude that the internal anti-PDL1 moiety in the bsAb has a three-fold lower affinity for PDL1 in comparison to the mAb, and that this interaction is slightly reduced by previous BCMA binding. Moreover, the anti-PDL1 moiety in the bsAb appears 5-fold less accessible than the anti-BCMA moiety. Nonetheless, overall affinity and binding to native PDL1 was in the sub-nM range, similar to that to BCMA, which should allow blocking of PDL1 in the tumor microenvironment.

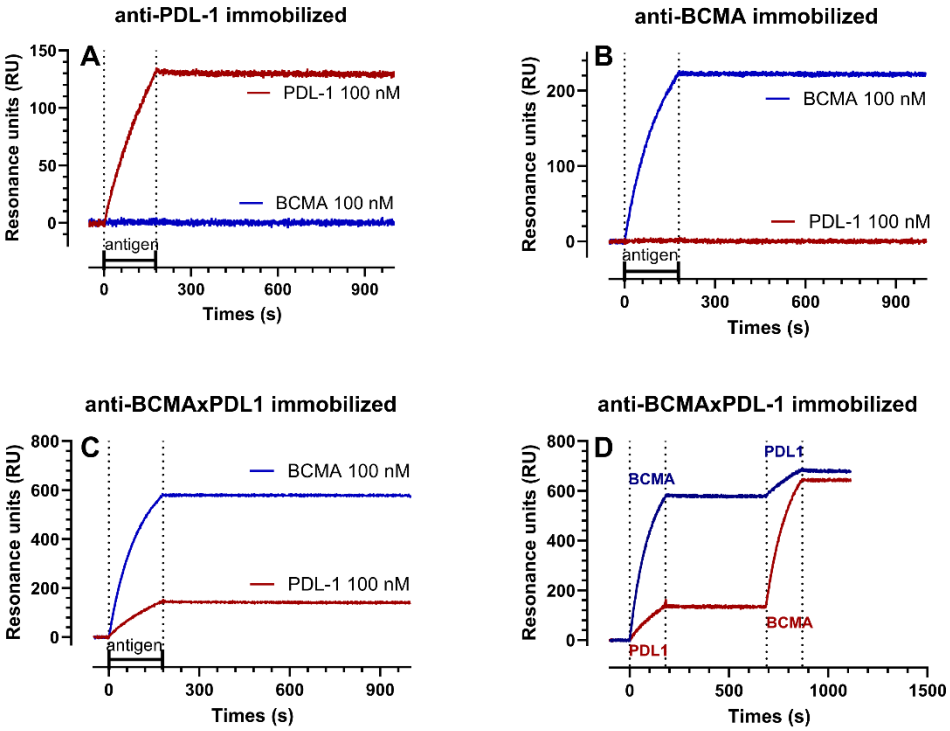


Fig. 5. Surface Plasmon Resonance analysis. *The sensorgrams shown were obtained injecting recPDL1 or recBCMA, alone (A-C) or in succession (D) over immobilized anti-PDL1 (A), anti-BCMA (B) or BCMAxPDL1 bsAb (C and D). The antigens were flowed for three min, as indicated by the dashed line.*

Table 1. Binding constants determined by SPR studies

	ka	**kd**	**KD**	**Rmax**
	1/Ms	**1/s**	**nM**	**RU**
recPDL1 on PDL1 mAb	**4.9-5.5 x 10^4**	**1.1-5.9 x 10^{-6}**	**0.02-0.12**	**222-227**
recPDL1 on BCMAxPDL1 bsAb	**3.8-6.2 x 10^4**	**0.4-1.8 x 10^{-5}**	**0.11-0.29**	**212-271**
recBCMA on BCMA mAB	**5.9-8.1 x 10^4**	**5.9-8.6 x 10^{-6}**	**0.11-0.10**	**289-312**
recBCMA on BCMAxPDL1 bsAb	**1.0-1.0 x 10^5**	**1.3-2.7 x 10^{-6}**	**0.01-0.03**	**683-700**

The sensorgrams were fitted with a 1:1 Langmuir equation to obtain association and dissociation rate constants (ka and kd), from which KD is calculated (kd/ka), and Rmax values, i.e. the maximum possible signal (in RU). The results of two independent experiments are shown.

The BCMAxPDL1 bsAb blocks PD1-PDL1 interaction and April binding to BCMA

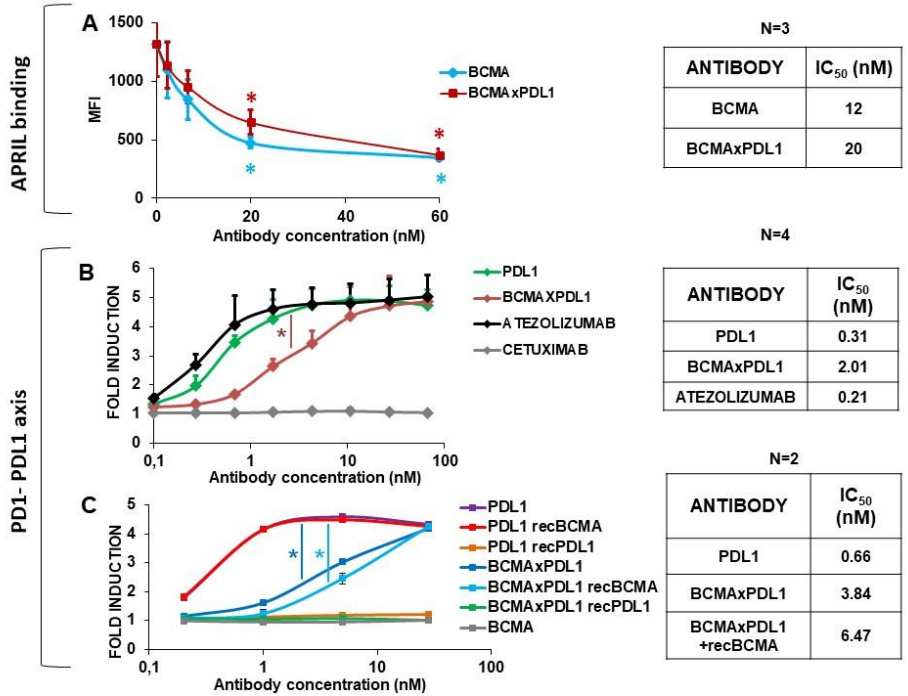
We next determined whether the bsAb could block the functions of its two target antigens. To investigate APRIL binding to BCMA, we used the CEM-BCMA+ cell line, a recombinant Flag-tagged April protein and an anti-Flag antibody. As shown in Figure 6A, both anti-BCMA mAb and BCMAxPDL1 BsAb were able to block April binding to BCMA, with similar IC_{50} of 12-20 nM. This suggests that BsAb is able to block BCMA function.

We next tested the capacity of the PDL1 mAb and bsAb to induce T cell activation, using a commercial TCR-mediated, luminescent T cell activation assay. T cell activation is specifically blocked by the PDL1-PD1 interaction. As shown in Figure 6B, the anti-PDL1 mAb induced T cell activation with an efficacy similar to commercially available atezolizumab, with IC_{50} of about 0.3 nM. Also the BCMAxPDL1 bsAb could block the PD1-PDL1 inhibitory pathway and induce T cell activation, but at a 8-10 fold higher concentration (2 nM and $p<0.05$) (Figure 6B). This is expected from the lower affinity/occupancy of PDL1 moiety in the bsAb compared to mAb, as described above. Irrelevant control antibody cetuximab had no effect (Figure 6B).

We also investigated whether occupancy of the BCMA moiety of BCMAxPDL1 bsAb reduced PDL1 binding and functional efficacy, as suggested above by the SPR data. To do this, we added recBCMA to the antibody before addition to the T cells in the PD1/PDL1 Blockade Bioassay. As shown in Figure 6C,

addition of recBCMA reduced the T cell activation by about 15 %, in line with the SPR data, suggesting a reduction in the capacity of BCMAxPDL1 to block PDL1-PD1 interaction in presence of BCMA target. The effect was not significant. Nonetheless, even with occupancy of the BCMA arm by excess recBCMA, the bsAb was able to block PDL1 and strongly induced T cell activation with an IC_{50} of about 8 nM.

We conclude that BCMAxPDL1 has the capacity to bind specifically to both targets with nM affinity and to effectively block the function of both BCMA and PDL1 *in vitro*, also in the nM range. Inhibition of PDL1 function was observed even if the BCMA arm was occupied by BCMA.



