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Targeting the crosstalk between tumor cells and microenvironment: a new therapeutic approach for the treatment of lymphoid neoplasms

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Targeting the crosstalk between tumor cells and microenvironment: a new therapeutic approach for the treatment of lymphoid neoplasms

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CERTIFIES THAT:

- The doctoral thesis entitled "Targeting the crosstalk between tumor cells and microenvironment: a new therapeutic approach for the treatment of lymphoid neoplasms" submitted for the award of Doctor in Biomedicine to the University of Barcelona is a record of authentic, original research conducted by Anna Vidal Crespo under her supervision and guidance, and it is eligible to be defended.

- The first work of this thesis was published in the scientific journal *Clinical Cancer Research* in 2016. The impact factor was 8.738 in 2016.

- This paper published in *Clinical Cancer Research*, where Anna Vidal Crespo shares co-first authorship, has been submitted previously for the award of Alba Matas Céspedes doctoral degree, and this work was conducted in equal contribution.

Barcelona, March 2017

To everyone who has helped me along my PhD journey...

INDEX

ABBREVIATIONS

A

ABC: Activated B cell-like

ACK: Ammonium chloride potassium

ADC: Antibody drug conjugate

ADCC: Antibody dependent cell cytotoxicity

ADCP: Antibody dependent cell mediated phagocytosis

ALCL: Anaplastic large cell lymphoma

ALL: Acute lymphoblastic leukemia

APC: Antigen presenting cell

APRIL: A proliferation-inducing ligand

ARF: ADP-ribosylation factor

ASCT: Autologous stem cell transplantation

ATM: Ataxia telangiectasia mutated

ATRA: All-trans retinoic acid

B

BAFF: B-cell-activating factor

BCL2: B-cell CLL/lymphoma 2

BCL2L11: Bcl-2-like protein 11

BCR: B cell receptor

BH3: Bcl-2 homology 3

BIM: Bcl-2 interacting mediator of cell death

BIRC3: Baculoviral IAP repeat containing 3

BiTE: Bispecific T cell engager

BL: Burkitt's lymphoma

BLK: B lymphocyte kinase

BLNK: B-cell linker

BM: Bone marrow

BMSC: Bone marrow stromal cell

BMX: Bone marrow tyrosine kinase

BR: Bendamustine and rituximab

B_{regs}: Regulatory B cells

BSA: Bovine serum albumin

bsAb: Bispecific antibody

BTK: Brutons's tyrosine kinase

C

cADPR: cyclic ADP ribose

CAR T-cells: Chimeric antigen receptor T-cells

CARD11: Caspase recruitment domain family member 11

CCL: Chemokine (C-C motif) ligand

CCND1: Cyclin D1 gene

CD40L: CD40 ligand

CDC: Complement-dependent cytotoxicity

CDK: Cyclin-dependent kinase

CDKN2A: Cyclin-dependent kinase inhibitor 2A

cDNA: Complementary deoxyribonucleic acid

CDR: Hypervariable complementarity-determining region

cIAP: Cellular inhibitor of apoptosis

CLL: Chronic lymphocytic leukemia

CR: Complete response

CRBN: Cereblon

CTL: Cytotoxic T lymphocyte

CTLA-4: Cytotoxic T-lymphocyte-associated protein 4

CXCL: CXC chemokine ligand

CXCR: CXC chemokine receptor

D

DAPK1: Death-associated protein kinase 1

DLBCL: Diffuse large B cell lymphoma

DMSO: Dimethyl sulfoxide

DNA: Deoxyribonucleic acid

E

E2F: E2 promoter binding factor

ECM: Extracellular matrix

EDTA: Ethylenediaminetetraacetic acid

EGFR: Epidermal growth factor receptor

ELISA: Enzyme linked immunosorbent Assay

EMA: European medicines agency

ERK: Extracellular regulated kinase

EZH2: Enhancer of zeste homolog 2

F

Fab: Immunoglobulin antigen binding fragment

FBS: Fetal bovine serum

Fc: Immunoglobulin constant fragment

FCR: Fludarabine, Cyclophosphamide, and Rituximab

FcγR: Immunoglobulin constant fragment-γ receptor

FDA: American food and drug administration

FDC: Follicular dendritic cell

FL: Follicular lymphoma

FN: Fibronectin

FR: Fludarabine and rituximab

Fv: Immunoglobulin variable fragment

G

GC: Germinal centre

GCB: Germinal centre B cell-like

GEP: Gene expression profiling

GSEA: Gene set enrichment analysis

H

HCDR3: Third complementary-determining region of the heavy chain

HDAC: High-dose cytarabine

HDACs: Histone deacetylases

HER2: Human epidermal growth factor receptor 2

HIP1R: Huntingtin-interacting protein 1 related

HIV: Human immunodeficiency virus

HL: Hodgkin lymphoma

HLA: Human leukocyte antigen

HLA-DR: Human leukocyte antigen - antigen D related

HLA-G: Human leukocyte antigen G

HSP90: Heat shock protein 90

I

i.p: Intraperitoneal

Ig: Immunoglobulin

IGH: Immunoglobulin heavy chain complex

IGHV: Immunoglobulin heavy variable chain

IHC: Immunohistochemistry

IKBKB: Nuclear factor NF-κB Inhibitor kinase beta

IL: Interleukin

INK4: Inhibitor of cyclin-dependent Kinase 4

IRB: Institutional review board

IRF: Interferon regulatory transcription factor

ITAM: Immunoreceptor tyrosine-based activation motif

ITK: Inducible tyrosine kinase

IWCLL: International workshop on chronic lymphocytic leukemia

J

JAK: Janus kinase

K

KIR: Killer Ig-like receptor

KMT2D: Lysine (K)-specific methyltransferase 2D

KRAS: Kirsten rat sarcoma viral oncogene homolog

L

LDH: Lactic acid dehydrogenase

LDT: Lymphocyte doubling time

LN: Lymph node

LYN: Lck/Yes novel tyrosine kinase

M

M: Mutated

M1: Classically activated macrophages

M2: Alternatively activated macrophages

mAb: Monoclonal antibody

MAC: Membrane attack complex

MALT: Mucosa-associated lymphoid tissue

MAP3K14: Mitogen-activated protein kinase kinase kinase 14

MBL: Monoclonal B-cell lymphocytosis

MCL: Mantle cell lymphoma

Mcl-1: Myeloid cell leukemia 1

mCRP: Membrane bound complement regulatory protein

M-CSF: Macrophage colony-stimulating factor

MDM: Murine double minute

MDSC: Myeloid-derived suppressor cell

MEF2B: Myocyte enhancer binding factor 2B

MFI: Mean fluorescence intensity

MFIR: Mean fluorescence intensity ratio

MHC: Major histocompatibility complex

MIPI: MCL international prognostic index

MM: Multiple myeloma

MMP9: Matrix metalloproteinase-9

MSC: Mesenchymal stromal cell

mTOR: Mammalian target of rapamycin

MTT: 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide

MYD88: Myeloid differentiation primary response gene 88

MYF6: Myogenic factor 6

N

NAADP: Nicotinic acid adenine dinucleotide phosphate

NAD^+: Nicotine adenine dinucleotide

NCR: Natural cytotoxicity receptor

NES: Normalized enrichment score

NFAT: Nuclear receptor of activated T cells

NF-κB: Nuclear factor kB

NHL: Non-Hodgkin lymphoma

NIK: Nuclear factor κB-inducing kinase

NK: Natural killer

NLC: Nurselike cells

NOTCH1: Notch homolog 1, translocation-associated (Drosophila)

NSD2: Nuclear set domain-containing 2

NSG: NOD/SCID gamma null

O

OCT: Optimal cutting temperature

ORR: Overall response rate

OS: Overall survival

P

PARP: Poly ADP-ribose polymerase

PAX: Paired box

PB: Peripheral blood

PBMC: Peripheral blood mononuclear cell

PBS: Phosphate buffered saline

PCA: Principal component analysis

PCR: Polymerase chain reaction

PD-1: Programmed cell death protein

PDGFA: Platelet-derived growth factor A

PDX: Patient derived mouse xenograft

PECAM-1: Platelet and endothelial cell adhesion molecule 1

PH: Pleckstrin homology

PI3K: Phosphatidylinositol 3-Kinase

PIP3: PhosphatidylInositol 3,4,5-trisPhosphate

PKCβ: Protein kinase Cβ

PL: Peritoneal lavage

PLCγ2: Phospholipase Cγ2

PLL: Prolymphocytic leukemia

PMN: Polymorphonuclear neutrophil

POT1: Protection of telomeres protein 1

PTEN: Phosphatase and tensin homolog

R

R/R: Relapsed/refractory

RA: Rheumatoid arthritis

RB: Rituximab, Bendamustine

RB1: Retinoblastoma 1

R-CHOP: Rituximab + Cyclophosphamide, Doxorubicin, Vincristine, and Prednisone

RIPA: Radio-immunoprecipitation assay

RLK: Receptor-like kinase

RNA: Ribonucleic acid

ROS: Reactive oxygen species

RS: Richter syndrome

RT-PCR: Real time polymerase chain reaction

S

sABC: Surface antibodies bound per cell

scFv: Single chain fragments variable

SCID: Severe combined immunodeficiency

SDF-1α: Stroma-derived factor-1 alpha

SDS-PAGE: Sodium dodecyl sulfate polyacrylamide gel electrophoresis

SF3B1: Splicing factor 3b subunit 1

SHIP1: SH2 domain-containing inositol 5-phosphatase 1

SHM: Somatic hypermutation

SIRPα: Signal-regulatory protein α

SLL: Small lymphocytic lymphoma

SOX: Sry-related HMG box

SYK: Spleen tyrosine kinase

T

TAM: Tumor associated macrophage

TCL1: T-cell leukemia/lymphoma 1

TCR: T cell receptor

TEK: Endothelial tyrosine kinase

TGF: Tumor growth factor

T_H: Helper T cell

TKI: Tyrosine kinase inhibitor

TLR: Toll-like receptors

TME: Tumor microenvironment

TNF: Tumor necrosis factor

TNFL: Tumor necrosis factor ligand

TP53: Tumor protein 53

T_R: Regulatory T cell

TRAF: Tumor necrosis factor receptor-associated

U

UM: Unmutated

V

V: Immunoglobulin variable regions

VCAM-1: Vascular cell adhesion molecule-1

VEC: Vascular endothelial cell

VEGF: Vascular endothelial growth factor

VLA-4: Very late antigen-4

W

WHO: World health organization

WM: Waldenström macroglublinemia

X

XLA: Immunodeficiency disease X-linked agammaglobulinemia

Z

ZAP-70: Zeta associated protein of 70 KDa

INTRODUCTION

INTRODUCTION

1. B- CELL LYMPHOID NEOPLASMS

Lymphomas are a heterogeneous group of cancers that arise from developing lymphocytes, which produce tumors predominantly in lymphoid structures but also in extranodal tissues. The current World Health Organization (WHO) classification categorizes lymphoma in more than 35 distinct entities, primarily on the basis of morphology, immunophenotype, genetic alterations and clinical features. Most lymphomas originate from B lymphocytes and their diversity can often be traced to the developmental stage of a normal precursor B cell.[1]

1.1 B-cell development

B cells are lymphocytes that confer efficient and long-lasting adaptive immunity by the generation of high affinity antibodies against antigens. All mature B cells express a membrane-bound antibody with individual specificity. This immunoglobulin (Ig) is associated with cofactors, and together these molecules form the B cell receptor (BCR). Early B-cell development, which occurs in the bone marrow (BM), concludes when a B-cell precursor successfully rearranges Ig heavy- and light-chain genes and is equipped with a functional surface antigen receptor. Cells that express a functional (and non-autoreactive) BCR differentiate into mature naïve B cells and leave the BM and circulate as small, resting lymphocytes in peripheral blood (PB) and secondary lymphoid tissues. Upon encounter with an antigen, naïve B cells become activated by interaction with CD4+ T cells in the T cell-rich area of the lymphoid tissues and aggregate into primary follicles to form germinal centres (GCs). GCs are the site of affinity maturation, which involves antigen-specific B cell proliferation, somatic hypermutation of the Ig genes, selection of cells expressing high affinity BCR variants and Ig class switching. After several cycles of proliferation, mutation, and positive selection, GC B cells differentiate into either antibody secreting plasma cells or resting memory B cells and leave the GC microenvironment.[2,3]

1.2 Origin of B cell lymphomas

Each lymphoma subtype bears a phenotypic resemblance to B cells at a particular stage of differentiation as judged by similarity of immunophenotype, histological appearance and gene expression profiles. Detecting the presence of somatic hypermutation (SHM) allows determination that the lymphoma cell has experienced the GC. The putative normal B cell counterpart of the B cell lymphomas is shown in Figure 1. Burkitt's lymphoma (BL), follicular lymphoma (FL) and germinal centre B cell-like (GCB) diffuse large B cell lymphoma

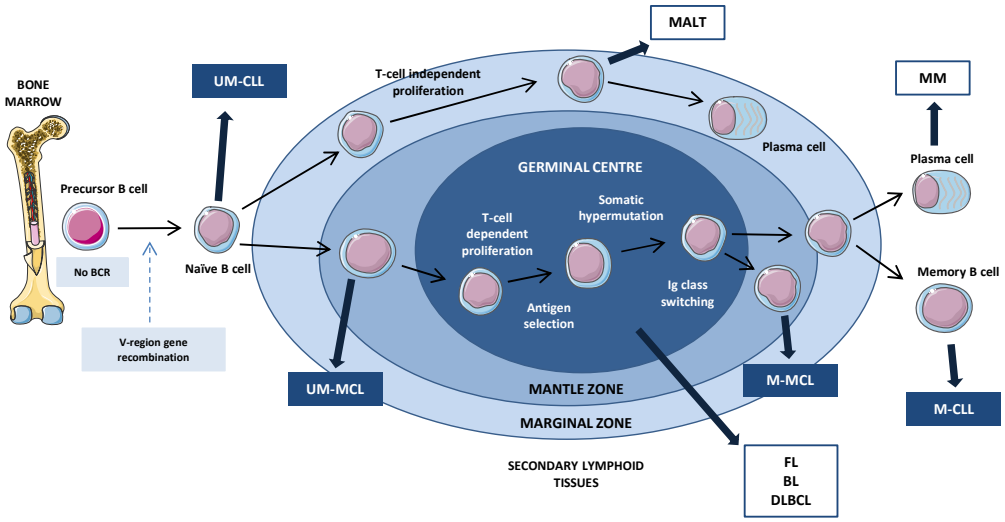


Figure 1: Cell of origin of mature B cell lymphomas. UM-CLL, chronic lymphocytic leukemia with no *IGHV* mutations; UM-MCL, mantle cell lymphoma with no *IGHV* mutations; MALT, mucosa-associated lymphoid tissue lymphoma; FL, follicular lymphoma; BL, Burkitt lymphoma; DLBCL, diffuse large B cell lymphoma; M-MCL, mantle cell lymphoma with *IGHV* mutations; M-CLL, chronic lymphocytic leukemia with *IGHV* mutations; MM, multiple myeloma.

(DLBCL) show features that are consistent with GC B cell derivation. The majority of mantle cell lymphomas (MCLs) are more reminiscent of naïve B cells (UM-MCL). However, 15-40% of cases show SHM, indicating that they have experienced the GC reaction (M-MCL). The unmutated subtype of chronic lymphocytic leukemia (UM-CLL) bears no mutation in the immunoglobulin heavy variable chain (*IGHV*) and thus is pregerminal centre in derivation. The other CLL subtype (M-CLL, 60%) has mutated *IGHV* regions and thus may derive from a postgerminal centre B cell. Mucosa-associated lymphoid tissue (MALT) lymphomas are extranodal in origin and phenotypically related to postgerminal centre marginal zone B cells. Multiple myeloma (MM) is characterized by the accumulation of malignant plasma

cells. It is important to note that the actual cell of origin may be a B cell at an earlier stage of differentiation than its normal B cell counterpart. This can occur if the initiating oncogenic event happens early in hematopoietic development but allows further differentiation to take place before subsequent oncogenic hits are sustained.[3–5]

2. MANTLE CELL LYMPHOMA

MCL is a rare B-cell lymphoma accounting for 3% to 6% of non-Hodgkin lymphomas (NHLs) and the median age at diagnosis is 68, with male predominance (3:1). The course of the disease is heterogeneous, with aggressive clinical evolution in most patients. Although a response to therapy is initially observed in the majority of patients, relapse is common and few patients are cured with current therapies.[1,6]

2.1 Pathobiology of MCL

The t(11;14)(q13;q32) translocation that juxtaposes the proto-oncogene *CCND1* at 11q13 to the immunoglobulin heavy chain complex (*IGH*) at chromosome 14q32 is considered the primary oncogenic mechanism in the development of MCL. The role of cyclin D1 in promoting MCL lymphomagenesis is related to its function in the cell cycle regulating the cyclin-dependent kinases CDK4 and CDK6. Cyclin D1 binding to CDK4/6 activates the transcription factor E2F by phosphorylating its inhibitor, retinoblastoma 1 (RB1), and further promotes cyclin E/CDK2 activation to trigger entry into the S phase of the cell cycle (Figure 2).[7] This translocation is acquired in pre-B cells of the BM and forces the constitutive overexpression of cyclin D1,

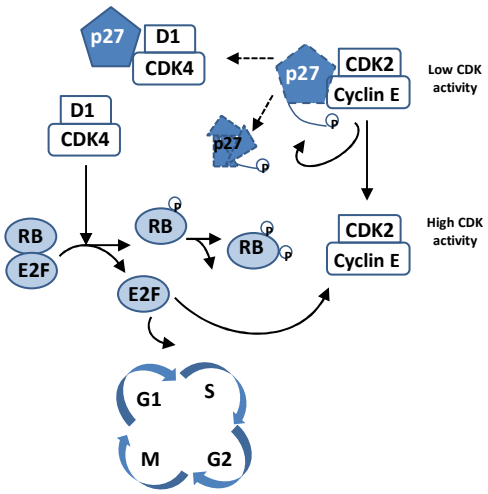


Figure 2: Potential role of cyclin D1 in G1/S phase transition. Cyclin D1 binds to CDK4 and controls the G1/S-phase transition by initiating the hyperphosphorylation of RB1 allowing the accumulation of cyclin E. In addition, the titration of p27 into cyclin D1/CDK4 complexes will promote the raising of active cyclin E/CDK2 complexes that will enhance p27 degradation and further phosphorylation of RB1 allowing the cell to progress into S phase. Adapted from Jares and Campo, Br J Haematol, 2008.[369]

which is not detected in normal B lymphocytes. Cyclin D1 overexpression is not sufficient for cell transformation[8] and the acquisition of secondary genetic alterations account for the aggressive behavior of some cases of MCL, with INK4a/CDK4/RB1 and ARF/MDM2/p53 cellular pathways frequently affected.[9,10]

Molecular profiling has further characterized two subsets of MCL. The SOX11-positive variant features minimal *IGHV* hypermutation, genetic instability and nodal dissemination; whereas the less common SOX-11 negative variant (14% to 32% of cases) derives from postgerminal centre B cells and features *IGVH* hypermutation, genetic stability, nonnodal clinical presentation and longer survival (Figure 3). Therefore, SOX11 expression is considered a biomarker that may help to recognize a subtype of MCL with more indolent behavior but should not be considered a prognostic parameter as some SOX11-negative tumors may progress to an aggressive form of MCL after the acquisition of mutations in genes such as *TP53*.[9,11] SOX11 has been reported to block terminal B-cell differentiation in aggressive MCL by regulating PAX5 expression[12] and there is also data demonstrating a role for SOX11 as a driver of proangiogenic signals in MCL through regulation of platelet-derived growth factor A (PDGFA).[13]

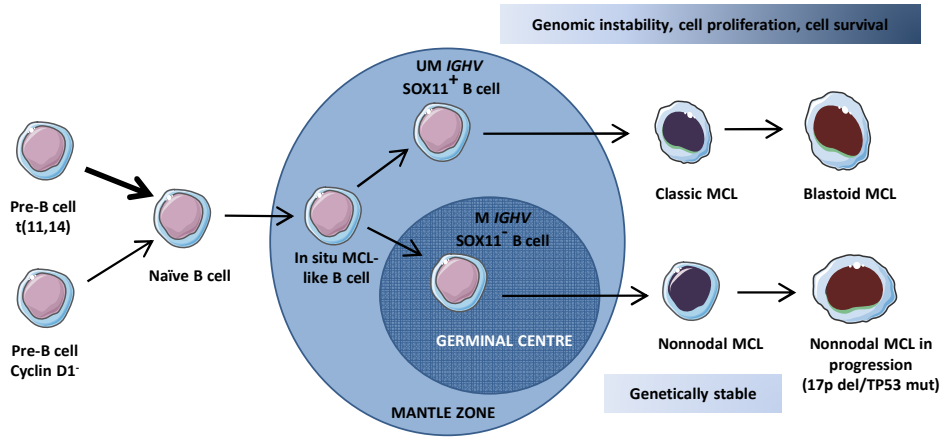


Figure 3: Hypothetical models of two different molecular subtypes of MCL. The naïve B cell carrying the t(11;14) colonizes the mantle zone of the lymphoid follicle and generates an in situ MCL lesion. Most MCLs evolve from these cells or cells in the marginal zone with no or limited *IGHV* somatic mutations. These tumors express SOX11, are genetically unstable, and tend to accumulate alterations. Alternatively, some cells with the t(11;14) may enter the germinal center and undergo *IGHV* somatic hypermutations. These cells are genetically stable and do not express SOX11. The disease seems to be stable for long periods of time, but some of these tumors may acquire mutations in genes such as *TP53* that lead to disease progression. Adapted from Jares et al., J Clin Invest, 2012.[9]

A recent study on the DNA methylome comparing 82 MCLs with cell subpopulations spanning the entire B cell lineage has identified two MCL subgroups. One carrying epigenetic imprints of GC inexperienced B cells and the other of GC experienced B cells. These subgroups showed significant clinicobiological differences in, for example, *IGHV* mutation levels, SOX11 expression, number of copy number alterations and requirement of treatment at diagnosis.[14]

The genetic landscape of MCL has been defined through exome sequencing and the DNA repair genes *ATM (42-55%)*, *CCND1 (35%)*, and *TP53 (19-28%)* were confirmed as the most frequently mutated (Table 1). Novel genes recurrently mutated that affect the epigenetic modifiers *NSD2 (10%)*, *KMT2D (14%)*, *MEF2B (3%)* were also identified. Somatic mutations in regulatory genes of the nuclear factor kB (NF-κB) pathway have been identified in 10% to 15% of MCLs. *BIRC3* is the most commonly affected gene (6-10%), and the mutations are preferentially truncating. Other alterations in both canonical and alternative NF-κB pathways include recurrent inactivating mutations of *TRAF2*, activating mutations of *TLR2*, and occasional mutations in *CARD11*, *MAP3K14* (NIK), and *IKBKB* (IKK-B).[15–17] *NOTCH1/2* mutations (reported in approximately 5% to 10% of patients with MCL) are associated with aggressive clinical behavior.[15,18]

Gene	Class/product	Lesion
Cell cycle		
CCND1	Cyclin D1	+
CDK4	Cyclin-dependent kinase 4	+
MDM2	Negative regulator of p53	+
RB1	Tumor suppressor	-
CDKN2A	p14 and p16	-
Apoptosis		
BCL2	Antiapoptotic regulator	+
BCL2L11	BIM (proapoptotic)	-
DNA damage/epigenetic modifiers		
TP53	Tumor suppressor	-
ATM	Role in DNA damage checkpoints	-
NSD2 ***KMT2D*** ***MEF2B***	Chromatin modifiers	-

Gene	Class/product	Lesion
Cell survival pathways		
SYK	BCR tyrosine kinase	+
PIK3CA	PI3K (p110α) catalytic subunit	+
PTEN	PI3K phosphatase	-
BIRC3	NF-κB negative regulator	-
TRAF2	NF-κB negative regulator	-
MAP3K14	NIK (NF-κB activator)	+
CARD11	NF-κB activator	+
IKBKB	IKKB (NF-κB activator)	+
NOTCH1/2	NOTCH family members	+
TLR2	Member of the TLR family	+

Table 1: Genetic alterations in MCL. **+** indicates amplification or activating mutations; **-** indicates deletion or inactivating mutations.

2.2 Clinical presentation and diagnosis

Patients with MCL typically present with generalized lymphadenopathy (75%), although extranodal involvement is common at sites such as the PB, BM and the gastrointestinal tract. Morphologically, two main variants are recognized, the classic and the blastoid (Figure 4).[19] In the blastoid category two subgroups are recognized-classic blastoid and pleomorphic blastoid and both are clinically relevant because of their aggressive behavior.[20] MCL cells express relatively intense surface IgM/IgD and by immunophenotyping, CD19, CD20, CD22, CD43, CD79a, CD5, and FMC7 typically are positive, whereas CD23, CD10, CD200, and BCL6 are negative.[1] Demonstration of either t(11;14)(q13;q32) by fluorescent in situ hybridization or cyclin D1 expression by immunohistochemistry (IHC) is generally required to diagnose MCL, although a small number of cases with clinical, phenotypic, and gene expression profiling consistent with MCL are cyclin D1 negative.[21] Overexpression of cyclin D2 or cyclin D3 may be observed in these rare cases of MCL negative for cyclin D1, however, IHC for cyclin D2 or cyclin D3 is not helpful in establishing the diagnosis because these proteins are also expressed in other B-cell malignancies. SOX11 expression may be useful in these cases because it is a highly specific marker for both cyclin D1-positive and negative MCL.[22,23]

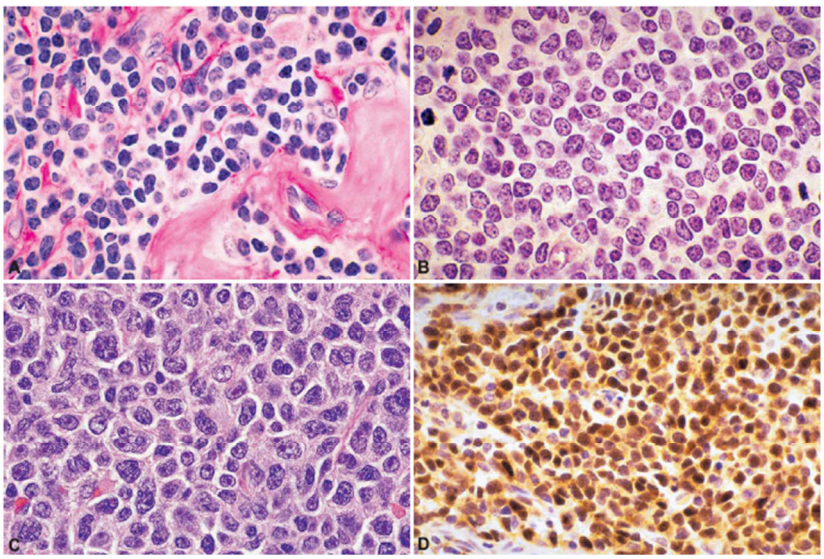


Figure 4: Mantle cell lymphoma. (A) Classic variant, (B) Blastoid variant, (C) Pleomorphic variant, (D) Cyclin D1 immunostain. Adapted from Swerdlow et al., WHO Classification of Tumours, 2008.[19]

2.3 Prognostic factors

Clinical outcome of MCL is highly heterogeneous and may be predicted by the MCL International Prognostic Index (MIPI) based on age, performance status, normalized LDH level, and white blood cell counts. MIPI stratifies patients into 3 risk groups, low, intermediate, and high. It acts as an important tool to apply risk-adapted therapy and should be used in clinical routine before decision about treatment strategy.[24] Ki-67 (or MIB1) has been used as a surrogate marker of proliferation (</≥30%), showing prognosis value independently of MIPI.[25] Recently, a new study on DNA methylation of MCL revealed that the magnitude of DNA methylation changes per case is highly variable and serves as an independent prognostic factor for MCL outcome, having a worse clinical outcome those patients with more epigenetic changes.[14]

2.4 Treatments

2.4.1 Watch and wait strategy

As mentioned above, a subset of MCL patients may have an indolent course of the disease. Such cases are characterized by nonnodal presentation (leukemic phase and splenomegaly), hypermutated *IGHV* genes, genetic stability, SOX11 negativity, and noncomplex karyotype.[26] Although patients with MCL are commonly treated immediately at diagnosis, delayed treatment in these patients with indolent MCL does not adversely affect overall survival (OS) from time of treatment initiation.[27]

2.4.2 Choice of induction therapy (first line)

Patient age and comorbidities are the primary factors that influence the initial approach. For patients younger than 65 years without significant comorbidities, regimens that contain high-dose cytarabine (HDAC) have achieved the best results, typically consolidated by high dose chemotherapy and autologous stem cell transplantation (ASCT).[28–30] In elderly patients or in young but biologically unfit patients, treatment should be less aggressive. R-CHOP (rituximab with cyclophosphamide, doxorubicin, vincristine and prednisone) or RB (rituximab with bendamustine) regimens are more feasible for this group of patients with an overall response rate (ORR) of 78%[31] and 97%[32], respectively. Post-induction strategies as maintenance/consolidation, even following HDAC-ASCT, are now supported by

randomized trials showing the benefit of rituximab in both young and elderly MCL patients.[31,33,34]

2.4.3 Relapsed/Refractory disease (second line)

The majority of patients experience relapses after initial response, with some cases being primarily resistant to first-line treatment. The treatment of patients with relapsed/refractory (R/R) MCL remains a major challenge with no clear standard of care established to date. Induction strategies may be carried over into the relapsed setting in an attempt to achieve better response with a different treatment that was initially received.[35] The use of novel drugs (Table 3) may also provide an opportunity to control the disease. Four newer agents have received regulatory approval for patients with MCL (Table 2): the proteasome inhibitor bortezomib (Velcade), the Brutons's tyrosine kinase (BTK) inhibitor ibrutinib (Imbruvica), the immunomodulatory agent lenalidomide (Revlimid), and the mTOR inhibitor temsirolimus (Torisel). In monotherapy, the ORR and complete responses (CR) are respectively, 33% and 8% with bortezomib[36], 22% and 2% with temsirolimus[37], 28% and 8% with lenalidomide[38], and 68% and 21% with ibrutinib[39]. With the possible exception of ibrutinib, it seems unlikely that these agents will be used as single agents for the treatment of MCL outside of maintenance strategies.[17,40]

Treatment	Nº patients	ORR	CR	Ref
Ibrutinib	111	68%	21%	39
Bortezomib	155	33%	8%	36
Lenalidomide	134	28%	8%	38
Temsirolimus	54	22%	2%	37

Table 2: Comparison of the 4 drugs licensed for use in MCL. ORR, overall response rate; CR, complete response.

2.4.4 Novel therapies

Several novel agents including BH3 mimetic-type BCL2 inhibitors such as venetoclax (ABT-199) has shown impressive single-agent activity in R/R MCL and is being tested in combination.[41] PI3Kδ inhibitors (e.g. idelalisib[42]), Histone deacetylases (HDACs) inhibitors (e.g. vorinostat[43]) or other small molecules including second-generation BTK inhibitors are being developed and explored in MCL and other lymphomas.[44] CDK4/6 inhibitors (e.g.

palbociclib[45], abemaciclib[46]) are also an attractive therapeutic option given the role of cell-cycle deregulation in the pathogenesis of MCL. Moreover, subsequently developed anti-CD20 monoclonal antibodies (mAbs), such as ofatumumab[47] and obinutuzumab[48], have single-agent activity in rituximab treated patients and will be used in combination with other therapies. On the other hand, an exciting recent advance in the management of B-cell malignancies has been the development of CAR T-cells, genetically engineered to express anti-CD19 specificity.[49] Other strategies to induce T-cell responses through checkpoint inhibitors (e.g. anti-PD-1 antibodies) show extremely promising results in a number of tumors and are currently being explored in different lymphoma subtypes.[33,34]

Agent	Mechanism	Phase	Nº of patients	%ORR (%CR)
Idelalisib	PI3Kδ inhibitor	I	40	40 (5)
Everolimus	mTOR inhibitor	II	58	8.6 (0)
Vorinostat	HDAC inhibitor	II	9	0 (0)
Venetoclax	BH3 mimetic	I/II	12	75 (0)
Polatuzumab vedotin	Antibody drug conjugate	I/Ib	7	86 (0)
Abexinostat	HDAC inhibitor	I	14	27 (0)
Abemaciclib	Cell cycle inhibitor (CDK4/6)	II	22	22 (0)

Table 3: Novel agent studies in R/R MCL. Adapted from Atilla et al., Int J Hematol., 2017.[370]

3. CHRONIC LYMPHOCYTIC LEUKEMIA

CLL is the most common leukemia among adults in the Western world, accounting for 25% of NHLs and the median age at diagnosis is 70, with male predominance (1.5-2:1). There is a large variation in survival among individual patients, ranging from several months to a normal life expectancy.[50]

3.1 Pathobiology of CLL

At variance with other lymphoproliferative disorders (e.g. FL or MCL), in most of which a distinctive genomic hallmark is found, there is no CLL-specific genomic aberration. However, more than 80% of patients with CLL at diagnosis carry cytogenetically detectable recurrent chromosomal abnormalities (Table 4). These genomic aberrations are important independent predictors of disease progression and survival. The four most frequent

genomic alterations include: deletion of chromosome 13q14 (55%), trisomy 12 (15%), deletion 11q22-23 (20%) and deletion 17p13 (10%).[51]

Alteration	Effect	Lesion
Cytogenetic alterations		
Del 13q14	MIR15A and MIR16A suppression	-
Trisomy 12	*P27, CDK4, HIP1R, MYF6* and *MDM2* (potential upregulated genes)	+
Del 11q22-23	*ATM*, *BIRC3* inactivation	-
Del 17p13	Inactivation of tumor suppressor p53	-
Genomic alterations		
NOTCH1	NOTCH1 pathway activation	+
SF3B1	Alterations of pre-mRNA splicing	-
BIRC3	Constitutive activation of NF-κB pathway	-
MYD88	Activation of TLR pathway	+
POT1	Telomere dysfunction	-
TP53	Inactivation of tumor suppressor p53	-
ATM	Inactivation of ATM	-

Table 4: Recurrent genetic alterations in CLL.

Whole exome sequencing studies have revealed recurrent genetic lesions (Table 4) that affect genes implicated in different biological pathways including: NOTCH signaling (*NOTCH1*, 10%)[52,53], mRNA processing (*SF3B1*, 10%)[54], inflammatory pathways (*MYD88*, 3%)[55,56], survival pathways (*BIRC3*, 4%)[57], cell cycle control (*TP53*, 10%)[58], telomeres integrity (*POT1*, 3.5%)[59] and DNA damage (*ATM*, 11%)[58].

There are two main CLL subtypes defined by the *IGHV* mutational status, CLL with mutated *IGHV* genes (*IGHV*-M) derived from an antigen-experienced postgerminal centre B cell, and CLL with unmutated *IGHV* genes (*IGHV*-UM) derived from antigen-experienced cells that acquire a memory phenotype in a T-, GC- independent fashion (Figure 5). Both CLL subtypes display a highly restricted and biased repertoire of Ig genes, suggesting that both *IGHV*-M *and IGHV*-UM derive from progenitors that are reminiscent of antigen-experienced B cells.[60,61]

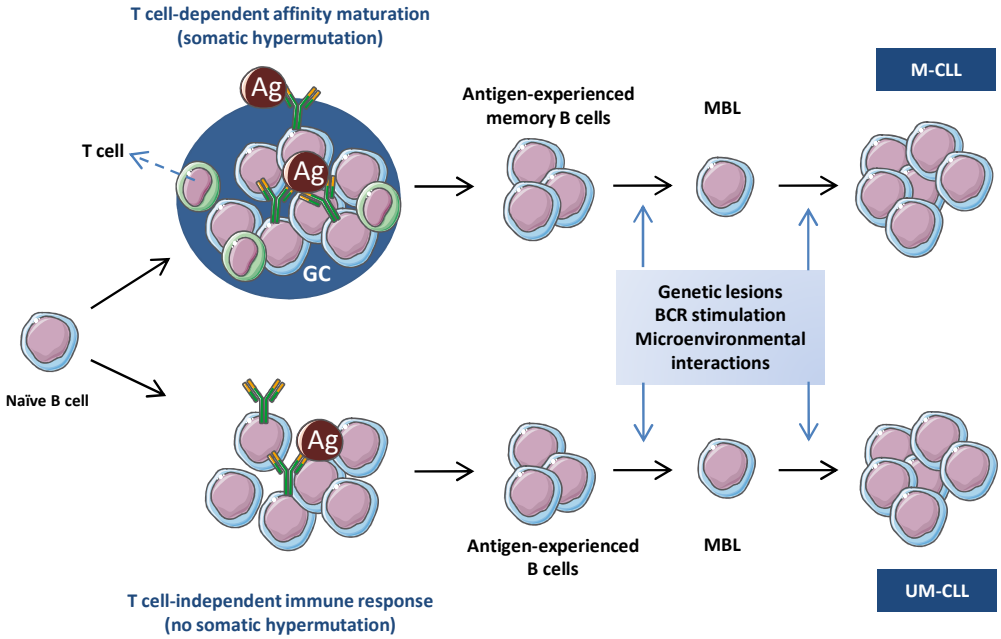


Figure 5: A model for the cellular origin of CLL. Encounter of naïve B cells with antigen may proceed either through a T cell–dependent reaction occurring in the GC or in T cell–independent immune responses. CLL, and the preceding MBL phase, may originate from both of these subsets of antigen-experienced B cells. CLL originating from B cells that have experienced somatic hypermutation carry mutated *IGHV* genes and are defined as M-CLL. Conversely, CLL originating from B cells that have been involved in T cell–independent immune reactions harbor germline *IGHV* genes and are defined as UM-CLL. MBL: monoclonal B cell lymphocytosis. Adapted from Gaidano et al., J Clin Invest, 2012.[371]

3.2 Clinical presentation and diagnosis

CLL is defined as a lymphoproliferative disorder composed by monomorphic round B lymphocytes involving PB, BM and lymphoid organs.[1] According to the International Workshop on Chronic Lymphocytic Leukemia (IWCLL) 2008[62], CLL diagnosis requires the following criteria:

1. the presence of at least 5 x 10^9 B lymphocytes/L in the PB for a duration of at least 3 months;
2. a peculiar immunophenotypic profile as detected by flow cytometry, showing:
   a) clonal light chain restriction (either kappa or lambda)

b) CD5 and CD23 expression

c) low levels of CD20, CD79b and surface immunoglobulin expression.