N=3

ANTIBODY	IC_{50} (nM)
BCMA	12
BCMAxPDL1	20

N=4

ANTIBODY	IC_{50} (nM)
PDL1	0.31
BCMAxPDL1	2.01
ATEZOLIZUMAB	0.21

N=2

ANTIBODY	IC_{50} (nM)
PDL1	0.66
BCMAxPDL1	3.84
BCMAxPDL1 +recBCMA	6.47

Fig. 6. The BCMAxPDL1 bsAb blocks April binding to BCMA and blocks PD1-PDL1 interaction. (A) To test the ability of bsAb to block APRIL binding to BCMA, we used CEM-BCMA$^+$ cell line, increasing concentrations of bsAb and anti-BCMA mAb, a Flag-tagged April protein and an anti-Flag antibody. *: $p<0.05$. (B) For assessing inhibition of the PD1-PDL1 axis, we tested increasing concentrations of BCMAxPDL1 bsAb or anti-PDL1 mAb in the cell-based PD1/PDL1 Blockade Bioassay. (C) Cell-based PD1/PDL1 Blockade Bioassay in presence of recBCMA. Atezolizumab and Cetuximab were used as positive and negative controls, respectively. *: $p<0.05$ vs PDL1.

BCMAxPDL1 induces CDC

We next investigated whether the Fc portion of bsAb has functional activity. We first tested complement dependent cytotoxicity (CDC) using human serum as source of complement and the BJAB cell line stably transduced with BCMA as a target. Anti-BCMA mAb induced 34% CDC at 24 nM, similar to what is observed with the gold standard anti-CD20 rituximab antibody (Figure 7). Also BCMAxPDL1 showed 19% and 15% CDC at 66 nM and 24 nM, respectively (Figure 7). Anti-PDL1 did not induce CDC even at high concentrations, as expected since the BJAB cell line is negative for PDL1 (data not shown). The data demonstrate that BCMAxPDL1 bsAb can induce CDC of appropriate targets. BsAb requires 3-10 fold higher concentrations than anti-BCMA mAb for similar efficacy. Nonetheless CDC is induced by BCMAxPDL1 at optimal concentrations to similar levels as that triggered by rituximab.

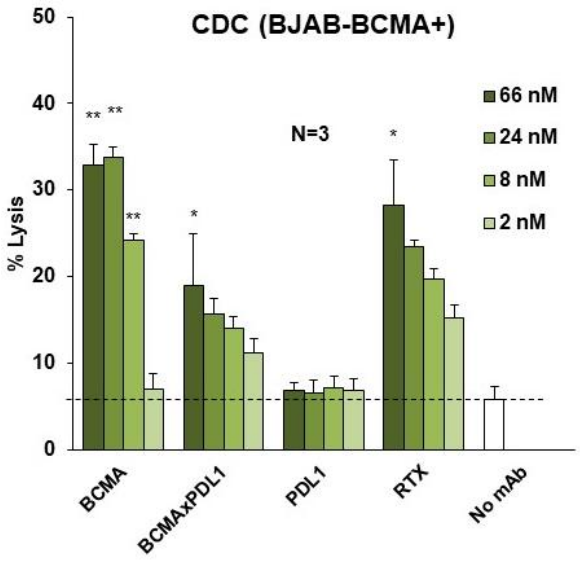


Fig. 7. The BCMAxPDL1 bsAb mediates CDC of BCMA$^+$ cells. *The BJAB-BCMA$^+$ cell line was incubated with increasing concentrations of bsAb, mAbs or rituximab (RTX) as positive control and in the presence of 50% HS as a source of complement. CDC was measured after 4 h by 7-AAD staining and flow cytometry. *:p<0.05 and **: p<0.01 versus No mAb.*

BCMAxPDL1 bsAb induces ADCC by NK cells

To probe the cell mediated cytotoxic activity of the bsAb, we first performed standard ADCC assays, using PBMCs as source of NK cells and three different MM target cell lines as targets (KMS11, OPM2, KMS12) expressing different levels of BCMA (MFI ranging from about 31000 to 8500 following DAPT treatment, data not shown). As shown in Figure 8A, both anti-BCMA mAb and BCMAxPDL1 bsAb were able to induce cytotoxicity of the $BCMA^{+++}$ cell line KMS11 (MFI 30877) up to 40% and 30%, respectively. The bsAb showed the same efficacy as the BCMA mAb, but at a 3-10 fold higher concentration compared to mAb, presumably due to the conformation of the bispecific, with Fc further distant from the antigen in the bsAb compared to the mAb format. ADCC of the OPM2 and KMS12 cells lines expressing lower levels of BCMA (MFI 13928 and 8477, respectively) was also observed with BCMAxPDL1 bsAb and BCMA mAb, albeit to a lower level (17-24% and 13-18% respectively, Figure 8B and 8C). As expected, anti-PDL1 mAb did not induce ADCC, as the 3 cell lines do not express this antigen (Figure 8 and data not shown). The fact that NK cells mediated cytotoxicity in this assay was confirmed by the induction of CD107a on NK cells in presence of anti-BCMA mAb and BCMAxPDL1 bsAb (Suppl. Figure 2). Similar ADCC was observed using a $BCMA^{+}$ B-NHL cell line as target (PA698, data not shown).

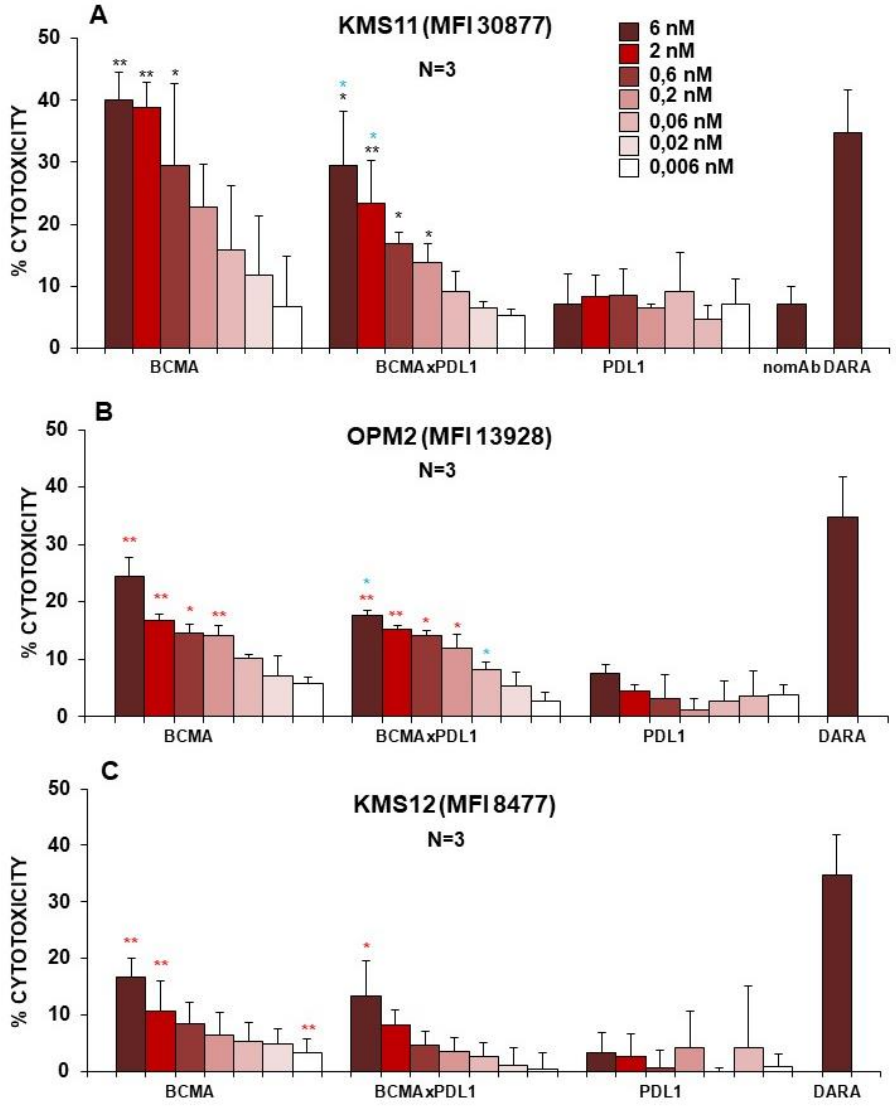
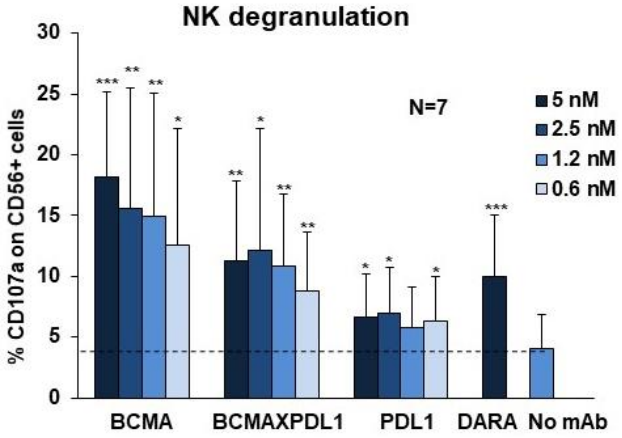


Fig. 8. BCMAxPDL1 bsAb mediates ADCC in 24 hours killing assays. *PBMCs were incubated with three different MM target cell lines (KMS11, OPM2, KMS12) in presence or absence of bsAb or control mAbs at the indicated concentrations. After 24 hours, target cell death was measured using an Apoptosis/Necrosis detection kit and flow cytometry. DARA: daratumumab. *:p<0.05 and **:p<0.01. black: * versus no mAb, red: * versus PDL1, blue: * versus BCMA.*



Supplementary Fig.2. BCMAxPDL1 bsAb activates NK cells.
*PBMCs as source of NK cells were incubated with KMS11 cells in presence or absence of bsAb or control mAbs at the indicated concentrations. NK cell activation was measured as the percentage of CD107a on CD56⁺ cells by flow cytometry. DARA: daratumumab; *p<0.05, **p<0.01 and ***p<0.01 versus No mAb.*

Cytotoxicity mediated by BCMAxPDL1 in 7-days killing assays

In order to mimic a more physiological condition and probe also the effect of the combination of Fc and anti-PDL1 moiety in mediating target cell killing, we performed cytotoxicity experiments over a 7-day period, using the KMS11 MM cell line as target and PBMCs as effectors. We could indeed observe a strong cytotoxic effect of the BCMA mAb and BCMAxPDL1 bsAb at optimal concentrations (75% decrease in live cells at day 7, Figure 9). In contrast anti-PDL1 mAb alone had more limited, albeit significant cytotoxic effect, with 45% reduction in live cells at the same time point (Figure 9).

We conclude that the BCMAxPDL1 bsAb is able to specifically kill BCMA$^+$ tumor cells *in vitro* in presence of different immune effector cells.

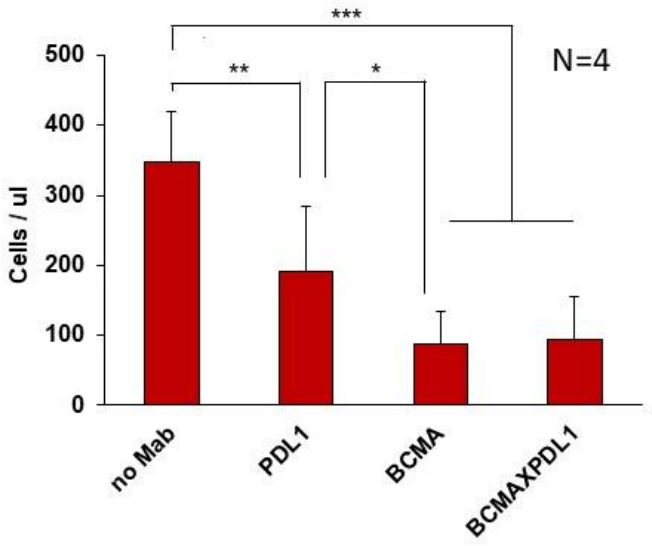


Fig. 9. BCMAxPDL1 bsAb mediates ADCC in the long term (7 days). PBMCs were co-cultured with KMS11 target cells and incubated with 6 nM of bsAb or mAbs. ADCC of target cells was assessed at 7 days by absolute counting live CD138$^+$ KMS11 target cells by flow cytometry. *: $p<0.05$, **: $p<0.01$ and ***: $p<0.001$.

DISCUSSION

We report here the detailed *in vitro* characterization of a novel IgG1-like BCMAxPDL1 bsAb, both in terms of antigen binding and function. We show that the bsAb, which is bivalent for each antigen and has a fully functional Fc, can bind specifically and with high affinity (nM range) to both target antigens. The affinity for both BCMA and PDL1 was similar, with IC_{50} of about 15 nM in binding assays for the membrane bound native antigens and KD of about 100 pM in SPR assays using recombinant protein

fragments. The affinity for BCMA was similar or even higher in the bsAb and corresponding mAb, whereas affinity for PDL1 apparently decreased about 3-10 fold in the bsAb compared to parent mAb. A similar decreased affinity of the internal moiety has previously been observed for other bsAbs designed on the same scaffold[6,14]. More detailed SPR analysis suggested that this apparent reduction in affinity for native protein was probably due to more limited access of the internal Fabs to the target antigen by steric hindrance, i.e. a reduction in the maximal number of molecules able to bind to PDL1, rather than a decreased affinity per se. This is likely due to the more internal position of the anti-PDL1 moiety within the bsAb structure.

SPR assays investigating the binding to both antigens in succession demonstrated that the same bsAb molecule can bind both antigens simultaneously, even though a further small decrease in maximal binding to PDL1 was observed after addition of recombinant BCMA. This is again probably due to the partial steric hindrance of the PDL1 moiety, that somewhat increased after BCMA binding, which may induce some conformational change to the molecule. In contrast, bsAb bound recombinant BCMA with at least as high affinity as the parent mAb. The small difference (~20 vs ~100 pM) may be due to the particular 3-D conformation of anti-BCMA Fabs in bsAb compared to mAb.

We also investigated the capacity of bsAb to block APRIL ligand binding to BCMA. We could show that both bsAb and anti-BCMA

parent mAb were able to specifically block APRIL binding to a BCMA positive cell line, with similar IC_{50}, confirming the cell binding and SPR data. Inhibition of APRIL binding should also therefore inhibit NFkB activation which is induced by APRIL[24] (data not shown) and supports mature B cell survival and proliferation. The block of APRIL therefore adds an additional function to the bsAb, which may participate in tumor control.

We also demonstrated that the BCMAxPDL1 bsAb could functionally block the PD1-PDL1 axis and induce TCR-dependent T cell activation *in vitro*. We observed a good capacity of the bsAb to activate T cells, at a concentration about 10-fold higher than that required by anti-PDL1 mAb, as expected from the reduced binding to PDL1 of bsAb compared to parent mAb. Nonetheless, T cell activation was maximal using the antibody at 8 nM, even in presence of simultaneous binding of recombinant BCMA, which corresponds to a concentration of 2 µg/ml, a concentration that may be reached *in vivo* in the circulation as well as locally[25,26].

We also found that the Fc portion of bsAb is functional. Indeed, the BCMA mAb and BCMAxPDL1 bsAb were both able to induce CDC of appropriate targets, as well as induce efficient ADCC in 24 hours and 7-days killing assays, using PBMC as effector cells. We could demonstrate NK activation and killing in 24 hours killing assays, showing that this cell type was indeed activated by the bsAb IgG1 Fc. In the 7-days killing assays, we could demonstrate greater efficacy of BCMAxPDL1 compared to anti-PDL1 alone.

Altogether, these *in vitro* studies characterize in considerable detail the binding characteristics and function of the new BCMAxPDL1 bsAb *in vitro*, compared to the parent mAbs. The aim of the work was to design a therapeutic bsAb that would be directed towards a MM BM through its anti-tumor associated antigen moiety (TAA, anti-BCMA) and then induce immune cell activation and killing by blocking *in situ* the PD1-PDL1 axis. Such inhibition of PD1-PDL1 signaling may be more effective because it is mediated locally, within the tumor microenvironment and close to the tumor site. This may also avoid the non-specific and toxic effect that is often observed with anti-PDL1/PD1 mAbs systemically. In our view, such a molecule should have an affinity for the TAA at least as high as that to the ICI, in order to optimally achieve this goal. This was the case for our bsAb, which showed similar affinity for both antigens, in conditions of binding to a single antigen or to both simultaneously. Furthermore, the affinity and functional activity of the bsAb in the nM range demonstrated here suggests that an effective concentration of bsAb may be achieved in vivo. The bsAb was indeed able to induce some CDC, ADCC and target cell killing in presence of different immune cells. One possible disadvantage of the present molecule is the partial steric hindrance that was observed for PDL1 binding, which may reduce efficacy of the anti-ICI moiety. We also tested a different molecule with different linker, but the behavior of this molecule was very similar to the one presented here (data not shown). On the basis of the detailed analyses presented here, future work may investigate other formats of the

bsAb with longer and more flexible linkers, in order to reduce steric hindrance, but such molecules should also foresee to reduce the affinity of the anti-ICI moiety, compared to the anti-TAA moiety, to ensure the delivery of the bsAb towards tumor cells.