Most, if not all, cases of CLL are preceded by an asymptomatic condition termed monoclonal B cell lymphocytosis (MBL), in which CLL-like cells are found in the PB with fewer than 5 x 10^9 B lymphocytes/L and no other signs of a lymphoproliferative disorder. Progression to CLL occurs in 1-2% of individuals with MBL per year, probably as a result of progressive accumulation of genetic lesions, epigenetic changes and environmental factors.[63]

CLL diagnosis does not require LN (lymph node) biopsy nor BM aspirate/biopsy, since a typical immunophenotype performed on the PB is sufficient for a conclusive diagnosis (Figure 6). Most patients are asymptomatic at diagnosis and the disease is detected due to increased lymphocyte count at blood evaluations performed for unrelated reasons.[64]

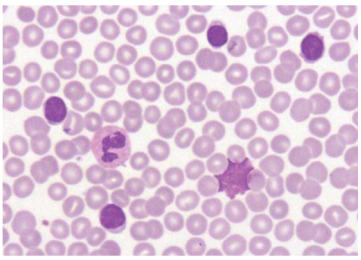


Figure 6: CLL in the peripheral blood. From Swerdlow et al., WHO classification of tumors of hematopoietic and lymphoid tissues, 2008.[19]

Two staging systems are currently applied in CLL patients to define disease burden and treatment indication: Rai (Low, Intermediate, High)[65] and Binet (A, B, C)[66] staging system. These systems provide a simple inexpensive tool to identify those patients who are suitable for observation alone vs. those patients who require systemic therapy and are based on the extent of involvement of PB, LNs, spleen, liver and BM by CLL lymphocytes.[65,66]

3.3 Prognostic factors

Among traditional clinical prognostic factors, Rai and Binet systems and lymphocyte doubling time (LDT, the time required for doubling the absolute lymphocyte count) are the most widely applied and are also reported as indication for treatment. In the last 15 years novel prognostic factors have been identified:[67]

1. The four most frequently detected alterations by FISH analysis bear also a prognostic value:[51]

a) Del13q14: it is the most frequent alteration at it is often found as a single lesion. Two non-coding microRNA genes, *MIR15A* and *MIR16A*, are located in this region and its deletion increases the expression of the antiapoptotic protein BCL2.[68]

b) Trisomy 12: it is associated with a less favorable clinical course and shorter survival. Potential candidate genes that are upregulated by this trisomy include *P27, CDK4, HIP1R, MYF6* and *MDM2*.[69]

c) Del11q22-23: often associated with aggressive clinical course and reduced overall survival. *ATM*, a known tumor suppressor gene, is located in this region and is affected by the deletion.[70] Other potentially affected genes include *BIRC3*.[57]

d) Del17p13: This alteration involves the tumor suppressor gene *TP53* and it is associated with a dismal clinical outcome, being linked to fludarabine-refractoriness, treatment resistance and early disease relapse.[71]

2. The *IGHV*-UM group of CLL is traditionally associated with an aggressive clinical course with clonal evolution and resistance to therapy, which translates into a shorter overall survival when compared with *IGHV*-M subtype.[72] The cut-off to distinguish among these two subgroups was established arbitrarily at 98% of sequence homology for *IGHV* genes in CLL compared to germ line genes.[73]

3. Expression of CD49d (the integrin alpha 4 subunit of VLA-4 receptor) and ZAP70 (an intracellular kinase) has been proposed as independent prognostic markers. A higher level of CD49d (more than 30%)[74,75], and ZAP70 (more than 20%)[76] were associated with worse prognosis. These two markers are dynamic and tend to increase with disease progression, suggesting that they may be a read-out of the response of CLL cells to microenvironment signals.[77,78] Moreover, it has recently been shown that CD49d is the strongest predictor marker of overall survival that can be analyzed by flow cytometry.[74]

4. CD38 is a cell surface protein involved in the interaction with the microenvironment that can function as a receptor and as an ectoenzyme and its expression changes as a result of contact with activated $CD4^+$ T-cells in pseudofollicles.[79] CD38 has been

proposed as an independent prognostic marker and a higher level of CD38 (more than 30%) was associated with worse prognosis in CLL.[80,81]

5. Other markers associated with a prognostic value are:
   a) Mutations which are shown to have an adverse prognostic impact include *TP53*, *NOTCH1*, and *SF3B1* while presence of *MYD88* mutations has a favorable impact.[82–84] TP53 disruption (50%-60%), NOTCH1 activation (30%), and MYC abnormalities (20%) have emerged as the most frequent recurrent genetic lesions in Richter syndrome (RS), which represents the clinicopathologic transformation of CLL into DLBCL.[85]
   b) DNA methylation status in CLL is remarkably stable over time and is characterized by widespread hypomethylation of DNA in genes and enhancer loci, combined with local hypermethylation.[86] Aberrant methylation has been described in some key genes related to CLL such as: *BCL2*, *ZAP70*, *TCL1*, *DAPK1* and *NOTCH1*. Recently, it has been shown that high levels of intra-sample methylation heterogeneity correlate with high-risk genetic lesions, CLL clonal evolution and poor prognosis.[87,88]

3.4 Treatments

At the time of initial diagnosis, patients with CLL, generally, are free of disease-related symptoms and virtually all patients are followed on a wait and watch regimen. International guidelines clearly specify that treatment indication is based on the presence of active disease.[64] The last 5 to 10 years have been marked by considerable advances in the treatment of CLL. Figure 7 shows the overview of the progress made in the treatment of CLL since late 1950s till date.[50]

3.4.1 Choice of induction therapy (first line)

The treatment choice requires a careful evaluation of different factors including: patient fitness status, the genetic profile (e.g. the presence *vs.* absence of *TP53* alterations), and the disease status.[64]

Chemoimmunotherapy combination with fludarabine (purine analogue), cyclophosphamide (alkylating agent) and rituximab (anti-CD20 mAb) (FCR) is currently considered the standard of care for first line therapy in young fit patients with an ORR of 95%.[89] Alternative treatment options include the use of bendamustine and rituximab (BR) with and ORR of 89%.[90] The vast majority of CLL patients is characterized by an impaired physical condition and may be offered a mild chemotherapy regimen containing chlorambucil for symptom control, but also dose-reduced fludarabine or bendamustine can be considered.

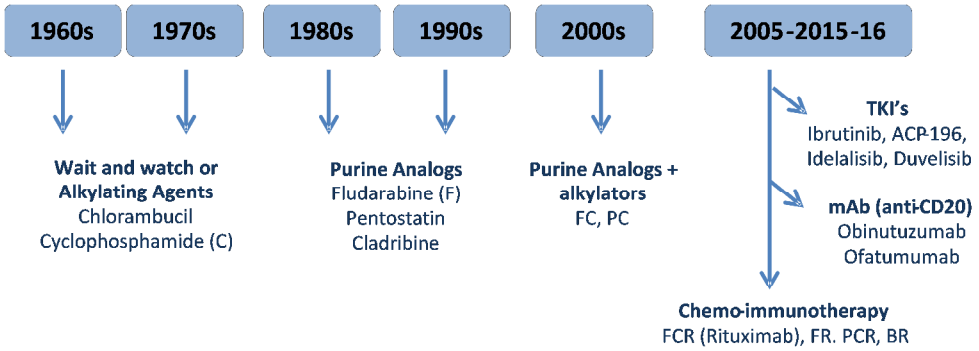


Figure 7: Timeline of development of various treatment modalities in CLL. TKI's: Tyrosine kinase inhibitors; mAb: Monoclonal antibody; B: Bendamustine. Adapted from Rai and Jain, Am J Hematol, 2016.[50]

Anti-CD20 antibodies (rituximab, obinutuzumab and ofatumumab) are also widely used in combination with chlorambucil. The monoclonal antibodies that have been approved in CLL are listed in table 5. The combination chlorambucil + ofatumumab significantly improved ORR compared to chlorambucil monotherapy (82% *vs.* 69%) and was also approved for the first-line treatment of CLL patients.[91] Moreover, a new type II anti-CD20 mAb, obinutuzumab, has recently been approved in combination with chlorambucil in previously untreated CLL, after demonstrating its higher ORR (78.4%) compared to the chlorambucil + rituximab regimen (65.1%).[92]

Finally, there is a high-risk category of patients mainly represented by bearing del17p and/or *TP53* mutations who have a very poor prognosis with alkylating and purine analogue-based chemoimmunotherapy.[93] Patients with these alterations and progressive disease requiring treatment are recommended that enroll in clinical trials with novel

agents. Allogenic stem cell transplantation should also be considered. Patients can also benefit from alemtuzumab (a recombinant humanized anti-CD52 antibody) and high-dose steroids. More recently, novel kinase inhibitors have been FDA (American Food and Drug Administration) approved in first-line for patients with 17p deletion: the BTK inhibitor ibrutinib and the PI3Kδ inhibitor idelalisib.[64,94]

Compound	Structure	Indication	Mechanisms of action
Rituximab	Chimeric, anti-CD20	Previously-untreated and previously-treated patients with CLL in combination with chemotherapy	Induction of CDC, ADCC and apoptosis
Ofatumumab	Human, anti-CD20	Fludarabine- and alemtuzumab-refractory CLL patients, first-line treatment in combination with chlorambucil for patients with CLL for whom fludarabine-based-therapy is inappropriate	Greater CDC activity and similar ADCC activity compared to rituximab
Obinutuzumab	Humanized and glyco-engineered anti-CD20	Older patients with previously untreated CLL in combination with chlorambucil	Increase ADCC and direct cell death compared to rituximab and/or ofatumumab
Alemtuzumab	Humanized, anti-CD52	CLL patients with (del)17p/TP53 defective and/or refractory to fludarabine	ADCC and CDC activity

Table 5: Monoclonal antibodies approved in CLL. ADCC: Antibody-dependent cell cytotoxicity; CDC: Complement-dependent cytotoxicity; mAb: Monoclonal antibody. Adapted from Robak et al., Expert Opin Emerg Drugs, 2015.[372]

3.4.2 Relapsed/Refractory disease (second line)

There is no standard treatment for R/R CLL patients. For those who previously achieved a durable remission, repeating the same regimen at relapse may be efficacious, whereas new agents have demonstrated significant activity for patients with early R/R disease.[64]

New therapies that have been recently approved by the FDA for patients with R/R CLL include: the BTK inhibitor ibrutinib (ORR of 71%)[95], the pan-PI3K inhibitor idelalisib (in combination with rituximab; ORR of 81%)[96], the human anti-CD20 mAb ofatumumab (ORR 45%)[97], and the BCL2 inhibitor venetoclax (for patients harboring del17p13 who have received one prior therapy; ORR 85%).[98]

3.4.3 Novel therapies

Beside the recently approved agents, other treatment approaches are under development (Table 6) and include: new generation BTK inhibitors (e.g. acalabrutinib[99], ONO-4059[100], CC-292[101]); new generation PI3K inhibitors (e.g. TGR-1202[102], IPI-145[103]); new generation SYK inhibitors (e.g. GS-9973[104]); new mAbs (e.g. otlertuzumab, an anti-CD37 mAb[105]); CAR T-cells; and checkpoint inhibitors among others.[64,94]

Agent	Mechanism	Phase	Nº of patients	%ORR (%CR)
ONO-4059	BTK inhibitor	I	25	96 (0)
Acalabrutinib (ACP-196)	BTK inhibitor	I-II	62	95 (0)
Duvelisib (IPI-145)	PI3Kγ/δ inhibitor	I	54	58 (2)-49 evaluable
TGR-1202	PI3Kδ inhibitor	I	16	94 (0)
Otlertuzumab (TRU-016)	anti-CD37	Ib	56	54 (8)
Ublituximab (TG-1101)	anti-CD20	I	6	67 (0)
		II	16	80 (10)-10 evaluable
CAR T cells	CTL019	I	14	57 (28.5)
Pembrolizumab (MK-3475)	Anti-PD-1	II	15 CLL 6 RS	2 SD-2CLL evaluable 80 (20)-5 RS evaluable

Table 6: Phase I-II studies of selected promising novel agents under development for treatment of CLL. CR: complete remission, ORR: overall response rate, RD: Richter syndrome, SD: stable disease. Adapted from Jamroziak et al., Curr Treat Options Oncol., 2017.[373]

4. TUMOR MICROENVIRONMENT

The tumor microenvironment (TME) is the cellular and molecular environment in which the tumor exists and is highly variable with regards to both spatial arrangement and composition of cells. It contains highly variable numbers of immune cells, stromal cells, blood vessels and extracellular matrix.[106]

Malignant B cells recruit and activate TME stromal cells and, in turn, TME stromal cells profoundly influence the behavior of B-cell malignancies. The collaborative interaction between neoplastic B cells and their supporting stromal cells enable and sustain the hallmarks of cancer, including resistance to cell death (anti-apoptosis and drug resistance), sustaining cell proliferation, angiogenesis, immune suppression, stemness and self-renewal and cell homing and invasion, thus promoting progression (Table 7).[107] *In vitro* co-culture experiments and xenotransplantation of lymphoma cells with stromal elements are some of

the models that are used to model interactions of the lymphoma cells and TME and are helping to elucidate this biology.

Resistance to cell death	TME facilitates EMDR contributing to minimal residual disease (MRD) through the modulation of extrinsic and intrinsic survival determinants.
Cell homing & invasion	TME-activated chemokine and adhesive networks are central to the migration and invasion of malignant B cells via coordinated signaling loops.
Angiogenesis	Malignant B cells collaborate with the TME to induce secretion of pro-angiogenic factors from TME and tumor cells leading to sustained angiogenesis.
Sustained proliferation	TME-induced persistent signaling via intercellular and intracellular network sustains lymphoma cell growth and proliferation.
Stemness & self-renewal	Malignat B cells and TME facilitate and maintains tumor cell "stemness" via the coordination of genetic and epigenetic means.
Immune suppression	Interaction of lymphoma cells, stromal cells and immune cells create an immunosuppressive TME that promotes tumor growth, protects the tumor from immune attack.

Table 7: TME shapes the hallmarks of B-cell malignancies. EMDR: Environment-mediated drug resistance. Adapted from Shain et al., Oncogene, 2015.[107]

4.1 The microenvironment in MCL

Constitutive activation of signaling pathways related to external signals from the microenvironment, such as BCR[108–110] and PI3K signaling[111,112] in the absence of activating mutations suggest that the microenvironment plays an important role in MCL pathogenesis. Saba et al provided *in vivo* evidence for BCR and canonical NF-κB activation in MCL cells within the LN microenvironment. The activity of BCR signaling directly correlated with increased tumor proliferation and identified a subset a patients with inferior survival.[113] Relative to other lymphoma subtypes, the precise composition of the MCL TME is not well characterized. MCLs that carry somatic hypermutations in the *IGHV* gene have transitioned through the follicular GC where MCL cells can be in contact with accessory cells, such as

mesenchymal stromal cells (MSCs), follicular dendritic cells (FDCs), macrophages and T cells (Figure 8).[114]

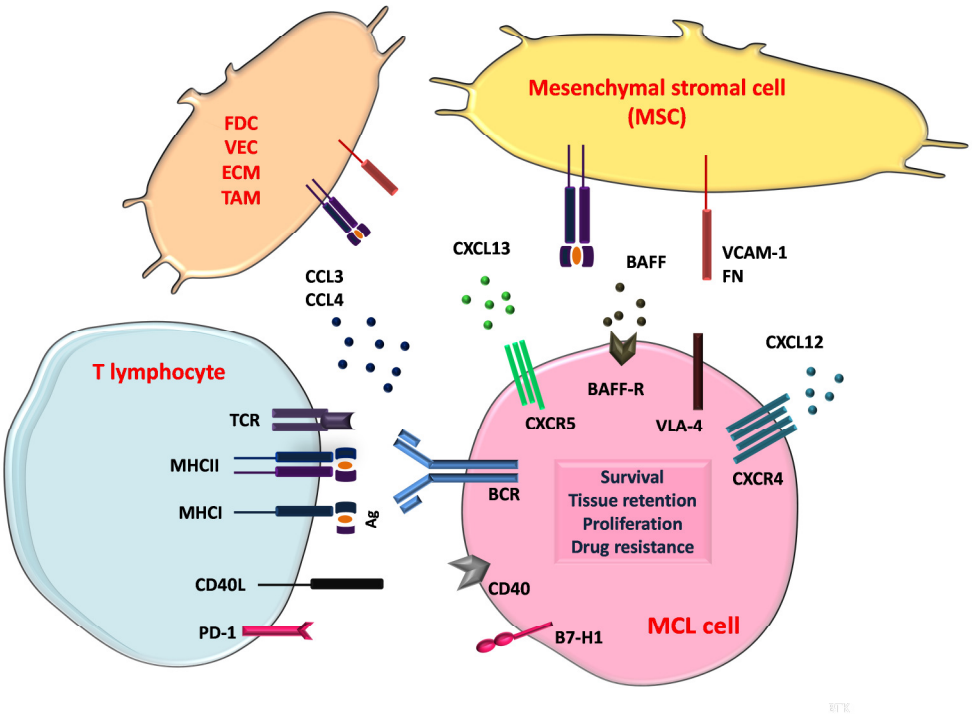


Figure 8: Crosstalk between MCL cells and surrounding microenvironment. Contact between MCL cells and MSCs (mesenchymal stromal cells), FDCs (follicular dendritic cells), VECs (vascular endothelial cells), T cells, ECM (extracellular matrix), and TAMs (tumor associated macrophages) is established and maintained by chemokine/cytokine receptors, adhesion molecules and receptor-receptor interactions. MSCs, FDCs, VECs and TAMs attract MCL cells via G coupled chemokine rectors (CXCR4, CXCR5) that are highly expressed on MCL cells.[115] VLA-4 integrins (CD49d) cooperate with chemokine receptors in adhesion and tissue retention through binding to respective stromal ligands (VCAM-1 and FN-fibronectin). MSCs, FDCs and TAMs can also express the TNF family member BAFF providing survival signals to MCL cells via BAFF-R[374]. T cells provide MCL cells with CD40L leading to prosurvival signaling.[375] Self-and/or environmental antigens allow BCR stimulation, which among other cellular effects, induces MCL cells to secrete chemokines (CCL3, CCL4) for the recruitment of monocytes and T-cells.[154] Lymphoma cell-expressed B7-H1 may lead to the suppression of host anti-tumor immune responses in MCL.[376] Collectively, this crosstalk between MCL cells and accessory cells results in activation of survival and drug-resistance-pathways.

MCL cells express high levels of functional CXCR4 and CXCR5 chemokine receptors and VLA-4 adhesion molecules (CD49d).[115] Adhesion to stroma elements is an important mechanism of chemoresistance that could, at least in part, be due to adhesion-mediated cell cycle arrest.[116] Medina and colleagues demonstrated that MCL contact with MSCs resulted in a significant increase in MCL drug resistance, associated with increased expression of BAFF with concomitant activation of the NF-κB survival pathway and enhanced chemotaxis response to CXCL12 and CXCL13 chemokines. In the same work the authors demonstrated that MCL cells can be maintained *ex vivo* for at least 7 months and that the MCL cells retain their original phenotype and continue to require MCL-MSCs interactions for proliferation and survival.[117] Recently, a study showed that co-culture of primary MCL with lymphoid like cells (CD40L), but not stromal cells, induced cell-cycle progression, which was amplified by MCL-specific cytokines (IGF-1, BAFF, IL-6, IL-10). It further demonstrated that proliferating MCL harbored an imbalance in BCL2 family expression leading to a consequent loss of mitochondrial priming that directly correlated with the sensitivity toward venetoclax and alkylating drugs.[118]

The relevance of the presence of macrophages in MCL remains ill-defined, although similarly to other lymphomas, a correlation between high numbers of macrophages and more aggressive disease has been found.[114]

4.2 The microenvironment in CLL

The majority of tumor cells in the blood are resting. However, heavy-water experiments have shown that CLL contains a small fraction of actively proliferating cells, with approximately 2% of cells newly generated each day.[119] The LN seems to be the key site of CLL proliferation, as documented by substantial activation of cascades such as NF-κB and BCR compared with that observed in BM and PB CLL cells.[120] Recent heavy-water experiments in CLL patients identified the LN as the anatomical site harboring the largest fraction of newly born cells.[121]

As in MCL, CLL trafficking and homing to tissue microenvironment is tightly regulated, involving activation of chemokine receptors and adhesion molecules on the CLL cells. High levels of $CXCR4^{+}$ cells have been associated with higher risk of lymphoid organ infiltration

and poorer disease outcome.[122] The cellular and molecular components of CLL microenvironment have been well characterized (Figure 9).

MSCs, such as BM stromal cells (BMSCs), are commonly found in secondary lymphatic tissues of CLL patients. Adhesion to BMSCs is mediated by VCAM-1 or FN interaction with VLA-4 integrins[123], and chemotaxis towards BMSCs involves the CXCR4-CXCL12 axis.[124] They induce up-regulation of aggressive disease markers in CLL cells, including ZAP70 and CD38, as well as down-regulation of CXCR4.[125,126]

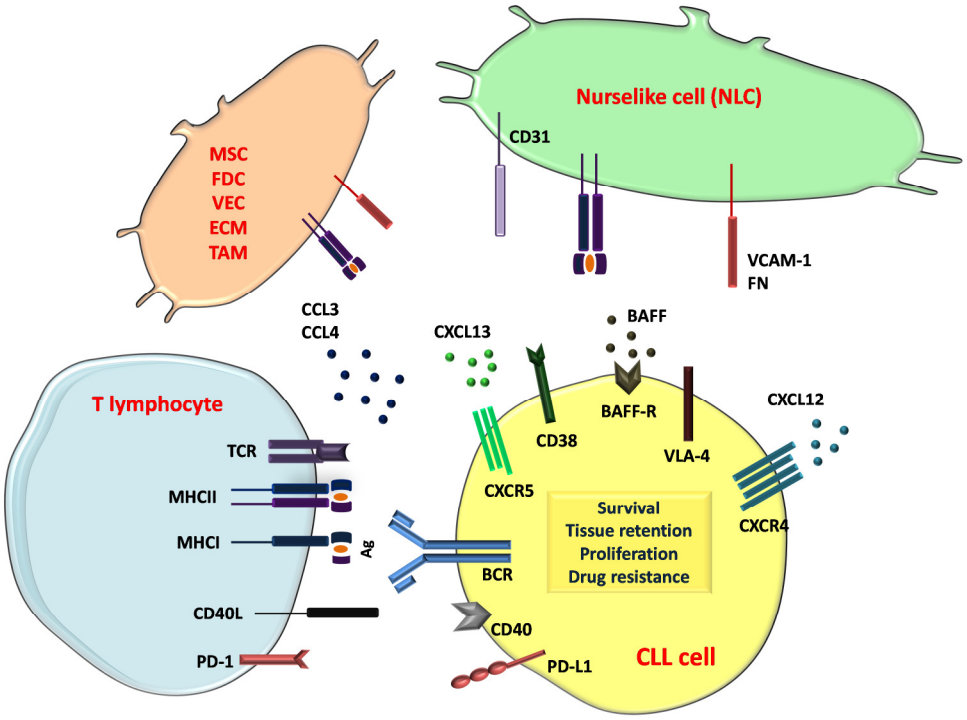


Figure 9: Crosstalk between CLL cells and surrounding microenvironment. Contact between CLL cells and MSCs (mesenchymal stromal cells), FDCs (follicular dendritic cells), VECs (vascular endothelial cells), T cells, ECM (extracellular matrix), NLC (nurselike cells), and TAMs (tumor associated macrophages) is established and maintained by chemokine/cytokine receptors, adhesion molecules and receptor-receptor interactions. Pro-survival pathways activated by the crosstalk between CLL cells and microenvironment cells include the CD38-CD31 axis[129] and the TNF family member BAFF, which interact with BAFF-R[130]. BCR stimulation induces CCL3 and CCL4 chemokine secretion[128], which recruit T cells and monocytes to tissue microenvironments. The CD40/CD40L axis favors survival and proliferation of CLL cells[132] and interaction of PD-L1 with PD-1, which is expressed at high levels on the surface of T cells from CLL patients, favors immune evasion of CLL cells from T-cell cytotoxicity.[377]

Another critical component of the CLL microenvironment is represented by nurselike cells (NLCs). Contact between CLL cells and NLCs is established and maintained by chemokine receptors and adhesion molecules expressed on CLL cells and corresponding ligands on NLCs.[127] The BCR pathway[128], the CD38-CD31 axis[129] and the TNF family members APRIL and BAFF[130] are activated in CLL cells after co-culture with NLCs. NLCs attract CLL cells by secreting CXCL12 and CXCL13 chemokines, which interact with their cognate receptors CXCR4 and CXCR5, which are expressed at high levels on CLL cells. NLCs share lineage and functions with tumor-associated macrophages (TAMs) described in other B cell tumors and have been identified as CLL-specific TAMs.[131] BCR stimulation induces CCL3 and CCL4 chemokine secretion, which recruit T cells and monocytes to tissue microenvironments.[128] The CD40/CD40L axis favors survival and proliferation of CLL cells[132], although the T cell population in CLL is dysfunctional because of the expression of inhibitory molecules such as CD200, CD270, programmed death (PD)-L1 and B7-H3 on tumor cells. Expression of these molecules is linked to a poor prognosis in patients with CLL.[133] There are also results suggesting that myeloid cells in leukemic mice contribute to T-cell defects on different levels. For example CLL cells induce skewing of myeloid cells toward immunosuppressive phenotypes with upregulated PD-L1 expression that might contribute to T-cell exhaustion in CLL.[134] Recent studies demonstrate that CLL cell growth *in vivo* is critically dependent on the support of TAMs. In CLL animal models, macrophages depletion sensitized CLL cells to apoptosis suggesting that these accompanying cells might induce survival benefit.[134,135]

Some studies have also reported defective natural killer (NK)-cell function in CLL because of high levels of human leukocyte antigen G (HLA-G) in the plasma of CLL patients that induces NK cell apoptosis and impairs NK cell lysis.[136] Likewise, several factors contribute to reduced NK-cell cytotoxicity, including low expression of NK-cell activating receptors, such as NKp30.[137] So, both the T and NK-cell compartments have overall reduced effector activities in CLL. Other components of the CLL microenvironment include FDCs and endothelial cells which are essential for tissue homing and CLL retention to tissues.[138]

5. B CELL RECEPTOR (BCR)

B cells depend on signals mediated through the BCR to govern a variety of cellular processes including survival, proliferation, differentiation, and production of antibodies.[139] B cells require an intact BCR for survival and its depletion leads to rapid cell death.[140]

The BCR consists of two Ig heavy and two Ig light chains forming the extracellular, antigen binding part of the BCR and it is complexed with CD79A and CD79B (Igα and Igβ, respectively) that form the cytoplasmatic tail of the BCR (Figure 10). Within the cytoplasmatic part of the BCR, immunoreceptor tyrosine-based activation motifs (ITAMs) are critical for intracellular signal generation. Binding of antigen to the extracellular part of the BCR activates upstream SRC family kinases, most probably LYN, that phosphorylate ITAMs, which then serve as docking site for SYK. In parallel, LYN phosphorylates tyrosine residues in the cytoplasmatic tail of the BCR co-receptor CD19, which enables the binding and activation of PI3K and VAV. PI3K generates PIP3, which is an important second messenger for activating downstream pathways. PI3K attracts BTK to the cell membrane through a PIP3-PH domain interaction, which allows SYK and LYN to fully activate BTK. BTK activation can be regulated by the phosphatases PTEN and SHIP1, which dephosphorylate PIP3 and thereby inhibit BTK membrane association. Once SYK is activated, signaling is propagated to downstream effectors by the recruitment and phosphorylation of BLNK and CIN85, which serves as a scaffold for various signaling molecules, including SYK, BTK and its crucial substrate PLCγ2. BTK is mostly responsible for the phosphorylation and activation of PLCγ2, which leads to an influx of Ca^{2+}, the activation of PKCβ and subsequent activation of the transcription factors nuclear receptor of activated T cells (NFAT) and NF-κB, as well as ERK1 or ERK2 activation, which promote proliferation and survival of normal and malignant cells.[141]

There are two main modes of BCR signaling: tonic signaling, which supports the survival of resting mature B cells through pathways that are activated by PI3K[142], and chronic signaling, which occurs after antigen engagement and activates NF-κB via the caspase recruitment domain-containing protein 11 (CARD11)-BCL10-MALT lymphoma-associated translocation (MALT1) complex.[143]

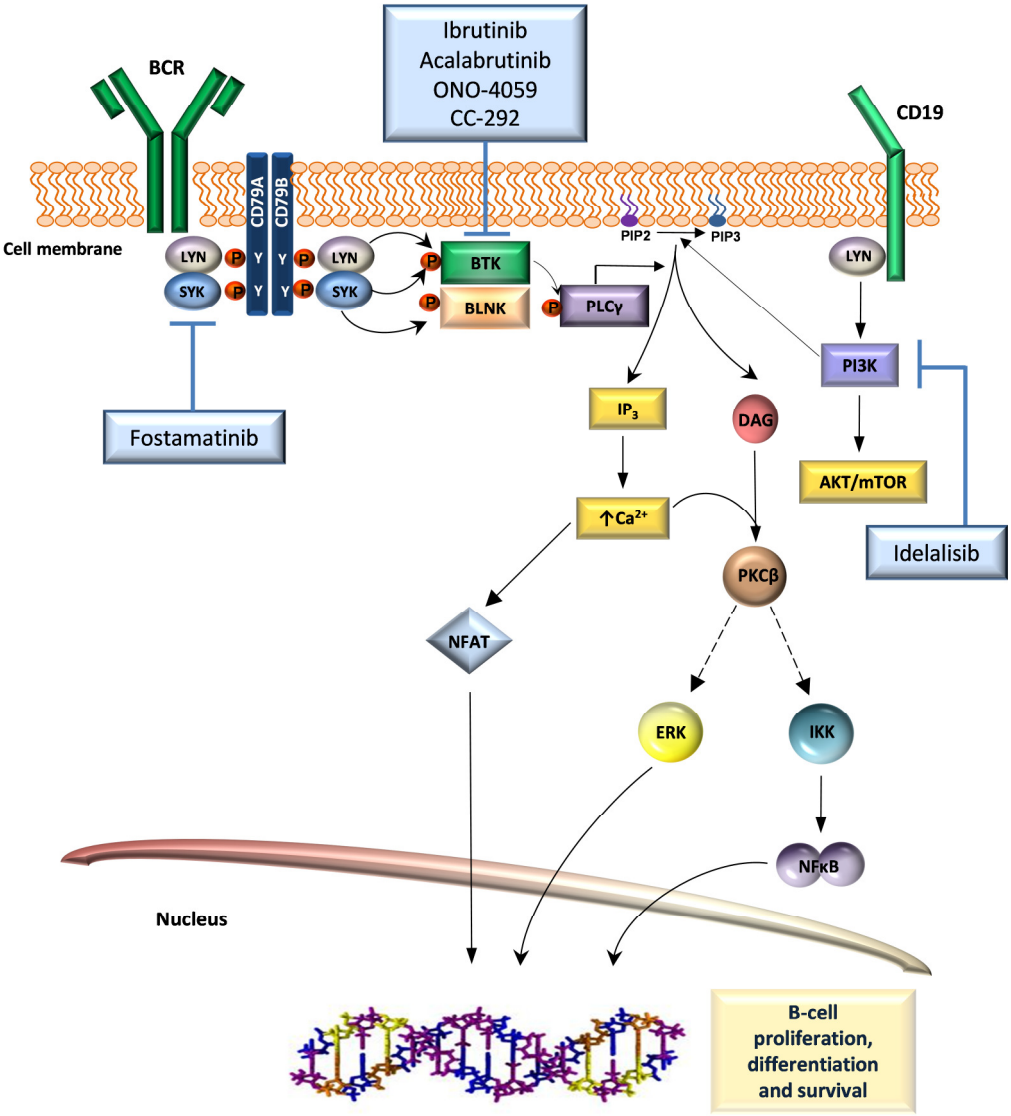


Figure 10: Role of BTK in B cell receptor signaling. BLNK, B-cell linker protein; Ca, calcium; DAG, diacylglycerol; IgH, immunoglobulin heavy chain; IgL, immunoglobulin light chain; IKK, inhibitor of NF-κB kinase; IP3, inositol-1,4,5-trisphosphate; PKC, protein kinase C; PIP2, phosphatidylinositol-4,5-biphosphate; PIP3, phosphatidylinositol-3,4,5-triphosphate; PLC, phospholipase C. Adapted from Herrera and Jacobsen, Clin Cancer Res, 2014.[378]

5.1 BCR signaling in B cell malignancies

The BCR signaling pathway, critical to the development and maturation of normal B cells, is emerging as a central oncogenic pathway that promotes growth and survival in various lymphoma subtypes. Several mechanisms activating this pathway have been identified in different B-cell malignancies.[144]

There are two major hypotheses regarding the microenvironmental source of ongoing stimulation of the BCR in lymphoma. The first is that the BCR recognizes foreign or self-antigens in the tumor microenvironment and is supported by the observation that CLL and MCL cells use a restricted repertoire of *IGHV* genes and some cases express virtually identical BCRs, so called "stereotyped BCRs".[145,146] The second hypothesis is that BCR stimulation is independent of antigen engagement. More than 80% of BCR genes in FL tumors contain sequence motifs that can function as acceptor sites for *N*-linked glycosylation.[147] This glycan chains terminate in a high mannose that can interact with mannose-binding lectins present on stromal cells in the TME, initiating BCR signaling.[148]

BCR can be activated through extrinsic signaling but also through intrinsic signaling due to acquired mutations. Some of the aggressive lymphomas, harbor genetic mutations that amplify external BCR signals, such as mutations in *CD79B*[149], or provide autonomy from these external signals, such as mutations in *CARD11*[150,151], giving rise to chronic NF-κB activation.

It is remarkable that most mature B-cell malignancies express an IgM type BCR despite the fact that most of these lymphomas are derived from GC cells that typically switch their BCRs from IgM to IgG. This observation may be explained by the fact that IgM signaling promotes primarily NF-κB activation and cell proliferation while IgG signaling promotes plasmacytic differentiation.[152]

Several small molecules that interfere with BCR signaling axis have been generated (Figure 10), including inhibitors of SYK kinase (e.g. fostamatinib, GS-9973), of BTK kinase (e.g. ibrutinib, ACP-196, ONO-4059, CC-292), and of PI3K kinases (e.g. idelalisib, duvelisib, pilaralisib, GS-9820, TGR-1202, ACP-319).[138]

5.1.1 BCR signaling in MCL

As mentioned above, the remarkably biased BCR repertoire of MCL suggests a crucial role for antigenic selection in the pathogenesis of at least a subset of MCL, although the specific mechanisms and downstream effectors potentially involved are not well characterized.[145] A pro-survival role of BCR signaling is suggested by the observation of constitutive phosphorylation in primary and MCL cell lines of different kinases of the BCR signaling, including LYN, SYK and BTK.[108] Furthermore, an amplification of the *SYK* gene and SYK overexpression is found in some primary samples[109], and MCL cells show constitutive activation of NF-κB and AKT, which might reflect BCR or toll-like receptor (TLR) signaling.[9] In addition, BTK is strongly expressed in MCL[153], and increased BTK autophosphorylation is observed in unstimulated primary MCL cells.[154] Finally, a recent study shows that malignant B cells from MCL display a potentiated α-BCR-induced signaling response compared to other lymphoma types that is strongly associated with surface expression of BCR subunits (IgM and CD79B).[155]

5.1.2 BCR signaling in CLL

There is increasing evidence that BCR plays a relevant role in CLL pathogenesis.[156] BCR proximal kinases such as Lyn[157], Syk[158] and PI3K[159] are constitutively active in CLL. BTK protein and mRNA are significantly over expressed in CLL compared with normal B cells and, although BTK is not always constitutively active in CLL cells, BCR or CD40 signaling is accompanied by effective activation of this pathway.[160] One third of CLL patients express quasi-identical ("stereotyped") BCRs, suggesting that common antigens may be relevant to disease pathogenesis.[161] Cells expressing CD38, ZAP70 and carrying unmutated *IGHV* genes are generally more responsive to IgM stimulation.[162,163] Accordingly, UM-CLL patients display gene expression profiles suggesting the activation of genes downstream of the BCRs.[164] Some reports have shown that UM-CLL BCRs are polyreactive and mostly recognize autoantigens and other environmental antigens including single stranded or double stranded DNA, vimentin, lipopolysaccharide and hepatitis C viral antigens.[165–167] In contrast, BCR from M-BCRs bind to a restricted set of more specific antigens, including for example β-(1,6)-glucans from yeast and fungi.[168] In addition to antigen-dependent signaling responses, autonomous signaling capacity of CLL-BCRs due to self-recognition of epitopes

within the BCR third complementary-determining region of the heavy chain (HCDR3) has been described.[169,170] This antigen-independent signaling response appears to be involved in leukemia development *in vivo* in the EμTCL1 mouse model of CLL.[171]

5.2 Bruton's tyrosine kinase (BTK)

BTK was originally identified in 1993 as a non-receptor protein tyrosine kinase that is defective in the inherited immunodeficiency disease X-linked agammaglobulinemia (XLA). XLA is a rare disease characterized by an almost complete block of B cell development at the pre-B cell stage and, consequently, B lymphocytes and immunoglobulins are almost completely absent from the circulation. Patients with XLA have no significant developmental defects in other immune cells, and this is consistent with the restriction of clinical features to B cell immunity. Moreover, patients who are maintained on sufficient Ig therapy are generally quite healthy, which suggests that BTK is dispensable outside the B cell compartment.[141] BTK belongs to the Tec kinase family and is expressed in most cells of the hematopoietic system, especially in B cells, myeloid cells, and platelets, whereas T lymphocytes and plasma cells have low or undetectable BTK levels.[172,173]

BTK activation is initiated by cell membrane association and phosphorylation of Y551 in the kinase domain that promotes the catalytic activity of BTK and results in its autophosphorylation at position Y223. Although predominantly cytoplasmic, a small proportion of BTK is detected in the nucleus and its importance remains elusive.[141] BTK transmits, diversifies, and amplifies signals from a wide variety of surface molecules that cells use to communicate with their microenvironment. These include G protein-coupled receptors such as chemokine receptors, antigen receptors (especially the BCR), and integrins.[174]

5.3 BTK inhibitors

The crucial role of BTK in B-cell malignancies makes this protein an interesting therapeutic target. The antitumor activity of BTK inhibitors is not solely dependent on the role of BTK in BCR signaling. Moreover, BTK inhibition also targets TLR signaling, B cell adhesion and migration, and cells in the TME.[152]

A common observation seen in the clinical practice with BCR inhibitors is the mobilization of lymphoma cells away from their tumor-supportive niche, thereby reducing their viability an increasing their susceptibility to immunochemotherapy drugs.[4] It was first described in the treatment of CLL patients with SYK, BTK and PI3K inhibitors. On commencing treatment, the CLL cells mobilized from the BM, LNs and spleen into the blood stream. This lymphocytosis is typically transient and must not be confused with disease progression.[175]

To date, ibrutinib (Imbruvica) remains the only BTK inhibitor approved for several lymphoproliferative malignancies but emerging resistances and off-target effects have led to active development of second generation and more specific BTK inhibitors.[44]

5.3.1 Ibrutinib

Ibrutinib (Figure 11) is a selective, small molecule that irreversibly binds to BTK (Cys-481) and its initial development at Celera and subsequently at Pharmacyclics was focused on rheumatoid arthritis (RA).[176,177] The *in vivo* activity of ibrutinib in B-cell lymphoma was first demonstrated in spontaneous canine B-cell lymphomas.[177]

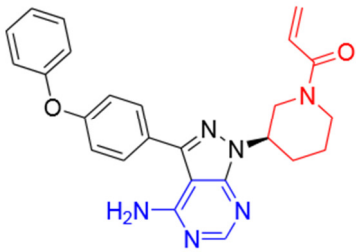


Figure 11: Ibrutinib structure. Adapted from Zhao et al., Eur J Med Chem, 2016.[379]

Ibrutinib has gained regulatory approval for the treatment of R/R patients with CLL, MCL and Waldenström macroglublinemia (WM), and also for first-line therapy in patients with del(17p) CLL. In a follow-up Phase II study performed by Byrd et al., 85 patients with R/R CLL or small lymphocytic lymphoma (SLL) received continuous ibrutinib treatment. An ORR of 71% was reported and the response was independent of clinical and genomic risk factors present before treatment, including advanced-stage disease, the number of previous therapies, and the 17p13.1 deletion.[95] Byrd et al. also reported the results of a three-year follow-up of treatment-naïve and previously treated patients with CLL and SLL receiving ibrutinib monotherapy.[178] The estimated 30-month ORR for previously untreated patients was 97% and that for previously treated patients was 79%. In a phase II study in which 111 patients with R/R MCL were treated, an ORR of 68% was reported by Wang et al.[39] Paralleling the

Phase II study in CLL[95], the response to ibrutinib treatment was independent of disease severity, risk factors and patient characteristics.[39]

Ibrutinib is given orally as a once-per day dose (560mg in MCL and 420mg in CLL) on a continuous schedule until progression or toxicity. It is rapidly absorbed and eliminated after oral administration with an effective half-life of 2-3 hours, and pharmacokinetics appear to be the same in patients with CLL and MCL. Despite rapid clearance from plasma, BTK remains covalently bound to ibrutinib for at least 24 hours.[174]

Ibrutinib has an acceptable tolerability profile in clinical studies. The most common treatment-related adverse events are grade 1 or 2 in severity and include diarrhea, fatigue, nausea, bleeding and infections. Grade 3 or higher hematologic events are infrequent and include neutropenia, thrombocytopenia, and anemia. Some of these events are off-target-related adverse effects.[179] Ibrutinib exhibits remarkable selectivity for BTK. However, nine other kinases have a corresponding cysteine residue in the ATP-binding site. These include four TFK members (ITK, TEC, BMX and RLK/TXK), three EGFR family kinases (EGFR, ErbB2/HER2 and ErbB4/HER4) and two other kinases, BLK and JAK3. Ibrutinib shows different affinity for these kinases (Table 8).[180]