The analyses of BCMA and PD1/PDL1 expression performed here confirmed that anti-BCMA mAbs and BCMAxPDL1 bsAbs may be useful therapeutic drugs not only for MM, but also for a proportion of more differentiated B-NHL, which in some cases express BCMA to a similar degree as MM cells, at least after inhibition of ɣ-secretase. This is in agreement with previous data on BCMA expression in MM and B-NHL [7,8,22,23]. We also analyzed PD1 and PDL1 expression in MM and B-NHL primary BM or PB samples and demonstrated that one of these molecules is frequently expressed either on the tumor cells or on immune cells (T lymphocytes/monocytes/macrophages) in the tumor microenvironment, suggesting that BCMAxPDL1 may indeed be a useful therapeutic tool in both MM and B-NHL.

Other bsAbs targeting checkpoint inhibitors have reached the clinic and showed in some cases very promising activities[27]. Most of these bsAbs target 2 different ICI or an ICI and an immune activator. Only few have been designed to target a TAA as well as an ICI. The latter include EGFRxPD1, HER2xPD1[28] PDL1xEGFR[29]. Also a TGFbxPDL1 bsAb has shown promising activity in clinical studies[30].

Clearly the next step will involve testing the present bsAb in immunocompetent humanized models *in vivo*, since these are the only models that are suitable for investigation of the therapeutic activity of anti-checkpoint inhibitors also targeting a human TAA [31,32].

ACKNOWLEDGEMENTS

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CHAPTER 3

GENETIC DEFECTS OF GAMMA-SECRETASE GENES IN A MULTIPLE MYELOMA PATIENT WITH HIGH AND DYSREGULATED BCMA SURFACE DENSITY: A CASE REPORT

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We analyzed BCMA surface density in bone marrow (BM) cells from multiple myeloma (MM) patients, isolated after informed consent. We observed that plasma cells (PCs) from 19/20 consecutive MM and 3 MGUS patients expressed membrane BCMA protein (mBCMA) at low-medium intensity (median MFI 753, range 315 to 1729), and this increased by a mean 3-fold after treatment with by ɣ-secretase inhibitor DAPT (GSI-IX)(Fig.1A-B, median MFI 2455, range 897 to 9845)($p<0.001$). This pattern is expected from the known role of this enzyme in shedding the BCMA ectodomain from the surface of PCs[1,2]. In contrast, the $CD38^+$ MM cells from one patient (pt#12) showed high mBCMA levels even without DAPT treatment (MFI 5612) and this did not increase upon gamma-secretase inhibition (MFI 5481; Fig.1A, bright red lines and Fig.1B).

Pt#12 was a 74-year old female diagnosed with MM in 2018 (Durie & Salmon stage IIIA). Her BM showed 50% infiltration with MM cells positive for IgAƛ/CD19/cytCD79b/CD38/BCMA, but negative for CD20/CD138. Karyotyping of purified MM cells revealed amplification of *CKS1B* (1q21), trisomy of chromosomes 4 and 5 and tetrasomy of chromosomes 9, 15 and 17 with deletion of *TP53*; *IGH* or *c-MYC* was not rearranged. Pt#12 was treated with lenalidomide, dexamethasone and bortezomib (EFC12522 study), with reduced dosing of bortezomib and lenalidomide from the 2nd and 4th cycle, respectively, due to peripheral neuropathy. The patient reached stringent complete remission (CR) after 4 chemotherapy cycles and continued with maintenance with dexamethasone and

lenalidomide. After 4 years of CR, the patient became positive for COVID-19. Dexamethasone and lenalidomide were temporarily suspended, but disease progressed, and the patient is currently treated with cyclophosphamide and dexamethasone.

The fact that basal levels of mBCMA in pt#12 MM cells were high and not increased by ɣ-secretase inhibition suggested that the neoplastic cells of this patient had some defect in BCMA ectodomain shedding. This could be due to a mutation in the BCMA amino acid sequence recognized by ɣ-secretase. The 4 complete exons of the *BCMA* gene *(TNFRSF17)* were therefore PCR amplified from the diagnostic BM genomic DNA and sequenced, but none of the exons showed any mutation with respect to the consensus genomic sequence for caucasians (NC_000016.10) (Supplementary Fig.1).

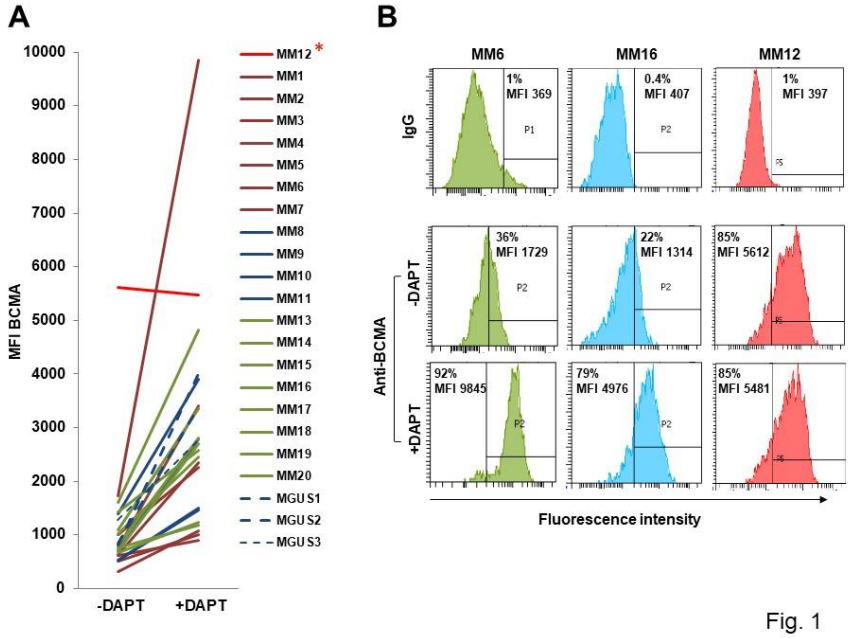


Fig. 1

Figure 1. MM cells BCMA surface density in presence or absence of γ-secretase inhibition. *Mononuclear cells from BM samples of 20 MM and 3 MGUS patients were cultured overnight with DAPT. BCMA surface density on $CD19^+CD38^+$ or $CD138^+$ MM cells was then analyzed by flow cytometry. Panel A: The data show the MFI of mBCMA expression on plasma cells of the 23 patients. The color code indicates the fluorochrome used for BCMA detection (red: PE-Cy7; blue: PE; green: APC). Panel B shows the histograms for mBCMA expression in MM cells from pt#12 as well as 2 representative patients showing the expected upregulation of BCMA by DAPT (pt#6 and pt#16).*

Supplementary Fig.1: Sequencing of BCMA gene of Pt#12.

Primers are evidenced in yellow or green. Introns. EXONS (NON CODIFYING). EXON (CODIFYING).
Ex2B: tttattta is an intron polymorphism. Ex2: g is a variant present in 99% of caucasians.

BCMA Ex1:

gcagtttctgttTCAGATGTGATATGCCCTGATATTtacaccctgtctcttaccccatccAAGACTCAAACTTAGAAACTTGAATTAGATGTGGTAT
TCAAATCCTTAGCTGCCGCGAAGACACAGACAGCCCCCGTAAGAACCCACGAAGCAGGCGAAGTTCATTGTTCTCAACATTCTAGCTGCTCTTGCTG
CATTTGCTCTGGAATTCTTGTAGAGATATTACTTGTCCTTCCAGGCTGTTCTTTCTGTAGCTCCCTTGTTTTCTTTTTGTGATCATGTTGCAGATGG
CTGGGCAGTGCTCCCAAAATGAATATTTTGACAGTTTGTTGCATGCTTGCATACCTTGTCAACTTCGATGTTCTTCTAATACTCCTCCTCTAACATG
TCAGCGTTATTGTAATGCAAgtaagtaatattgcttgaacgattattcattggtgtgaactattctgtctatatggactgcttattcagagaatcaa
cataatgggcatgatggtgagttttcttgaatcaaaaagagaa

BCMA Ex2:

gagccgagatcgcgccactgcactctagcctgggcaacagagcaagactttgtctcaaaataaataaataaataataacaataaagtatgtgaatat
tatgttatcagctcattatctgtctgatgttcttttcataaagGTGTGACCAATTCAGTGAAAGGAACGAATGCGATTCTCTGGACCTGTTTGGGAC
TGAGCTTAATAATTTCTTTGGCAGTTTTCGTGCTAATGTTTTTGCTAAGGAAGATAAgCTCTGAACCATTAAAGGACGAGTTTAAAAACACAGgttg
gtttgatggtgaatctttgaaatctatttccaggggatggctattgtgagttcagttccttttcttttttagcgttgactatttcacttcgttac
agccctttcgaatgtgttagaacattgttacattaaatgaacttggtagaggtgagccatcttcattctgattttgaca

BCMA Ex2B:

tcatctaaaaaataattatttttaagcaatagagaaccagtcccagaaaaatgaatgttctacttaagtgcctcttaagataaaaatacttctgcag
caccttgctcatgattggattcccaagcatgtacagccactgccctatttctgtatgcatttatttatttatttatttatttatttattttattt
ttaGAGATGGAGTCTCGCTCTGTGGCCCAGGCTGGAGTGCAGTGGCGTGATCTCAACTCACTGCAGCCTCTGCCTCTTGGGTTCAAACAATTCTCCC
ATCTCAGCCTCCTGAGTAActgggactataggtatgtgccatcacctccgactaatttttgtacttttggtagagacagggtttcatcatgttggc
caggatggtctcaagctcctgaccacaagtgatctgcccgcctcagcttcccaaagtgctgggattacaggcgtgagccacagggcccagcacatac
tcattctttttactgaaaagatctgtttcaagctgggtgtggtggcat

BCMA Ex3:

gctttgagtcccgatgtgtactgctaagactctcatgaccacattctctgtgaagtttgggttaatgtttccgtttctacataattagGATCAGGTC
TCCTGGGCATGGCTAACATTGACCTGGAAAAGAGCAGGACTGGTGATGAAATTATTCTTCCGAGAGGCCTCGAGTACACGGTGGAAGAATGCACCTG
TGAAGACTGCATCAAGAGCAAACCGAAGGTCGACTCTGACCATTGCTTTCCACTCCCAGCTATGGAGGAAGGCGCAACCATTCTTGTCACCACGAAA
ACGAATGACTATTGCAAGAGCCTGCCAGCTGCTTTGAGTGCTACGGAGATAGAGAAATCAATTTCTGCTAGGTAATTAACCATTTCGACTCGAGCAG
TGCCACTTTAAAAATCTTTTGTCAGAATAGATGATGTGTCAGATCTCTTTAGGATGACTGTATTTTTCAGTTGCCGATACAGCTTTTTGTCCTCTAA
CTGTGGAAACTCTTTATGTTAGATATATTTCTCTAGGTTACTGTTGGGAGCTTAATGGTAGAAACTTCCTTGGTTTCATGATTAAACTCTTTTTTTT
CCTGAcatctaagttttattaacgtgagttttaaaaacaagcatgtataccagtgtgggggtgagggtgggagagaaaggtgggaggggaaag
aattctaacctattgataataaagctccagttttggccaggcgcggtggctcatgcct

Lack of *BCMA* gene *(TNFRSF17)* mutation suggested that the shedding enzyme itself may be defective in pt#12 MM cells. γ-secretase is a membrane-associated multi-unit enzyme. The major catalytic unit is presenilin 1 (*PSEN1*, chr14q24.2). Alternative catalytic protein is presenilin 2 (*PSEN2*, chr1q42.13). Other components of the complex are nicastrin (*NCSTN*, chr1q23.2), anterior pharynx 1 (*APH1A*, chr1q21.2 and *APH1B*, chr15q22.2) which stabilizes, and presenilin enhancer (*PSENEN*, chr19q13.12), which activates PSEN1/2[3]. Given the role of *PSEN1* and *PSEN2*, the whole coding region and the exon junction of these 2 genes, were analyzed by targeted next-generation sequencing (NGS), using the Neurodegeneration Illumina panel and Sequencer (NextSeq), as previously described[4]. Whole DNA from pt#12 BM at diagnosis and from the peripheral blood (PB) at CR were used. The results did not reveal any mutation in the *PSEN1/2* genomic sequences, but depth of coverage using the Genome Analysis Toolkit and normalization to the control *PRNP* gene showed that coverage ratio in the MM vs remission sample was 0.69 for *PSEN1* and 2.85 for *PSEN2*. This suggested at least partial deletion of *PSEN1* exons and amplification of *PSEN2* sequences in pt#12 MM cells.

To corroborate these findings, *PSEN1/2* copy number variation (CNV) was analyzed in duplicate, using multiplex ligation-dependent probe amplification (MLPA), which relies on the binding and subsequent amplification of probe sets covering the *PSEN1* and 2 exons. The results confirmed that, in the diagnostic samples, *PSEN2* exons were >1 copy with respect to the

reference gene for most probes covering the 13 *PSEN2* exons (Fig.2A). In contrast, several *PSEN1* exons showed <1 copy number and the exon 8 signal (ex8) was below detection level. In contrast, remission sample showed no CNV, confirming that the alterations are specific for MM cells. Control amyloid beta precursor (*APP*) gene showed no CNV in either diagnostic or remission sample, as expected (Fig.2A).

The above data suggest that one *PSEN1* allele may be deleted in MM cells (giving a relative signal of about 0.75, since the BM sample contained ~50% MM cells, as well as ~50% normal cells that presumably carry 2 normal *PSEN1* alleles). In addition, the lack of ex8 signal indicates the possible presence of an ex8 defect in the non-deleted *PSEN1* allele in MM cells. Interestingly ex8 covers the active site of *PSEN1* and has been reported to be alternatively spliced in some cell types (e.g. PBMCs), producing a dominant negative molecule[5,6]. To verify whether Pt#12 MM cells bear deletion or alteration of ex8 in their single *PSEN1* allele, we first set up the PCR methods for detection and quantification of full length (FL) and ex8$^-$ alternatively spliced *PSEN1* transcripts. Control PBMC showed the expected high expression of both ex8$^-$ and FL *PSEN1* mRNA by non-quantitative and quantitative RT-PCR (Fig.2B-C)[5]. In contrast, MM cell lines such as OPM2 expressed ≤1% *PSEN1* ex8$^-$ transcript compared to FL (Fig.2B-C and Supplementary Fig.2). We then purified the MM cells from the last cryopreserved diagnostic BM samples (95% purity) and analyzed the expression of FL or ex8$^-$ *PSEN1* mRNA transcripts by RTQ-PCR

in both BCMA positive and negative fractions. We detected FL *PSEN1* transcript as well as 6% and 11% ex8^{-} mRNA in pt#12 MM cells and the negative fraction, respectively, similarly to what is observed in BM-MNCs from other leukemia patients in remission used as controls (Fig.2D). This suggests that a full-length *PSEN1* transcript is produced in Pt#12 patients MM cells, as well as a small amount of ex8- spliced transcript. Therefore, the apparent lack of ex8 signal in the MLPA assay of pt#12 BM may have been spurious.

We finally quantified *PSEN1* mRNA in the purified pt#12 MM cells compared to the negative fraction or to control BM-MNCs from other donors. We used an RNA sample from a pool of PBMC to normalize expression of *PSEN1* in the different samples. The level of *PSEN1* FL mRNA expression in pt#12 MM cells was about half that observed in the negative fraction and was also significantly less than that observed in control BM-MNCs (Fig.2E). Similar results were obtained after normalization to a house keeping gene (*GUSB*, Supplementary Fig.3). These data support a reduced *PSEN1* mRNA expression in pt#12 MM cells due to deletion of one *PSEN1* allele.