Proteins	Expression	Biological function
Tec family kinases		
BTK	B cells, platelets, erythrocytes, macrophages, neutrophils, mast cells, dendritic cells, NK cells	TEC family kinases, non-receptor tyrosine kinases playing an important role in signalling pathways in hematopoietic cells
TEC	B cells, T cells, platelets, erythrocytes, macrophages, neutrophils, mast cells, liver, heart	
ITK	T cells, NK cells, mast cells	
BMX	Macrophages, neutrophils, endothelial cells, arterial endothelium	
RLK/TXK	T cells, NK cells, mast cells	
Other kinases		
BLK	B cells, thymocytes, plasmacytoid dendritic cells	Non-receptor tyrosine kinase of the SRC-family kinases, involved in B-lymphocyte development
EGFR	Epithelial cells	Epidermal growth factor receptor (EGFR) family of receptor tyrosine kinases involved in cell growth, proliferation and differentiation
ErbB2/HER2		
ErbB4/HER4		
JAK3	B cells, T cells, NK cells, myeloid cells, vacular smooth muscle cells, endothelium	Janus family of kinases, involved in cytokine receptor-mediated intracellular signal transduction, cell proliferation and differentiation

Table 8: The expression and biological functions of proteins with ibrutinib binding sites. Adapted from Berglöf et al., Scand J Immunol, 2015.[180]

		IBRUTINIB:
NK cell		
Btk, Ikt		
- Maturation and activation - TLR-triggered and FcR-stimulated immune response - Citotoxicity, degranulation and cytokine release		- Does not impair direct cell death or CDC by anti-CD20 antibodies [365] - Interferes with NK-mediated ADCC by anti-CD20 antibodies [365] - Reduces rituximab-mediated NK cytotoxicity, degranulation and cytokine release [380] - Down-regulates CD20 expression [380]
T cell		
Btk, Ikt, Rik, Tec		
- TCR-mediated cell activation		- Promotes Th1 cytotoxic population [381] - Decreases levels of Th2-cytokines in treated patients [381]
Macrophage		
Btk, Tec, Bmx		
- FcR-mediated phagocytosis - Induction of reactive metabolites - Production of inflammatory cytokines - M1 polarization		- Impairs rituximab-mediated phagocytosis [364,365] - No data about effect on polarization

Table 9: Schematic view of the role of Btk in non-malignant cells. The table summarizes the main cellular functions, in which Btk is involved in NK cells, T cells, and macrophages. On the right section, some data concerning the effects of ibrutinib on these cells are indicated. Adapted from Maffei et al., J Hematol Oncol, 2015.[182]

The mechanism of action of ibrutinib involves direct effects on malignant B cells, including inhibition of cell proliferation, induction of apoptosis and disruption of cell adhesion and migration.[181] However, the effects of ibrutinib on the tumor microenvironment are also important as the molecular targets of ibrutinib are not restricted to B cell compartment but regulate key functions in other cellular elements, e.g. NK, T cells, and macrophages. (Table 9).[182]

5.3.1.1 Ibrutinib resistances in CLL and MCL

Despite ibrutinib's promising activity across multiple lymphoid malignancies, cases of primary and secondary or acquired resistances have emerged and poor outcomes have been seen in patients with MCL after ibrutinib failure.[183] In both MCL and CLL patients, a cysteine-to-serine missense mutation at the ibrutinib binding site of BTK (BTK^{C481S}) has been identified at relapse after a durable response. This mutation prevents irreversible binding of ibrutinib to BTK and results in a reduced degree of BCR signaling inhibition.[184,185] In addition to BTK^{C481S}, PLCγ2 mutations that can activate the BCR pathway independently from BTK have been identified in CLL patients with acquired resistance to ibrutinib.[184,186]

Recently, a study showed that single *CCND1* mutations increase cyclin D1 protein stability in MCL cell lines and primary samples, and this regulation of cyclin D1 protein levels is directly associated with primary ibrutinib resistance in MCL.[187] Finally, on *in vitro* cell line-based experiments, overexpression of CARD11 mutants was demonstrated to confer resistance to ibrutinib and lenalidomide.[150]

In MCL, recent studies described the presence of alternative mechanisms for primary resistance unrelated to BTK mutations involving sustained distal BCR signaling, specifically via PI3K-AKT activation.[185] Furthermore, ibrutinib resistance in MCL may depend on the alternative NF-κB signaling pathway, rather than the BCR-BTK signaling pathway. Rahal et al. observed that MCL cell lines exhibiting ibrutinib sensitivity bear chronic activation of the BCR leading to the activation of the classical NF-κB pathway via BTK. In contrast, ibrutinib-

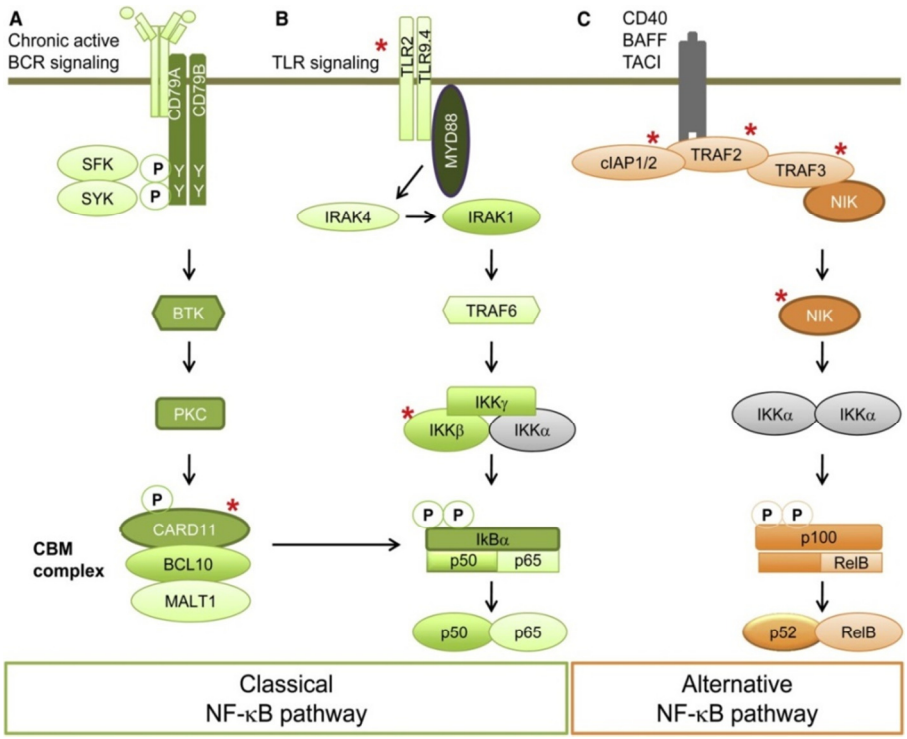


Figure 12: Somatic mutation and activation of the NF-κB pathway in MCL. Genomic sequencing studies have identified mutations in several elements of the NF-κB pathway (denoted by red asterisks). Chronic active BCR **(A)** or Toll-like receptor (TLR) **(B)** signaling activate classical NF-κB pathway. Somatic mutations in the inhibitors of the alternative pathways **(C)** cIAP1 and cIAP2 (gene products of *BIRC2* and *BIRC3*, respectively) and TRAF2/3 activate the alternative NF-κB pathway. Somatic mutations in other elements of these pathways (*CARD11*, *IKKβ*, encoded by *IKBKB*, *TLR2*, and NIK, encoded by *MAP3K14*) have been also found in MCL by whole exome or genome sequencing. From Colomer and Campo, Cancer Cell, 2014.[357]

resistant MCL cell lines are dependent on the alternative NF-κB pathway not mediated by BTK. The authors found inactivating mutations of *TRAF2* and *TRAF3*, which are negative regulators of the alternative NF-κB pathway, in two of the resistant cell lines.[16]

The NF-κB family of transcription factors, (p50/p105, p65/RELA, c-REL, RELB, and p52/p100) binds DNA as heterodimers and homodimers that activate the transcription of genes involved in survival, proliferation, and apoptosis (Figure 12).[188] A wide range of stimuli such as pro-inflammatory cytokines (TNFα, IL-1β, IL-6, CD40L, BAFF), chronic active BCR, DNA damaging agents, TLR agonists and viruses trigger the classical NF-κB pathway. This pathway involves the phosphorylation and consequent degradation of IκBα, a cytosolic inhibitor that sequesters p50 and RELA. Constitutive activation of the classical NF-κB pathway has been reported in MCL cell lines evidenced by the presence of pIκBα and nuclear p65, p50, and c-REL. The alternative NF-κB pathway is induced by a subset of tumor necrosis factor ligand (TNFL) family members (inlcuding BAFF and CD40L) as well as by some viral proteins. This pathway is dependent on stabilization and activation of the NF-κB inducing kinase (NIK). The half-life of this kinase is negatively controlled by TRAF-2, TRAF-3, c-IAP-1 and c-IAP-2 (gene products of *BIRC2* and *BIRC3*, respectively). Upon activation of receptors like CD40 or BAFF, the inhibitory function of TRAF-2 and TRAF-3 is alleviated. Then, stabilized NIK activates IKKα leading to the processing of p100 into p52.[10,189] As mentioned above, mutations in the alternative NF-κB pathway have been associated with ibrutinib resistance.[16]

5.3.2 CC-292

CC-292 (Spebrutinib) (Figure 13) is another covalent irreversible BTK inhibitor developed by Celgene that also binds with high affinity to cysteine 481 in BTK. CC-292 appears to have greater specificity for BTK than ibrutinib (Table 10) and effectively inhibits constitutive and induced BTK and PLCγ2 phosphorylation.[190] The effect of CC-292 on MM cells and osteoclast function has been studied by Eda et al.[191] Recently, the results of a phase I study of CC-292 in patients with R/R CLL/SLL, B-NHL and WM were reported.[192] CC-292 was well tolerated as a

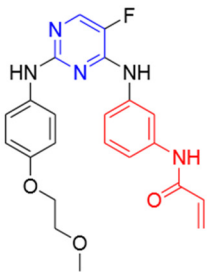


Figure 13: CC-292 structure. Adapted from Zhao et al., Eur J Med Chem, 2016.[379]

daily oral monotherapy at doses up to 1000mg once daily or 500mg twice daily. Twice-daily administration was instituted to improve sustained BTK occupancy and resulted in >90% BTK receptor occupancy at 24 hours post-dose time point. CC-292 was readily absorbed and reached maximum concentration within 1-2 hours post-dose. The most common grade 1-2 adverse events were diarrhea and fatigue, while neutropenia and thrombocytopenia were the most frequent grade 3-4 adverse events. Efficacy in the CLL/SLL population, including patients with poor-risk cytogenetics, showed an ORR of 53% in patients receiving twice-daily dosing. However, its clinical activity (in particular, durability of response) was inferior to that of ibrutinib and acalabrutinib.[99,178,192] The completed and ongoing clinical trials with CC-292 are listed in Table 11.

	Ibrutinib IC_{50} nM	CC-292 IC_{50} nM
BTK*	0.5	0.5
BMX*	0.8	0.7
CSK	2.3	>10000
BRK	3.3	2430
HCK	3.7	14460
YES	6.5	723
ITK*	10.7	36
LCK	33.2	9079
TEC*	78	6.2
FYN	96	7146
c-SRC	171	1729
LYN	200	4401

Table 10: Kinase inhibitory biochemical activity of ibrutinib and CC-292. *Kinases that contain a cysteine residue aligning with Cys-481 in BTK. Data from Honigberg et al.[177] and Evans et al.[190] IC_{50}, half maximal concentration. Adapted from Arnason and Brown, Immunotargets Ther, 2014.[349]

NCT number	Recruitment	Intervention	Condition	Phase
NCT02031419	**Recruiting**	**(CC-122 or CC-223 or CC-292) + rituximab**	**DLBCL**	**Phase 1**
NCT01732861	**Active, not recruiting**	**CC-292 + lenalidomide**	**CLL**	**Phase 1**
NCT01766583	**Active, not recruiting**	**CC-292 + lenalidomide**	**R/R B-NHL**	**Phase 1**
NCT01744626	**Completed**	**CC-292 + rituximab**	**CLL**	**Phase 1**
NCT02433457	**Completed**	**CC-292**	**Healthy Volunteers**	**Phase 1**
NCT01351935	**Completed**	**CC-292**	**R/R CLL, B-NHL, or WM**	**Phase 1**
NCT01975610	**Completed**	**CC-292**	**Rheumatoid Arthritis**	**Phase 2**

Table 11: Clinical trials of CC-292 with available data or ongoing.

5.3.3 Acalabrutinib (ACP-196)

It is a novel irreversible second generation BTK inhibitor and it is more potent and selective than ibrutinib with reduced off-target side effects.[193] In CLL mouse models, acalabrutinib was demonstrated to be able to reduce tumor proliferation and increase survival.[194] In R/R CLL patients an ORR of 95% was reported as monotherapy, and was effective in patients harboring del17p13.[195] Currently, a phase III study (NCT02477696) directly comparing acalabrutinib with ibrutinib in high-risk patients with relapsed CLL has commenced. In addition, multiple trials of ACP-196 on other hematological malignancies and solid tumors are underway.[193]

6. CD38

6.1 Biology of human CD38

CD38 is a single chain type II transmembrane molecule localized in lipid microdomains of the plasma membrane in close proximity with other receptors, forming large supramolecular structures.[196] In addition to the existence of a membrane form, a soluble form of CD38 has also been identified as well as in exosomes, probably forming an intercellular communication network.[197,198] It is expressed in numerous cell types of the hematopoietic system, such as lymphocytes, myeloid cells, NK cells, platelets, and erythrocytes, as well as in solid tissues, including various cell types of the brain, the eye, in pancreatic islet cells, smooth muscle cells, and osteoclasts and osteoblasts. However, CD38 is expressed outside the immune system, often as a cytoplasmic and nuclear molecule.[199]

CD38 behaves simultaneously as an enzyme and as a receptor (Figure 14). The extracellular domain of CD38 contains an enzymatic site that can generate cyclic ADP ribose (cADPR) and ADPR from nicotine adenine dinucleotide (NAD^+) and nicotinic acid adenine dinucleotide phosphate (NAADP) from $NADP^+$. The products of the enzymatic reactions are all involved in the release of different intracellular calcium stores, playing key role on physiologic processes, including cell proliferation, muscle contraction, stem cell regeneration, hormone secretion, T cell activation, neutrophil chemotaxis, dendritic cell (DC) migration, and monocyte chemokine production.[200,201] As a receptor, CD38 is characterized by the property of establishing strong lateral associations with signaling

complexes, varying according to cell lineage, differentiation steps, and microenvironment.[196] CD31 (also known as PECAM-1) has been identified as a ligand for CD38 and is expressed on endothelial cells, DCs, lymphoid cells, in the lungs, and in the kidney.[202] Another ligand proposed for CD38 include hyaluronic acid, which regulate cell-matrix interactions.[203]

6.2 CD38 in normal B lymphocytes

CD38 expression is tightly regulated during B-cell ontogenesis and is present at high levels in BM precursors and is downregulated in resting normal B cells. The molecule is reexpressed at high density once naïve B lymphocytes are activated and peaks when B cells enter the GC. Terminally differentiated plasma cells and their pathological counterparts express the highest surface density among human cells, while it is completely absent in memory B cells.[200] Thus, CD38 expression is one of the early markers of mature naïve B cell activation.

CD38-associated molecules in human B cells include the BCR complex, the chemokine receptor CXCR4, and adhesion molecules, such as CD49d. Ligation of CD38 by agonistic antibodies triggers different *in vitro* responses depending on the differentiation stage of the cells.[81]

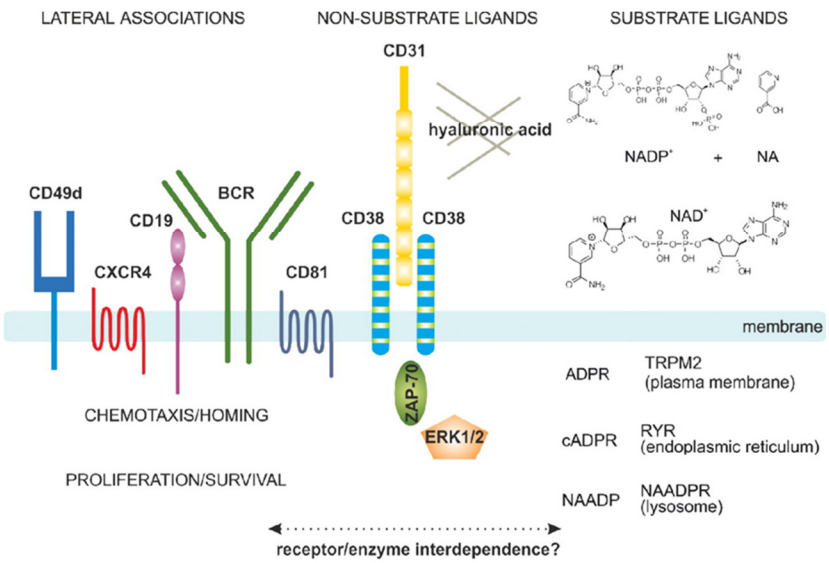


Figure 14: Structural and functional characteristics of the human CD38 molecule. From Malavasi et al., Blood, 2011.[81]

6.3 CD38 in CLL

CD38 is accepted as an independent marker of unfavorable prognosis in CLL with relevance in clinical practice to predict disease outcome (Figure 15).[204] An association between CD38 expression by CLL cells and a more aggressive clinical behavior was first reported in 1999.[205] Consequently, determination of the percentage of CD38-expressing cells within a CLL clone has become part of the workup of CLL patients in many clinical centers and in the majority of studies the threshold is considered as ≥30% $CD38^+$ clonal members.[206] It is generally accepted that $CD38^+$ patients will have a shorter progression-free interval, require earlier and more frequent treatments, and ultimately die sooner.[81,205] $CD38^+$ CLL cells present several molecular differences compared with $CD38^-$ cells of the same clone, including:

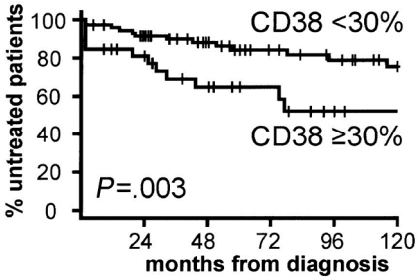


Figure 15: Effect of CD38 protein expression with cutoff of 30% on the clinical course of CLL. Adapted from Wiestner et al., Blood, 2003.[382]

1) Express higher levels of CD69 and HLA-DR, both indicative of recent activation.[207,208]
2) This subset is enriched in Ki-67^+ and ZAP-70^+ cells.[122,209]
3) Express higher VEGF and Mcl-1 levels.[210]

Furthermore, $CD38^+$ CLL clones display enhanced ability to:

1) Migrate in response to CXCL12.[211]
2) Adhere via CD49d integrin.[212]
3) Digest extracellular matrix via metalloproteinase-9 (MMP9).[213]
4) Transduce BCR-mediated signals and to respond to anti-IgM and anti-IgD antibody mediated crosslinking.[162,214,215]

Adhesion to CD49d/CD29 ligands leads to the coagulation of a CLL invadosome, a functional structure characterized by the simultaneous presence of the chemokine receptor CXCR4, of the CD49d/CD29 integrin and of the enzyme MMP9. The additional presence of CD38 in this domain acts as an enhancement factor of chemotaxis, adhesion and matrix digestion, ultimately making homing more efficient.[213]

Additionally, there are results suggesting that the enzymatic activities of CD38 enhances CLL proliferation and signaling mediated via chemokine receptors or integrin, determining a more aggressive clinical course of CLL.[216]

Taken together, the above differences contribute to the adverse impact of CD38 expression in CLL and may help to explain why $CD38^+$ CLL patients have a worse outcome.

CD38 expression in CLL is dynamic and changes as a result of contact with activated $CD4^+$ T cells in proliferation centres, being CD38 specifically expressed on cells that are primed to proliferate in the LN.[79] As a consequence, surface CD38 expression in LNs is significantly higher than in PB or BM[217], and in the proliferating fraction of the tumor.[208] In addition, NLCs derived from CLL patients express high levels of functional CD31.[218] Thus, CD38/CD31 crosstalk occurring in the lymphoid tissues may contribute to create an actively proliferating pool of cells that acts as a tumor reservoir.

6.4 CD38 as a target

High expression levels in combination with its role in cell signaling points to CD38 as an attractive therapeutic antibody target, in particular for MM and CD38-positive NHL. Indeed, various CD38 mAbs have been developed being the human anti-CD38 IgG1 daratumumab[219] the most advanced in development. Two other CD38 antibodies are in clinical development: the humanized mAb isatuximab (previously known as SAR650984)[220] and the human antibody MOR202.[221] Furthermore, several other CD38-targeting antibodies are in preclinical development including: human mAbs Ab79 and Ab19, and an anti-CD38 antibody-drug conjugate (MT-4019).[222]

7. ANTIBODY-BASED IMMUNOTHERAPY

The advent of hybridoma technology in 1975 enabled the production of mAbs. Antibodies are important therapeutic agents for cancer and, recently, it has become clear that antibodies possess several clinically relevant mechanisms of action.[223]

7.1 Structural features of antibodies

Antibodies are grouped into five classes based on the sequence of their heavy chain constant regions: IgM, IgD, IgG, IgE and IgA. Of the five classes, IgG is the most frequently used for cancer immunotherapy. IgG is composed of two heavy ($_H$) and two light ($_L$) chains. These chains comprise one constant fragment (Fc) and two antigen binding fragments (Fab) (Figure 16). The Fab contains the variable regions (V), which consist of three hypervariable complementarity-determining regions (CDRs) that form the antigen binding site of the antibody and confer antigen specificity. The Fc domain connects IgG antibodies to immune effector mechanisms by engaging Fcγ receptors (FcγRs) on NK cells, neutrophils, monocytes, DCs and eosinophils.[224]

Mouse monoclonal antibodies were typically immunogenic in humans and had poor abilities to induce human effector responses, thereby limiting their clinical applicability. Later advances in antibody engineering provided platforms for the development of modified antibodies to increase the similarity to human antibodies. There are three different types of modified mAbs to prevent a mouse-specific antibody response in humans (Figure 16):[225]

1) Chimeric mAbs are constructed with variable regions (VL and VH) derived from a murine source and constant regions derived from a human source.
2) Humanized therapeutic mAbs are predominantly derived from a human source except for the CDRs, which are murine.
3) Human mAbs are entirely derived from a human source by using mice that are either genetically engineered to carry humanized Ig genes or engrafted with a human immune system.

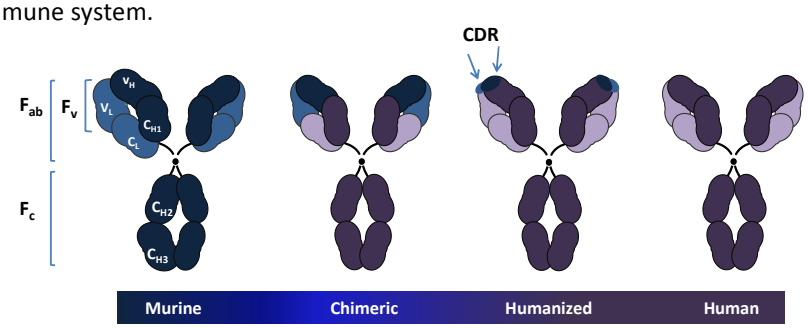


Figure 16: Different mAb types- murine, chimeric, humanized and human. Adapted from Imai and Takaoka, Nat Rev Cancer, 2006.[225]

7.2 Functional features of antibodies

Anticancer mAbs can be directed against the TME. For instance, anti- VEGF mAbs inhibit new blood vessel formation, whereas the checkpoint inhibitors anti-cytotoxic T-lymphocyte-associated protein (CTLA)-4 and anti-PD-1 mAbs target the immune system. However, most mAbs, like anti- HER-2 or anti- CD20 mAbs, are directed against tumor cells. mAbs can have direct effects on tumor cells including the induction of apoptosis or inhibition of cell growth by blocking the binding of a ligand to its growth factor receptor. The main mechanisms proposed to explain the clinical antitumor activity of monoclonal antibodies are the following (Figure 17):[224,226]

1) Complement dependent cytotoxicity (CDC)

   When two or more antibodies bind to a cell, the classical complement pathway is activated through the binding of the C1 complex, a serine protease consisting of C1q, C1r and C1s, to the antibody's Fc domains. This activates a proteolytic cascade that leads to the formation of the membrane attack complex (MAC) and the release of potent anaphylatoxins and opsonins resulting in cell lysis.[224]

2) Antibody dependent cell-mediated cytotoxicity (ADCC)

   The Fc domain of antibodies can activate ADCC through interactions with FcγR on effector immune cells. There are three activating FcγRs: FcγRI (CD64), FcγRIIA (CD32A), and FcγRIIIA (CD16A) and one inhibitory receptor, FcγRIIB (CD32B). NK cells, which predominantly express FcγRIIIA are the main effector cells of ADCC, although macrophages and granulocyte cells have been shown to mediate ADCC to a lesser extent. These effector cells, through FcγRs, recognize an antibody coated target cell and cause direct lysis of the target cell through release of granzymes and perforin. [224]

3) Antibody dependent cell-mediated phagocytosis (ADCP)

   Macrophages, which differentiate from monocytes, are capable of performing phagocytosis, a process that involves the engulfment and degradation of material such as debris, dead cells, or pathogens. They express all classes of FcγRs. TAMs, often found in high numbers within tumors, are often considered protumor, promoting tumor growth and dissemination, and are broadly characterized as M2-like. On the other hand, M1 macropahges are pro-inflammatory or "classically

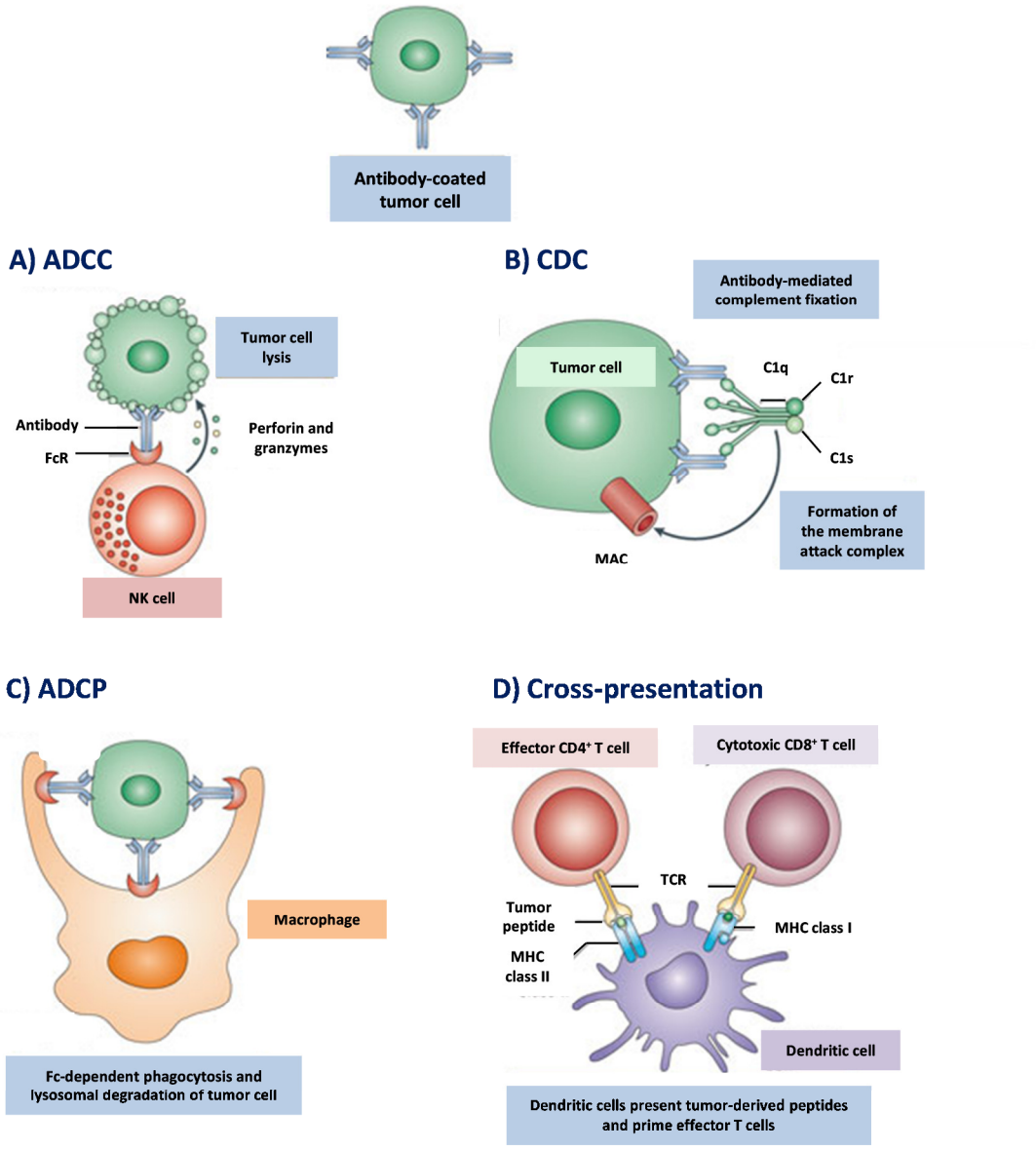


Figure 17: Immune-mediated effects of tumor-specific IgG. Monoclonal antibodies (mAbs) that bind directly to cancer cells can mediate their antitumor effects through various mechanisms. A) Antibody-dependent cell cytotoxicity (ADCC), B) Complement-dependent cytotoxicity (CDC), C) Antibody-dependent phagocytosis (ADCP), and D) Cross-presentation. Adapted from Weiner, Surana and Wang, Nat Rev Immunol, 2010.[226]

activated" macrophages and mediates innate immunity. However, although TAMs have protumor properties, they also maintain Fc-dependent antitumor function.[227,228] Indeed, although in lymphoma patients treated with conventional therapy, the degree of macrophage infiltration correlates with poor prognosis[229]; macrophage infiltration appears to be a favorable prognostic indicator when rituximab is added to conventional therapy.[230]

4) Induction of adaptative immunity

Several groups have suggested that maximal benefit of antibody therapy is achieved through induction of adaptative immunity. In support of this hypothesis is the amount of time it takes to see clinical benefits of antibody therapy. CDC and ADCC generate tumor cell fragments and release tumor antigens that can be taken up by professional antigen presenting cells (APCs), such as DCs, to initiate tumor directed adaptative immunity. In addition, antibodies can trigger adaptative immunity by acting as an opsonin and triggering Fc dependent phagocytosis of tumor cells by APCs. APCs can present tumor-derived peptides on major histocompatibility complex (MHC) class II molecules and promote $CD4^+$ T cell activation. Aditionally, in a process known as cross-presentation, tumor-derived peptides can be presented on MHC class I molecules, resulting in activation of $CD8^+$ cytotoxic T cells. However, the TME is a key determinant of whether this adaptative immune response promotes tolerance or anti-tumor response. [224,226]

7.3 Mechanisms of resistance to antibody therapy

One mechanism of resistance to antibody therapy is due to amplification of downstream signaling and alternative signaling pathways. The most thoroughly described example of molecular determinant of primary resistance to antibody therapy is KRAS mutational status and sensitivity to the anti-EGFR antibodies cetuximab and panitumumab.[224] Another mechanism of resistance is explained by the fact that tumor cells may upregulate expression of inhibitory receptors, such as HLA-E and HLA-G, which engage inhibitory receptors on NK cells to inhibit ADCC.[224] Similarly, tumor cells may express membrane bound complement regulatory proteins (mCRPs), such as CD55, CD59, and CD46, which inhibit the activation of C3 and subsequent generation of the membrane attack complex.

Thus, mCRPs actively inhibit CDC and they are expressed by a wide range of cancers including breast, colon, lung and hematological malignancies.[231] Impairment of proper antigen presentation is another putative mechanism of resistance to antibody therapy. Tumor cells often downregulate expression of MHC I and as a result evade destruction by MHC-restricted cytotoxic T lymphocytes (CTLs).[232] Moreover, loss of antigen expression is also a mode of mAb resistance. Two key modes of antigen expression loss have been proposed (Figure 18); internalization/modulation and "shaving". In the former, a cell intrinsic process occurs whereby mAb binds to the target antigen (e.g. CD20) and its Fc is engaged by the inhibitory FcγRIIB, precipitating internalization of the tripartite complex. In contrast, shaving is performed by a secondary phagocytic cell. Under certain conditions, such as target cell saturation, the activatory FcγR rather than mediating phagocytosis of the target cell, rip a portion of the cell membrane containing the target antigen and the antibody from the target cell, stripping it of mAb and antigen.[233,234] Finally, the tumor microenvironment is enriched with myeloid-derived suppressor cells and T_{reg} cells that produce IL-10, TGF-beta and reactive oxygen species (ROS) to inhibit tumor cell killing by CTLs.[235]

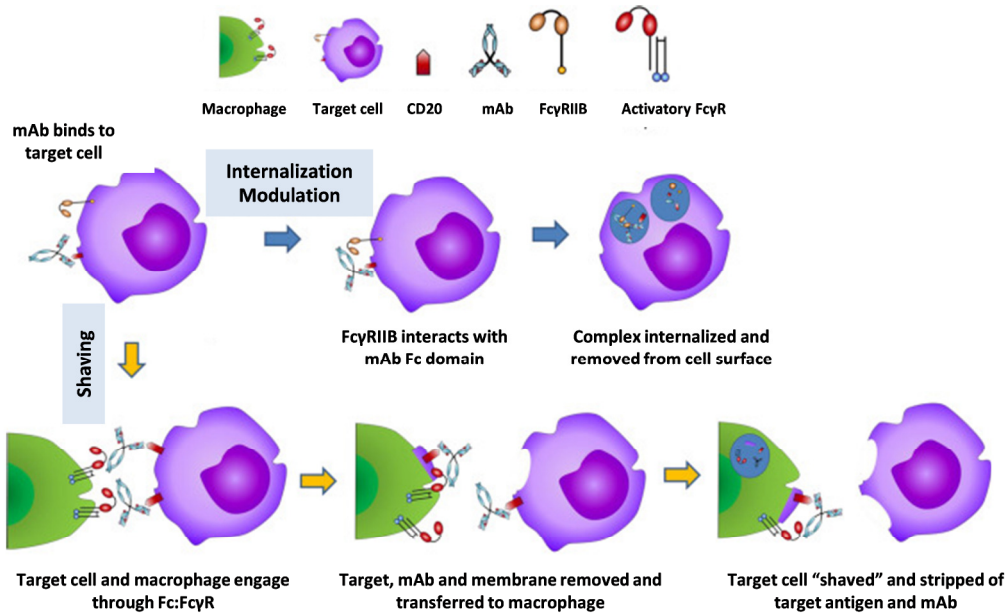


Figure 18: mAb resistance through internalization/modulation and shaving. Adapted from Vaughan et al., Pharmacological Research, 2015.[234]

7.4 mAbs in hematological malignancies

There are currently several mAbs approved by the FDA for the treatment of hematological malignancies (Table 12).

Agent	Target	Type	Indication
Rituximab[382]	CD20	Chimeric	B-NHL, CLL
Ofatumumab[383]	CD20	Human	CLL
Obinutuzumab[260]	CD20	Humanized	CLL, R/R FL
Ibritumomab[266]	CD20	Murine/radiolabeled	R/R B-NHL
Brentuximab[384]	CD30	Chimeric/radiolabeled	R/R HL, R/R ALCL
Daratumumab[270]	CD38	Human	R/R MM
Alemtuzumab[385]	CD52	Humanized	CLL

Table 12: mAbs approved by the FDA against hematological malignancies.

7.4.1 Anti-CD20 mAbs

The CD20 molecule is a transmembrane protein comprising a large loop and smaller loop that serves as a calcium channel initiating intracellular signals such as proliferation, activation, differentiation and cell survival.[236] It is present during all stages of B-cell development except in pro-B cells and antibody-producing plasma cells.[237] Monocytes, T-cells, non-lymphoid cells and stem cells are devoid of CD20.[238] The antigen is expressed at high density on 90% of all B-cell NHLs, although in CLL the antigen density is lower.[239] mAbs to this molecule are classified as type I or type II. Type I antibodies translocate CD20 into detergent-insoluble fractions, or 'lipid rafts' which function as platforms for cell signaling and receptor trafficking.[240,241] They are most effective in activating CDC. Type II antibodies do not induce lipid rafts but efficiently induce ADCC via the recruitment of cells displaying FcγRs. They also play an important role in the induction of direct cell death via apopototic or non-apoptotic mechanisms.[242] There are currently several anti-CD20 mAbs approved by the FDA for the treatment of hematological malignancies (Table 12).[242]

7.4.1.1 Rituximab

Rituximab is a type I chimeric anti-CD20 mAb and was the first mAb to be approved for treatment in cancer patients, being aproved by the FDA in 1997 for the treatment of R/R $CD20^+$ B-cell NHL.[243] The mechanisms of antitumor action of rituximab are CDC[244], ADCC[245],

and apoptosis induced via cross-linking of rituximab by FcγR-expressing cells.[246] Preclinical and clinical studies have shown antitumor efficacy of rituximab in monotherapy, but especially in combination with standard chemotherapy, in several B-cell NHLs.[247] Moreover, several clinical studies have evaluated the role of rituximab as maintenance therapy after rituximab monotherapy[248,249], standard chemotherapy[250], or following combined rituximab-chemotherapy.[251]

7.4.1.2 Ofatumumab

Success of rituximab initiated development of new generations of anti-CD20 mAbs, which are currently under several steps of clinical trials or have already been approved for B-cell NHL treatment. An example of an emerging anti-CD20 mAb is ofatumumab (HuMax-CD20). It is a human type I anti-CD20 mAb that recognizes a distinct small-loop epitope on the CD20 molecule, resulting in increased binding affinity to CD20, prolonged dissociation rate, and increased cell killing due to greater CDC activity and similar ADCC activity compared to rituximab.[252] This enhanced CDC translated into a modest clinical benefit when used as a single agent therapy in rituximab-resistant B-NHL. The combination chlorambucil + ofatumumab is approved for the first-line treatment of CLL patients, reporting an ORR of 82%.[91] As monotherapy in patients with R/R CLL, ofatumumab demonstrated an ORR of 45% and was FDA approved for CLL patients refractory to fludarabine and alemtuzumab.[253] Phase II trial results suggest that ofatumumab finds its place in combination with chemotherapy and phase III comparisons with rituximab and other regimens are currently ongoing.[242]

7.4.1.3 Obinutuzumab

Obinutuzumab is a humanized type II anti-CD20 mAb (GA101) that binds to CD20 in a completely different orientation than type I antibodies, forming different molecular assemblies that elicit different cellular responses.[254] When it binds to CD20 it induces intracellular signaling transduction cascades that mediate homotypic cell adhesions which rearrange the actin cytoskeleton causing the lysosome membrane to increase in permeability.[255,256] This proposed mechanism culminates in the generation of reactive oxygen species (ROS), which compromise the integrity of the plasma membrane causing

non-apoptotic cytoplasmic cell death.[257] Additionally, obinutuzumab was also glycoengineered to have an enhanced afucosylated Fc segment which more effectively engaged effector cells displaying CD16 receptors (e.g. FcγRIIIA in NK cells). The resultant design increased its affinity by up to 50-fold.[258] *In vitro* studies have also demonstrated that obinutuzumab induces human polymorphonuclear neutrophils (PMNs) activation more efficiently compared to rituximab in the phagocytosis of CLL targets.[259] Moreover, it has shown superior ADCC, direct cell death and B-cell depleting activities compared to rituximab and ofatumumab, although it exhibits lower CDC activity because unlike type I antibodies, type II antibodies do not stabilize CD20 into lipid rafts.[260] This novel antibody received approval as first-line treatment in CLL patients in combination with chlorambucil, after being demonstrated to improve the ORR (78.4%) compared to chlorambucil + rituximab (65.1%).[92] When used as monotherapy in R/R CLL, a phase I/II study revealed an ORR of 62% in phase I and 30% in phase II.[261] In FL, it has been recently approved for its use in combination with bendamustine followed by obinutuzumab monotherapy for R/R FL patients.[262] Several phase III trials exploring obinutuzumab in various combination therapies are ongoing, including: a study of obinutuzumab + chemotherapy *vs.* rituximab + chemotherapy in untreated indolent NHL patients.