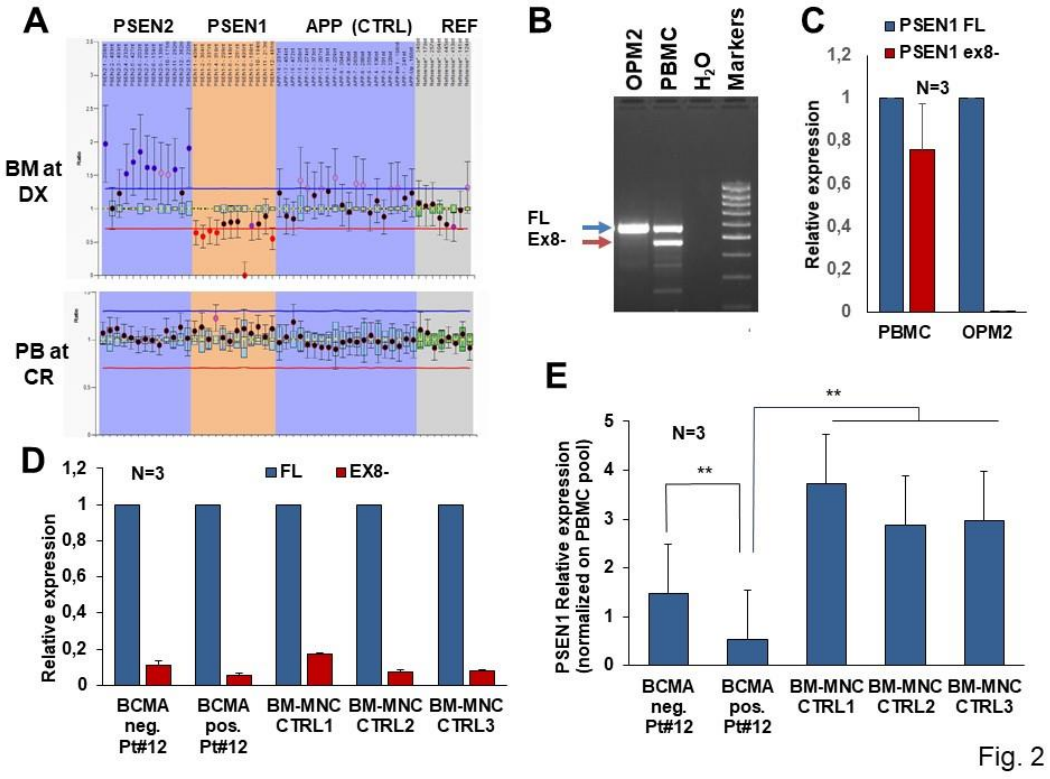
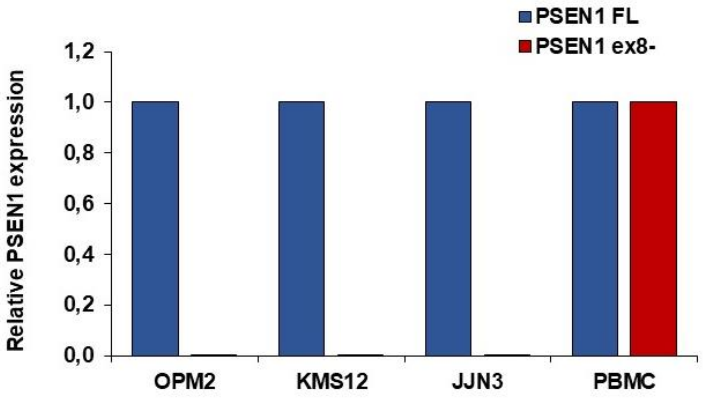
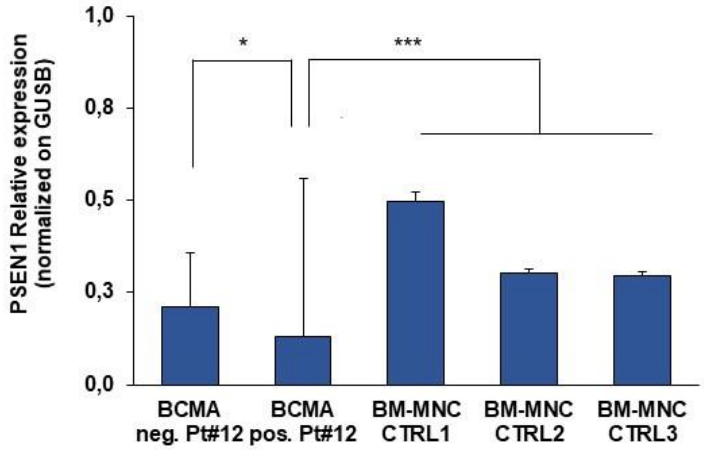


Fig. 2

Figure 2. Analysis of PSEN1/2 DNA and mRNA in pt#12 and control samples (A): Genomic DNA from duplicate pt#12 diagnostic BM (~50% MM cells) and PB remission samples was analyzed for CNV of *PSEN1* and *PSEN2* by MLPA, using the SALSA P471 EOFAD probe mix (MRC-Holland, Amsterdam, the Netherlands, www.mrcholland.com). One of two representative experiments is shown. (B,C): FL and ex8$^-$ alternatively spliced PSEN1 mRNA was analyzed by RT-PCR (B) and quantified by RTQ-PCR (C). (D): Pt#12 MM cells were immunoselected with anti-BCMA and FL or ex8$^-$ PSEN1 mRNA analyzed by RTQ-PCR. (E): The major FL PSEN1 was quantified in Pt#12 MM cells (BCMA positive) and negative BM fractions, compared to total bone marrow cells (BM-MNC) from 3 different control donors.PSEN1 levels were normalized to that observed in a PBMC pool used as standard. **: $p<0.01$.



Supplementary Fig. 2.
Relative expression of PSEN1 FL and ex8- mRNAs analyzed by RTQ-PCR in several MM cell lines compared to a PBMC sample



Supplementary Fig.3.
PSEN1 FL mRNA expression measured by RTQ-PCR in Patient #12 purified MM cells (BCMA pos) compared to the negative fraction or total bone marrow cells from 3 different control donors. PSEN1 levels were normalized to the GUSB house keeping gene.

The limited amount of pt#12 MM sample available precluded further dissection of *PSEN1* or other components of ɣ-secretase. We cannot therefore demonstrate that under-expression of *PSEN1* is causative or sufficient for ɣ-secretase dysregulation and BCMA high surface density in pt#12 MM cells. Nor is it possible to fully understand whether and how the amplification of *PSEN2*, and possibly that of *APH1a* and *NCSTN* since their localization (chr1q21.2 and chr1q23.2, respectively), near the amplified *CKS1B* genes (1q21.3) and *PSEN2* (ch1q42.13), indicates they may also have suffered genetic alterations in this patient MM cells. Indeed *PSEN2, APH1A and NCSTN* are all located in a region frequently amplified in MM cases (40%). ɣ-secretase has >70 substrates, but most studies have concentrated on its role in APP degradation in Alzheimer disease, and in NOTCH-1 and/or WNT signaling in skin diseases. Mutations of *PSEN1* and *PSEN2* have variable effects on these different substrates[7–9]. PSEN1 has been shown to be sufficient for BCMA ectodomain shedding[1], but the differential role of PSEN1 vs PSEN2 is not known[3,8,10]. Interestingly, a recent report describes a deletion and point mutation in the *PSENEN* gene, an activator of PSEN1/2, in another case of MM[11]. Basal mBCMA levels in this patient was also higher than in most other MM cases. The effect of ɣ-secretase inhibitors was not analyzed, but the *PSENEN* mutation was demonstrated in MM cell lines to abolish the activating function of PSENEN on ɣ-secretase and BCMA shedding. The patient also showed low levels of serum soluble BCMA, indicating poor shedding of the protein *in vivo*[11].

The case presented here suggests therefore that several mechanisms for ɣ-secretase dysregulation may occur in MM patients leading to high mBCMA levels.

It is worth noting that a quantitative loss of *PSEN1* (only one allele present), in mice lacking *PSEN2*, has been reported to lead to hyperactivation and proliferation of B cells and a severe autoimmune phenotype[12]. Deletion of *NCSTN* leads to defective development of marginal zone B1 B cells in mice and of T-independent antibody responses, perhaps due to alterations in NOTCH2 signaling[13].