7.4.1.4 Ibritumomab

Radiolabeled mAbs represent a novel way of targeting lymphoma cells and taking advantage of its inherent radiosensitivity. Ibritumomab (90 Y-ibritumomab tiuxetan; Zevalin®) is the murine equivalent of rituximab that targets the same epitope on the CD20 molecule. It is covalently bonded to tiuxetan, which chelates the radioactive particle yttrium-90, therefore, ibritumomab is able to deliver radioactivity specifically to tumor sites.[263] Ibritumomab tiuxetan is indicated for treatment of patients with R/R low-grade, follicular NHL, including patients who are refractory to rituximab, and as consolidation therapy in previously untreated follicular NHL in patients who achieve a partial or complete response to first-line chemotherapy.[264,265] It has proven safe with relative efficacy, however, common hematologic toxicities have been reported after treatment, and its use should be limited to patients with adequate BM function.[266]

7.4.2 Anti-CD38 mAbs

As previously described, CD38 is a transmembrane glycoprotein that functions as a receptor and as an ectoenzyme. It is expressed by most cells of the hematopoietic lineage at some time during their differentiation, and more specifically on CLL cells that are primed to proliferate in the LN.[79] Moreover, CD38 is expressed at high levels on some hematological malignancies such as MM, NHL, and B and T Acute Lymphoblastic Leukemia (ALL).[267,268] At present, Daratumumab is the only anti-CD38 mAb that has been granted FDA approval, although there are several other anti-CD38 mAbs under clinical development for the treatment of hematological malignancies, such as isatuximab and MOR202.[222]

7.4.2.1 Daratumumab

Daratumumab (Darzalex) is a first-in-class, human IgG1κ mAb that targets the CD38 epitope and was developed by Genmab and Janssen Biotech for the treatment of MM and NHLs.[269] It was the first antibody approved by the FDA for the treatment of refractory MM. It is indicated for patients with MM who have received at least three prior lines of therapy, including a proteasome inhibitor and an immunomodulatory agent, or who are double-refractory to a proteasome inhibitor and an immunomodulatory agent.[270] In a phase II trial in patients with previously treated (as described above) R/R MM, monotherapy with daratumumab 16 mg/kg achieved an ORR of approximately 30%. Patients received 16mg/kg once weekly for 8 weeks (cycles 1 and 2), then every 2 weeks for 16 weeks (cycles 3-6), and then every 4 weeks thereafter (cycle 7 and higher). Intravenous daratumumab monotherapy 16 mg/kg was generally well tolerated and fatigue, anemia, nausea, thrombocytopenia, and neutropenia were the most common adverse events.[271] FDA has also recently approved daratumumab in combination with lenalidomide and dexamethasone (ORR: 93%)[272], or bortezomib and dexamethasone (ORR: 83%)[273], for the treatment of patients with MM who have received at least one prior therapy.

The favorable efficacy results in heavily pretreated patients with advanced MM have provided the rationale for the investigation of daratumumab in a number of ongoing and future phase II and phase III trials. Trials with daratumumab are also ongoing in NHL, including a phase II trial in R/R MCL, DLBCL and FL (NCT02413489). There are also plans to

explore daratumumab as a maintenance agent, similarly to the role of rituximab in maintenance therapy.[274]

Daratumumab has a broad-spectrum killing activity in MM. In addition to CDC, the binding of daratumumab to CD38 on the surface of tumor cells has been shown to induce ADCC[219], ADCP[275] and tumor cell apoptosis.[276] It has also been shown that daratumumab can modulate the enzymatic activity of CD38 and potentially may lead to a reduction in immunosuppressive adenosine levels.[277,278] Moreover, it has been suggested that Daratumumab has also an immunomodulatory role in MM by depleting $CD38^+$ immune suppressive cells (Figure 19).[279]

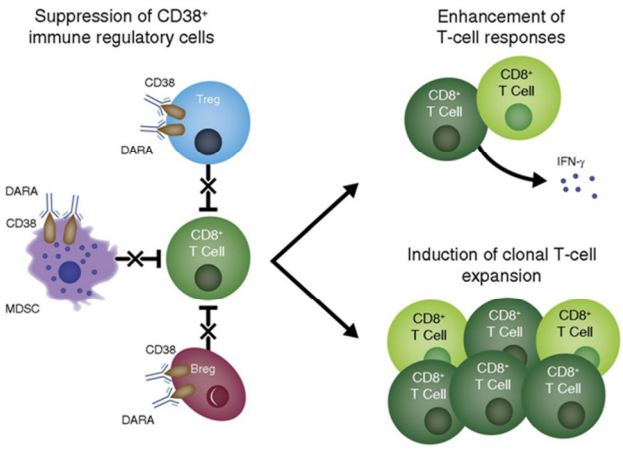


Figure 19: Daratumumab depletes $CD38^+$ immunosuppressive cells (Tregs, T-cell MDSCs, Bregs) and thus stimulates T-cell effector functions. From Krejcik et al., Blood, 2016.[279]

There is convincing preclinical data showing the potential synergism of daratumumab with lenalidomide, which occurs largely via NK cell activation by lenalidomide.[280] Moreover, recent preclinical results provide evidence for the benefit of daratumumab plus lenalidomide combination for lenalidomide- and bortezomib- refractory MM patients.[281] In addition, in another study, the combination of IPH2102, an anti-KIR antibody, with daratumumab and lenalidomide enhanced daratumumab-induced ADCC in MM.[282]

Finally, a recent study demonstrated that CD38 expression is associated with response to daratumumab monotherapy and also that the development of daratumumab resistance may occur by acquisition of a new drug-induced phenotype with higher CD55 and CD59 expression levels, or as a result of a pre-existing subpopulation that is already relatively resistant to DARA prior to initiation of therapy.[283]

7.4.3 Bispecific antibodies

The natural properties of antibodies that enable specific antigen engagement can be improved by engineering approaches that increase antitumor activity. Bispecific T cell engager (BiTE) molecules, which use a new format of bsAb that targets both CD3 and another antigenic marker, are formed by linking two Ig variable fragments (Fv) using flexible linkers. Thus, it is possible to activate a T cell when it physically engages a tumor cell.[223] Blinatumomab, a murine anti-human CD3 anti-human CD19 single-chain antibody (Figure 20), demonstrates significant clinical promise in patients with advanced NHL. It has been approved for the treatment of R/R B precursor ALL by the FDA and EMA (European Medicines Agency).[284] It is currently being tested as monotherapy in a phase II trial, and also in a phase I trial combined with lenalidomide, in patients with R/R NHL. The results of a phase I study revealed an ORR of 71% in R/R MCL, 80% in R/R FL, 55% in R/R DLBCL, with a good safety profile.[285]

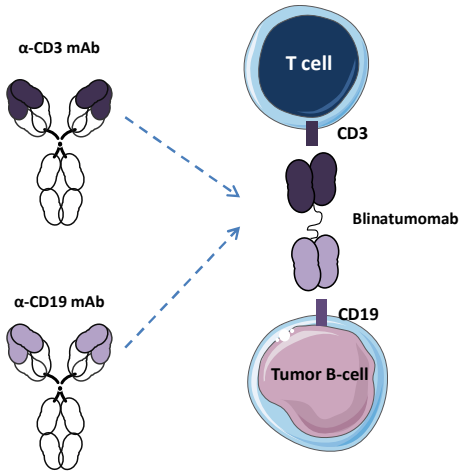


Figure 20: Mechanism of action of Blinatumomab.

8. OTHER IMMUNOTHERAPIES: CAR T-CELLS

Another manner to redirect T-cells in a targeted and MHC-independent manner is by adoptive cell transfer therapy with genetically engineered CAR T-cells. These synthetic receptors use an antibody fragment (single chain fragments variable, or scFv) fused to a transmembrane region and signaling domains. T-cells are obtained from the patient and then genetically engineered to specifically recognize a target antigen on the surface of

tumor B-cells. These modified T-cells are returned to the patient where they will recognize the specific antigen, resulting in T-cell activation and consequent killing of the tumor B-cell.[286] The most clinically studied CARs are those that target B-cell malignancies, in particular CARs against the antigen CD19.[287] To date, CAR T-cell therapies have been most efficacious in patients with B-cell ALL with an ORR of 80% achieved across various CD19-targeted CAR T-cell designs, clinical trials and institutions.[288–291] Less impressive results were achieved in R/R CLL patients, with an ORR ranging from 35-57% among different studies.[292,293] In other NHL, predominantly in R/R FL and R/R DLBCL, an ORR ranging from 50-100% was observed among different studies.[294,295] The adverse events associated with CAR T-cell therapy correlate with the expansion or the persistence of the cells after tumor elimination.[290,296]

AIMS

AIMS

The CD38 molecule is gaining importance as a novel therapeutic target for patients with hematological malignancies, with several anti-CD38 mAbs in clinical trials showing promising results. CD38 appears to be a global molecular bridge to the environment, promoting survival/proliferation of B cells on their way to and after neoplastic transformation.

Aim 1: **To evaluate the antitumor profile of the first-in-class anti-CD38 antibody daratumumab in the poor prognosis CD38^{+} CLL subtype.**

1.1. To identify the mechanisms of action of DARA in CLL primary samples and cell lines analyzing CDC, ADCC and ADCP.
1.2. To characterize the effect of DARA on CLL homing.
1.3. To validate DARA antitumor activity using *in vivo* models.

BCR signaling contributes to the pathogenesis of B cell malignancies and has emerged as a new target for therapy with special relevance in tumor cell-microenvironment crosstalk. Recently, blockade of the BCR-related kinase BTK with the first-in-class inhibitor ibrutinib has shown impressive clinical responses in MCL and CLL.

Aim 2: **To evaluate the antitumor profile of a novel and highly selective BTK inhibitor CC-292 in MCL.**

2.1 To analyze the cytotoxic/cytostatic effect of CC-292 in a panel of MCL cell lines.
2.2 To determine the ability of CC-292 to inhibit constitutive and IgM-induced BTK activation.
2.3 To determine the effect of CC-292 on BCR derived cellular activation.
2.4 To assess the impact of CC-292 on MCL cell migration.
2.5 To identify potential therapeutic combinations.

MATERIALS AND METHODS

STUDY 1: DARA in CLL

1. Cell lines and patient samples

Primary tumor cells from 18 CLL patients diagnosed according to the WHO classification criteria were used.[19] Written informed consents of the patients were granted following the guidelines of the Hospital Clínic Ethic Committee (IRB) and the Declaration of Helsinki. Mononuclear cells were isolated from PB by gradient centrifugation on Ficoll (GE Healthcare) (Figure 21) and used fresh or cryopreserved in liquid nitrogen in RPMI 1640 containing 10% DMSO (Sigma-Aldrich) and 60% heat-inactivated fetal bovine serum (FBS; Life Technologies) and maintained within the Hematopathology collection of the institution (IDIBAPS-Hospital Clínic Biobank, R121001-094). The Prolymphocytic Leukemia (PLL) cell lines, MEC1, MEC2 and JVM13 as well as the BL Daudi cell line were obtained from DSMZ. CLL primary samples and cell lines were cultured in RPMI 1640 or IMDM supplemented with 10% FBS, 2 mM L-glutamine, 50 µg/mL penicillin/streptomycin (Life Technologies) and were maintained in a humidified atmosphere at 37ºC containing 5% CO^2. Normocin (100 µg/mL) (Invivo Gen) was added to the cell line cultures to prevent Mycoplasma contamination in cell lines that were routinely tested for Mycoplasma infection by PCR. The identity of all cell lines was verified by using AmpFISTR identifier kit (Life Technologies).

2. Therapeutic and CRPs blocking antibodies

A human IgG1 targeting CD38 (Daratumumab, DARA) was generated by immunization in a HuMAb mouse.[269] The human mAb IgG1-b12, specific for the HIV-1 gp120 envelope glycoprotein[297] was included in all experiments as an isotype control mAb. Both antibodies were provided by Genmab.

Anti-CD46 (clone TRA-2-10, Biolegend), anti-CD55 (clone 1C6, Hycult Biotech) and anti-CD59 (clone YTH 53.1, AbD Serotech) antibodies were used to block mCRPs.

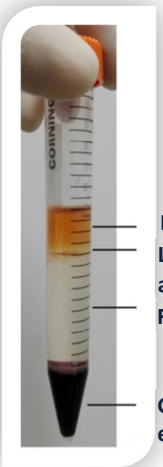


Figure 21: Isolation of mononuclear cells from peripheral blood by gradient centrifugation on Ficoll-Paque.

3. CD38 molecule per cell quantification

CLL samples were stained with QuantiBRITE CD38-PE (BD Biosciences) and the mean fluorescence intensities were assessed in an Attune acoustic focusing cytometer (Life Technologies). The surface antibodies bound per cell (sABC) were calculated using a standard curve obtained by QuantiBRITE PE Beads (BD Biosciences).

4. Antibody dependent cell cytotoxicity (ADCC)

Target cells were labeled with 1 µM Calcein-AM (Life Technologies) for 30 minutes (min) at 37ºC. Afterwards, cells were washed thrice with PBS, plated in triplicate at 1×10^4 cells/well in 96-well round bottom plates, and pre-incubated (room temperature for 15 min) with 10-fold serial dilutions of either isotype control (IgG1-b12) or DARA (range: 1 to 0.0001 µg/mL) in RPMI 1640. DARA doses for *in vitro* studies were previously established.[269] Culture medium was added instead of mAb to determine the spontaneous calcein release and 1% Triton X-100 was used to determine the maximal calcein release. Thereafter, fresh human peripheral blood mononuclear cells (PBMCs) were added at an effector:target (E:T) ratio of 50:1, optimized in a previous report [269] and cells were incubated for 4 hours (hr) at 37°C. The plates were centrifuged, supernatant transferred into black plates (Thermo Scientific) and fluorescence was measured in a Synergy spectrophotometer (Bio-Tek) (excitation filter: 485 ± 20 nm; band-pass filter: 530 ± 20 nm). The percentage of cellular cytotoxicity was calculated using the following formula:

$$\text{specific lysis} = 100 \times \frac{\text{experimental release (RFU)} - \text{spontaneous release (RFU)}}{\text{maximal release (RFU)} - \text{spontaneous release (RFU)}}$$

5. Complement-dependent cytotoxicity (CDC)

Target cells were labeled with 1µM Calcein-AM (Life Technologies) for 30 min at 37ºC. Afterwards, cells were washed thrice with PBS, plated in triplicate at 1×10^5 cells/well in 96-well round bottom plates, and preincubated (room temperature, 15 min) with 10-fold serial dilutions of either isotype control (IgG1-b12) or DARA (range: 10 to 0.01 µg/mL) in RPMI 1640. Culture medium was added instead of mAb to determine the spontaneous calcein release and 1% Triton X-100 was used to determine the maximal calcein release. Thereafter, 10% normal human AB serum was added and incubated for 45 min at 37°C. The

plates were centrifuged, supernatants transferred into black plates (Thermo Scientific) and fluorescence measured in a Synergy spectrophotometer (Bio-Tek) (excitation filter: 485 ± 20 nm; band-pass filter: 530 ± 20 nm). The percentage of cellular cytotoxicity was calculated using the following formula:

$$specific\ lysis\ = 100 \times \frac{experimental\ release\ (RFU) - spontaneous\ release\ (RFU)}{maximal\ release\ (RFU) - spontaneous\ release\ (RFU)}$$

6. Antibody dependent cell mediated phagocytosis (ADCP)

Macrophages (mϕ) were generated from monocytes isolated from BM of the hind legs of female severe combined immunodeficiency (SCID) mice (C.B-17/Icr-Prkdcscid/Crl) (Janvier Labs) by flushing the femurs. The cells were cultured for 7 days in DMEM supplemented with 10% FBS, 2 mM L-glutamine, 50 µg/mL penicillin/streptomycin, and 50 U/ml M-CSF (Cell Guidance), and the culture medium was renewed every 3 days. On day 7, mϕ were detached with 0.1% trypsin-EDTA and characterized by flow cytometry (CD11b+, F4/80+) (mouse antibodies obtained from eBiosciences and Invitrogen, respectively) (Figure 22). The mϕ were seeded at 2.5×10^5 cells per well into non-tissue cultured treated 24-well plates and allowed to adhere overnight. Target cells (primary CLL and cell lines) were labeled with 0.01µM Calcein-AM and added to the mϕ at an E:T ratio of 1:1 in the presence of a fixed mAb concentration of 1 µg/mL. After 4hr of incubation, the non-phagocytosed target cells were collected. The mϕ were detached with 0.1% trypsin-EDTA, added to the non-phagocytosed target cells and stained for F4/80 expression. The amount of remaining target cells (calcein+ F4/80-) was determined on an Attune acoustic cytometer, and the percentage of killed target cells in the presence of DARA compared to isotype control was calculated using the following formula:

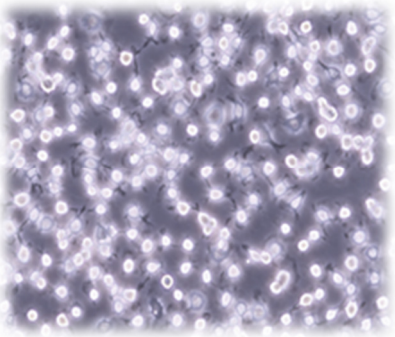


Figure 22: BM-derived M1 macrophages/monocytes isolated from the hind legs of female SCID mice and cultured for 7 days.

$$\%\ \text{eliminated target cells}\ = 100 - [100 \times \frac{\text{remaining target cells after DARA treatment}}{\text{remaining target cells after isotype control treatment}}]$$

7. *In vivo* phagocytosis assay

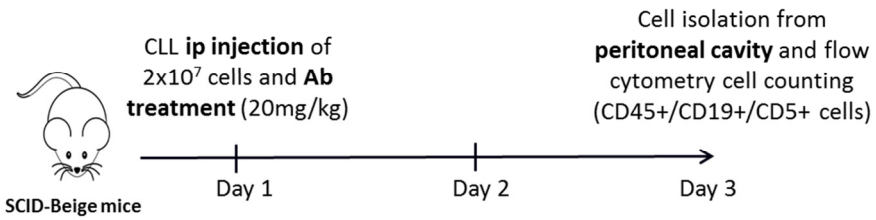


Figure 23: Schematic summary of the *in vivo* phagocytosis assay.

In vivo phagocytosis assay was carried out as described by Overdijk et al.[275] SCID beige mice (CB17.CG-PRKDC-LYST/CR, Charles River Laboratories) which lack NK cells (Table 13), were inoculated with primary CLL cells or MEC2 cells (2×10^7 cells per mouse) into their peritoneal cavity, following a protocol approved by the Animal Testing Ethic committee of the University of Barcelona (Figure 23). Mice were randomly assigned into cohorts of three to five mice and received one intraperitoneal (i.p.) injection of 20 mg/kg of DARA or isotype control. 48 hrs later, mice were sacrificed and peritoneal lavage (PL) done by injecting the cavity with 5 mL of cold PBS. Total recovery of the peritoneal cells was evaluated by flow cytometry after staining with huCD45/CD19/CD5 antibodies (provided by Invitrogen and BD-Pharmigen, respectively). The relative percentage of remaining CLL cells from DARA-treated mice was derived from the isotype control group, which was set at 100%.

$$\%\ \text{remaining target cells} = 100 \times \frac{\text{remaining target cells after DARA treatment}}{\text{remaining target cells after isotype control treatment}}$$

8. Migration assay

SDF-1α/CXCL12-induced migration was evaluated in 24-well chemotaxis chambers containing 5 µm pore size inserts (Corning, NY, US) (Figure 24). The lower chamber contained 200ng/ml CXCL12. The cells pretreated with the antibodies for 30min at 4ºC (30µg/ml for isotype control and DARA and 25µg/ml

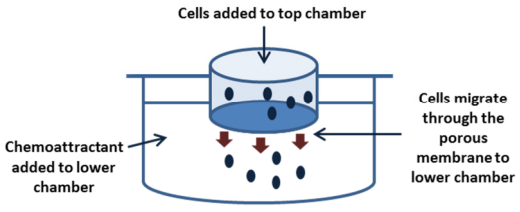


Figure 24: Chemotaxis chamber system.

anti-CXCR4) were assayed for migration. Anti-CXCR4 (R&D Systems) was used as a positive control for the inhibition of migration. After 4h, CLL cells ($CD19^+$ $CD5^+$) in the lower chamber were counted in triplicates in a flow cytometer at fix flow rate.

9. *In vivo* homing

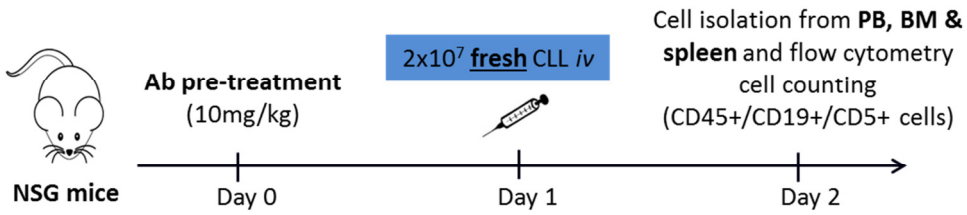


Figure 25: Schematic summary of the *in vivo* homing assay.

Homing experiment was done as previously described by Vaisitti et al.[211] Briefly, NOD/SCID gamma null (NSG) mice (bred in-house, animal facility, University of Barcelona) were randomly assigned into cohorts of four mice and pre-treated i.p. with 10 mg/kg of DARA, isotype control or anti-CXCR4 (R&D Systems) (Figure 25). 24 hr later, mice were inoculated with fresh primary CLL (2×10^7 cells per mouse) via the tail vein following a protocol approved by the Animal Testing Ethic committee of the University of Barcelona. Mice were sacrificed 24 hr after tumor cell inoculation; PB, spleen and BM were recovered and the presence of tumor cells evaluated by flow cytometry after staining with huCD45/CD19/CD5 antibodies.

	NSG, NOD scid gamma	SCID	NOD scid beige
Mature B cells	Absent	Absent	Absent
Mature T cells	Absent	Absent	Absent
Macrophages	Defective	Active	Active
Natural killer cells	Absent	Active	Defective
Complement	Absent	Absent	Absent

Table 13: Mice strains used in this thesis.

10. Adhesion assay

EIA/RIA 96-well plates (Costar), were coated in triplicate with 100 µL of RPMI 1640 (Lonza) containing BSA 1% (non-specific adhesion), or 500ng/mL of VCAM-1 (R&D Systems) and incubated overnight at 4ºC. CLL cells were labeled with 1µM Calcein, AM (Invitrogen) for 30 minutes and washed thrice with PBS. Subsequently, cells were treated with isotype control (IgG1-b12), DARA or anti-CD49d (AbD Serotec) for 30 min at 4ºC. Anti-CD49d was used as a positive control for inhibition of adhesion. Next, cells were plated in triplicates in 100µL RPMI1640 and incubated for 30 minutes at 37ºC. Then, the plate was washed extensively with RPMI 1640 to remove non-adhering cells, 100µL of medium plus 1% Triton X-100 was added into each well and incubated for 15 minutes at 37ºC. Each supernatant was transferred into black plates (Thermo Scientific, Nunc) and fluorescence was measured in a spectrophotometer (Bio-Tek Instrument) (excitation filter: 485 ± 20 nm; band-pass filter: 530 ± 20 nm).

11. RNA isolation and RT-PCR

Total RNA was isolated from CLL cells using the TRIzol reagent (Life Technologies) according to manufacturer's instructions. cDNA was obtained from 0.5-1 µg of RNA using the High Capacity cDNA Reverse Transcription kit (Life Technologies). RT-PCR quantitative assays for MMP9 (assay ID Hs00234579_m1) were run on a 7900 Fast Real-Time PCR using mGUSB (IDHs00939627_m1) as endogenous control. The results were analyzed with RQ manager software (Applied Biosystems).

12. Protein isolation and western blot

CLL cells were lysed in Triton buffer [1% Triton X-100, 50mM Tris (trishydroxymethyl-aminomethane)–HCl [pH 7.6], 150mM NaCl, 1mM EDTA (ethylene diaminetetraacetic acid), 10 µg/mL leupeptin, 10 µg/mL aprotinin, 1mM phenylmethanesulfonyl fluoride, 5mM NaF, and 2mM Na3VO4)] and whole-cell lysates (50 µg/lane), were subjected to 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and transferred onto phenylmethylsulfonyl fluoride membranes (Immobilon-P; Millipore). Membranes were then probed with antibodies for pERK (p42/p44) and ERK (Signaling Technology) and α-tubulin (Sigma). Chemiluminiscence detection was done by using ECL system (Thermo Fisher,

Waltham, MA, US) in a mini-LAS4000 device with Image Gauge software (Fujifilm, Tokyo, Japan).

13. Systemic MEC2 xenograft mouse model

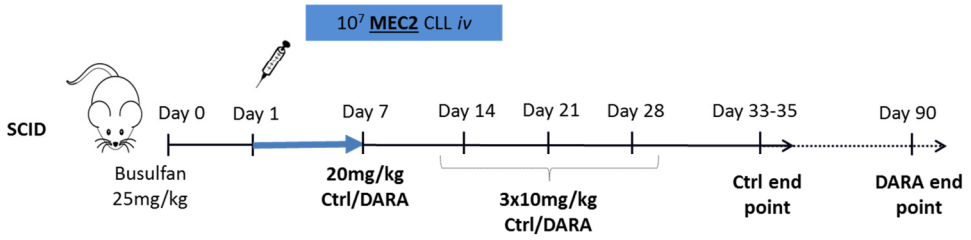


Figure 26: Schematic summary of the systemic MEC2 xenograft mouse model.

SCID mice (Janvier Labs, Dijon, France) were preconditioned with 25 mg/kg of busulfan 24hr before inoculation via tail vein of MEC2 cells (10^7 cells per mouse), following a protocol approved by the Animal Testing Ethic committee of the University of Barcelona (Figure 26). One week later, mice were randomly assigned into cohorts of 6-7 mice. A saturating loading dose of 20 mg/kg DARA or isotype control i.p. was given on day 7 and thereafter 10 mg/kg weekly for 3 weeks. Mice were sacrificed if they lost 15-20% of weight and/or showed signs of disease. Survival studies were extended up to day 90 when the study was terminated. The presence of tumor cells was evaluated first macroscopically and then by flow cytometry. Cells from infiltrated organs were obtained by tissue homogenization. BM cells were obtained after flushing the femoral and tibia bones with RPMI 1640 media. These samples were filtered through 70 µm nylon sieves (BD Falcon). Erythrocytes were lysed using ACK buffer (Quality Biological Inc.). The cells were labeled with huCD45/CD19/CD5 antibodies and analyzed by flow cytometry. Organ samples were snap-frozen in OCT medium (Sakura Tissue Tek) or formalin fixed and embedded in paraffin. Tissue sections were stained with H&E and CD19 (Dako) antibody and evaluated by Cell B Basic Imaging Software (Olympus).

14. Detection of daratumumab in mouse plasma by ELISA

Mouse blood was collected in EDTA micro-containers (Sarstedt), serum isolated by centrifugation at 10.000g for 5 minutes, and kept at -80ºC until ELISA was performed. Then, serums were allowed to thaw on ice and quantification of human IgG was performed using

the Human IgG total Ready-SET-Go (eBioscience, Affymetrix) following manufacturer instructions.

15. CLL-Patient derived mouse xenograft (CLL-PDX)

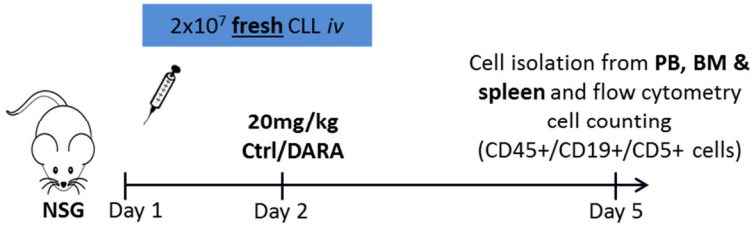


Figure 27: Schematic summary of the CLL-Patient derived mouse xenograft (CLL-PDX).

On day 1, NSG mice were inoculated iv with fresh PBMCs from CLL ($2x10^7$ cells/mouse) (Figure 27). On day 2, mice were randomly assigned to two groups (3-4 mice per group) and dosed i.p. with 20mg/kg of DARA or control isotype. Mice were sacrificed on day 5, PB, spleen and BM were recovered and the presence of tumor cells was evaluated by flow cytometry after staining with huCD45/CD19/CD5 antibodies.

STUDY 2: CC-292 in MCL

1. Cell lines and patient samples

Primary tumor cells from 11 MCL patients diagnosed according to the WHO classification criteria were used.[19] Written informed consents of the patients were granted following the guidelines of the Hospital Clínic Ethics Committee (IRB) and the Declaration of Helsinki. Mononuclear cells were isolated from PB by gradient centrifugation on Ficoll (GE Healthcare, Little Chalfont, UK) and used fresh or cryopreserved in liquid nitrogen in RPMI 1640 containing 10% DMSO (Sigma-Aldrich, St Louis, MO, US) and 60% heat-inactivated FBS (Life Technologies, Carlsbad, CA, US) and maintained within the Hematopathology collection of the institution (IDIBAPS-Hospital Clínic Biobank, R121001-094). MCL cell lines REC-1 and MINO were obtained from ATCC (LGC Standards, Barcelona, Spain). UPN-1, MAVER-1 and Z138 were kindly provided by Dr A. Turhan (Institut Gustave Roussy, Villejuif, France) (2004), Dr A. Zamo (University of Verona, Verona, Italy) (2008) and Dr E. Ortega-Paino (Lund University, Lund, Sweden) (2006), respectively. MCL primary samples and cell lines were cultured in RPMI 1640 or DMEM supplemented with 10% FBS, 2 mM L-glutamine, 50 µg/mL penicillin/streptomycin (Life Technologies) and were maintained in a humidified atmosphere at 37ºC containing 5% CO_2. Normocin (100 µg/mL) (InvivoGen, San Diego, US) was added to the cell line cultures to prevent Mycoplasma contamination in cell lines, which were routinely tested for Mycoplasma infection by PCR. The identity of all cell lines was verified using AmpFISTR identifier kit (Thermo Fisher Scientific, Waltham, MA, US). The mesenchymal stromal cell line StromaNKtert was obtained from Riken Cell Bank (Tsukuba, Ibaraki, Japan) and cultured as described previously.[298]

2. Treatments

The BTK inhibitor CC-292 and lenalidomide were kindly provided by Celgene (San Diego, CA, US). Two NIK inhibitors [AM-0216 and AM-0561] and an isomeric control of AM-0216 [AM-0650] were kindly provided by Amgen (Seattle, WA, US). AM-0216 inhibits NIK with a Ki of 2 nM. AM-0650 is the enantiomer of AM-0216, with a Ki of 290 nM against NIK. AM-0561 is a more potent active analog with a Ki of 0.3 nM.[299]

3. Cell proliferation assay and apoptosis quantification

MCL cells ($5x10^4$) were treated with CC-292, lenalidomide or NIK inhibitors for the times indicated and 0.5 mg/mL MTT (3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium

bromide) reagent (Sigma-Aldrich) was added for 2–6 additional hours before spectrophotometric measurement. Each measurement was made in triplicate. Values were represented using untreated control cells as reference. Apoptosis induction was evaluated by flow cytometry in an Attune acoustic focusing cytometer (Thermo Fisher Scientific) after staining MCL cells with Annexin V-FITC (BD-Pharmingen, Franklin Lakes, New Jersey, US) and co-stained with CD19-PE (BD-Pharmingen) in the case of primary cells.

4. Gene expression profiling (GEP) and data meta-analysis

Total RNA was isolated from MCL cells using the TRIzol reagent (Thermo Fisher Scientific) followed by a cleaning step using the RNAeasy kit (Qiagen, Hilden, Germany). RNA integrity was examined with the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, US). Only high quality RNA was then retrotranscribed to cDNA (Qiagen) and hybridized on HGU219 microarray (Affymetrix, Santa Clara, CA, US). All samples were simultaneously run in a GeneTITAN platform (Affymetrix). Principal component analysis (PCA) was done with Partek Genomics Suite. For the identification CC-292 modulated pathways, Gene Set enrichment Analysis (GSEA) v2.0 (Broad Institute) was performed using experimentally derived custom gene sets[16,300] and the Human NFκB Signaling pathway and Targets from PCR Array gene list (SABiosciences, Qiagen). A two class analysis with 1000 permutations of gene sets and a weighted metric was used. Bonferroni correction for multiple testing was applied and only gene sets with FDR ≤0.10 and a normalized enrichment score (NES) of $\geq$1.5 were considered significant. The leading edge genes were displayed using Cluster (v2.11) and TreeView (v1.6) softwares (Eisen Laboratory, Berkeley, CA, US). Microarray data were deposited in the NCBI´s Gene Expression Omnibus and is accessible through GEO series accession number GSE94328.

5. Protein isolation and western blot

MCL cells were lysed in 1% Triton or RIPA buffer as indicated, containing protease and phosphatase inhibitors. Protein lysates were resolved by SDS-PAGE electrophoresis as described previously[301], developed with enhanced chemiluminescence substrate (SuperSignal, Thermo Fisher Scientific) and visualized on a LAS4000 Fujifilm device (Fujifilm, Tokyo, Japan). BTK, phospho-BTK (Y223), ERK, phospho-ERK (Thr202/Tyr204), p52/100, and phospho-IκB(Ser32) antibodies were from Cell Signaling Technology (Danvers, MA, US); IRF4 antibody from Santa Cruz (CA, US); and α-tubulin and β-actin from Sigma-Aldrich.

6. Flow cytometry

Cells were pretreated with 1µM CC-292 at 37ºC for 1h and subsequently stimulated with 10µg/ml anti-IgM (Jackson Immunoresearch Laboratories, Grove, PA, US) for 24h. Cellular activation was evaluated by co-staining of MCL cells with CD69-PC7/CD86-FITC, including CD19/CD5 and Annexin V-FITC in the case of primary cells, followed by flow cytometry evaluation in an Attune cytometer (Life Technologies).

7. Migration assay

SDF-1α/CXCL12-induced migration was evaluated using 24-well chemotaxis chambers containing 5 µm pore size inserts (Corning, NY, US) coated with 1 µg/ml VCAM-1. The lower chamber contained 200ng/ml CXCL12. The cells, pretreated with 1µM CC-292 at 37ºC for 1h, were applied to the upper compartment and allowed to migrate for 3h at 37ºC and enumerated by flow cytometry.

8. Co-culture experiments

StromaNKtert cells were seeded on day 0, and MCL cell lines or primary cells were added the following day at 1:5 ratio (stromaNKtert:MCL) and cultured in the presence or absence of the drug (Figure 28). After 3 or 6 days viable MCL cells were counted by flow cytometry labeling with CD19-PE/Annexin V-FITC.

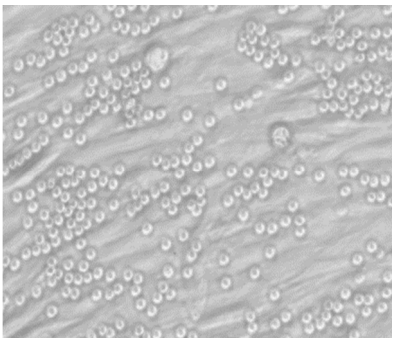


Figure 28: MCL-StromaNKtert co-culture.

9. Cytokine quantification by ELISA

CCL3 and CCL4 levels were assessed in duplicates using ELISA kits (eBioscience, San Diego, US) in supernatants harvested from cells that had been pretreated with 1µM CC-292 at 37ºC for 1h and subsequently stimulated with 10µg/ml of anti-IgM for 24h.

RESULTS

STUDY 1:

To evaluate the antitumor profile of the first-in-class anti-CD38 antibody Daratumumab in the poor prognosis $CD38^{+}$ CLL subtype

1. Daratumumab induces ADCC.

Ab-dependent killing via ADCC by FcγR-bearing effector cells accounts for the anti-tumor activity of DARA in models of MM and BL.[269,280] The ability of DARA to induce ADCC on CLL cells was assessed by calcein-AM release assay using PBMCs from healthy donors as a source of effector cells (mainly NK cells and monocytes). CLL cell lines and primary cells were treated with increasing concentrations of DARA or isotype control. DARA induced significant cell lysis starting at doses as low as 0.01μg/mL in $CD38^+$ CLL cell lines (Figure 29A), and at 0.001μg/mL in primary CLL cells (Figure 29B), reaching its maximum killing activity at 0.1-1μg/mL (mean±SD=31.9±11.6%). In contrast, the isotype control antibody did not induce significant cell lysis, tested at the maximum concentration of 1μg/mL (mean±SD=12.4±11.6%) (Figure 29B). No ADCC induction was detected in $CD38^-$ CLL cases (Table 14-15: CLL7, CLL11 and MEC1 cell line). A summary of ADCC induction in $CD38^+$ vs $CD38^-$ CLL primary cases is shown in Figure 29C. The degree of ADCC induction did not correlate with CD38 sABC for CLL cell lines and primary cells (r^2=0.088) (Figure 29D). Altogether, these data indicates that ADCC constitutes a mechanism of DARA activity in $CD38^+$ CLL cells, but the extent of ADCC does not strictly correlate with CD38 expression.

2. Daratumumab promotes CLL cell clearance by phagocytosis *in vitro* and *in vivo.*

Recent results indicate that ADCP is a potent mechanism of action for DARA.[275] We explored ADCP of CLL both *in vitro* and *in vivo*. To assess ADCP *in vitro* macrophages were generated from BM mouse monocytes stimulated with M-CSF. DARA induced ADCP in primary CLL cells (mean±SD=23±4%) (Figure 30A). Representative flow cytometry profiles of CLL cells and macrophages after DARA treatment are depicted for CLL1 (Figure 30B). As observed for ADCC, phagocytosis was specimen-dependent and not strictly related to CD38 expression levels.

We next demonstrated the occurrence of ADCP *in vivo*. SCID beige mice, devoid of NK cells but with active macrophages were inoculated i.p. with primary CLL cells or the MEC2 cell line as described in materials and methods. As shown in Figure 30C, the percentage of remaining viable CLL cells after DARA treatment was significantly reduced (mean±SD= 46±5%;$p<0.05$,unpaired t-test) compared to the isotype control group at 100%. Remarkably, this decrease in cell number was detectable as early as 2hrs after DARA

administration (data not shown). Figure 30D represents flow cytometry profiles showing the number of CLL cells (huCD45^{+}/CD19^{+}/CD5^{+}) recovered from the intraperitoneal cavity after isotype control or DARA treatment.

Taken together, these results demonstrate that ADCP may contribute to DARA anti-tumor activity against CLL cells both in *in vitro* and *in vivo* settings.

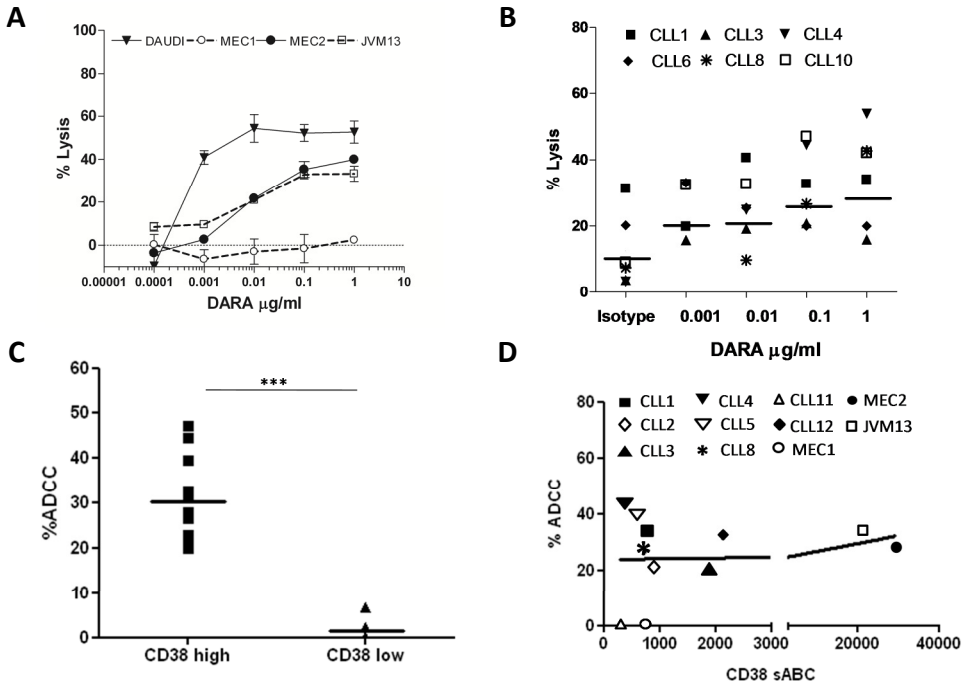


Figure 29. Daratumumab induces ADCC in the presence of external effectors. Daudi cells, CLL cell lines, both CD38high (MEC2 and JVM13) and CD38low (MEC1) **(A)** and primary CLL cells **(B)** were treated with increasing daratumumab (DARA) doses (0.0001-1µg/mL) in the presence of PBMC from healthy donors at a E:T ratio of 50:1 for 4 hours. Viability was then evaluated by calcein release assay. In panel B, ADCC induced by isotype control at the maximal dose of 1µg/mL is also depicted and the horizontal line represents the mean lysis. **(C)** ADCC induction by daratumumab (0,1 µg/mL) in CD38 high (>30%) vs CD38 low (<30%) CLL primary cases. **(D)** The number of surface antibodies bound per cell (sABC) of CD38 was quantified in primary CLL cells and cell lines and plotted for correlation with ADCC induction by DARA (cell lines 0.1µg/mL and primary CLL 0.01µg/mL).