To conclude, to the best of our knowledge, this is the first description of alterations of *PSEN1* and *PSEN2* genes in MM, which are accompanied by high surface density and dysregulated BCMA in an MM patient. Given the role of BCMA in PC survival and growth[1], the observed genetic defect may also have a pathogenetic role in this patient by increasing NFkB signaling[1]. In addition, PSEN1/2 modulation may also affect other pathways involved in MM pathogenesis, such as the NOTCH/WNT pathways. Finally, high levels of mBCMA may also have therapeutic implications, since high mBCMA may favor the clinical response to anti-BCMA CAR-T or antibody-mediated immunotherapy[11,14–16].

To conclude, future studies should investigate more systematically the genetic integrity and fine regulation of ɣ-secretase subunits in MM patients with high BCMA surface

density and correlate these findings with the response to anti-BCMA immunotherapy or standard chemotherapy.

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CHAPTER 4

CONCLUSIONS AND FUTURE PERSPECTIVES

In the spectrum of new agents in development for the treatment of MM, mAbs, bsAbs and CAR-T cells directed against specific surface antigens highly expressed on malignant cells have emerged as a potentially efficacious strategy[1]. BsAbs have two main advantages over other modalities. First, they are off-the-shelf products, compared to CAR-T cells which are complex to produce and are often patient-specific. Second, they allow to simultaneously target two different antigens, thus adding another function to the drug when compared to mAbs.

MM is a common neoplasia of plasma cells and still an incurable disease, despite the advent of several novel therapeutic agents, in particular biological drugs, directed against MM specific antigens, such a mAbs, ADCs, CAR-T and bsAbs.

In this work we have set to define suitable targets to construct a novel bsAb for MM treatment. We have chosen a TAA, BCMA (CD269) to direct the bsAb towards MM cells, since this molecule is known to be expressed only by normal and neoplastic plasma cells[2]. In addition, it is essential for the survival of long-lived PCs in the BM[3]. High serum levels of soluble BCMA (sBCMA) are reported in MM patients[4] and sBCMA is considered a marker of prognosis and treatment response[5,6]. As second specificity for the bsAb, we have chosen PDL1, the ligand for PD1; PDL1 and PD1 are immune checkpoint inhibitors known to be expressed by tumor cells and immune cells within the tumor microenvironment.

More specifically, PD1 is highly expressed on T and NK cells in MM patients[7], while PDL1 may be induced on MM-PCs[8], suggesting that by PDL1 expression MM-PCs escape the host immune response and expand the tumor burden.

In the first part of the work we have focused on a more systematic analysis of mBCMA expression in different B cell neoplasias, including MM[9–12], and of its regulation (i.e. its shedding) by the ɣ-secretase enzyme[13,14]. We therefore investigated the expression of mBCMA in a panel of B cell lines, from immature pre-B to B-NHL and to mature MM, and on primary samples from MM and B-NHL patients. Expression of mBCMA was investigated in presence or absence of the ɣ-secretase inhibitor DAPT. A weak basal expression of mBCMA was observed in all MM cell lines tested, strongly upregulated by treatment with DAPT. Among the other 21 B cell lines analyzed we observed that mBCMA was low or negative in most cell lines but was strongly induced by DAPT treatment. Analysis of primary samples from MM and B-NHL patients revealed that most MM cells were positive for mBCMA but at a relatively low density, which was significantly increased by DAPT treatment. The two primary Mantle Zone Lymphoma (MZL) samples that could be analyzed also revealed low density of mBCMA expression, which could be increased by ɣ-secretase inhibitor. These data are consistent with the literature, since mBCMA is known to be expressed on most MM samples and cell lines, but to be strongly upregulated by inhibition of its shedding[13–15]. Some literature data also suggest, as shown here, that more mature B-NHL cells may in some cases express

mBCMA at levels similar to MM cells. We show here that inhibition of ɣ-secretase induces BCMA also in B-NHL, thereby indicating that this molecule is also expressed in some more differentiated B-NHL samples and cell lines. This suggest that anti-BCMA drugs may also be used to treat some B-NHL cases[15,16].

During the screening of BM samples of MM patients for mBCMA expression in presence or absence of ɣ-secretase inhibitor, we came across an interesting case of MM (pt#12), which showed unusually high expression levels of mBCMA, even in absence of DAPT, and no induction in presence of this inhibitor. In contrast, all other 19 cases showed low basal levels of mBCMA and a mean 3-fold induction after *in vitro* DAPT treatment, as is observed also in most MM cell lines and reported in the literature[14,17].These data suggested that some alteration, either within the BCMA gene, or within the ɣ-secretase complex inhibited shedding of BCMA on pt#12 MM cells. We therefore set out to further analyze this case genetically. Sequencing of pt#12 BM genomic DNA showed that the patient had no mutations of the 4 BCMA exons and exon-intron junctions. The genes encoding the most important ɣ-secretase units of this complex multi-protein enzyme were therefore also investigated. The major catalytic unit of ɣ-secretase is presenilin 1 (*PSEN1*, chr14q24.2). Alternative catalytic protein is presenilin 2 (*PSEN2*, chr1q42.13). Other components of the complex are nicastrin (*NCSTN*, chr1q23.2), anterior pharynx 1 (*APH1a*, chr1q21.2 and *APH1b*, chr15q22.2) which stabilizes, and presenilin enhancer

(*PSENEN*, chr19q13.12), which activates PSEN1/2[18,19]. Given the role of PSEN1 and PSEN2, as major and alternative catalytic unit of ɣ-secretase respectively[18], the whole coding region and the exon junction of these 2 genes were analyzed by next generation sequencing (NGS)[20], as well as multiplex ligation-dependent probe amplification (MLPA). Results obtained using the diagnostic samples containing 50% MM cells, suggested genetic defects in both the PSEN1 and PSEN2 genes, i.e. deletion of one PSEN1 allele and amplification of PSEN2 exons, which were not observed in the remission samples, free of disease. [21,22]. Using quantitative PCR, we quantified lower levels of PSEN1 full length mRNA expression in pt#12 purified MM cells compared to the negative fraction or other BM samples from MM patients, consistent with the deletion of one allele of PSEN1. Altogether our data suggest that pt#12 MM cells express high mBCMA with no shedding due to genetic alterations of one or more ɣ-secretase subunits, in particular PSEN1 and PSEN2. The limited amount of pt#12 MM samples available precluded further dissection of the role of PSEN1 or of the other ɣ-secretase components. We could not therefore directly demonstrate if under-expression of PSEN1 or PSEN2 amplification were responsible for reduced ɣ-secretase activity and mBCMA overexpression in pt#12, nor whether other components of the ɣ-secretase enzyme were also affected. Indeed *NCSTN* (chr1q23.2) and *APH1a* (chr1q21.2) are 2 other important regulatory components of ɣ-secretase and their genome are located close to the area of chromosome 1 which is amplified in

pt#12 MM cells. These genes could potentially also be altered in this pt#12, although, again, insufficient material was available to investigate this point.

To conclude, to the best of our knowledge, this is the first description of alterations of PSEN1 and PSEN2 genes in MM, which are accompanied by high surface density and dysregulated BCMA in a MM patient. Interestingly, a recent report describes a deletion and point mutation in the PSENEN gene, a ɣ-secretase subunit which activates PSEN1/2, in another case of MM[23]. Basal mBCMA levels in this patient were also higher compared to other MM cases. The authors demonstrated that PSENEN mutation in MM cell lines suppressed the activating function of PSENEN on ɣ-secretase and BCMA shedding, suggesting that several mechanisms for ɣ-secretase dysregulation may occur in MM patients leading to high mBCMA levels. Future studies should investigate more systematically the genetic integrity and regulation of ɣ-secretase subunits in MM patients with high BCMA surface density.

In parallel with BCMA analysis, we investigated the expression of PD1 and PDL1 in primary samples of MM and B-NHL patients, in order to define whether the planned bsAb would be indeed able to target BCMA and the PDL1/PD1 axis in the same tumor microenvironment. We demonstrated that either PD1 or PDL1 are frequently expressed either on the tumor cells or on immune cells (T lymphocytes/monocytes/macrophages) in the BM MM

microenvironment, suggesting that indeed targeting BCMA and PDL1 may be effective in MM or B-NHL.

Having determined that BCMA and PDL1 were appropriate targets in MM as well as some B-NHL, we set out to construct a novel tetravalent IgG-like, human IgG1 Fc-bearing bsAb, directed against BCMA and PDL1 (bsAb BCMAxPDL1). The novel bsAb BCMAxPDL1 is based on a patented platform and structure previously described by our group[24,25] (patent no. WO/2013/005194). The plasmids and strategies already used to generate other bsAbs on the same format were used for genetic engineering, using published sequences for the anti-BCMA and anti-PDL1 VH and CL sequences. Transfection of plasmids expressing the fused heavy chain and 2 light chains, selection of clones, production of bsAb on large scale and purification was performed in the laboratory of Martine Cerutti and Muriel Roth in France. The purified mAbs and bsAbs were then sent for analyses in our laboratory.

BsAb BCMAxPDL1 bound to both BCMA and PDL1 naturally expressed on the surface of cells positive for these antigens. The relative binding affinity of bsAb BCMAxPDL1 to native antigens was about 15 nM for BCMA, similar to that observed with the corresponding anti-BCMA mAb. The affinity of bsAb for PDL1 was 13 nM, while it was 1 nM for corresponding anti-PDL1 mAb. A similar decrease in binding affinity of the internal moiety has previously been observed for other bsAbs designed on the same scaffold by our group[24,25]. The anti-PDL1 moiety in the bsAb is more internal to the scaffold, a position that can result in some

steric hindrance. SPR data confirm this supposition showing that the internal anti-PDL1 moiety in the bsAb has an apparent 3-fold lower affinity for PDL1 in comparison to the mAb, and that the anti-PDL1 moiety in the bsAb appears 5-fold less accessible than the anti-BCMA moiety. The simultaneous binding of both antigens was also investigated by SPR. The data showed that both antigens can bind simultaneously and that previous BCMA binding reduces PDL1 binding by about 20%, due presumably to the steric hindrance stated above and to some conformational change taking place after BCMA binding. Nonetheless, overall affinity to native PDL1 is in the sub-nM range, similar to that of BCMA, which should allow blocking of PDL1 in the tumor microenvironment *in vivo*. In our conception, the plan was to generate a bsAb that would have at least as high an affinity for the TAA as for the ICI, in order to bring the bsAb most effectively towards the tumor bed and avoid ICI inhibition systemically. As it turned out, the BCMAxPDL1 bsAb produced has similar affinity for both antigens. We believe that this may be adequate for *in vivo* use, but this will need to be verified, by testing the efficacy and toxicity of the BCMAxPDL1 described here in humanized animal models[26].

We have also demonstrated that the BCMAxPDL1 bsAb was able to block *in vitro* the binding of the APRIL growth factor to BCMA, with an IC_{50} in the nM range. These data suggest that bsAb-BCMAxPDL1 may have a direct anti-myeloma activity by inhibiting MM cell survival and proliferation induced by APRIL[27,28]. We have also shown that the bsAb was able to block

the PD1-PDL1 interaction and unleash TCR-mediated T cell activation, with an IC_{50} also in the nM range, even though about 10-fold higher than anti-PDL1 mAb, as expected from cell binding and SPR data. Worth noting is that maximal T cell activation was reached with a 8nM concentration of bsAb, corresponding to 2 µg/ml, a concentration that may be reached *in vivo*[29,30]. Alltogether these data suggested that, despite a lower binding capacity of bsAb to PDL1, compared to anti-PDL1 mAb, a sufficient functionality of bsAb in blocking the PD1-PDL1 axis may be reached *in vivo*. Such block, performed close to $BCMA^+$ target cells within the tumor microenvironment, may avoid the non-specific and toxic effect often observed with anti-PDL1/PD1 mAbs therapy[31].

We additionally analyzed whether the Fc domain of the bsAb BCMAxPDL1 construct was able to activate immune-mediated mechanisms in presence of $BCMA^+$ tumor targets. BsAb could activate complement and NK cells to mediate ADCC in 24 hours killing assays. We also demonstrated the ability of bsAb to significantly kill MM target cells in 7-days killing assays, using PBMC as effectors. In these tests, the bsAb had greater efficacy compared to anti-PDL1 mAb. Further studies *in vivo* will be needed to demonstrate therapeutic activity in animal models of MM and investigate a possible advantage of the bsAb compared to BCMA or PDL1 mAbs alone or in combination.

Other bsAbs targeting checkpoint inhibitors (ICI) are now under investigation in clinical studies. Promising outcomes have been

reported for bsAbs targeting 2 different ICI[32] (such as CTLA-4 and PDL1) or an ICI with a co-stimulatory checkpoints[33] (e.g. OX40xPD-L1 and ICOSxPDL1). Only few bsAbs have been designed to simultaneously target a tumor antigen and an ICI, like our bsAb BCMAxPDL1 construct. Published examples are PDL1xEGFR[34], GD2xPD1[35] and HER2xPD1[36]. Others include other regulatory molecules and angiogenic factors combined with ICI, such as TGFβxPDL1[37].

Currently, bsAbs targeting BCMA approved (Teclistamab, BCMAxCD3[9]) or under investigation (Elranatamab[38], TNB-383B[39] and Alnuctamab[40]) for MM treatment are all bispecific T cell engagers (TAAxCD3). Only recently, a novel tri-specific molecule CD3xBCMAxPDL1 has been developed and assessed in a preclinical study[41]. BCMA is also a target of ADCs and CAR-T, the latter having shown very promising results in the clinic[11,12]. BCMA is therefore widely considered as a suitable target for novel biological drugs in MM. An interesting point to note is that several groups are investigating the combination of anti-BCMA immunotherapy with ɣ-secretase inhibitors[42].

To conclude, to the best of our knowledge, our bsAb is one of the first constructs targeting a TAA (BCMA) and an ICI (PDL1) proposed for the treatment of MM. Our bsAb molecule presents the limitation of partial steric hindrance observed for PDL1 binding, which may reduce the efficacy of the anti-ICI moiety. As previously reported by our group[24], in our bsAb the hinge of anti-BCMA, contains a couple of cysteines that mediate dimerization of the IgG H chains, creating a relatively rigid and closed

structure. A strategy to potentially produce more flexible bsAbs may be replace the pair of cysteines, in the anti-BCMA hinge, with two serines[24]. Our group has already demonstrated that this cysteine-free-hinge version of bsAb showed slightly lower binding and effectiveness in inducing CDC, is effective in mediating ADCC and ADCP compared to bsAb with unmodified hinge[24]. However, this molecule was also shown to be less stable that the cysteine version of the molecule (Golay et al[24] unpublished results). In contrast the present bsAb format has shown extremely high stability at 4°C, presumably due to its structure being very similar to a natural IgG1. This is an important point to consider when developing bsAbs, which often show problems of aggregation and instability upon storage[43]. Hence, future work may investigate other formats of the bsAb with longer and more flexible linkers, in order to reduce steric hindrance, but such molecules should also plan to work on the anti-PDL1 VH sequence to reduce the affinity of the anti-ICI compared to the anti-BCMA moiety**,** to ensure the delivery of the bsAb towards tumors cells.

In addition, the present bsAb should be investigated in human tumor xenografts, cell-line-derived or patient-derived, growing in humanized mouse models *in vivo*[26]. Possible model to employ for this purpose may be the Hu-SRC-SCID mice (Humanized - Scid Repopulating Cell - Severe Combined Immunodeficiency mice) established by injecting irradiated SCID mice with CD34+ human hematopoietic stem cells from a variety of sources as human BM, leading to the engraftment of a human hemopoietic

system in the murine recipient[26]. Recently a human MM (NCI-H929) xenograft model in NPG mice has been found to be adequate to study the *in vivo* efficacy of a trispecific bsAb CD3xBCMAxPDL1 [41].

Finally the best molecule identified will need to be produced in a standard mammalian expression system such as CHO. The BCMAxPDL1 bsAb and corresponding mAb, validated in this thesis, were produced with a baculovirus/insect cells system [24,44]. Indeed this system enables the rapid production of antibodies at relatively low cost. Baculoviruses are not pathogenic to vertebrates or plants and can be used to rapidly obtain stable recombinant viruses, capable of producing fully active proteins[44]. In addition, the insect cells used for production lack the fucosylation system and can generate afucosylated antibodies, that have been shown to control tumor growth more efficiently than their fully fucosylated parent molecules in mice[45]. However, in terms of glycosylation, insect cell-derived antibodies differ from mammalian cell-derived[46]. Insect cell lines lack the ability to provide complex type N-glycan structures and some insect specific proteins represent possible immunogenic epitopes, suggesting the need to produce the bsAb BCMAxPDL1 using a mammalian expression system, choosing among the available systems that produce defucosylated antibodies.

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