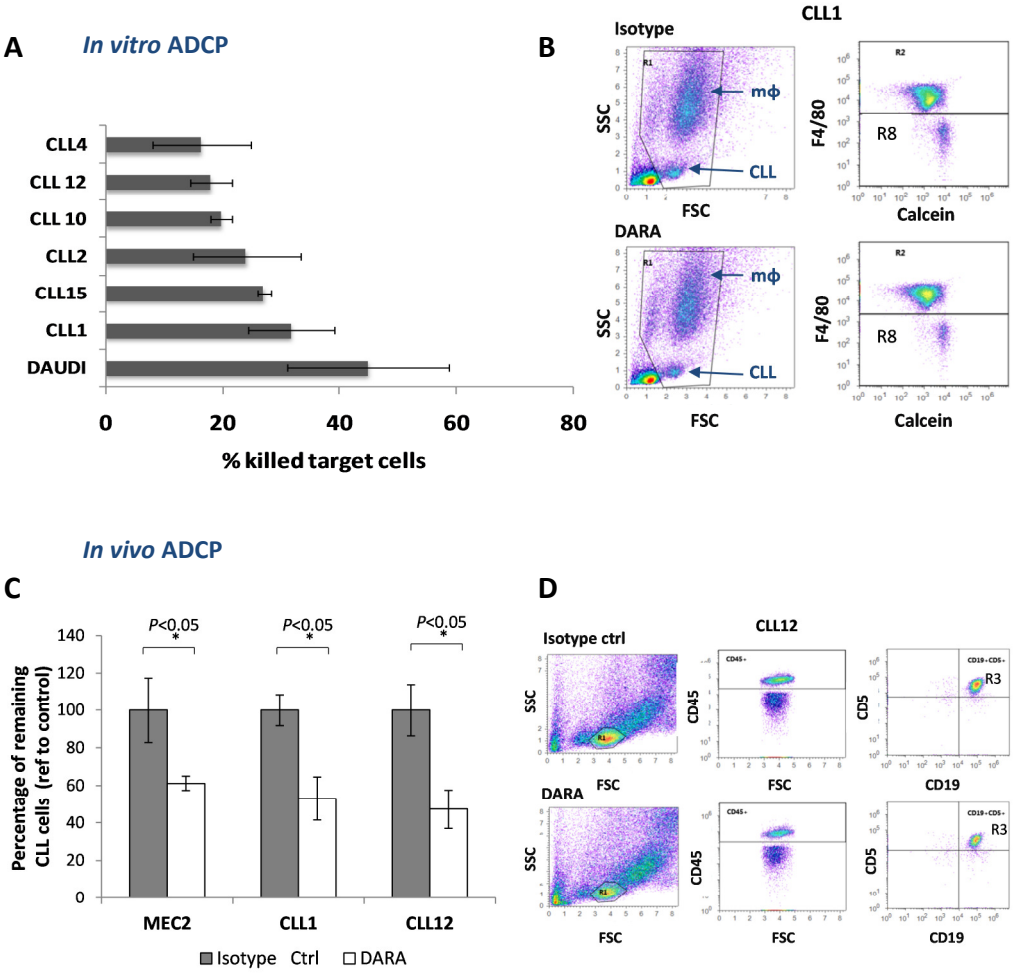


Figure 30. Daratumumab induces ADCP *in vitro* and *in vivo*. **(A)** CLL cells were treated in triplicates with a fixed concentration (1µg/mL) of DARA or isotype control in the presence of mouse macrophages at a E:T ratio of 1:1, for 4 hours. Percentage of killed target cells was calculated by flow cytometry. CLL cells were identified as Calcein^{+} F4/80^{-} and the percentage of killing by DARA was calculated according to the formula included in materials and methods. Daudi cell line was used as positive control. **(B)** Representative flow cytometry plots of *in vitro* ADCP. CLL cells and macrophages (mϕ) are clearly seen in the FSC/SSC density plot. The number of cells in the R8 gate was used to calculate the percentage of killed tumor cells. **(C)** *In vivo* phagocytosis was evaluated in SCID *beige* mice (n=3-5 per group) that were inoculated *i.p*. with primary CLL cells or MEC2 cell line (2x10^{7} cells per mouse) and subsequently treated *i.p* with one dose of 20 mg/kg of DARA or isotype control. The mice were sacrificed 48 hours later and cells from the peritoneum recovered and counted by flow cytometry as huCD45^{+}CD19^{+}CD5^{+} for primary CLL cells and CD45^{+}CD19^{+}CD5^{-} for MEC2 cells. The percentage of residual leukemia cells after DARA treatment is plotted where the total number of cells remaining treated with the isotype control was set to 100% ($p<0.05$, unpaired t-test). **(D)** Representative flow cytometry plots where the R3 gate was used to calculate the percentage of killed tumor cells. The gating strategy started with cells in FSC/SSC (R1), then gating on CD45^{+}, and finally CD19^{+}CD5^{+}.

3. Daratumumab induces limited CDC in CLL cells.

DARA was selected from a panel of human antibodies for its broad-spectrum killing activity against hematological cell lines. DARA was particularly differentiated for its potent CDC activity.[269] We evaluated DARA-induced CDC activity in a panel of CLL primary cells and cell lines (Tables 14 and 15). In the majority of primary CLL samples, $CD38^+$ PLL cell lines (MEC2 and JVM13) and in $CD38^-$ cell line (MEC1), DARA did not induce significant cell death in the presence of normal human serum (10%). In five out of eighteen primary CLL cells, DARA induced just over 10% CDC (range: 10.4-25.6%). This limited CDC induction was not increased in the presence of higher human serum concentrations (data not shown). To explain the poor induction of CDC, we assessed the expression of mCRPs and the number of CD38 molecules/cell (CD38 sABC) on CLL cells. High expression of the CRPs: CD46, CD55, CD59 (mean± SD= 94±3%; 92±4%; 92±8%, respectively) was detected by flow cytometry in all CLL cell lines and primary cases, while only CD46 (88%) was highly expressed in the BL cell line Daudi (Tables 14 and 15) which was used as a positive control for CDC. Blocking antibodies against these CRPs, probed to increase CDC induction by DARA in Daudi, while no effect was observed in CLL cells. However, blockade of CRPs increased CDC induced by the anti-CD20 antibodies rituximab (RITUX) or ofatumumab (OFA) in some CLL cases (Figure 31).

Study label	%CD38[1]	CD38 sABC[2]	%CD46	%CD55	%CD59	%CD49d	%CDC[3]	%ADCC[4]
JVM13	68	20,760	98	98	100	72	0	34.8
MEC1	14	738	100	99	99	100	0	0
MEC2	95	29,289	97	96	99	98	4	28.3
DAUDI*	100	292,131	88	17	9	nd	95.9	55.1

Table 14. Cell line characteristics. [1]CD38 was considered positive when the percentage of positive cells exceeded 30% [2]sABC: number of surface antibodies bound per cell evaluated by QuantiBRITETM CD38-PE. [3]Percentage of CDC induction at 10 µg/mL DARA. [4]Percentage of ADCC induction at 0.1 µg/mL DARA. * Burkitt lymphoma

Study label	Gender[1]	Binet/Rai stage	%tumor cells[2]	IgVH status[3]	CD38[4]	CD38 sABC[5]	CD46[4]	CD55[4]	CD59[4]	CD49d[4]	%CDC[6]	%ADCC[7]
CLL1	M	C/IV	95	nd	98	816	98	91	9	4	nd	32.7
CLL2	M	A/I	96	UM	63	897	889	88	96	6	6.4	20.8
CLL3	M	C/IV	86	UM	80	1875	90	88	79	68	8.4	20.8
CLL4	M	A/I	97	UM	31	355	92	90	75	79	nd	44.5
CLL5	M	B/II	97	UM	44	562	92	90	97	1	0.1	39.4
CLL6	F	C/III	86	M	50	nd	nd	nd	nd	nd	25.1	20
CLL7	M	C/III	96	UM	2	nd	98	99	90	0.7	5.6	0.2
CLL8	M	A/0	95	M	53	736	93	94	83	5	3.4	26.6
CLL9	M	A/I	82	nd	73	nd	98	97	98	63	0.9	22.9
CLL10	M	B/III	93	M	64	nd	92	91	94	100	25.6	47
CLL 11	M	A/0	97	M	0.5	294	95	94	98	0.1	0	0
CLL 12	F	A/III	94	UM	95	2132	97	87	90	80	17.4	31.4
CLL 13	M	A/0	60	nd	55	nd	99	100	99	98	10.4	nd
CLL14	M	B/II	98	UM	66	nd	97	93	86	nd	nd	27.9
CLL15	F	A/I	86	UM	71	1531	96	98	88	83	nd	27.2
CLL 16	M	A/I	53	nd	75	nd	98	94	96	95	23.2	38.1
CLL17	M	A/0	85	UM	83	nd	99	98	96	nd	0.3	nd
CLL 18	M	A/0	91	UM	70	nd	98	98	97	92	0	22

Table 15: CLL patient characteristics. [1]M: male; F: female. [2]Percentage of tumor cells assessed by flow cytometry based on $CD19^+CD5^+CD23^+$ cells; [3]Determined by direct sequencing. UM: unmutated, sequence homology > 98%. M: mutated, sequence homology < 98%. nd: not determined. [4]Percentage of positive cells for CD38, CD46, CD55, CD59 and CD49d determined by flow cytometry in $CD19^+$ $CD5^+$ population, referred to isotype control. CD38 was considered positive when the percentage of positive cells exceeded 30%. [5]sABC: number of surface antibodies bound per cell evaluated by QuantiBRITE CD38-PE. [6]Percentage of CDC induction at 10 µg/mL DARA. [7]Percentage of ADCC induction at 0.1 µg/mL DARA.

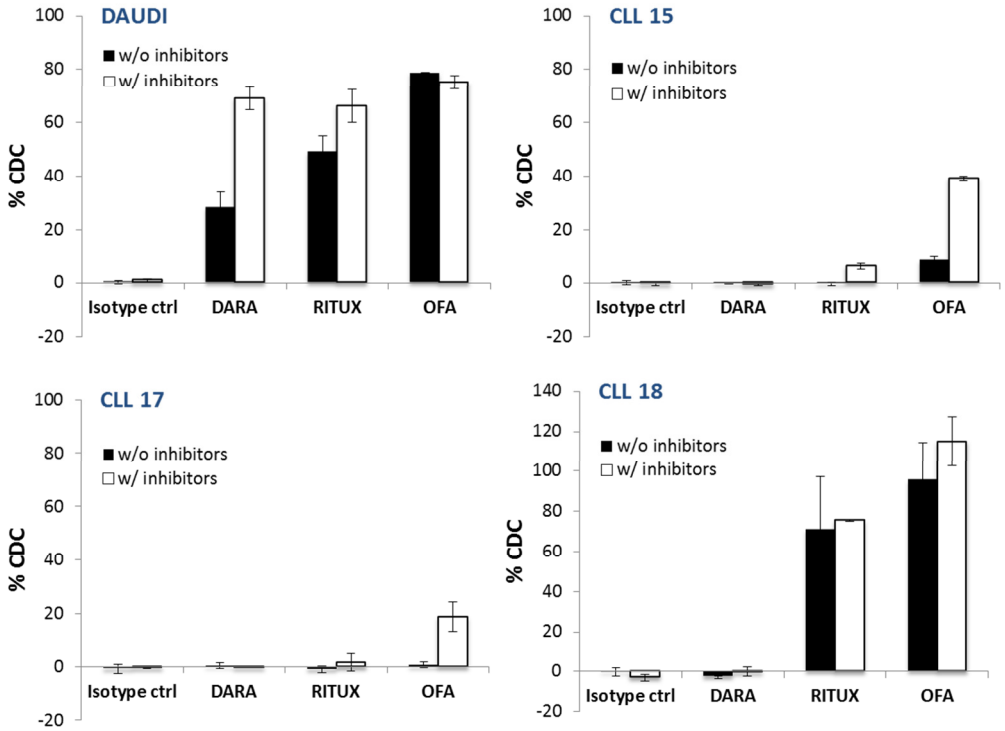


Figure 31. Effect of CRPs blockade on daratumumab-induced CDC. Daudi and CLL cells were labeled with Calcein-AM (1μM) and challenged to CDC induction by daratumumab (DARA), rituximab (RITUX), ofatumumab (OFA) or isotype control, all of them at 10μg/mL, in the presence of 10% Normal Human Serum (NHS). When indicated, 1μg/mL of mixed mCRPs inhibitors (anti-CD46, anti-CD55 and anti-CD59) were added.

CD38 expression in $CD38^+$ CLL primary tumor samples (mean sABC±SD=1053±677) was lower than in $CD38^+$CLL cell lines (mean sABC±SD=25,024±6,031), which was roughly ten-fold below that detected in Daudi (mean sABC±SD=292,131). A summary of Mean Fluorescence Ratio (MFIR) for CD38 and mCRPs in CLL cells and cell lines is included in Table 16. Previous results in MM have demonstrated that all-trans retinoic acid (ATRA) increases CD38 expression.[302] We have analyzed this possibility in CLL. CLL cells (n=6) were pretreated with ATRA or left untreated for 48h. CD38 expression was analyzed subsequently on these CLL cells and challenged to CDC assay. As shown in Figure 32A, CD38 MFI of $CD19^+CD5^+$ cells, was significantly ($p<0.05$) increased after ATRA treatment (average increase 30%), in a similar proportion than that shown for MM patient samples. A representative example is

shown (Figure 32B). However, no CDC induction by DARA was observed in the cases analyzed (Figure 32C), indicating that this increase in CD38 expression was not sufficient to engage CDC.

In conclusion, these results indicate that DARA did not induce significant CDC in either CLL cell lines or primary CLL cells, and is probably due to high expression of CRPs and insufficient CD38 expression.

	Mean Fluorescence Ratio (MFIR)			
study code	**CD38**	**CD46**	**CD55**	**CD59**
CLL1	2.5	7.6	4.6	11.0
CLL2	2.3	6.2	6.4	6.4
CLL3	4.9	4.2	4.7	4.4
CLL4	1.4	4.8	4.9	3.1
CLL5	1.8	5.5	4.6	5.4
CLL6	nd	4.8	4.9	3.1
CLL7	1.1	12.0	17.9	11.6
CLL8	2.0	4.1	5.8	4.2
CLL9	3.3	12.7	10.3	12.0
CLL10	3.3	6.0	10.0	7.6
CLL 11	1.2	9.5	10.3	11.5
CLL 12	4.6	10.0	8.4	8.8
CLL 13	4.6	8.2	4.5	5.9
CLL14	3.7	15.2	7.1	5.8
CLL15	4.3	7.1	10.5	5.4
CLL16	9.2	11.2	10.1	8.7
CLL17	5.5	11.3	13.3	6.5
CLL18	4.0	10.5	16.4	9.7
JVM13	7.6	9.8	11.4	39.1
MEC1	0.4	6.1	7.0	7.4
MEC2	6.3	13.5	12.5	35.9
DAUDI	276.6	3.3	1.4	1.2

Table 16. Mean Fluorescence Ratios (MFIR) of CD38 and CRPs. The expression of CD38, CD46, CD55 and CD59 was evaluated in the CLL population (CD19+ CD5+) by flow cytometry. MFIR=MFI of Abs of interest / MFI of Isotype. For the cell lines, CD19 CD5 labeling was not included.

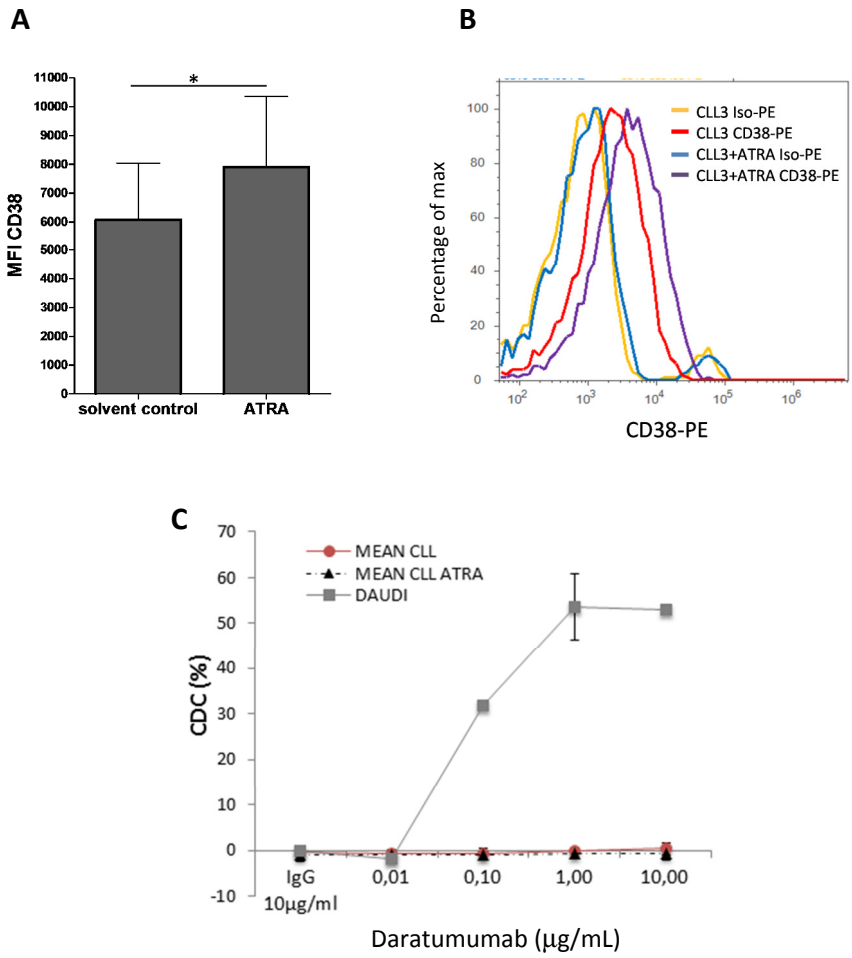


Figure 32. Effect of all-trans retinoic acid (ATRA) on daratumumab-induced CDC. CLL cells (n=6) were treated with 10nM ATRA or left untreated for 48h. CD38 expression was analyzed subsequently (CD19, CD5, CD38). A summary of CD38 MFI is shown in **(A)** and a representative CLL case in **(B)**. **(C)** Then, CLL cells treated w/wo ATRA were labeled with Calcein-AM (1µM) and challenged to CDC induction with daratumumab (0.01-10µg/mL) or isotype control (10µg/mL) and in the presence of 10% Normal Human Serum (NHS) as described in Material and Methods, including Daudi cells as positive control of CDC induction. (*p<0.05, paired t-test)

4. Daratumumab interferes with *in vitro* migration and *in vivo* homing.

Homing of CLL cells to secondary lymphoid organs is mainly coordinated by the CXCL12/CXCR4 axis.[124] Using CLL primary cells and a xenograft mouse model, Vaisitti et al. demonstrated that CD38 synergizes with the CXCR4 signaling pathway and controls chemotaxis/homing of CLL cells through a close interaction between CD38 and CXCR4 in the membrane.[211] Following this line of investigation, the effect of DARA on CLL cell migration

was evaluated using a CXCL12 gradient. An anti-CXCR4 antibody was used as a positive control of migration blockade. In $CD38^+$ CLL cells, DARA inhibited CXCL12-mediated migration up to 70% (mean±SD=44±16%; p<0.01;n=5), which was comparable to anti-CXCR4 treatment (Figure 33A). These results are in agreement with that previous report using the blocking anti-CD38 antibody SUN-4B7.[211] We next examined DARA-mediated signaling following CXCR4-CXCL12 interaction. The immediate early effect of stimulation for migration is the activation of ERK1/2.[211] Phosphorylation of ERK1/2 in CLL tumor cells occurred shortly after CXCR4-CXCL12 ligation and peaked at five minutes after CXCL12 addition. Treatment with DARA reduced ERK activation by CXCR4-CXCL12 in CLL1 and CLL12 while the ERK inhibition was less pronounced in CLL3 (Figure 33B), illustrating heterogeneity in primary tumor cells.

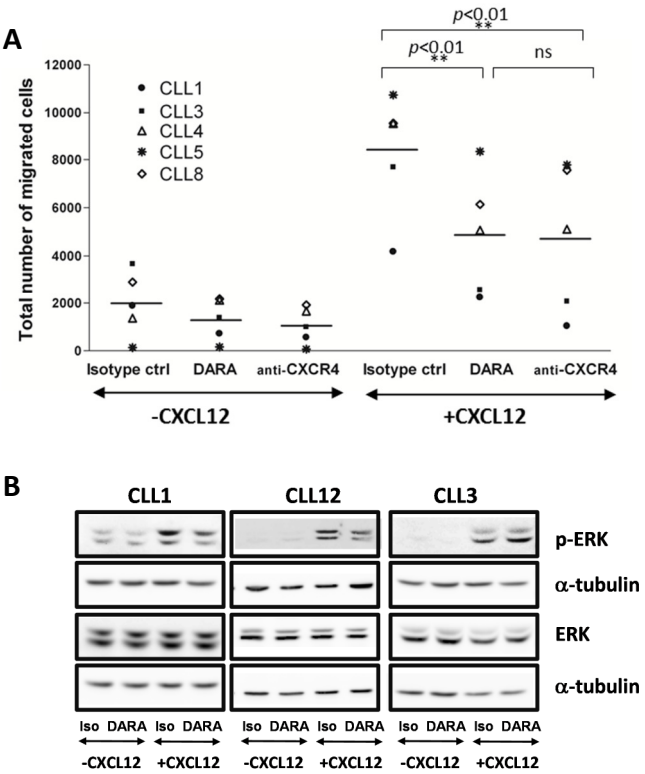
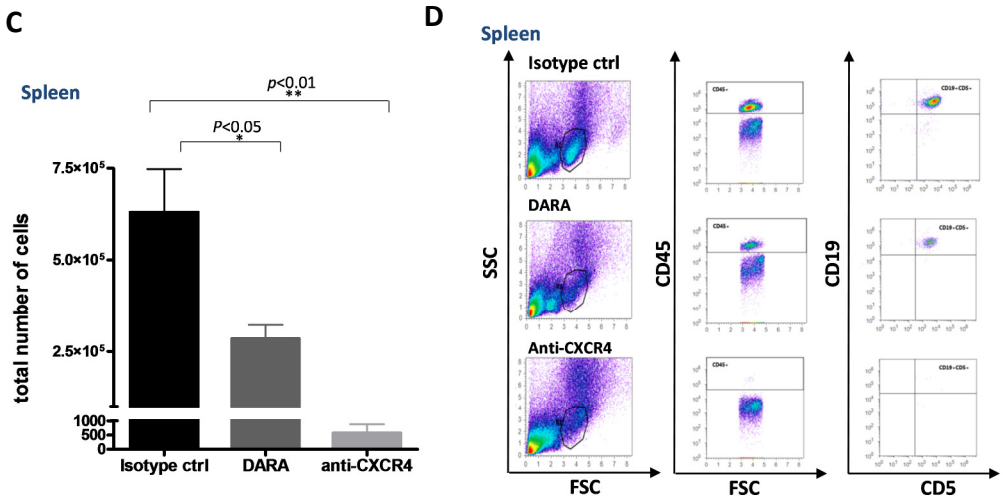


Figure 33. Daratumumab interferes with CLL cell migration and *in vivo* homing. (A) CLL cells were preincubated with the antibodies for 30 min at 4ºC (30µg/mL for isotype control and DARA and 25µg/mL anti-CXCR4) and then assayed for migration in a CXCL12 gradient. After 4h, CLL cells ($CD19^+$ $CD5^+$) in the lower chamber were counted in triplicates in a flow cytometer at fix flow rate. Total number of cells is graphed for representative CLL patients (n=5). Statistical differences between groups were assessed by paired t-test. **(B)** Western blot analysis of ERK activation after stimulation of CLL cells for 5 minutes with CXCL12 (200ng/mL). Before stimulation, cells were serum starved for 2h and pretreated for 30 min with the corresponding antibodies (30µg/mL).

We then validated these *in vitro* migration results using the *in vivo* homing mouse model described previously by Vaisitti et al.[211] Using NSG mice, which lack NK cells and active macrophages, we analyzed the effect of DARA on primary CLL cell migration from PB to BM and spleen. In this model, NSG mice were pretreated (day 0) with DARA, isotype control or anti-CXCR4, followed by fresh CLL cell inoculation on day 1. PB, BM and spleen cells were isolated on day 2 and CLL cells were identified as $CD45^+/CD19^+/CD5^+$. Representative flow cytometry profiles from a mouse spleen are shown in figure 33C. Cell enumeration showed that CLL cells rapidly move from PB and mainly migrated to the spleen and that DARA significantly reduced this migration (55% inhibition on average, $p<0.05$) (Figure 33D). Migration of CLL cells to BM was limited and was not affected by pretreatment of mice with DARA (data not shown). In conclusion, *in vivo* and *in vitro* results suggest that DARA hampers dissemination of CLL cells to secondary lymphoid organs.



(Cont. Figure 33) **(C-D)** *In vivo* homing was assessed by *iv* inoculation of fresh CLL cells via tail vein in NSG mice, previously pretreated with the corresponding antibodies (10mg/kg, n=4 mice/group). After 20h, cells were recovered from spleen, labeled with huCD45/CD19/CD5 and counted in a flow cytometer. Representative density plots for each treatment are shown **(C)**.The gating strategy started with cells in FSC/SSC (R1), then gating on $huCD45^+$, and finally $huCD19^+$ $CD5^+$. **(D)** Total number of $huCD45^+CD19^+CD5^+$ recovered from the spleen is plotted. Statistical differences between groups were assessed by unpaired t-test.

5. Daratumumab inhibits CD49d mediated CLL cell adhesion by reducing MMP9 levels.

In addition to migration, CD38 also plays a key role in cell adhesion through physical interaction with the integrin CD49d/CD29 (α4β1 integrin)[212] which is the strongest flow cytometry marker associated with poor prognosis in CLL together with *IGHV* mutational status[74] and MMP9.[303] In addition, the expression of CD38 correlates with that of CD49d. We analyzed the effect of DARA on CD49d/CD29-mediated adhesion of CLL cells VCAM-1, an essential component of extracellular matrix. As shown in Figures 34A and 34B, when compared to isotype control antibody, DARA significantly impeded the adhesion to VCAM-1 of CLL primary cells (n=4) and MEC2 cell line (mean±SD=38±11%, p<0.01), with no significant differences with anti-CD49d blocking antibody used here as a positive control (mean±SD=49±30%).

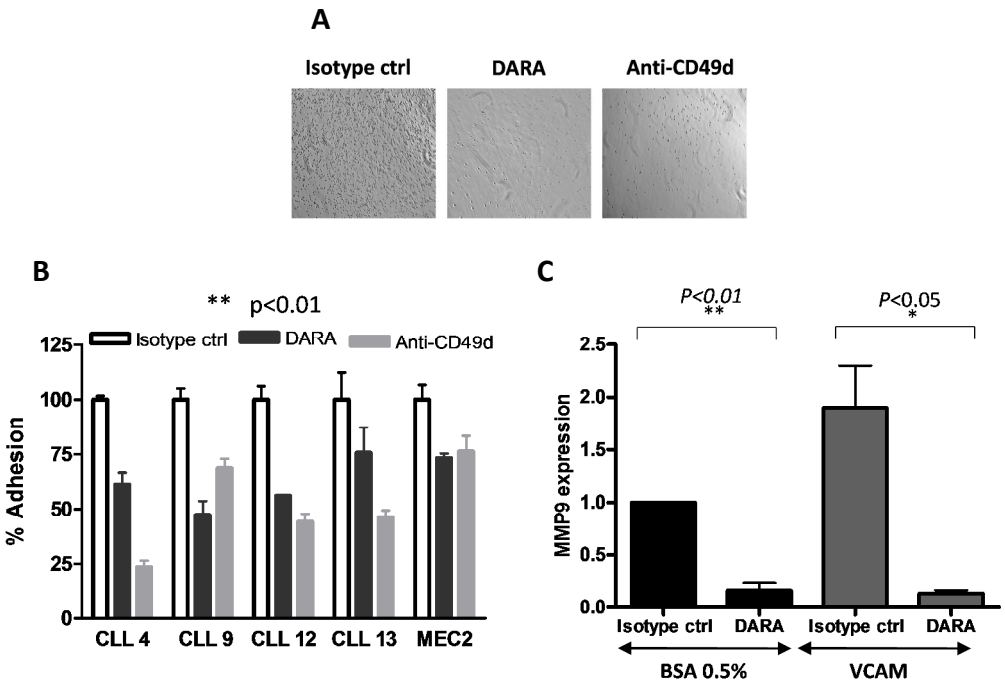


Figure 34. Daratumumab hampers CLL adhesion to VCAM. Calcein-labeled primary CLL and MEC2 cells were pre-incubated with the corresponding antibodies (30μg/mL) and left to adhere for 30 min to plates pre-coated with VCAM-1 or BSA (non-specific adhesion). Anti-CD49d was used as positive control for inhibition of adhesion. Non-adhered cells were removed by extensive washing. **(A)** Representative phase-contrast microscopy field images (x100) from adhesion to VCAM-1 of CLL9. **(B)** Adhered cells were then lysed and supernatants analyzed in a fluorimeter. Percentage of VCAM-1 adhesion is expressed normalized to isotype control and after subtraction of BSA non-specific adhesion (p<0.01, one way-ANOVA comparing the three groups together). **(C)** RT-PCR for MMP9 was performed on the adhered cells to VCAM, using mGUS as endogenous control. (n=3, CLL4, CLL12 and CLL13) Expression levels for each sample are normalized to the corresponding isotype control and adhesion to BSA. ** = p<0.01 and * = p<0.05, unpaired t-test).

Moreover, CD38-CD49d complex also recruits the matrix metalloproteinase MMP9 leading to the up-regulation and activation of this metalloproteinase.[212,213,303] Thus, we investigated the effect of DARA on MMP9 expression, by analyzing the variations in MMP9 transcripts levels in $CD38^{+}CD49d^{+}$ CLL cells. As depicted in Figure 34C, CLL cell adhesion to VCAM increased MMP9 mRNA levels (mean±SD=2±1), and DARA completely abrogated both constitutive ($p<0.01$) and VCAM-induced ($p<0.05$) MMP9 expression. Altogether, these results demonstrate that DARA counteracts VCAM-1-mediated adhesion of CLL cells and induces the transcriptional down-regulation of MMP9.

6. Daratumumab prolongs overall survival in a systemic CLL mouse model and reduces tumor burden in CLL-PDX.

We successfully established a systemic MEC2 model by intravenous cell inoculation in busulfan-preconditioned SCID mice, which retain NKs and macrophages that can function as effectors for DARA activity. Previous attempts to establish a MEC2 mouse model failed using subcutaneous cell inoculation in nude mice.[304] One week after cell inoculation, mice were randomly assigned into two groups and were administered a total of four doses of DARA or isotype control following a weekly schedule (20/10/10/10mg/kg). These doses were chosen based on dose escalation studies in MM.[305] The isotype control-treated mice started to show signs of disease (mainly weight loss>20% and rough hair) starting at day thirty-two post cell inoculation (Figures 35A-B), and all mice in the control group were sacrificed by day 40. These mice showed systemic dissemination of disease in lung, kidney, ovaries, parathyroid glands and enlarged lymph nodes (identified as $CD45^{+}/CD19^{+}$. Figure 35C-D and Figure 36), resembling aggressive CLL. Of note, a similar disseminated CLL mouse model was described by Bertilaccio et al using the CD38-PLL cell line MEC1 in Rag2-/-γ-/- mice, and the authors demonstrated its value as a tool to assess the efficacy of chemotherapeutic agents.[306] In several mice, BM and spleen infiltration was also observed (data not shown). In contrast, in the DARA-treated group, only one mouse harbored signs of illness and required euthanasia at day 41, while the remaining animals survived and did not develop life-threatening symptoms up to day 90, when the experiment was terminated (Figures 35A-B). By this day, the antibody concentrations in the serum of DARA-treated animals had dropped to 1.5µg/mL (Figure 37). Interestingly, in this group of mice no MEC2 cells were found by flow cytometry or IHC in the commonly infiltrated organs like lung and

kidney, contrasting with the remarkable predominance of malignant, human CD19^{+} cells observed in these secondary sites in control isotype-treated mice (Figure 35C-D), suggesting that these mice may be free of disease. These data suggest a strong anti-tumor activity and long-term survival of DARA-treated mice in this model.

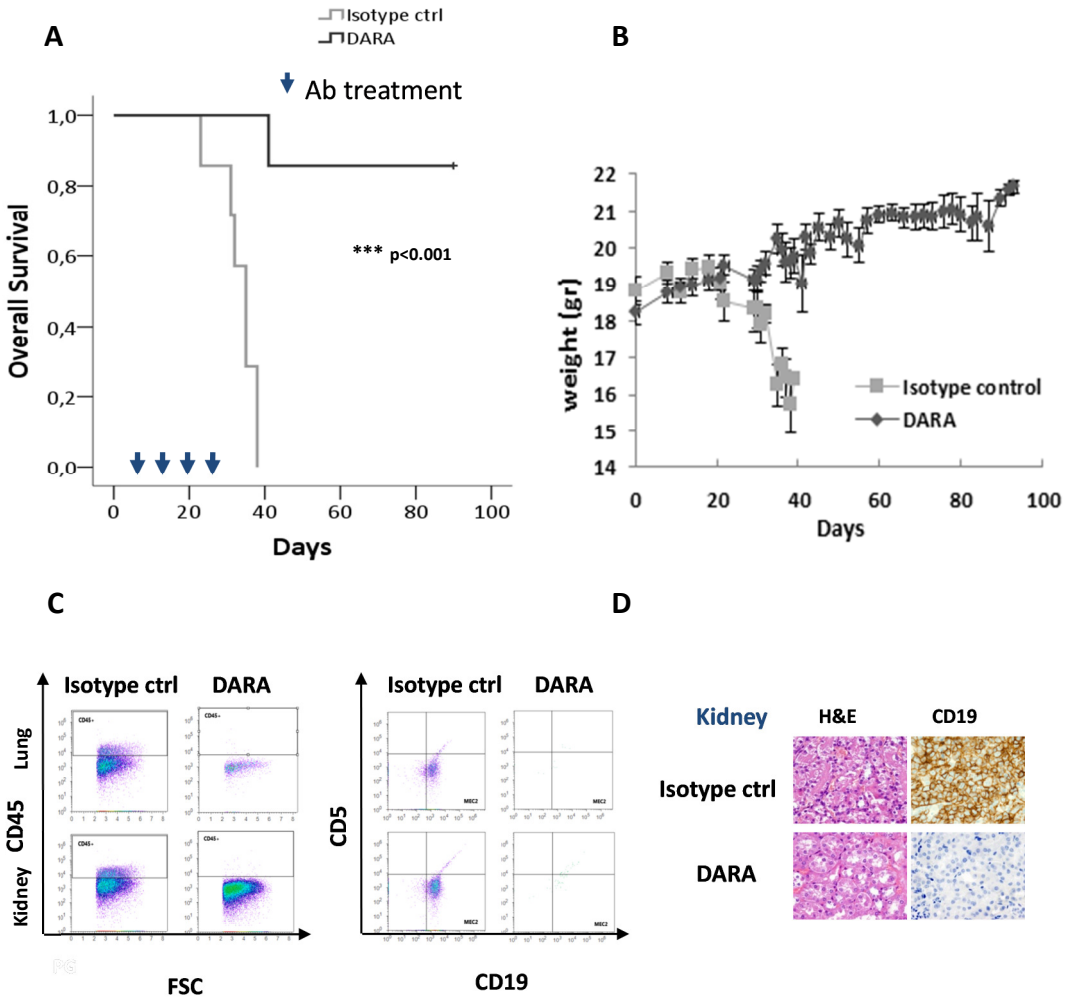


Figure 35. ***In vivo*** **efficacy of daratumumab. (A)** Kaplan-Meier survival curves for MEC2 systemic mouse xenografts. Mice received DARA (n=7) or isotype control (n=6) weekly for four weeks, starting one week after cell inoculation. Then, mice were monitored twice a week for any sign of disease and sacrificed when body weight decreased 15-20%. All control mice had to be sacrificed between days 23-38. One DARA-treated mouse became ill and was sacrificed on day 41. **(B)** Body weight changes in the isotype and DARA treated mice. **(C)** Cells from lungs and kidneys were isolated and labeled with huCD45/CD19/CD5. The presence of MEC2 cells was evaluated by CD19^{+}CD5^{-} (right panel) cells in the CD45^{+}population (left panel). Representative flow cytometry density plots for one mouse of each cohort sacrificed at day 30 are shown. **(D)** IHC staining of H&E and CD19 of kidneys from isotype and DARA treated mice (magnification x400).

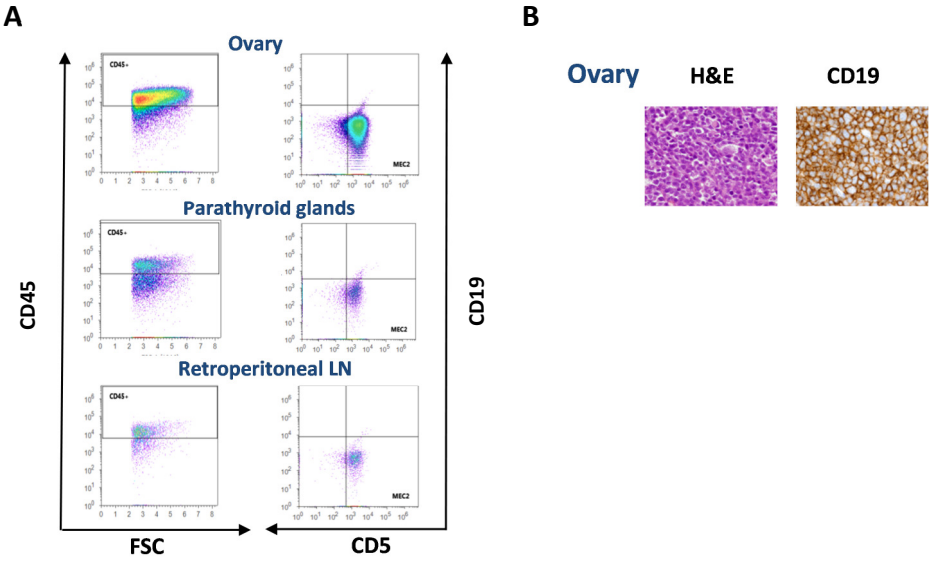


Figure 36. MEC2 mouse xenograft shows systemic organ infiltration. **(A)** Cells from ovary, parathyroid glands and retroperitoneal LN from a control mouse were isolated and labeled with CD45/CD19/CD5. The presence of MEC2 cells was evaluated by $CD19^{+}CD5^{-}$ (right panel) inside $CD45^{+}$population (left panel). Representative flow cytometry density plots are shown. **(B)** IHC staining of H&E and CD19 of an infiltrated ovary (magnification x400).

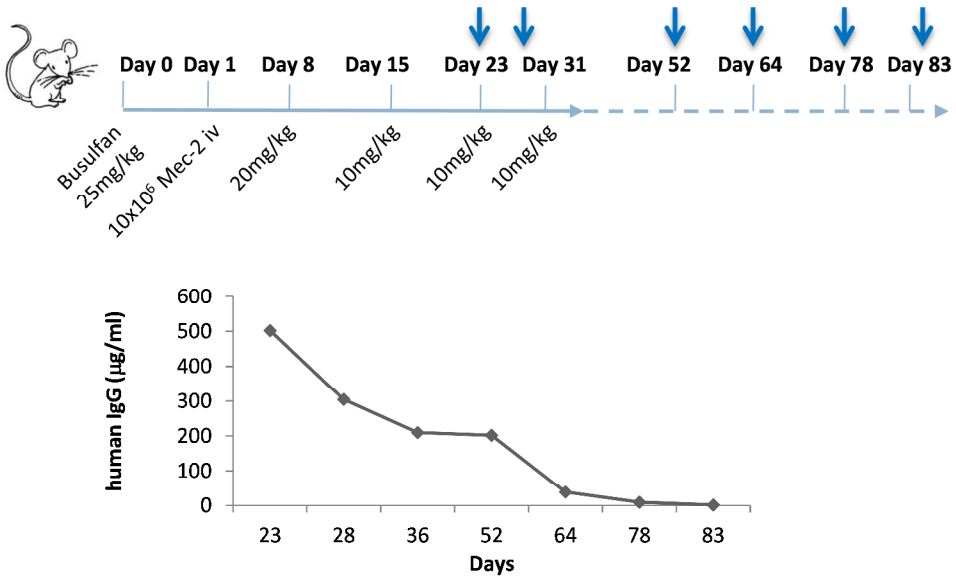


Figure 37. Dynamics of human IgG concentration in daratumumab treated mice. DARA treated mice were bled and serum was isolated before third and fourth (last) dose, and then every two weeks until the end of the experiment, as indicated in the diagram. ELISA was used to quantify human IgG in these sera. Results from a representative mouse are shown.

In order to develop a mouse model closer to CLL biology, we established a short time CLL-PDX model using NSG mice, needed to avoid CLL clearance by mouse NKs. To provide this mouse model with FcγR-bearing effector cells, we selected CLL cases enriched in NKs and monocytes (Figure 38A). Fresh PBMCs from these patients were *iv* inoculated (day 1) and treated the following day (day 2) with DARA or control isotype. On day 5 mice were sacrificed and cells recovered from PB, BM and spleen. As described previously, cells mainly homed to the spleen, where a significant (*p<0.05) decrease of CLL cells was found in the spleen of DARA-treated group (Figure 38B). No significant differences were found in BM or PB resident CLL cells between the two groups (data not shown). Off note, when the experiment was performed with a CLL sample with reduced numbers of effectors, the anti-tumoral effect of DARA was diminished (data not show).

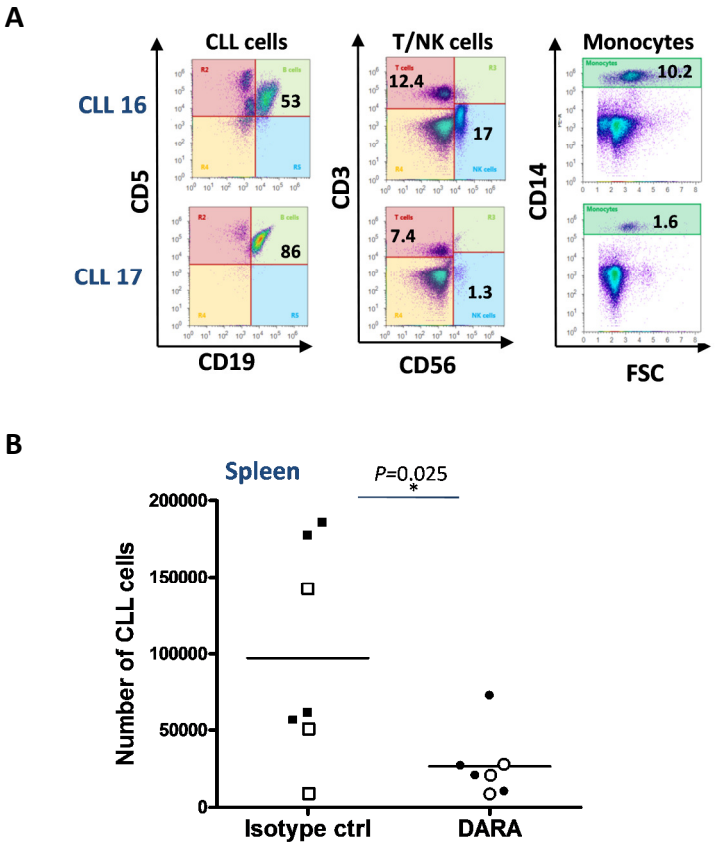


Figure 38. Daratumumab activity in a CLL-PDX model. **(A)** Density plots showing the percentage of CLL cells ($CD19^+$ $CD5^+$), T cells ($CD3^+$ $CD56^-$), NK cells ($CD3^-$ $CD56^+$) and monocytes ($CD14^+$). **(B)** Total number of $huCD45^+CD19^+CD5^+$ recovered from the spleen of isotype ctrl and DARA treated groups (CLL16: open symbols; CLL17: closed symbols). Statistical differences between groups were assessed by unpaired t-test.

STUDY 2:

To evaluate the antitumor profile of a novel and highly selective BTK inhibitor CC-292 in MCL

1. CC-292 shows differential anti-proliferative activity in MCL depending on NF-κB activation status.

We first investigated the anti-tumor effects of CC-292 in five MCL cell lines (REC-1, MINO, UPN-1, MAVER-1 and Z138) after 72h of treatment. CC-292 (10-1000nM) exerted cytostatic effect in a subset of cell lines, where REC-1, MINO and UPN-1 appeared the most sensitive, and MAVER-1 and Z138 were the most resistant to CC-292, following a similar trend than ibrutinib (Figure 39A-B). CC-292 marginally induced apoptosis in MCL cells, as shown by the low rates of Annexin-V+ cells (10-15%) observed in the most sensitive cell lines (UPN-1 and REC-1), exposed to a 1 µM dose of the compound (Figure 39C).

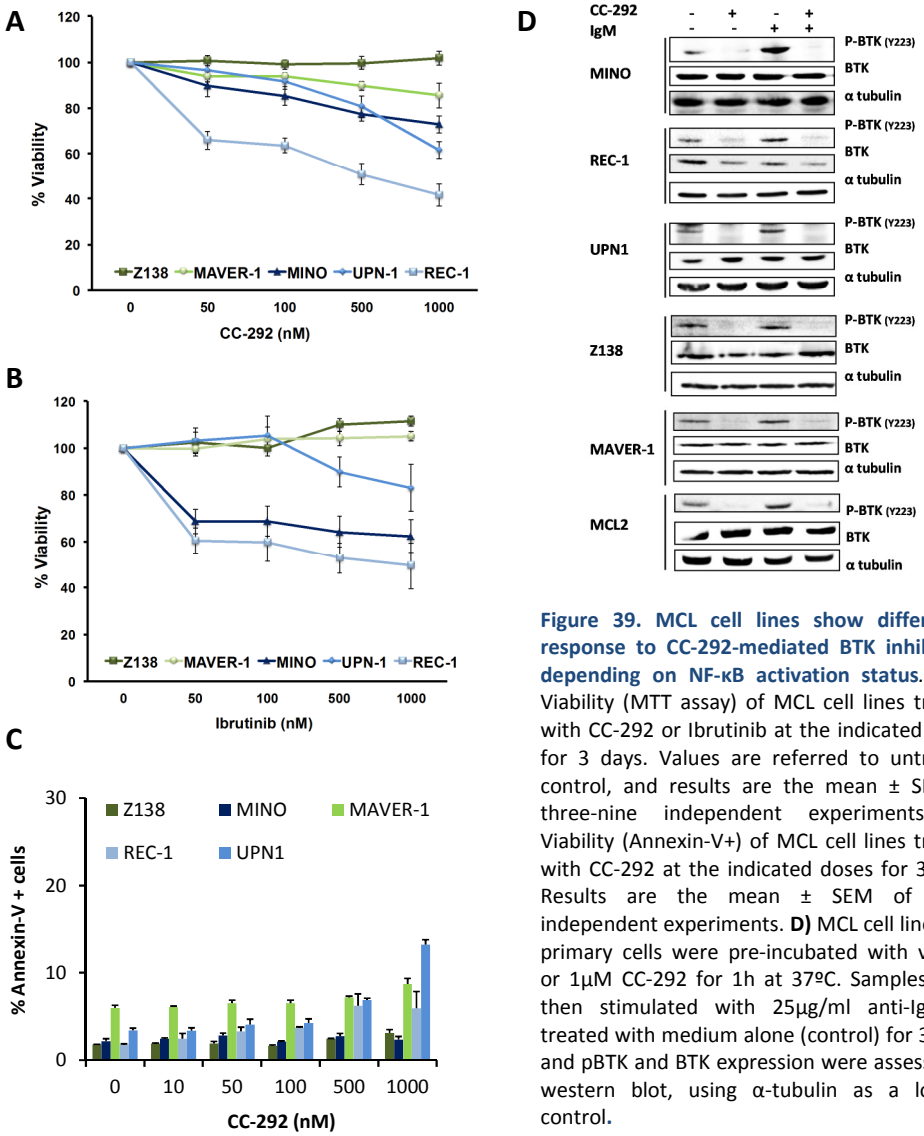
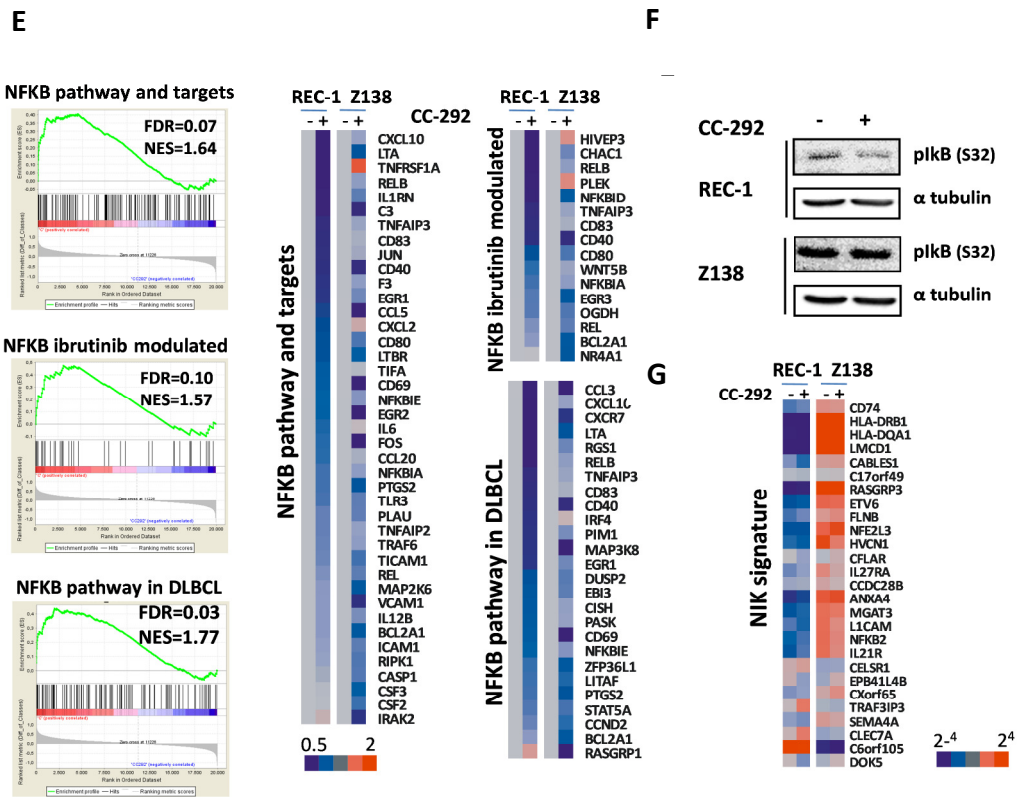


Figure 39. MCL cell lines show differential response to CC-292-mediated BTK inhibition, depending on NF-κB activation status. **(A,B)** Viability (MTT assay) of MCL cell lines treated with CC-292 or Ibrutinib at the indicated doses for 3 days. Values are referred to untreated control, and results are the mean ± SEM of three-nine independent experiments. **(C)** Viability (Annexin-V+) of MCL cell lines treated with CC-292 at the indicated doses for 3 days. Results are the mean ± SEM of three independent experiments. **D)** MCL cell lines and primary cells were pre-incubated with vehicle or 1µM CC-292 for 1h at 37ºC. Samples were then stimulated with 25µg/ml anti-IgM or treated with medium alone (control) for 30 min and pBTK and BTK expression were assessed by western blot, using α-tubulin as a loading control.

BCR-mediated phosphorylation of the BTK kinase at Tyr551 residue results in a 10-fold increase in its catalytic activity. Then, this form of BTK undergoes auto-phosphorylation at Tyr223 to become fully activated. Identification of Tyr223 pBTK is therefore considered a surrogate marker for kinase activity.[141] MCL cell lines were pre-incubated with CC-292 before IgM stimulation to mimic BCR activation. As displayed in figure 40D, CC-292 significantly reduced constitutive and IgM-induced BTK phosphorylation at Y223 residue, in MCL cell lines and primary cells, independently of their sensitivity to the compound (Figure 39D). These results indicate that the differential response to CC-292 does not rely on deficient pBTK inhibition in the CC-292 resistant cell lines.

To examine the molecular basis of CC-292 sensitivity and resistance, we then compared the molecular changes induced by this inhibitor in the sensitive cell line REC-1 and the resistant cell line Z138, by gene expression profiling. Using GSEA, we discovered that, NF-κB pathway appeared as one of the most prominently affected pathways by CC-292. Importantly, specific gene down-modulation of NF-κB target genes using signatures from 3 different sources was much more prominent in the sensitive cell line REC-1 than in the resistant cell line Z138 (Figure 39E). In accordance, the expression of the surrogate marker for NF-κB activation pIkB (Ser32), was higher in Z138 than in REC-1 and CC-292 was able to down-regulate this factor only in REC-1 cells (Figure 39F). These results are in agreement with a previous study with the first-in-class BTK inhibitor ibrutinib, reporting that MCL cell lines resistant to BCR inhibitors displayed activation of the alternative NF-KB pathway, associated to genetic lesions in this pathway. In this sense, the two cell lines resistant to CC-292, MAVER-1 and Z138, harbor a TRAF3 biallelic deletion and a nonsense mutation in *TRAF2* genes, respectively.[16] We confirmed in these cell lines a constitutive activation of the alternative NF-KB pathway, as shown by cleavage of p100 subunit into p52 by western blot (Figure 40A). In this regard we analyzed the expression of genes related to NF-κB inducing kinase (NIK) activity, a central kinase of NF-κB alternative pathway allowing this p100 processing, in REC and Z138 together with their regulation by CC-292. As displayed in figure 39G, genes belonging to NIK signature[113] were prominently expressed in Z138 compared to REC-1, and CC-292 did not significantly down-modulate their expression in any of these cell lines. In summary, CC-292 shows anti-proliferative activity in MCL cell lines without constitutive activation of NF-κB alternative pathway.



(cont. Figure 39) **(E)** REC-1 and Z138 cell lines were treated with 1μM CC-292 for 48h, RNA isolated and subjected to gene expression profiling (GEP) using Affymetrix platform (HG-U219). Gene Set Enrichment Analysis (GSEA) was performed using custom genes related to NF-κB pathway[16,383]. Enrichment plots and heatmaps of the leading edges are shown. NES indicates Normalized Enriched Score; FDR, False Discovery Rate. Threshold FDR<0.10 and NES>1.5. **(F)** REC-1 and Z138 cell lines were treated overnight with 1μM CC-292, and pIκB expression were assessed by western blot, using α-tubulin as a loading control. These samples were run in the same gel and were developed simultaneously, allowing comparison of pIkB baseline expression. **(G)** NIK gene signature expression[113] of REC-1 and Z138 cell lines treated as in (E).

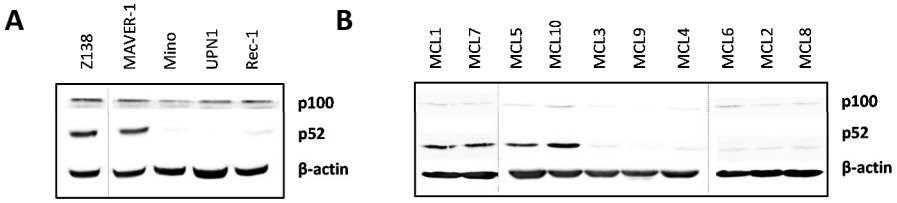


Figure 40. Activation of alternative NF-κB pathway in MCL. Constitutive expression of p52/100 in MCL cell lines **(A)** and primary MCL cases **(B)** was assessed by western blot using β-actin as loading control.

2. CC-292 inhibits cellular activation of MCL cells.

We then sought to determine the effect of CC-292 on cellular activation after BCR stimulation, as reflected in the expression of cell surface markers such as CD69 and CD86. CD69 is a classic and early lymphocyte activation marker[307] as well as CD86 which is regulated by NF-κB.[308] MCL cell lines, both sensitive and resistant to CC-292 (UPN-1 and MAVER-1) and primary cells bearing wt*BIRC3* (MCL3, MCL6) or mut*BIRC*3 (MCL1, MCL7) were stimulated with IgM for 24h in the presence or absence of 1μM CC-292. *BIRC3* gene encodes for cIAP2, a key component of alternative NF-κB pathway that regulates NIK protein degradation. *BIRC3* inactivation leads to NIK protein stabilization, allowing IKKα/IKKα complex activation, and p100 processing to p52.[309] As shown in figure 41A-B, CC-292 significantly decreased the expression of CD69 and CD86 in both MCL cell lines and primary samples, irrespective of NF-κB mutational status. Moreover, IgM stimulation increased the viability of primary samples that was significantly blocked by the treatment with CC-292 ($p<0.05$) (Figure 41C). As CCL3 and CCL4 are reported to be upregulated after BCR engagement in MCL cells[154], we investigated the effect of CC-292 on the secretion of these chemokines. As expected, IgM stimulation of MCL cell lines and primary cells induced the secretion of CCL3 and CCL4. CC-292 efficiently decreased this secretion both in CC-292 sensitive and resistant cell lines and in supernatants from primary MCL cells bearing inactivation of *BIRC3* by deletion of one allele and mutation of the other (Table 17), and consequent alternative NF-κB pathway activation (Figure 40B, Figure 41D). In summary, CC-292 efficiently blocks BCR-derived activation and chemokine secretion both in sensitive and resistant cell lines, and in primary cells bearing mut*BIRC3*.

3. CC-292 interferes with CXCL12-induced migration.

BTK is involved in B-cell trafficking mediated by the chemokine receptors CXCR4/CXCR5.[310] We thus measured the direct effect of CC-292 on migration of two MCL cell lines (UPN-1, Z138) and four primary cells (MCL1, MCL2, MCL10, MCL11). CXCL12-induced migration of MCL cell lines was diminished after CC-292 treatment, although the extent of inhibition was greater in the CC-292 sensitive cell line UPN-1 (34.4%) than in the CC-292 resistant cell line Z138 (18.8%) (Figure 42A). Regarding the primary cases, we found that CC-292 significantly

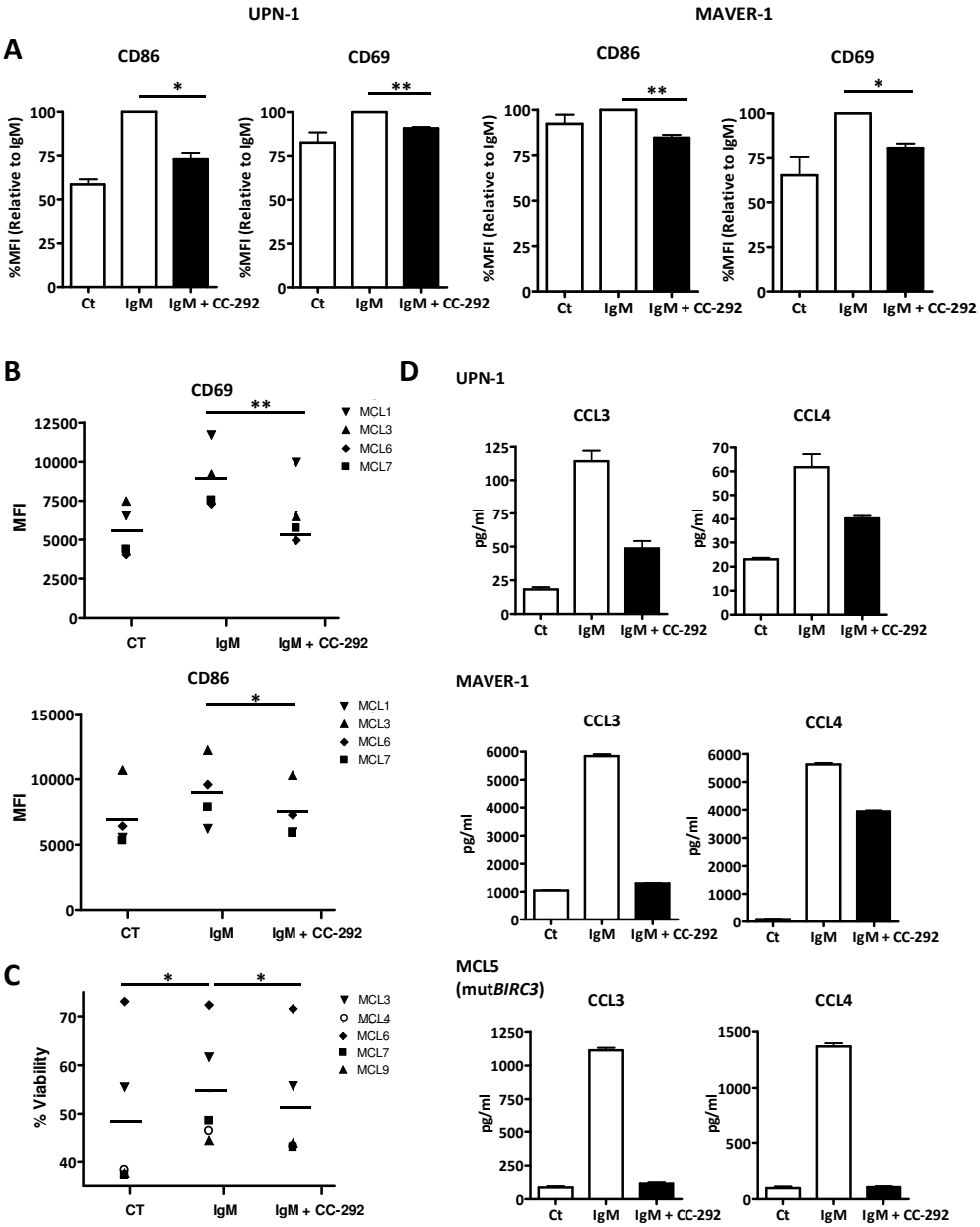


Figure 41. CC-292 inhibits BCR-induced cellular activation and secretion of CCL3 and CCL4 chemokines. **A)** The expression of CD69 and CD86 on UPN-1 and MAVER-1 cells was compared between cells pre-treated or not with 1μM CC-292 under anti-IgM stimulation (10μg/ml) for 24h. The bars in this graph represent mean ± SD expression relative to stimulated control of 3 independent experiments. **B)** Expression of CD69 and CD86 and **C)** cell viability of anti-IgM-stimulated CD19+CD5+ cells from 4-5 MCL primary samples, either pre-treated or not with 1μM CC-292 for 24h. **D)** CCL3 and CCL4 protein secretion was evaluated by ELISA in the culture supernatant of UPN-1, MAVER-1 and MCL5, in the absence (control) or upon BCR stimulation (anti-IgM 10μg/ml) for 24 hr. Bars correspond to the mean ± SD from duplicates of the concentrations in pg/ml.

inhibited CXCL12-induced migration of those cells with no mutations in the alternative NF-κB pathway (MCL2, MCL11) (22.2% and 55.1% of inhibition, respectively), whereas it was not able to interfere with CXCL12-induced migration of MCL1 and MCL10, bearing mut*BIRC3* (Figure 42A). Moreover, CXCL12 ligation to CXCR4 induced immediate and prominent pERK1/2 activation (phosphorylation at Thr202/Tyr204 residues). Treatment with CC-292 efficiently reduced ERK activation (Figure 42B) in UPN-1 and to a lesser extend in Z138 while had no effect in MCL10, accordingly to the results obtained in CXCL12-migration assay. In summary, CC-292 mainly interferes with CXCL12-induced migration of MCL cells bearing no mutations in the alternative NF-κB pathway.

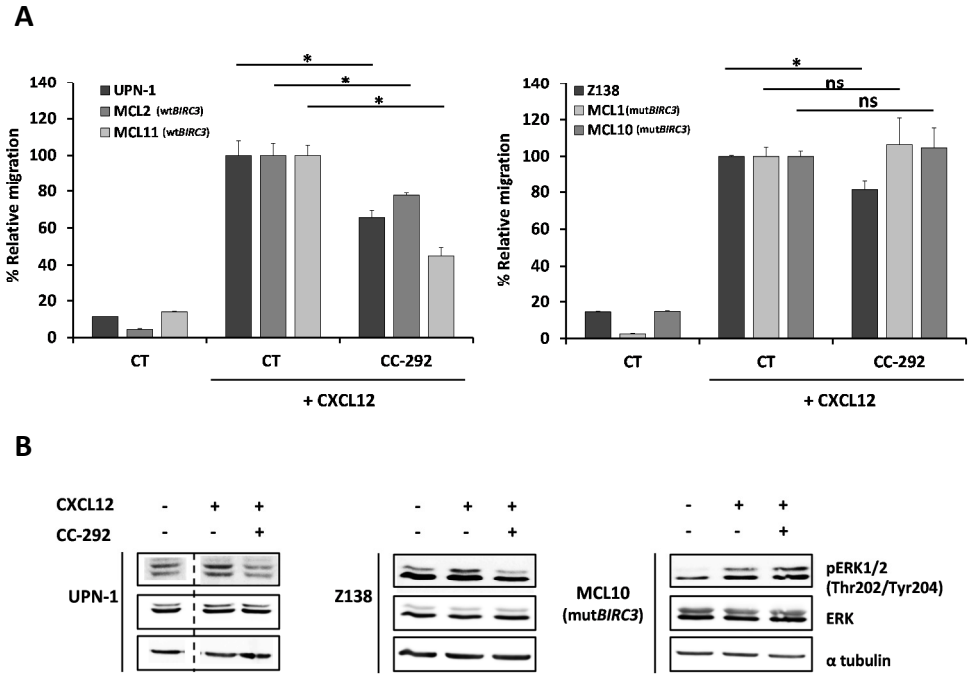


Figure 42. CC-292 interferes with CXCL12-induced migration. **A)** UPN-1, Z138 and four primary samples (MCL1, MCL2, MCL10 and MCL11) were pretreated with 1μM CC-292 or with vehicle (DMSO) for 1h. Then, cells were challenged to migration towards a CXCL12 gradient through a VCAM-coated chemotaxis chamber for 3h. The mean ± SD of primary samples and cell lines migrations referred to the CXCL12-stimulated control, are displayed. **B)** UPN-1, Z138 and MCL10 cells were pre-incubated with vehicle or 1μM CC-292 for 1hr followed by stimulation with CXCL12 for 5min. pERK and ERK expression were assessed by western blot, using α-tubulin as a loading control.

4. CC-292 cooperates with lenalidomide in the CC-292 sensitive cell lines.

Since CC-292 anti-tumor activity is limited, we sought to determine if we could achieve a greater therapeutic effect in combination with the immunomodulatory agent lenalidomide, already approved by FDA for the treatment of relapsed MCL. Lenalidomide is known to exert a plethora of effects that include antiproliferative effect on tumor cells and modulation of the immune microenvironment.[311,312] In addition, among other activities, lenalidomide is known to downregulate NF-κB activation.[313] Thus, we treated both CC-292 sensitive (MINO, REC-1, UPN-1) and resistant cell lines (MAVER-1 and Z138) with the combo and analyzed the effect on cell proliferation. In the cell lines with primary sensitivity to CC-292, lenalidomide significantly increased the anti-tumor activity of the BTK inhibitor when used both at low and high doses. On the contrary, no cooperation was found between these two agents in the CC-292 resistant cell lines (Figure 43A). It has been described that lenalidomide downregulates IRF4 expression in MCL sensitive cell lines.[314] In this line, the combination was very efficient in the inhibition of the downstream target IRF4 in CC-292 sensitive cases, while remained unaffected in CC-292 resistant cases (Figure 43B). It is noteworthy that this cooperation was maintained, both in terms of proliferation blockade and IRF4 downregulation, when MCL cells were in co-culture with the MSC StromaNKtert (Figure 43C-D). This cooperative effect was likewise observed in the MSC-MCL coculture system of primary cells (MCL4) with no mutations in the alternative NF-κB pathway regulators (Figure 43E, Figure 40B, Table 17). These results support the use of a combinatorial regimen of CC-292 and lenalidomide in those MCL cases with primary sensitivity to CC-292.

5. NIK inhibition overcomes MCL resistance to CC-292 driven by mutations in the alternative NF-κB pathway.

NIK is a member of the MAPK kinase kinase (MAP3K) family that causes activation of both canonical and non-canonical NF-κB signaling pathways.[315,316] NIK inhibitors have recently proved to be effective in multiple myeloma (MM) cells with activating mutations in this pathway.[299] These specific NIK inhibitors are selectively cytotoxic for cells with NIK-dependent activation of NF-κB. In addition, results in MM have demonstrated that combination therapy targeting both alternative and canonical NF-κB pathway results in improved anti tumoral activity.[299]

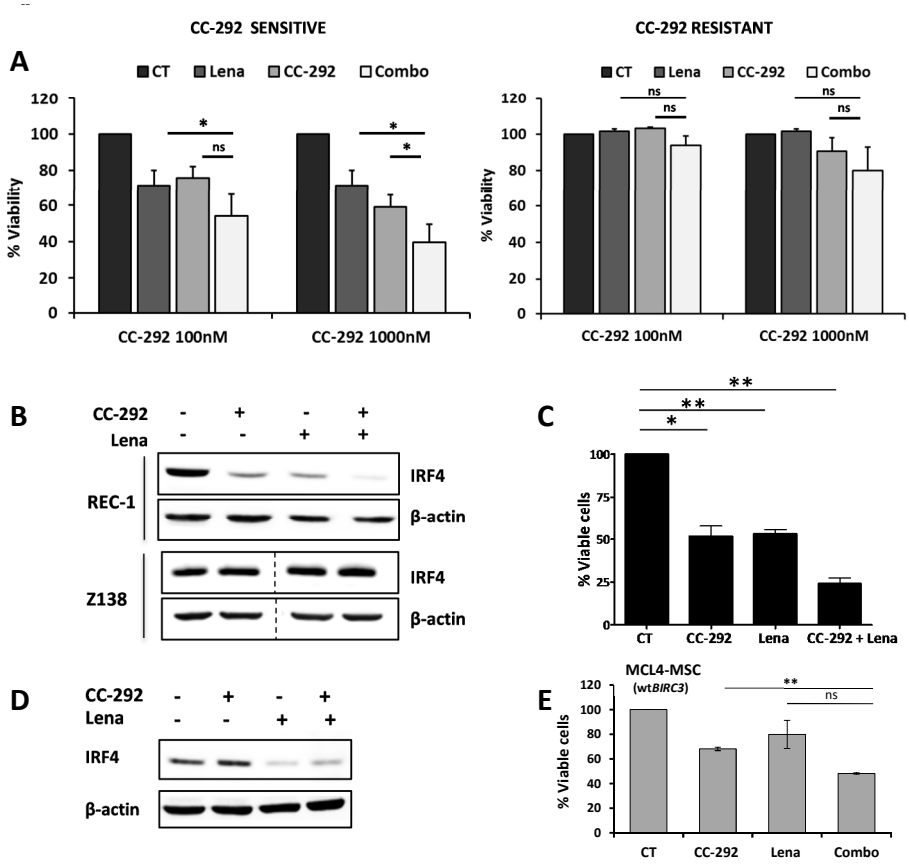


Figure 43. CC-292 cooperates with lenalidomide in CC-292 sensitive cell lines.
A) CC-292 sensitive (MINO, REC-1 and UPN-1) and CC-292 resistant (MAVER-1 and Z138) cell lines, were exposed to either CC-292 (100nM or 1000nM) or lenalidomide (5μM) or their combination for 3 days. Viability was assessed by MTT assay and results are displayed referred to the untreated control. **B)** REC-1 and Z138 cell lines were treated with 100nM CC-292 and/or 5μM lenalidomide for 3 days. IRF4 was assessed by western blot using β-actin as a loading control. **(C,D)** REC-1 was co-cultured with stromaNKtert cells in the presence or absence of 1μM CC-292 and/or 5μM lenalidomide for 72h. The number of viable MCL cells was quantified by Annexin-V and CD19 labeling; results are plotted referred to untreated control. CD20+ cells were then purified with MACS separation columns and the expression of IRF4 was assessed by western blot using β-actin as loading control **(D)**. **E)** Primary cells from a representative MCL patient with wt*BIRC3* (MCL4) were cultured with stromaNKtert feeder cells and treated with 1μM CC-292 with or without 5μM lenalidomide for 72h. The number of viable MCL cells was quantified by Annexin-V and CD19 labeling followed by flow cytomery analysis. Results are shown referred to untreated control.

As described previously, those MCL cell lines that are the most resistant to CC-292 (Z138 and MAVER-1) exhibit activation of the alternative NF-κB pathway due to genetic lesions in *TRAF2* and *TRAF3*, resulting in the cleavage of NF-κB p100 subunit to p52 (Figure 40A). Thus, we sought to determine the potential activity of those NIK inhibitors, in MCL cell lines refractory to BTK inhibition. Z138 and MAVER-1 cell lines were treated for six days with two

NIK inhibitors, AM-0216 (#16) and AM-0561 (#61), or with an isomeric control of AM-0216 (AM-0650 (#50)), in the presence or absence of 1μM CC-292. AM-0216 and AM-0561 were active in both cell lines, albeit in a different degree, with MAVER-1 being the most sensitive. Noteworthy, in agreement with the results described for MM, the combination of AM-0216 and AM-0561 with an inhibitor of the canonical NF-κB pathway, such as CC-292, resulted in a significant cooperative effect in terms of cell growth inhibition and apoptosis induction (Figure 44A). It is important to note that after six days of treatment MAVER-1, but not Z138, becomes more responsive to CC-292 (Figure 45), suggesting that the phenotypic results of alterations in the alternative NF-κB pathway may be different. Analysis of the p52 levels, as a surrogate maker of alternative NF-κB pathway activation, indicated that while CC-292 exerted no effect on p100 processing, AM-0216 and AM-0561 induced a remarkable decrease in p52 levels, while remained unaffected in the presence of the isomeric control AM-0650 (Figure 44B). More importantly, pIκB completely disappeared in the cells treated with the combination, indicating that total inhibition of NF-κB pathway was achieved (Figure 44B).

Microenvironmental stimuli play a determinant role on survival and activation of MCL cells as well as on response to therapy. Several studies have demonstrated that MSC protect MCL cells from spontaneous and drug-induced apoptosis through secretion of different growth factors such as BAFF, leading to the activation of the canonical and non-canonical NF-κB pathway.[117,317] Thus, we speculated if simultaneous targeting of these pathways with CC-292 and NIK inhibitors could show efficacy in a co-culture system. Z138 and MAVER-1 cell lines were co-cultured with stromaNKtert cells in the presence or absence of 1μM CC-292 and/or 1μM NIK inhibitors for 6 days. Viable MCL cells were identified as CD19+/Annexin V- and counted. As expected, both cell lines became less sensitive to NIK inhibitors in the presence of stromaNKtert, although the cooperation with the CC-292 and NIK inhibitors was maintained, leading to a significant anti-tumor effect (Figure 44C). We finally validated these results in MCL primary cases bearing inactivation of *BIRC3* by deletion of one allele and mutation of the other, as described previously.[15,309] In order to improve the cell viability of primary samples and to obtain more meaningful results, the combination was tested in the MCL-stromaNKtert co-culture system. As depicted in figure 44D, CC-292 and NIK inhibitors combination was significantly active in MCL primary cases

with *BIRC3* inactivation (MCL7 and MCL10; Table 17 and Figure 40B), confirming the results obtained with MCL cell lines.

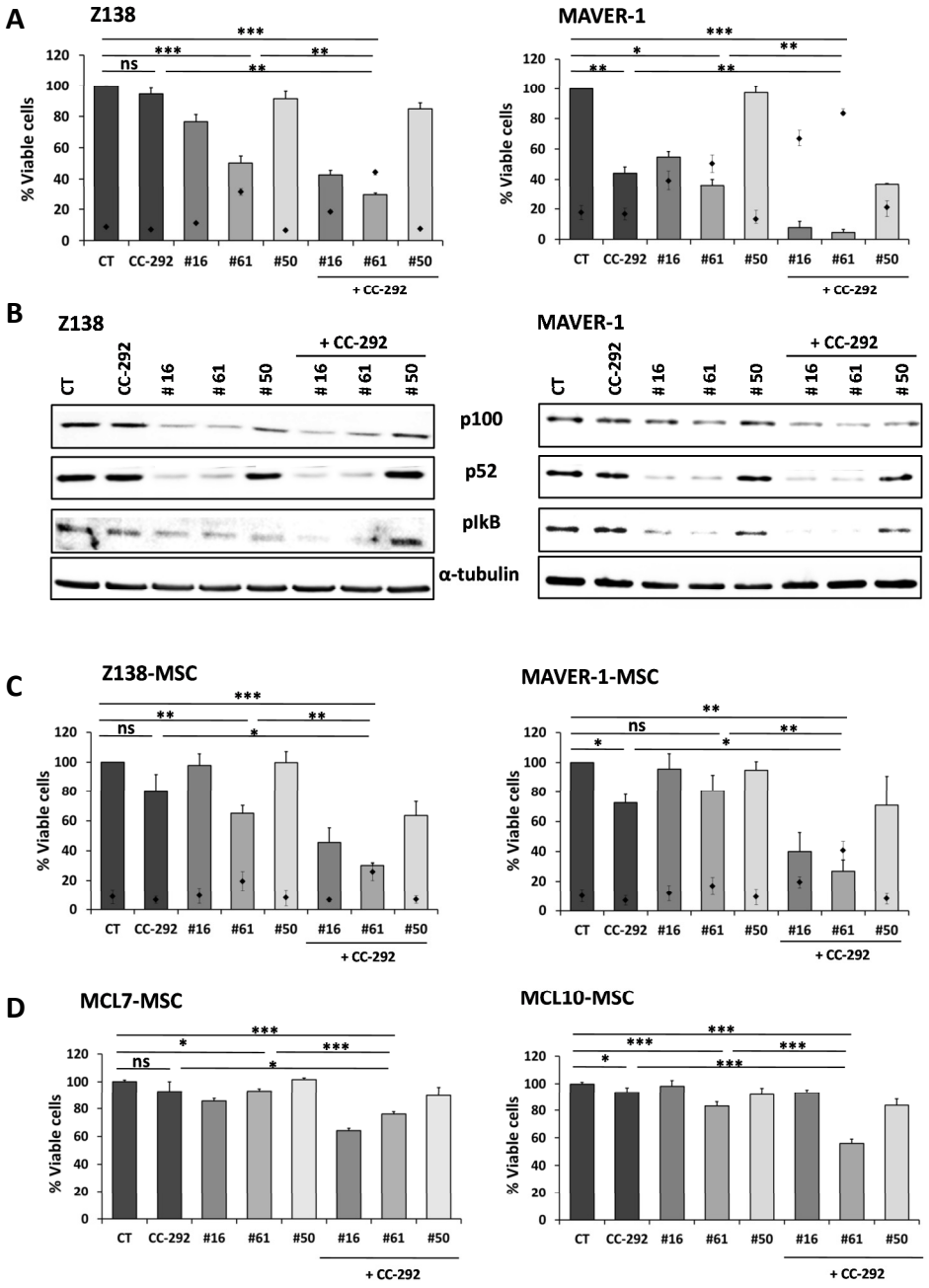


Figure 44 *(previous page)*. CC-292 cooperates with NIK inhibitors in CC-292 resistant MCL cells. **A)** Z138 and MAVER-1 were exposed to either 1µM CC-292 or the 1µM NIK inhibitors (AM-0216 (#16) and AM-0561 (#61); inactive isomer AM-0650 (#50)) or the combination for 6 days. Total number of viable cells was analyzed by MTT proliferation assay (bars) and viability by Annexin-V labeling (dots). Values are referred to untreated control and results are expressed as the mean ± SD of three independent experiments. **B)** Z138 and MAVER-1 were treated with either 1µM CC-292 or 1µM NIK inhibitors or the combination for 72h at 37ºC, and the expression of NF-κB proteins was assessed by western blot. **C)** MAVER-1 and Z138 were co-cultured with stromaNKtert in the presence or absence of 1µM CC-292 and 1µM NIK inhibitors for 6 days. The number of viable MCL cells was quantified by Annexin-V and CD19 labeling. Values are referred to untreated control and results were expressed as the mean ± SD of three independent experiments. **D)** Primary cells from MCL patients with mut*BIRC3* were cultured with stromaNKtert feeder cells and treated with 1µM CC-292 with or without 1µM NIK inhibitors for 6 days. The number of viable MCL cells was quantified as before. Results are shown referred to untreated control.

Study label	Gender[1]	Sample type[2]	%tumor cells[3]	MCL variant[4]	Stage[5]	MIPI[6]	*BIRC3* mutation[7]	11q loss/UPD[7]	*TP53* mutation[7]	*17p* loss/UPD[7]
MCL1	M	PB	83	C	IV-B	HR	1	1	0	1
MCL2	M	PB	85	SC	IV	HR	0	0	0	0
MCL3	M	PB	83	B	IV-B	ND	ND	0	ND	1
MCL4	F	PB	97	SC	IV	HR	0	0	1	1
MCL5	M	LN	71	C	IV-A	IR	1	1	0	0
MCL6	M	PB	94	B	IV-A	HR	0	0	0	0
MCL7	M	PB	69	B	IV-B	HR	1	1	0	0
MCL8	F	PB	78	B	IV-A	HR	ND	0	1	1
MCL9	M	PB	84	SC	IV-A	HR	ND	0	0	0
MCL10	M	PB	86	C	IV	HR	1	1	0	0
MCL11	M	PB	96	C	IV	HR	0	0	1	1

Table 17: MCL patient characteristics. [1]M:male, F:female. [2] PB: peripheral blood, LN: lymph node. [3]% of tumor cells assessed by flow cytometry based on CD19 CD5 positive cells. [4]C: Classic; B: Blastoid, SC: Small Cells. [5] Defined by Ann Arbor stage system. [6] MIPI: MCL International Prognostic Index: high-risk (H), intermediate risk (I)[24]; [7] 1: alteration present, 0: alteration absent, ND: not done. Whole exome sequencing and genomic data from Bea S, PNAS, 2013.[15]

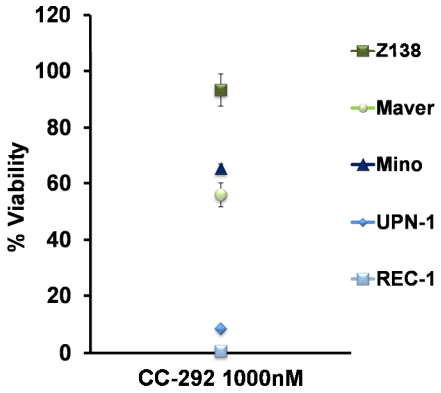


Figure 45. MCL cell lines response to CC-292 at six days. Proliferation of MCL cell lines treated with 1µM CC-292 for 6 days.

DISCUSSION

DISCUSSION

Targeted therapy against biomarker molecules has revolutionized drug development and cancer therapy. Nowadays, two main approaches for targeting specific molecules are available in the clinical practice: therapeutic mAbs and small-molecule agents.

The active role of the microenvironment in the pathogenesis of B cell lymphomas has been well recognized. We are now beginning to understand the complex crosstalk between neoplastic B cells and tissue microenvironment. Disrupting this crosstalk is an attractive, novel strategy for treating patients with B cell malignancies. In this thesis, we explore the targeting of two molecules that play important roles in these interactions between B cells and their microenvironment: CD38 and BTK.

DARATUMUMAB IN CLL

Targeted immunotherapy with mAbs has become the standard of care for successful treatment of many forms of cancer. In CLL, anti-CD20 antibodies (rituximab, ofatumumab and obinutuzumab), have demonstrated therapeutic benefit, alone and in combination with chemotherapy.[318] Identification of new targets with a broader spectrum and potential for distinctive mechanisms could yield novel antibody therapeutics for a wider range of hematologic malignancies.

In the past few years, CD38 has gained momentum as a novel therapeutic target for patients with hematologic malignancies, namely MM[281,302], CLL and NHLs. CD38 is an ectoenzyme belonging to the family of nucleotide-metabolizing enzymes, involved in the scavenging of extracellular nucleotides. CD38 catalyzes the synthesis of cyclic ADP-ribose and ADP-ribose from NAD, leading to an increase in cytoplasmic Ca^{2+} concentration. CD38 is used as a disease marker for leukemias and myeloma and is considered a negative prognostic marker for CLL.[204,205] Moreover, recent evidence indicates that CD38 forms a complex molecular network delivering growth and survival signals in CLL cells. CD38 cooperates with chemokines and their receptors to influence cell migratory responses.[211] These characteristics make CD38 an attractive target for CLL therapy. The use of an antibody such as DARA that specifically blocks CD38 might provide a new approach for

interfering with deleterious growth circuits and for increasing the susceptibility of leukemic cells to conventional chemotherapy.

In the first study of this thesis, we have analyzed the potential therapeutic activity of DARA in CLL. DARA showed limited CDC activity in both primary CLL cells and CLL cell lines. Complement activation is strongly regulated by mCRPs *in vivo* to prevent its uncontrolled amplification, including CD46, CD55, and CD59 that have been shown to mediate resistance to CDC induced by rituximab and to a lesser extent by ofatumumab.[319] In fact, these proteins are overexpressed in a number of tumor types, and their upregulation has been postulated to contribute to mAb resistance *in vivo*.[231] Our data show that CLL cell lines and primary cells display very high expression of the mCRP, which may explain the limited sensitivity to DARA-mediated CDC *in vitro*. On the other hand, CLLs express low numbers of CD38 molecules on the cell surface that may also contribute to the limited CDC, as target expression has been previously shown to significantly affect CDC.[320]

Our results are the first to provide strong evidence that DARA induces lysis of CLL cells by FcγR mediated ADCC and ADCP through NK cells and macrophages, respectively. This cytotoxic effect is noteworthy in CLL, where the mean ADCC induction was 35% at 0.1µg/mL, being in the same range as those published for primary MM.[271,304] In addition, ADCC does not correlate strictly with CD38 sABC in CLL cell lines or primary cells, indicating that at least in CLL the number of CD38 molecules on the cell surface may not be the only factor in driving Fc-mediated cytotoxicity of DARA. Other molecules within the immune synapse must control the extent of ADCC/ADCP by DARA. Activating receptors expressed on NK cells include FcγRIIIA, activating forms of KIRs (KIR2DS and KIR3DS), NKG2D, and the Natural Cytotoxicity Receptors (NCR) called NKp30, NKp44, and NKp46 that are critical for optimal ADCC activity.[321] There are observations that clinical response after therapeutic antibody treatment correlates with FcγRIIIA polymorphisms.[322,323] Moreover, KIR and HLA genotypes predictive of high-affinity NK cell receptor-ligand interactions have been associated with improved clinical outcome in MM patients treated with the anti-CD38 mAb isatuximab combined with lenalidomide and dexamethasone.[324] In addition, inhibitory receptors counteract activating receptors as a means to tolerate mature NK cells. A recent study in MM have shown that blocking inhibitory KIRs with IPH2102, a human IgG4 monoclonal antibody that blocks the interaction of the three main inhibitory KIR receptors

with their ligands, improves ADCC induced by DARA against MM cells.[325] Thus, the overall make-up of these activating and inhibitory molecules on each individual CLL tumor cell may dictate the extent of ADCC by DARA.[326]

Combination therapies simultaneously target multiple pathologic pathways and prevent escape and resistance mechanisms of tumor cells. This also applies for combinations of pharmacological agents with therapeutic mAbs. Because of the strong immunostimulating effects of lenalidomide including activation of NK cells, its combination with DARA has been evaluated in preclinical and clinical MM studies with promising results.[280,281] The results of a phase 3 trial showed that the addition of DARA to lenalidomide and dexamethasone significantly prolonged progression-free survival and was associated with a 63% lower risk of disease progression or death than lenalidomide and dexamethasone alone among patients with MM who had received one or more lines of therapy.[272] Recently, the FDA approved DARA in combination with lenalidomide and dexamethasone, or bortezomib and dexamethasone, for treatment of patients with MM who have received at least one prior therapy. We have explored its effects in DARA–induced ADCC of $CD38^{+}$ CLLs, and keeping with the results in MM, lenalidomide significantly increased ADCC induction by DARA (Annexes). Results of lenalidomide efficacy and toxicity in CLL were first reported by Chanan-Khan et al. in an R/R CLL setting, in which lenalidomide demonstrated good efficacy, with an ORR of 47% in a high risk, heavily-pretreated patient population, characterized by worse prognostic factors. Nevertheless, unexpected severe toxicity was reported representing an issue in the management of CLL patients treated with lenalidomide.[327] To date, lenalidomide has been used in CLL as an off-label drug both in Europe and in the US, and is generally considered among the possible clinical options only in advanced stage R/R patients.[312]

Macrophages are tissue-resident immune cells that play a critical role not only in maintaining homeostasis and fighting infection but also in the progression of many pathologies including cancer.[328] Human macrophages express both activating and inhibitory FcγR and are involved as the most prominent effector cell populations in mAb-mediated tumor elimination *in vivo*. We have demonstrated that DARA induces phagocytosis of CLL by macrophages both *in vitro* and *in vivo*. In the *in vitro* model, CLL cells and macrophages were co-cultured in the presence or absence of DARA. In the *in vivo* model, CLL cells were

injected into the peritoneum of SCID beige mice, which are devoid of NK cells, but possess active macrophages in their peritoneal cavity. ADCP has been described as a relevant mechanism of action that may contribute to the therapeutic activity of DARA in MM[275] and our results support the idea that it might also be an important mechanism of action in $CD38^+$ CLL. A number of approaches to augment macrophage responses to therapeutic antibodies are under investigation, including engineering Fc fragments for greater binding to activating Fc receptors, using either bsAbs that cross link macrophages and cancer cells or antibody-drug conjugates (ADCs) with immunostimulatory agents.[227] New targets have also been explored in order to augment macrophage responses. For example, the interaction of CD47 on tumor cells with signal-regulatory protein α (SIRPα) that is expressed on macrophages serves as a myeloid-specific immune checkpoint that limits the response to antibody therapies.[329] CD47 is described as being upregulated on leukemic cells to avoid phagocytosis.[330] Moreover, anti-CD47 mAb has been shown to synergize with rituximab in the treatment of NHL.[331] Thus, targeting the CD47-SIRPα axis may lower the threshold for macrophage phagocytosis and increase efficacy of several therapeutic antibodies, including DARA.

We then explored whether these *in vitro* mechanisms translate to *in vivo* tumor growth inhibition. To accomplish this task, we developed two approaches. Firstly, we successfully developed a MEC2 tumor model that showed 100% engraftment efficacy and systemic disease involving mostly lungs, kidneys, ovaries, parathyroid glands, enlarged lymph nodes and BM in a portion of the mice. This model is clinically relevant for CLL with leukemic infiltrates in isolated organs similar to that reported in CLL patients[332], presenting aggressive disease or transformation to RS. In this model, treatment with relevant pharmacological doses of DARA efficiently prevented tumor progression and significantly prolonged survival. Mice treated with DARA showed long-term survival, even though DARA dosing was stopped after 4 weeks and the antibody concentrations in the serum were minimal, suggesting that these mice were free of disease. Secondly, we developed a CLL-PDX model using NSG mice, to avoid CLL clearance by mouse NKs or phagocytes, and inoculating selected CLL enriched in NKs and monocytes to provide the system with FcγR-bearing effector cells. In this model, DARA proved to reduce tumor burden in the mouse spleen, which constitutes the main infiltrated organ in this model.[333] These results are the

first to provide evidence that anti-CD38 therapy with DARA may be relevant for $CD38^+$ CLL.

We have also demonstrated additional activities of DARA in addition to ADCC and ADCP in CLL. DARA has the potential to counteract microenvironment-derived signaling in protective cancer niches, such as LN and BM. We have demonstrated that DARA interferes with *in vitro* cell migration and *in vivo* homing of CLL cells to spleen in NSG mice. Transendothelial migration and organ invasion of malignant cells require proteolytic degradation of the vascular basement membrane and extracellular matrix of lymphoid tissues and MMPs play a key role in these processes. MMP9 is the predominant MMP expressed in CLL and is physiologically regulated by CD49d/CD29 and CXCL12, playing a key role in cell invasion and transendothelial migration.[334] Moreover, MMP9 correlates with advanced stage disease and poor patient survival.[335] We have demonstrated that DARA significantly reduces CD49d/CD29-mediated adhesion of CLL cells to VCAM-1 and, more importantly, downregulates both constitutive and adhesion-induced MMP9 expression. Based on the prominent role of MMP9 in CLL cell invasion, our results indicate that DARA treatment may impede CLL tissue infiltration that leads to progressive disease. Thus, in the era of BCR kinase inhibitors (such as the BTK inhibitor ibrutinib), DARA immunotherapy opens a new horizon offering unique effects on tumor dissemination against $CD38^+$ CLL cases. Both CD38 and BTK are part of molecular hubs integrating proliferative, migratory and adhesion signals on tumor cells. There are no clinical trials evaluating DARA in CLL yet, but taking into account our results suggesting that DARA interferes with *in vitro* cell migration/adhesion and *in vivo* homing of CLL cells, it may well be possible that lymphocytosis also occurs in CLL patients treated with DARA, similarly to what have been observed with BTK inhibitors.

As described in the introduction, DARA activity may extend beyond its effect on the tumor cells. Recent studies suggest a pivotal role of CD38-dependent adenosine production in immune suppression mediated by NK cells[336] and an involvement of CD38 in immune modulation mediated by MDSCs.[337] Thus, anti-CD38 monoclonal therapy may hold potential for targeting this immunosuppressive population in cancer treatment strategies. Indeed, there is data describing that DARA depletes $CD38^+$ immune suppressive cells, which is associated with an increase in T_H cells, cytotoxic T-cells, T-cell functional response, and TCR clonality.[279] This previously unknown, multidimensional, immunomodulatory role for DARA may contribute to deeper clinical responses and enhanced survival.

Taken together, our results support DARA as a novel therapeutic approach for CD38$^+$ CLL by not only inducing the classical FcγR-mediated cytotoxicity but also by harnessing microenvironment-derived survival signaling and blocking CLL dissemination to secondary lymphoid organs. The exact contribution of each mechanism needs to be elucidated but ADDC might not be the predominant mechanism of action *in vivo*. A phase I/II dose escalation study of DARA with R/R MM patients describes a dose-dependent decrease in PB NK cells, with full recovery after treatment.[338] Moreover, the NK-cell compartment has an overall reduced effector activity in CLL.[136] NK cells have generally been considered as the main effector cells in mAb therapy; however, increasing evidence supports a major role for macrophages in the elimination of tumor cells. In this regard, an association between polymorphism in human FcγRIIa (expressed by macrophages but not NK cells) and clinical responses to rituximab therapy has been reported.[339] Similarly, the efficacy of treatment with antitumor mAbs was improved in mice that did not express the inhibitory receptor FcγRIIb[340], which cannot be a consequence of enhanced ADCC, as NK cells do not express this receptor. Furthermore, in another study removal of lymphoma cells after anti-CD20 mAb treatment was dependent on the mononuclear phagocyte network and required expression of activating FcγRs.[341]

There are currently no clinical trials evaluating DARA in CLL but our results suggest that it may be active in this indication as well. It is important to consider that, unlike MM, only a subset of CLL patients is CD38$^+$, implying the need for selection of CD38-positive tumors that are eligible for treatment with DARA or other CD38 mAbs. Variables that could have an impact on clinical outcome of CD38$^+$ CLL patients treated with DARA include host-related factors such as frequency and activity of effector cells, which may be affected by various anti-CLL therapies. Furthermore, tumor-related factors such as target antigen expression levels, expression of mCRPs, expression of inhibitory receptors such as PD-L1, which inhibits PD-1-positive NK and T cells, or CD47, which impairs phagocytosis, may impact clinical outcome. Genetic variations in certain genes including FcγR polymorphisms and KIR may also influence response to DARA treatment. The expression pattern of CD38 in a broad range of cell types can raise concerns about potential adverse effects of anti-CD38 therapy. However, clinical studies of DARA in MM have demonstrated an acceptable safety profile, suggesting that an appropriate dosage and treatment schedule can minimize the effects of

CD38 targeting in normal tissue. Finally, novel therapeutic approaches are being explored that target CD38, including CD38-specific CAR T-cells[342] and CD3xCD38 bsAbs.[343] These strategies show promise in preclinical models, but their activity in the clinic remains to be tested.

CC-292 IN MCL

Over the last few decades, there has been significant improvement in the clinical outcome of MCL, from a median OS of 2.5 years initially to 5-7 years currently.[344] This condition usually responds to any one of standard regimens, which often consist of rituximab in combination with standard chemotherapy, and the responses can be durable. Nevertheless, after repeated treatment regimens the disease becomes more aggressive, therapy-resistant, and the patient becomes more intolerant to additional treatment.[1,6] Four new agents, including the BTK inhibitor ibrutinib, now have approval for MCL patients who have received at least one prior therapy.[40]

Constitutive or aberrant signaling of the BCR-signaling cascade has been implicated in the propagation and maintenance of a variety of B cell malignancies.[345] These observations have propelled the development of drugs targeting several elements of this pathway, including SYK, BTK, and PI3K.[152] Ibrutinib was the first BTK inhibitor and also the first BCR inhibitor approved for treatment of B cell malignancies.[39,95] It is a promising agent for patients with MCL with notable response rates; in fact, it is the drug that appears to have the greatest activity when used as a single agent in MCL.[39] However, as a single agent, acquired and primary resistances have been reported and nearly one third of patients relapse in the first two years of treatment.[346] The outcome of patients with MCL who experience disease progression following ibrutinib therapy is poor, with both low response rates to salvage therapy and short duration of responses.[347] It is not clear whether the poor outcome after ibrutinib failure represents the final common pathway of treating patients with advanced MCL or the result of ibrutinib selecting for a biologically more aggressive. The second assumption could have significant implications in frontline treatment, and would make it reasonable to “save” ibrutinib for use in patients that are refractory to other standard therapies given the low probability of response to those treatments following

ibrutinib failure. What is clear is that MCL patients with primary or secondary ibrutinib resistance represent the greatest unmet medical need in this disease at present.[348]

More selective and specific BTK inhibitors are being explored and developed. CC-292 is a highly potent small-molecule inhibitor of BTK (IC50=0.5 nM), that covalently/irreversibly binds to Cys481 in the BTK active site. It has superior specificity against BTK than ibrutinib with limited inhibition *vs.* other kinases with homologous cysteine residues including CSK, BRK, HCK, YES, ITK and LCK.[349] The second objective of this thesis was aimed at evaluating the antitumor profile of CC-292 in MCL, together with its impact on migration, adhesion and cellular activation processes. In addition, we have also explored possible combination strategies to enhance CC-292 activity.

Upon BCR activation, BTK becomes activated by other tyrosine kinases, such as Lyn and SYK, leading to the activation of downstream pathways that regulate B cell survival, proliferation and differentiation.[141] We evaluated herein the antiproliferative activity of CC-292 in five MCL cell lines. CC-292 demonstrated a differential anti-proliferative activity that did not rely on deficient pBTK inhibition, as both constitutive and IgM-induced BTK phosphorylation were reduced after treatment in all cell lines tested. In parallel to our observation, it has been described that ibrutinib activity in MCL cell lines does not strictly correlate with a decrease in pBTK levels.[350] Recently, Rahal et al. reported that ibrutinib sensitive MCL cell lines exhibited chronic activation of the BCR-driven classical NF-κB pathway, whereas insensitive cell lines displayed activation of the alternative NF-κB pathway.[16] Our study demonstrates that this mechanism of resistance is also applicable to CC-292, where sensitive cell lines (UPN-1, REC-1, MINO) display NF-κB classic activation and insensitive cell lines (Z138, MAVER-1) exhibit alternative NF-κB signaling.

BCR engagement initiates a cascade of intracellular signaling, which leads to the activation of the B cell and subsequent internalization of antigen for processing and presentation to T cells.[351] The intracellular signaling pathways initiated upon ligand binding results in the upregulated expression of some surface molecules, such as CD69 and CD86, and increased chemokine secretion, such as CCL3 and CCL4. In this vein, we evaluated MCL cell activation using CD69/CD86 expression and CCL3/4 secretion and found that CC-292 interfered with BCR cellular activation independently of the NF-κB mutational status. This is in agreement

with previous results showing that BTK inhibition reduced the secretion of CCL3/4 in Mino cell cultures and the plasma of MCL patients treated with ibrutinib, as well as reducing CD69 and CD86 expression on circulating and tissue-resident cells from CLL treated patients.[154,352]

BTK not only plays a role in the BCR signaling pathway, but also exercises a regulatory function in chemokine-controlled migration of B cells.[310] We evaluated the effect of CC-292 on MCL cell migration *in vitro* and found that BTK inhibition by CC-292 reduced CXCL12-induced chemotaxis and the degree of inhibition was greater in those cells without activation of the alternative NF-κB pathway. Our results show that the inhibition of migration was higher in UPN-1 than in Z138 (mutTRAF2), and, more importantly, that BTK inhibition by CC-292 showed no effect on CXCL12-mediated migration of mut*BIRC3* primary cases. The basis of these differential chemotactic responses may rely on the recently demonstrated requirement of both the canonical and alternative NF-κB pathway for this biological process. RelB and p52 subunits, pertaining to alternative NF-κB pathway, seem to be essential for cell migration towards CXCL12 and they are critically important for the initial polarization and velocity of cell movement towards a CXCL12 gradient.[353]Thus, this fact may explain the limited efficacy of BTK inhibition by CC-292 on CXCL12-induced migration in MCL cells with alternative NF-κB pathway activation.

There are very few complete responses with ibrutinib and progression is rapidly seen when treatment is interrupted.[347] Rapid disease progression on discontinuation with CC-292 has also been reported.[192] Thus, continued therapy with BTK inhibitors is required and it raises concerns about the affordability due to the excessive costs of ibrutinib. Additionally, some patients, especially those with adverse disease features may eventually develop resistance to these inhibitors and, therefore, designing strategies to avoid or overcome resistance is mandatory. Inhibitors of other kinases within the BCR pathway, such as SYK, would be potential replacements for BTK inhibition, while PI3K inhibitors could theoretically be additive/synergistic with the BTK inhibitors.[354] Combining BCR inhibitors with pro-apoptotic agents, for example, venetoclax (ABT-199) represents another rational strategy. There is also potential interest for combining BTK inhibitors with immunostimulatory agents, such as

lenalidomide, or with immunotherapy approaches, such as mAbs (e.g. PD-1 antibodies) or CAR T-cells.

Several clinical studies with the immunomodulatory agent lenalidomide as monotherapy have shown significant efficacy and tolerable safety profiles in patients with R/R MCL.[311] In fact, it is FDA-approved for the treatment of MCL that has relapsed or progressed after two prior therapies. Lenalidomide exerts pleiotropic effects including immune modulation, antiproliferative activity, and antineoplastic mechanisms of action. Amongst its multiple functions are the downregulation of NF-κB activity and several CDKs, the upregulation of the CDK inhibitor p21, leading to subsequent G1 cell-cycle arrest, and the decrease in cell proliferation.[355] These activities on tumor cells and the TME seem to be mediated by the downregulation of IRF4 and SPIB transcription factors requiring Cereblon (CRBN) expression.[313] The combination of CC-292 with lenalidomide in MCL is of interest given that both molecules have significant activity in this disease, and lenalidomide is already introduced in clinical practice. It is a mechanistically but also a financially logical combination, given that both lenalidomide and CC-292 are now owned by Celgene. We have demonstrated that lenalidomide shows antiproliferative activity in MCL cases sensitive to CC-292, and significantly improves its activity both in monoculture and in MSC-MCL co-culture. In these samples, both agents separately downregulated the expression of IRF4, and the combination led to a complete disappearance of this factor. According to these promising *in vitro* results, a phase IB trial (CLEAR, NCT01766583) is testing this combination in R/R B-cell lymphoma. Importantly, lenalidomide did not re-sensitize MCL cases insensitive to CC-292 due to mutation on alternative NF-κB regulators, suggesting that other therapeutic approaches should be considered.

MCL shows constitutive activation of NF-κB pathway as a result of genomic alterations in factors regulating both canonical (mutations in *TLR2*[15], *CARD11*[356] and *IKBKB*[235]or *A20* deletion[358]) and non-canonical pathways *(TRAF2*, *TRAF3*, *BIRC3* and *MAP3K14*).[15,16] However, their limited frequencies (3-10%) may not explain all MCL cases with intrinsic resistance to ibrutinib. An additional noteworthy fact to consider is that the loss of 11q22-q23, with a frequency of 11-57% depending on the patient cohorts,[359] involves both *ATM* and *BIRC3* genes and may also result in *BIRC3* inactivation, increasing the MCL cases with alternative NF-κB activation and potential resistance to BTK inhibitors. Furthermore, the

activation of this pathway may be engaged by the microenvironment, where MSC secretion of factors such as BAFF leads to activation of both the canonical and alternative NF-κB pathway.[117] One of the critical events in the alternative NF-κB pathway is the accumulation of NIK, which phosphorylates IKKα and p100, leading to the generation of the p52 subunit, which leads to accumulation of p52/RelB heterodimers in the nucleus. Under normal conditions, the level of NIK protein (encoded by *MAP3K14*) is extremely low, due to its constant degradation through a ubiquitination dependent mechanism that involves TRAF2/3 and cIAP1/2. Importantly, several cytokines and ligands present in the lymphoma microenvironment, such as CD40L[360] and BAFF[361], can activate the alternative pathway, mainly through the control of NIK turnover. In addition, NIK accumulation is known to activate the classical NF-κB.[188] We have analyzed herein the activity of two novel and highly specific NIK inhibitors in the CC-292 insensitive cell lines Z138 (mutation in *TRAF2*[16]) and MAVER-1 (biallelic *TRAF3* deletion[16]) and also in primary cases with deregulated alternative NF-κB signaling. AM-0216 (#16) and AM-0561 (#61) already have proven efficacy in MM with activating mutations of alternative NF-κB.[299] Both NIK inhibitors showed anti-proliferative activity and induced apoptosis in MCL cells accompanied by p52 subunit disappearance. It is worth mentioning that, these inhibitors significantly augmented the activity of CC-292 resulting in total clearance of pIκB, leading then to simultaneous inhibition of the alternative and canonical NF-κB pathways.

Another interesting feature of CC-292 that has been demonstrated is its activity in MCL cases bearing inactivation of TP53 either by mutation, deletion or both. CC-292 was active in those cases in terms of inhibition of BCR activation, CXCL12-mediated migration and cooperation with lenalidomide treatment. These results suggest that CC-292, similarly to ibrutinib in 17p del-CLL cases, may be effective in MCL patients bearing this genetic alteration.

Overall, in the second part of this thesis we have analyzed the effect of a second-generation BTK inhibitor on MCL viability and microenvironment crosstalk. Our study suggests that CC-292 shares a common mechanism of resistance with ibrutinib, associated with the activation of the alternative NF-κB signaling pathway allowing MCL cell proliferation when BTK is inactivated[16]. It will be important to ascertain whether mutations in the alternative

NF-κB pathway are identified in MCL patients who progress following ibrutinib or CC-292 therapy. It will also be important to genetically characterize the tumor genome prior to treatment choice in order to identify MCL patients with some oncogenic mutations (*CARD11*, *MAP3K14*, *BIRC3*, etc.) that make lymphomas resistant to BCR inhibitors. Regarding combination therapies, our results propose two strategies for MCL patients depending on NF-κB mutational status that warrant further investigation in the clinic. The combination with lenalidomide may be translated to the clinical practice soon, while the combination with NIK inhibitors warrants further investigation, and holds promise to be effective when combined with other BTK inhibitors.

Antibody therapies are typically used in conjunction with chemotherapeutic agents, so it is important to consider the effects of chemotherapy on effector cells. The combination of ibrutinib with different targeted therapies is now being explored and, although the addition of ibrutinib to rituximab regimens seems promising in clinical trials[362], there are preclinical findings that strongly suggest that ibrutinib antagonizes anti-CD20 mAb activity. Khort et al. showed that ibrutinib decreases ADCC by directly inhibiting Fc receptor-dependent NK cell activation and cytotoxicity *in vitro*.[363] Similarly, ibrutinib has also been shown to decrease ADCP of target cells by macrophages and neutrophils.[364,365] More recently, a new study suggested that ibrutinib promotes both negative and positive interactions with anti-CD20 mAbs. It demonstrates that (i) CD20 expression on ibrutinib is rapidly and reversibly downregulated with reduced NF-κB signaling; (ii) ibrutinib decreases anti-CD20 mediated CDC; (iii) opsonization by the complement protein C3d, which targets cells for phagocytosis, remained relatively stable probably because of decreased CD55 on ibrutinib; (iv) ibrutinib significantly inhibited trogocytosis, a major contributor to antigen loss and tumor escape during mAb therapy.[366] These last two beneficial interactions between ibrutinib and anti-CD20 mAbs could mitigate some of the negative effects. All these studies suggest that when combining these kinase inhibitors with antibodies, it may be best to optimize the timing of treatments to avoid unfavorable interactions. Sequential administration of BCR inhibitors followed by mAbs, and not the concurrent treatment with these agents, might enhance their anti-tumor activity *in vivo* and improve therapy. Indeed, administering ibrutinib before or after the rituximab yielded better antitumor activity in a mouse lymphoma model than concurrent administration.[363]

Unlike ibrutinib, CC-292 was found not to inhibit several kinases, including ITK.[367] It will be of great interest therefore to determine whether CC-292 has similar effects on ADCP and ADCC when combined with CD20-mAbs. ITK has been reported to be required for the activation of human NK cells, but BTK also has a direct role in NK cell activation and cytotoxicity[368], and consequently, CC-292 may still inhibit NK cells via BTK. Thus, it would be important to carry out preclinical studies evaluating the possible interactions between CC-292 and anti-CD20 mAbs before investigating this combination in cancer patients. The above observations about the interference of BTK inhibitors on CD20-mAbs would also be applicable to the combination with DARA.

The long-term toxic effects of BCR inhibition have not been established; moreover, combinations including several BCR inhibitors may yield increased or unexpected toxicities. The higher specificity of CC-292 for BTK, which is the primary characteristic that differentiate CC-292 from ibrutinib, may offer some advantages related to reduced adverse effects in other cell types such as T cells, platelets, and cardiomyocytes. It may also be of benefit in limiting emergent toxic side effects in combination trials. This is probably the most significant strength of CC-292.

To sum up, in this thesis we have focused on two novel therapeutic strategies that target the crosstalk between the tumor cells and the microenvironment. First, we have examined *in vitro* and *in vivo* the activity of a human monoclonal antibody against CD38 (Daratumumab) in CLL and demonstrated that it is effective in $CD38^+$ CLL. Our findings show that Daratumumab interferes with homing and dissemination processes and induces cell lysis (via ADCC and ADCP) of $CD38^+$ CLL cells. Secondly, we have evaluated a novel agent that modulates the BCR pathway by inhibiting BTK. Our results indicate that CC-292, a BTK inhibitor, has therapeutic activity in MCL in terms of inhibition of cell proliferation, activation and homing processes. Moreover, we provide a rational combination of CC-292 with lenalidomide or NIK inhibitors depending on NF-κB mutational status. All in all, the findings of the present thesis support the importance of developing strategies to target the microenvironment and its interaction with neoplastic cells.

CONCLUSIONS

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The main conclusions derived from this thesis are as follows:

First study: To evaluate the antitumor profile of the first-in-class anti-CD38 antibody Daratumumab in the poor prognosis CD38$^+$ CLL.

1. DARA demonstrated efficient lysis of patient-derived CLL cells and cell lines by ADCC *in vitro* and ADCP both *in vitro* and *in vivo*, while exhibited negligible CDC in these cells.
2. The effect of DARA on patient-derived CLL cell dissemination was demonstrated *in vitro* by its effect on CXCL12-induced migration and *in vivo* by interfering with CLL cell homing to spleen in NSG mice.
3. DARA reduced adhesion of CLL cells to VCAM-1, accompanied by down-regulation of the matrix metalloproteinase MMP9.
4. DARA significantly prolonged overall survival of MEC2 mice, completely eliminated cells from the infiltrated organs and significantly reduced disease burden in the spleen of CLL-PDX.

Second study: To evaluate the antitumor profile of a novel and highly selective BTK inhibitor CC-292 in MCL.

5. CC-292 shows differential anti-proliferative activity in MCL depending on NF-κB activation status.
6. CC-292 interferes with CXCL12-induced migration.
7. CC-292 inhibits cellular activation of MCL cells.
8. CC-292 cooperates with lenalidomide in the CC-292 sensitive cell lines.
9. CC-292 cooperates with novel NIK inhibitors in CC-292 resistant cell lines and in primary MCL cases with mutations in the alternative NF-κB pathway.

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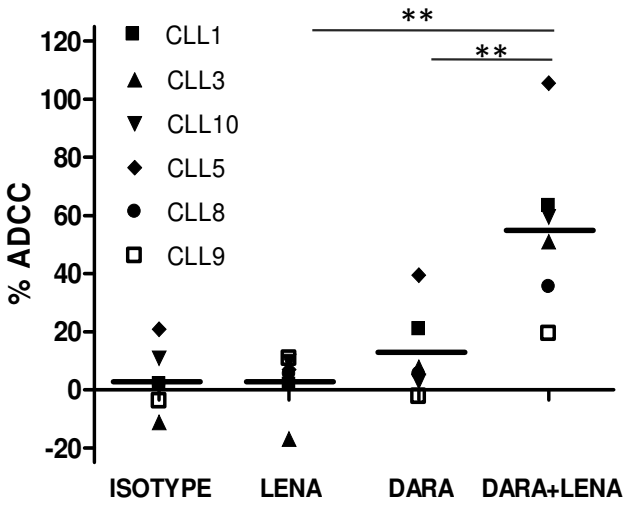
ANNEXES



Lenalidomide pretreatment of PBMCs significantly increases Daratumumab ADCC activity against CLL primary cells. Primary CLL cells (n=6) were treated with 0.1µg/mL Daratumumab (DARA) for 4 hours in the presence of PBMCs that had been pretreated or not with lenalidomide (LENA 3µM 72 hours), the ratio of effectors vs. target cells was 50:1. Cell death was assessed by Calcein release and fluorescence measured in a fluorimeter (Synergy HT, Biotek) (** =p<0.01, unpaired t-test).

Clinical Cancer Research

The Human CD38 Monoclonal Antibody Daratumumab Shows Antitumor Activity and Hampers Leukemia–Microenvironment Interactions in Chronic Lymphocytic Leukemia

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Abstract

Purpose: To establish a proof-of-concept for the efficacy of the anti-CD38 antibody daratumumab in the poor prognosis CD38$^+$ chronic lymphocytic leukemia (CLL) subtype.

Experimental Design: The mechanism of action of daratumumab was assessed in CLL primary cells and cell lines using peripheral blood mononuclear cells to analyze antibody-dependent cell cytotoxicity (ADCC), murine and human macrophages to study antibody-dependent cell phagocytosis (ADCP), or human serum to analyze complement-dependent cytotoxicity (CDC). The effect of daratumumab on CLL cell migration and adhesion to extracellular matrix was characterized. Daratumumab activity was validated in two *in vivo* models.

Results: Daratumumab demonstrated efficient lysis of patient-derived CLL cells and cell lines by ADCC *in vitro* and ADCP both *in vitro* and *in vivo* whereas exhibited negligible CDC in these cells. To demonstrate the therapeutic effect of daratumumab in CLL, we generated a disseminated CLL mouse model with the CD38$^+$ MEC2 cell line and CLL patient–derived xenografts (CLL-PDX). Daratumumab significantly prolonged overall survival of MEC2 mice, completely eliminated cells from the infiltrated organs, and significantly reduced disease burden in the spleen of CLL-PDX. The effect of daratumumab on patient-derived CLL cell dissemination was demonstrated *in vitro* by its effect on CXCL12-induced migration and *in vivo* by interfering with CLL cell homing to spleen in NSG mice. Daratumumab also reduced adhesion of CLL cells to VCAM-1, accompanied by downregulation of the matrix metalloproteinase MMP9.

Conclusions: These unique and substantial effects of daratumumab on CLL viability and dissemination support the investigation of its use in a clinical setting of CLL. *Clin Cancer Res; 1–13.*

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Note: Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

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Introduction

Chronic lymphocytic leukemia (CLL) is the most common leukemia in adults and is characterized by progressive accumulation of nonfunctional, apoptosis-resistant mature B cells in peripheral blood, bone marrow, and lymphoid tissues (1, 2). The majority of the tumor cells in the blood are resting. However, heavy-water experiments have shown that CLL contains a small fraction of actively proliferating cells, with approximately 2% of cells newly generated each day (3). This proliferation occurs in specific structures known as proliferation centers localized in the lymph nodes and in the bone marrow. Thus, CLL is considered a disease characterized by a dynamic balance between cells circulating in the blood and cells located in permissive niches in lymphoid organs (1, 2). The former are primarily mature-looking small lymphocytes resistant to apoptosis, whereas the latter are composed by lymphocytes that undergo either proliferation or apoptosis depending on the microenvironment.

CD38 was first reported to be associated with inferior outcome by Damle and colleagues in 1999 (4) and confirmed later as a

Translational Relevance

Chronic lymphocytic leukemia (CLL) remains an incurable disease where high CD38 expression is associated with poor prognosis and identifies cells that are prone to proliferate. CD38 cooperates in migration, adhesion, and invasion through its molecular association with CXCR4, MMP9, and CD49d. The human anti-CD38 monoclonal antibody daratumumab has shown efficient cell killing and a good safety profile in clinical trials in multiple myeloma. Here, we demonstrate that daratumumab also exerts significant cytotoxicity against patient-derived CLL cells, via ADCC and ADCP *in vitro* and *in vivo*. Furthermore, daratumumab interferes with CD38 signaling and reduces CLL cell adhesion, migration, and homing. Moreover, daratumumab shows therapeutic activity in two mouse models. Thus, daratumumab improves overall survival in a systemic $CD38^+$ MEC2 cell line mouse model and reduces tumor burden in CLL patient–derived xenografts. These results provide scientific rationale for the clinical development of daratumumab in poor prognosis $CD38^+$ CLL.

prognostic factor independent of *IGHV* mutation status (5). Patten and colleagues demonstrated that CD38 expression in CLL is dynamic and changes as a result of contact with activated $CD4^+$ T cells in proliferation centers, being CD38 specifically expressed on cells that are primed to proliferate in the lymph node (6). As a consequence, the expression of CD38 on CLL differs among lymphoid compartments, being higher in bone marrow and lymph node than in peripheral blood (7, 8) and in the proliferating fraction of the tumor (9). The functional importance of CD38 in CLL extends beyond proliferation, as it appears to be linked to the tyrosine kinase ZAP-70 and characterizes CLL cells with high migratory potential (10). CD38 cooperates with CXCR4-induced migration (11) and sustains B-cell receptor (BCR)-mediated signaling (12). Finally, a role of CD38 in adhesion and tissue invasion was recently recognized. CD38 forms a macromolecular complex with the integrin CD49d and the matrix metalloproteinase MMP9, enhancing CD49d-mediated cell adhesion as well as MMP9 expression and activity (13–15). This is of key relevance because CD49d surface expression strongly correlates with overall survival in CLL (16). All these properties make CD38 an attractive target for antibody therapy in CLL and other $CD38^+$ hematologic malignancies such as multiple myeloma (17), non–Hodgkin lymphoma (NHL), and B- and T-acute lymphoblastic leukemia.

The human anti-CD38 antibody daratumumab has progressed to phase III clinical trials in patients with multiple myeloma. Daratumumab is a human IgG1 therapeutic monoclonal antibody (mAb) that binds to CD38. In 2015, the U.S. FDA has approved daratumumab for patients with multiple myeloma, who have received at least 3 prior lines of therapy including a proteasome inhibitor and an immunomodulatory agent, or patients double refractory to these agents. Approval was based on 2 phase II studies of daratumumab monotherapy (16 mg/kg) in heavily treated patients (18, 19). A pooled analysis of these studies revealed an overall response rate of 31%, including responses that deepened over time, and median overall survival of 19.9 months. Daratumumab induces killing of tumor cells, mainly via complement-dependent cytotoxicity (CDC), antibody-dependent cellular cytotoxicity (ADCC; ref. 20), and antibody-dependent cellular phagocytosis (ADCP) by macrophages (21) in multiple myeloma and Burkitt lymphoma cell lines. In addition, daratumumab induces apoptosis upon secondary cross-linking (22). Recent studies have revealed previously unknown immunomodulatory effects of daratumumab where CD38-expressing immunosuppressive regulatory T and B cells and myeloid-derived suppressor cells are highly sensitive to daratumumab treatment (23). It has also been shown that daratumumab can modulate the enzymatic activity of CD38 and potentially may lead to a reduction in immunosuppressive adenosine levels (24, 25). This shift away from an immunosuppressive environment may lead to the generation of protective immune responses. Indeed, a concomitant increase of helper and cytotoxic T-cell absolute cell counts and production of IFNγ in response to viral peptides was observed. In addition, an increase in T-cell clonality in subjects who responded to daratumumab versus subjects who did not respond was observed, indicating an improved adaptive immune response (23).

Two additional anti-CD38 antibodies have also entered clinical trials for multiple myeloma and other $CD38^+$ hematologic malignancies, MOR202 (26) and isatuximab (SAR650984; ref. 27), that are being tested alone and in combination with standard therapy.

The aim of this study was to evaluate the cytotoxic effect of daratumumab on CLL cells via CDC, ADCC, and ADCP, as well as its effect on tumor cell–microenvironment interactions, using patient-derived CLL cells and CLL cell lines in *in vitro* and *in vivo* settings.

Materials and Methods

Cell lines and patient samples

Primary tumor cells from 18 patients with CLL (see clinical characteristics in Table 1), diagnosed according to the World Health Organization (WHO) classification criteria, were used. Written informed consents of the patients were granted following the guidelines of the Hospital Clínic Ethic Committee (IRB) and the Declaration of Helsinki. Mononuclear cells were isolated from peripheral blood by gradient centrifugation on Ficoll (GE Healthcare) and used fresh or cryopreserved in liquid nitrogen in RPMI-1640 containing 10% DMSO (Sigma-Aldrich) and 60% heat-inactivated FBS (Life Technologies) and maintained within the hematopathology collection of the institution (IDIBAPS-Hospital Clínic Biobank, R121001-094). The prolymphocytic leukemia (PLL) cell lines, MEC1, MEC2, and JVM13, as well as the Burkitt lymphoma Daudi cell line were obtained from DSMZ. CLL primary samples and cell lines were cultured in RPMI-1640 or IMDM supplemented with 10% FBS, 2 mmol/L L-glutamine, 50 μg/mL penicillin/streptomycin (Life Technologies) and were maintained in a humidified atmosphere at 37°C containing 5% CO_2. Normocin (100 μg/mL; InvivoGen) was added to the cell line cultures to prevent mycoplasma contamination in cell lines that were routinely tested for mycoplasma infection by PCR. The identity of all cell lines was verified by using AmpFISTR identifier kit (Life Technologies).

Therapeutic and complement regulatory proteins blocking antibodies

A human IgG1 targeting CD38 (daratumumab) was generated by immunization in a HuMAb mouse (20). The human mAb IgG1-b12, specific for the HIV-1 gp120 envelope glycoprotein

Table 1. CLL patient characteristics

Study label	Gender	Binet/Rai stage	% tumor cells[a]	IgVH status[b]	CD38[c]	CD38 sABC[d]	CD46[c]	CD55[c]	CD59[c]	CD49d[c]	%CDC[e]	%ADCC[f]
CLL1	M	C/IV	95	nd	98	816	98	91	9	4	nd	32.7
CLL2	M	A/I	96	UM	63	897	889	88	96	6	6.4	20.8
CLL3	M	C/IV	86	UM	80	1,875	90	88	79	68	8.4	20.8
CLL4	M	A/I	97	UM	31	355	92	90	75	79	nd	44.5
CLL5	M	B/II	97	UM	44	562	92	90	97	1	0.1	39.4
CLL6	F	C/III	86	M	50	nd	nd	nd	nd	nd	25.1	20
CLL7	M	C/III	96	UM	2	nd	98	99	90	0.7	5.6	0.2
CLL8	M	A/0	95	M	53	736	93	94	83	5	3.4	26.6
CLL9	M	A/I	82	nd	73	nd	98	97	98	63	0.9	22.9
CLL10	M	B/III	93	M	64	nd	92	91	94	100	25.6	47
CLL 11	M	A/0	97	M	0.5	294	95	94	98	0.1	0	0
CLL 12	F	A/III	94	UM	95	2,132	97	87	90	80	17.4	31.4
CLL 13	M	A/0	60	nd	55	nd	99	100	99	98	10.4	nd
CLL14	M	B/II	98	UM	66	nd	97	93	86	nd	nd	27.9
CLL15	F	A/I	86	UM	71	1,531	96	98	88	83	nd	27.2
CLL 16	M	A/I	53	nd	75	nd	98	94	96	95	23.2	38.1
CLL17	M	A/0	85	UM	83	nd	99	98	96	nd	0.3	nd
CLL 18	M	A/0	91	UM	70	nd	98	98	97	92	0	22

Abbreviations: F, female; M, male; nd, not determined.

[a]Percentage of tumor cells assessed by flow cytometry on the basis of $CD19^+CD5^+CD23^+$ cells.

[b]Determined by direct sequencing. M, mutated, sequence homology < 98%; UM, unmutated, sequence homology > 98%.

[c]Percentage of positive cells for CD38, CD46, CD55, CD59, and CD49d determined by flow cytometry in $CD19^+CD5^+$ population, referred to as isotype control. CD38 was considered positive when the percentage of positive cells exceeded 30%.

[d]sABC, number of surface antibodies bound per cell evaluated by QuantiBRITE CD38-PE.

[e]Percentage of CDC induction at 10 μg/mL daratumumab.

[f]Percentage of ADCC induction at 0.1 μg/mL daratumumab.

(28), was included in all experiments as an isotype control mAb. Both antibodies were provided by Genmab.

Anti-CD46 (clone TRA-2-10, Biolegend), anti-CD55 (clone 1C6, Hycult Biotech), and anti-CD59 (clone YTH 53.1, AbD Serotech) antibodies were used to block complement regulatory proteins (CRP).

Antibody-dependent cellular cytotoxicity

Target cells were labeled with 1 μmol/L Calcein-AM (Life Technologies) for 30 minutes at 37°C. Afterward, cells were washed 3 times with PBS, plated in triplicate at 1×10^4 cells per well in 96-well round-bottom plates, and preincubated (room temperature for 15 minutes) with 10-fold serial dilutions of either isotype control (IgG1-b12) or daratumumab (range, 1 to 0.0001 μg/mL) in RPMI-1640. Daratumumab doses for *in vitro* studies were previously established (20). Culture medium was added instead of mAb to determine the spontaneous calcein release, and 1% Triton X-100 was used to determine the maximal calcein release. Thereafter, fresh human peripheral blood mononuclear cells (PBMC) were added at an effector:target (E:T) ratio of 50:1, optimized in a previous report (20), and cells were incubated for 4 hours at 37°C. The plates were centrifuged, supernatant transferred into black plates (Thermo Scientific) and fluorescence was measured in a Synergy spectrophotometer (Bio-Tek; excitation filter: 485 ± 20 nm; bandpass filter: 530 ± 20 nm). The percentage of cellular cytotoxicity was calculated using the following formula:

$$\textit{specific lysis} = 100 \times \frac{\textit{experimental release (RFU)} - \textit{spontaneous release (RFU)}}{\textit{maximal release (RFU)} - \textit{spontaneous release (RFU)}}$$

Antibody-dependent cellular phagocytosis

Macrophages were generated from monocytes isolated from bone marrow of the hind legs of female SCID mice (C.B-17/Icr-Prkdc^{scid}/Crl; Janvier Labs) by flushing the femurs. The cells were cultured for 7 days in DMEM supplemented with 10% FBS, 2 mmol/L L-glutamine, 50 μg/mL penicillin/streptomycin, and 50 U/mL M-CSF (Cell Guidance), and the culture medium was renewed every 3 days. On day 7, macrophages were detached with 0.1% trypsin-EDTA and characterized by flow cytometry ($CD11b^+$, $F4/80^+$; mouse antibodies obtained from eBiosciences and Invitrogen, respectively). The macrophages were seeded at 2.5×10^5 cells per well into non–tissue cultured–treated 24-well plates and allowed to adhere overnight. Target cells (primary CLL and cell lines) were labeled with 0.01 μmol/L Calcein-AM and added to the macrophages at an E:T ratio of 1:1 in the presence of a fixed mAb concentration of 1 μg/mL. After 4 hours of incubation, the nonphagocytosed target cells were collected. The macrophages were detached with 0.1% trypsin-EDTA, added to the nonphagocytosed target cells, and stained for F4/80 expression. The amount of remaining target cells ($calcein^+$ $F4/80^-$) was determined on an Attune acoustic cytometer, and the percentage of killed target cells in the presence of daratumumab compared with isotype control was calculated using the following formula:

$$\%\ \textit{eliminated target cells} = 100 - \left[100 \times \frac{\textit{remaining target cells after DARA treatment}}{\textit{remaining target cells after isotype control treatment}}\right]$$

In vivo phagocytosis assay

In vivo phagocytosis assay was carried out as described by Overdijk and colleagues (21). SCID beige mice (CB17.CG-PRKDC-LYST/CR, Charles River Laboratories), which lack natural killer (NK) cells, were inoculated with primary CLL cells or MEC2 cells (2×10^7 cells per mouse) into their peritoneal cavity, following a protocol approved by the Animal Testing Ethic committee of the University of Barcelona (Barcelona, Spain). Mice were randomly assigned into cohorts of 3 to 5 mice and received one intraperitoneal injection of 20 mg/kg of daratumumab or isotype control. Forty-eight hours later, mice were

sacrificed and peritoneal lavage done by injecting the cavity with 5 mL of cold PBS. Total recovery of the peritoneal cells was evaluated by flow cytometry after staining with huCD45/CD19/CD5 antibodies (provided by Invitrogen and BD Pharmingen, respectively). The relative percentage of remaining CLL cells from daratumumab-treated mice was derived from the isotype control group, which was set at 100%.

$$\%\ remaining\ target\ cells = 100 \times \frac{remaining\ target\ cells\ after\ DARA\ treatment}{remaining\ target\ cells\ after\ isotype\ control\ treatment}$$

Complement-dependent cytotoxicity

Target cells were labeled with 1 μmol/L Calcein-AM (Life Technologies) for 30 minutes at 37°C. Afterward, cells were washed 3 times with PBS, plated in triplicate at 1×10^5 cells per well in 96-well round-bottom plates, and preincubated [room temperature (29), 15 minutes] with 10-fold serial dilutions of either isotype control (IgG1-b12) or daratumumab (range, 10 to 0.01 μg/mL) in RPMI-1640. Culture medium was added instead of mAb to determine the spontaneous calcein release and 1% Triton X-100 was used to determine the maximal calcein release. Thereafter, 10% normal human AB serum was added and incubated for 45 minutes at 37°C. The plates were centrifuged, supernatants transferred into black plates (Thermo Scientific) and fluorescence measured in a Synergy spectrophotometer (Bio-Tek; excitation filter: 485 ± 20 nm; bandpass filter: 530 ± 20 nm). The percentage of cellular cytotoxicity was calculated using the following formula:

$$specific\ lysis = 100 \times \frac{experimental\ release\ (RFU) - spontaneous\ release\ (RFU)}{maximal\ release\ (RFU) - spontaneous\ release\ (RFU)}$$

***In vivo* homing**

Homing experiment was done as previously described by Vaisitti and colleagues (11). Briefly, NOD/SCIDγ null (NSG) mice (bred in-house, animal facility, University of Barcelona) were randomly assigned into cohorts of 4 mice and pretreated intraperitoneally with 10 mg/kg of daratumumab, isotype control, or anti-CXCR4 (R&D Systems). Twenty-four hours later, mice were inoculated with fresh primary CLL (2×10^7 cells per mouse) via the tail vein following a protocol approved by the Animal Testing Ethic committee of the University of Barcelona. Mice were sacrificed 24 hours after tumor cell inoculation; peripheral blood, spleen, and bone marrow were recovered and the presence of tumor cells evaluated by flow cytometry after staining with huCD45/CD19/CD5 antibodies.

Systemic MEC2 xenograft mouse model

SCID mice were preconditioned with 25 mg/kg of busulfan 24 hours before inoculation via tail vein of MEC2 cells (10^7 cells per mouse), following a protocol approved by the Animal Testing Ethic committee of the University of Barcelona. One week later, mice were randomly assigned into cohorts of 6 to 7 mice. A saturating loading dose of 20 mg/kg daratumumab or isotype control intraperitoneally was given on day 7 and thereafter 10 mg/kg weekly for 3 weeks. Mice were sacrificed if they lost 15% to 20% of weight and/or showed signs of disease. Survival studies were extended up to day 90 when the study was terminated. The presence of tumor cells was evaluated first macroscopically and then by flow cytometry. Cells from infiltrated organs were obtained by tissue homogenization. Bone marrow cells were obtained after flushing the femoral and tibia bones with RPMI-1640 media. These samples were filtered through 70-μm nylon sieves (BD Falcon). Erythrocytes were lysed using ACK buffer (Quality Biological Inc.). The cells were labeled with huCD45/CD19/CD5 antibodies and analyzed by flow cytometry. Organ samples were snap-frozen in OCT medium (Sakura Tissue Tek) or formalin fixed and embedded in paraffin. Tissue sections were stained with hematoxylin and eosin (H&E) and CD19 (Dako) antibody and evaluated by Cell B Basic Imaging Software (Olympus).

CLL patient–derived mouse xenograft

On day 1, NSG mice were inoculated intravenously with fresh PBMCs from CLL (2×10^7 cells per mouse). On day 2, mice were randomly assigned to 2 groups (3–4 mice per group) and dosed intraperitoneally with 20 mg/kg of daratumumab or control isotype. Mice were sacrificed on day 5; peripheral blood, spleen, and bone marrow were recovered and the presence of tumor cells was evaluated by flow cytometry after staining with huCD45/CD19/CD5 antibodies.

Statistical analysis

Unpaired and paired *t* tests or one-way ANOVA were used to assess statistical differences between groups by means of GraphPad Prism software 4.0. For Kaplan–Meier survival curves, SPSS19 software was used.

Results

Daratumumab induces ADCC

Antibody-dependent killing via ADCC by FcγR-bearing effector cells accounts for the antitumor activity of daratumumab in models of multiple myeloma and Burkitt lymphoma (20, 30). The ability of daratumumab to induce ADCC on CLL cells was assessed by calcein-AM release assay using PBMCs from healthy donors as a source of effector cells (mainly NK cells and monocytes). CLL cell lines and primary cells were treated with increasing concentrations of daratumumab or isotype control. Daratumumab induced significant cell lysis starting at doses as low as 0.01 μg/mL in $CD38^+$ CLL cell lines (Fig. 1A) and at 0.001 μg/mL in primary CLL cells (Fig. 1B), reaching its maximum killing activity at 0.1 to 1 μg/mL (mean ± SD = 31.9% ± 11.6%). In contrast, the isotype control antibody did not induce significant cell lysis, tested at the maximum concentration of 1 μg/mL (mean ± SD = 12.4% ± 11.6%; Fig. 1B). No ADCC induction was detected in $CD38^-$ CLL cases (Table 1: CLL7, CLL11, and MEC1 cell line [Table S1]). A summary of ADCC induction in $CD38^+$ versus $CD38^-$ CLL primary cases is shown in Fig. 1C. The degree of ADCC induction did not correlate with CD38 sABC for CLL cell lines and primary cells ($r^2 = 0.088$; Fig. 1D). Altogether, these data indicate that ADCC constitutes a mechanism of daratumumab activity in $CD38^+$ CLL cells, but the extent of ADCC does not strictly correlate with CD38 expression.

Daratumumab promotes CLL cell clearance by phagocytosis *in vitro* and *in vivo*

Recent results indicate that ADCP is a potent mechanism of action for daratumumab (21). We explored ADCP of CLL both

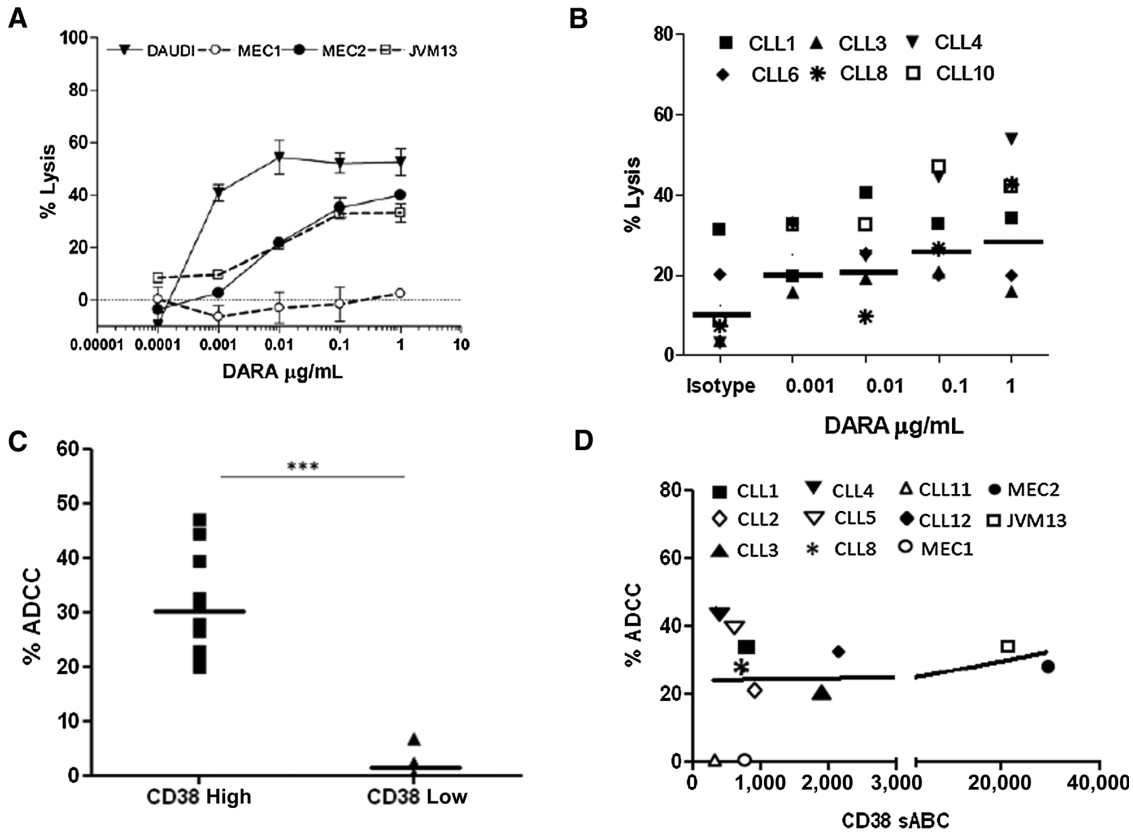

Figure 1.

Daratumumab (DARA) induces ADCC in the presence of external effectors. **A,** Daudi cells, CLL cell lines, both CD38^{high} (MEC2 and JVM13) and CD38^{low} (MEC1) were treated with increasing daratumumab doses (0.0001–1 μg/mL) in the presence of PBMCs from healthy donors at an E:T ratio of 50:1 for 4 hours. Viability was then evaluated by calcein release assay. **B,** Results in primary CLL cells with daratumumab doses (0.001–1 μg/mL). ADCC induced by isotype control at the maximal dose of 1 μg/mL is also depicted and the horizontal line represents the mean lysis. **C,** ADCC induction by daratumumab (0.1 μg/mL) in CD38 high (≥30%) versus CD38 low (<30%) CLL primary cases. **D,** Number of surface antibodies bound per cell (sABC) of CD38 was quantified in primary CLL cells and cell lines and plotted for correlation with ADCC induction by daratumumab (cell lines, 0.1 μg/mL and primary CLL, 0.01 μg/mL).

in vitro and *in vivo*. To assess ADCP *in vitro*, macrophages were generated from bone marrow mouse monocytes stimulated with M-CSF. Daratumumab induced ADCP in primary CLL cells (mean ± SD = 23% ± 4%; Fig. 2A). Representative flow cytometry profiles of CLL cells and macrophages after daratumumab treatment are depicted for CLL1 (Fig. 2B). As observed for ADCC, phagocytosis was specimen-dependent and not strictly related to CD38 expression levels.

We next demonstrated the occurrence of ADCP *in vivo*. SCID *beige* mice, devoid of NK cells but with active macrophages, were inoculated intraperitoneally with primary CLL cells or the MEC2 cell line as described in Materials and Methods. As shown in Fig. 2C, the percentage of remaining viable CLL cells after daratumumab treatment was significantly reduced (mean ± SD = 46% ± 5%; $P < 0.05$, unpaired t test) compared with the isotype control group at 100%. Remarkably, this decrease in cell number was detectable as early as 2 hours after daratumumab administration (data not shown). Figure 2D represents flow cytometry profiles showing the number of CLL cells (huCD45$^+$/CD19$^+$/CD5$^+$) recovered from the intraperitoneal cavity after isotype control or daratumumab treatment.

Taken together, these results demonstrate that ADCP may contribute to daratumumab antitumor activity against CLL cells both in *in vitro* and *in vivo* settings.

Daratumumab induces limited CDC of CLL cells

Daratumumab was selected from a panel of human antibodies for its broad-spectrum killing activity against hematologic cell lines. Daratumumab was particularly differentiated for its potent CDC activity (20). We evaluated daratumumab-induced CDC activity in a panel of CLL primary cells and cell lines (Table 1 and Supplementary Table S1). In the majority of primary CLL samples, CD38$^+$ PLL cell lines (MEC2 and JVM13) and in CD38$^-$ cell line (MEC1), daratumumab did not induce significant cell death in the presence of normal human serum (10%). In 5 of 18 primary CLL cells,

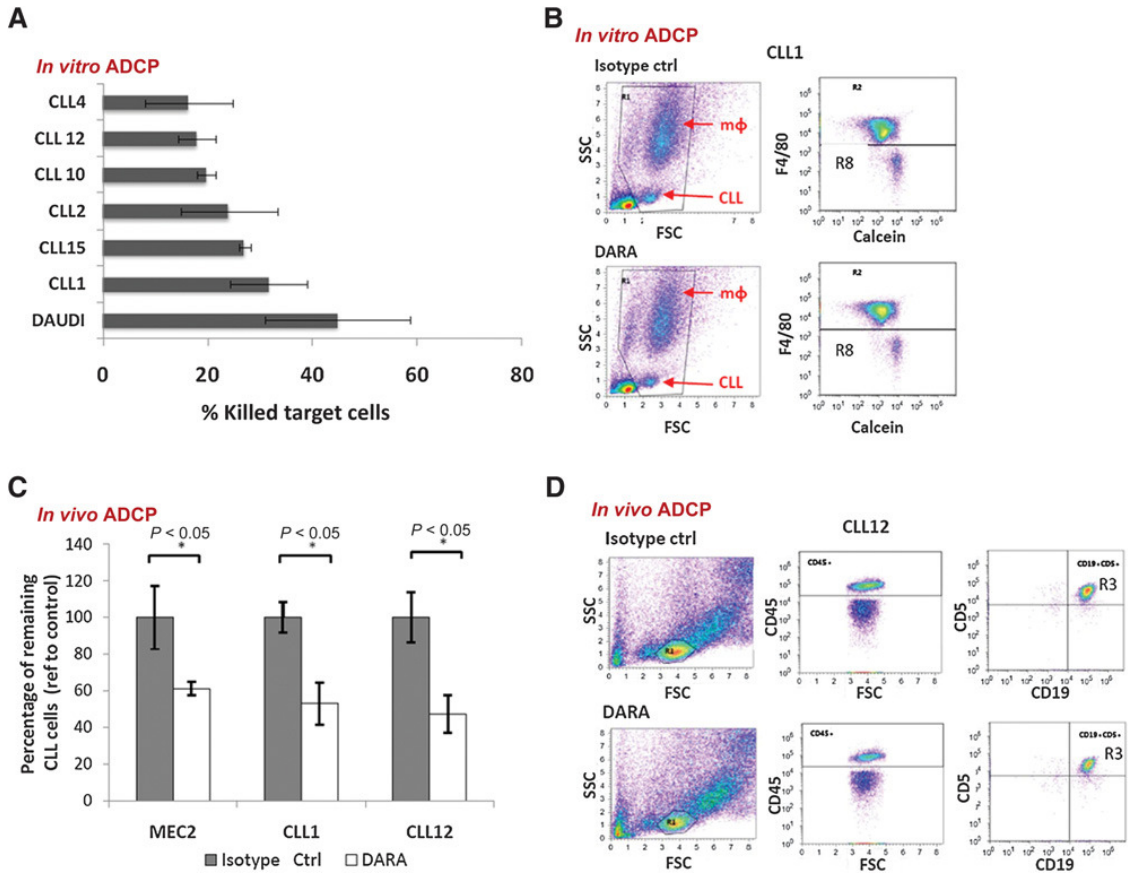

Figure 2.

Daratumumab (DARA) induces ADCP *in vitro* and *in vivo*. **A,** CLL cells were treated in triplicates with a fixed concentration (1 μg/mL) of daratumumab or isotype control in the presence of mouse macrophages at a E:T ratio of 1:1 for 4 hours. Percentage of killed target cells was calculated by flow cytometry. CLL cells were identified as calcein$^+$ F4/80$^-$, and the percentage of killing by daratumumab was calculated according to the formula included in Materials and Methods. Daudi cell line was used as positive control. **B,** Representative flow cytometric plots of *in vitro* ADCP. CLL cells and macrophages (mφ) are clearly seen in the forward scatter (FSC)/side scatter (SSC) density plot. The number of cells in the R8 gate was used to calculate the percentage of killed tumor cells. **C,** *In vivo* phagocytosis was evaluated in SCID *beige* mice (n = 3–5 per group) that were inoculated intraperitoneally with primary CLL cells or MEC2 cell line (2×10^7 cells per mouse) and subsequently treated intraperitoneally with one dose of 20 mg/kg of daratumumab or isotype control. The mice were sacrificed 48 hours later and cells from the peritoneum recovered and counted by flow cytometry as huCD45$^+$CD19$^+$CD5$^+$ for primary CLL cells and CD45$^+$CD19$^+$CD5$^-$ for MEC2 cells. The percentage of residual leukemia cells after daratumumab treatment is plotted where the total number of cells remaining treated with the isotype control was set to 100% ($P < 0.05$, unpaired t test). **D,** Representative flow cytometric plots where the R3 gate was used to calculate the percentage of killed tumor cells. The gating strategy started with cells in FSC/SSC (R1), then gating on CD45$^+$, and finally CD19$^+$CD5$^+$.

daratumumab induced just more than 10% CDC (range, 10.4%–25.6%). This limited CDC induction was not increased in the presence of higher human serum concentrations (data not shown). To explain the poor induction of CDC, we assessed the expression of CRPs and the number of CD38 molecules per cell (CD38 sABC) on CLL cells. High expression of the CRPs [CD46, CD55, CD59 (mean ± SD = 94% ± 3%, 92% ± 4%, 92% ± 8%, respectively)] was detected by flow cytometry in all CLL cell lines and primary cases, whereas only CD46 (88%) was highly expressed in the Burkitt lymphoma cell line Daudi (Table 1 and Supplementary Table S1) which was used as a positive control for CDC. Blocking antibodies against these CRPs probed to increase CDC induction by daratumumab in Daudi, whereas no effect was observed in CLL cells. However, blockade of CRPs increased CDC induced by the anti-CD20 antibodies rituximab (RITUX) or ofatumumab (OFA) in some CLL cases (Supplementary Fig. S1).

CD38 expression in CD38$^+$ CLL primary tumor samples (mean sABC ± SD = 1,053 ± 677) was lower than in CD38$^+$ CLL cell lines (mean sABC ± SD = 25,024 ± 6,031), which was roughly 10-fold below that detected in Daudi (mean sABC ± SD = 292,131). A summary of mean fluorescence intensity ratio (MFIR) for CD38 and CRPs in CLL cells and cell lines is included in Supplementary Table S2. Previous results in multiple myeloma by Nijhof and colleagues have demonstrated that all-trans retinoic acid (ATRA) increases CD38 expression. We have analyzed this

possibility in CLL. CLL cells ($n = 6$) were pretreated with ATRA or left untreated for 48 hours. CD38 expression was analyzed subsequently on these CLL cells and challenged to CDC assay. As shown in Supplementary Fig. S2A, CD38 MFI of $CD19^+CD5^+$ cells, was significantly ($P < 0.05$) increased after ATRA treatment (average increase, 30%), in a similar proportion than that shown for multiple myeloma patient samples. A representative example is shown (Supplementary Fig. S2B). However, no CDC induction by daratumumab was observed in the cases analyzed (Supplementary Fig. S2C), indicating that this increase in CD38 expression was not sufficient to engage CDC.

In conclusion, these results indicate that daratumumab did not induce significant CDC in either CLL cell lines or primary CLL cells and is probably due to high expression of CRPs and insufficient CD38 expression.

Daratumumab interferes with *in vitro* migration and *in vivo* homing

Homing of CLL cells to secondary lymphoid organs is mainly coordinated by the CXCL12/CXCR4 axis (31). Using CLL primary cells and a xenograft mouse model, Vaisitti and colleagues demonstrated that CD38 synergizes with the CXCR4 signaling pathway and controls chemotaxis/homing of CLL cells through a close interaction between CD38 and CXCR4 in the membrane (11). Following this line of investigation, the effect of daratumumab on CLL cell migration was evaluated using a CXCL12 gradient. An anti-CXCR4 antibody was used as a positive control of migration blockade. In $CD38^+$ CLL cells, daratumumab inhibited CXCL12-mediated migration up to 70% (mean ± SD = 44% ± 16%; $P < 0.01$; $n = 5$), which was comparable with anti-CXCR4 treatment (Fig. 3A). These results are in agreement with that previous report using the blocking anti-CD38 antibody SUN-4B7 (11). We next examined daratumumab-mediated signaling following CXCR4–CXCL12 interaction. The immediate early effect of stimulation for migration is the activation of ERK1/2 (11). Phosphorylation of ERK1/2 in CLL tumor cells occurred shortly after CXCR4–CXCL12 ligation and peaked at 5 minutes after CXCL12 addition. Treatment with daratumumab reduced ERK activation by CXCR4–CXCL12 in CLL1 and CLL12, whereas the ERK inhibition was less pronounced in CLL3 (Fig. 3B), illustrating heterogeneity in primary tumor cells.

We then validated these *in vitro* migration results using the *in vivo* homing mouse model described previously by Vaisitti and colleagues (11). Using NSG mice, which lack NK cells and active macrophages, we analyzed the effect of daratumumab on primary CLL cell migration from peripheral blood to bone marrow and spleen. In this model, NSG mice were pretreated (day 0) with daratumumab, isotype control, or anti-CXCR4, followed by fresh CLL cell inoculation on day 1. Peripheral blood, bone marrow, and spleen cells were isolated on day 2, and CLL cells were identified as $CD45^+/CD19^+/CD5^+$. Representative flow cytometric profiles from a mouse spleen are shown in Fig. 3C. Cell enumeration showed that CLL cells rapidly move from peripheral blood and mainly migrated to the spleen and that daratumumab significantly reduced this migration (55% inhibition on average, $P < 0.05$; Fig. 3D). Migration of CLL cells to bone marrow was limited and was not affected by pretreatment of mice with daratumumab (data not shown). In conclusion, *in vivo* and *in vitro* results suggest that daratumumab hampers dissemination of CLL cells to secondary lymphoid organs.

Daratumumab inhibits CD49d-mediated CLL cell adhesion by reducing MMP9 levels

In addition to migration, CD38 also plays a key role in cell adhesion through physical interaction with the integrin CD49d/CD29 (α4β1 integrin; ref. 15) which is the strongest flow cytometry marker associated with poor prognosis in CLL together with *IGVH* mutational status (16) and MMP9 (13). In addition, the expression of CD38 correlates with that of CD49d. We analyzed the effect of daratumumab on CD49d/CD29-mediated adhesion of CLL cells to vascular cell adhesion molecule-1 (VCAM-1), an essential component of extracellular matrix. As shown in Fig. 4A and B, when compared with isotype control antibody, daratumumab significantly impeded the adhesion to VCAM-1 of CLL primary cells ($n = 4$) and MEC2 cell line (mean ± SD = 38% ± 11%, $P < 0.01$), with no significant differences with anti-CD49d blocking antibody used here as a positive control (mean ± SD = 49% ± 30%).

Moreover, CD38–CD49d complex also recruits MMP9 leading to the upregulation and activation of this metalloproteinase (13–15). Thus, we investigated the effect of daratumumab on MMP9 expression, by analyzing the variations in *MMP9* transcripts levels in $CD38^+CD49d^+$ CLL cells. As depicted in Fig. 4C, CLL cell adhesion to VCAM increased *MMP9* mRNA levels (mean ± SD = 2 ± 1), and daratumumab completely abrogated both constitutive ($P < 0.01$) and VCAM-induced ($P < 0.05$) *MMP9* expression. Altogether, these results demonstrate that daratumumab counteracts VCAM-1–mediated adhesion of CLL cells and induces the transcriptional downregulation of *MMP9*.

Daratumumab prolongs overall survival in a systemic CLL mouse model and reduces tumor burden in CLL-PDX

We successfully established a systemic MEC2 model by intravenous cell inoculation in busulfan-preconditioned SCID mice, which retain NKs and macrophages that can function as effectors for daratumumab activity. Previous attempts to establish a MEC2 mouse model failed using subcutaneous cell inoculation in nude mice (32).

One week after cell inoculation, mice were randomly assigned into 2 groups and were administered a total of 4 doses of daratumumab or isotype control following a weekly schedule (20/10/10/10 mg/kg). These doses were chosen on the basis of dose escalation studies in multiple myeloma (33). The isotype control–treated mice started to show signs of disease (mainly weight loss > 20% and rough hair) starting at day 32 post cell inoculation (Fig. 5A and B), and all mice in the control group were sacrificed by day 40. These mice showed systemic dissemination of disease in lung, kidney, ovaries, parathyroid glands, and enlarged lymph nodes (identified as $CD45^+/CD19^+$; Fig. 5C and D and Supplementary Fig. S3), resembling aggressive CLL. In several mice, bone marrow and spleen infiltration was also observed (data not shown). Of note, a similar disseminated CLL mouse model was described by Bertilaccio and colleagues using the $CD38^-$PLL cell line MEC1 in $Rag2^{-/-}\gamma^{-/-}$ mice, and the authors demonstrated its value as a tool to assess the efficacy of chemotherapeutic agents (34). In several mice, bone marrow and spleen infiltration was also observed (data not shown). In contrast, in the daratumumab-treated group, only one mouse harbored signs of illness and required euthanasia at day 41, whereas the remaining animals survived and did not develop life-threatening symptoms up to day 90, when the experiment was terminated (Fig. 5A and B).

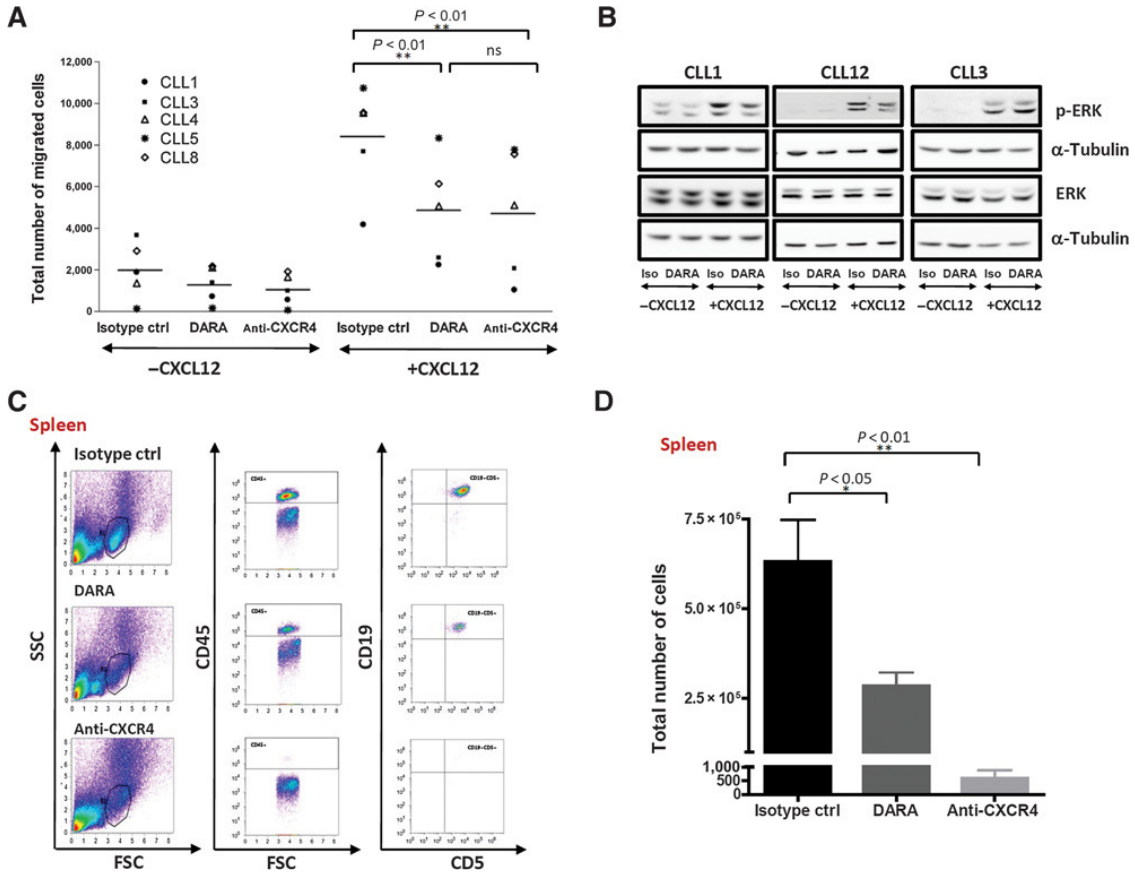

Figure 3.

Daratumumab interferes with CLL cell migration and *in vivo* homing. **A,** CLL cells were preincubated with the antibodies for 30 minutes at 4°C (30 μg/mL for isotype control and daratumumab and 25 μg/mL anti-CXCR4) and then assayed for migration in a CXCL12 gradient. After 4 hours, CLL cells ($CD19^+CD5^+$) in the lower chamber were counted in triplicates in a flow cytometer at fix flow rate. Total number of cells is graphed for representative patients with CLL ($n = 5$). Statistical differences between groups were assessed by paired *t* test. **B,** Western blot analysis of ERK activation after stimulation of CLL cells for 5 minutes with CXCL12 (200 ng/mL). Before stimulation, cells were serum-starved for 2 hours and pretreated for 30 minutes with the corresponding antibodies (30 μg/mL). **C** and **D,** *In vivo* homing was assessed by intravenous inoculation of fresh CLL cells via tail vein in NSG mice, previously pretreated with the corresponding antibodies (10 mg/kg, $n = 4$ mice/group). After 20 hours, cells were recovered from spleen, labeled with huCD45/CD19/CD5, and counted in a flow cytometer. Representative density plots for each treatment are shown (**C**). The gating strategy started with cells in forward scatter (FSC)/side scatter (SSC; R1), then gating on $huCD45^+$, and finally $huCD19^+CD5^+$. **D,** Total number of $huCD45^+CD19^+CD5^+$ recovered from the spleen is plotted. Statistical differences between groups were assessed by unpaired *t* test.

By this day, the antibody concentrations in the serum of daratumumab-treated animals had dropped to 1.5 μg/mL (Supplementary Fig. S4). Interestingly, in this group of mice, no MEC2 cells were found by flow cytometry or immunohistochemistry in the commonly infiltrated organs like lung and kidney, contrasting with the remarkable predominance of malignant, human $CD19^+$ cells observed in these secondary sites in control isotype–treated mice (Fig. 5C and D), suggesting that these mice may be free of disease. These data suggest a strong antitumor activity and long-term survival of daratumumab-treated mice in this model.

To develop a mouse model closer to CLL biology, we established a short time CLL-PDX model using NSG mice, needed to avoid CLL clearance by mouse NKs. To provide this mouse model with FcγR-bearing effector cells, we selected CLL cases enriched in NKs and monocytes (Fig. 5E). Fresh PBMCs from these patients were intravenously inoculated (day 1) and treated the following day (day 2) with daratumumab or control isotype. On day 5, mice were sacrificed and cells recovered from peripheral blood, bone marrow, and spleen. As described previously, cells mainly homed to the spleen, where a significant (*, $P < 0.05$) decrease of CLL cells was found in the spleen of daratumumab-treated group. No significant differences were found in bone marrow or peripheral blood resident CLL cells between the 2 groups (Supplementary Fig. S5A). Off note, when the experiment was performed with a CLL sample with reduced numbers of effectors, the antitumoral effect of daratumumab was diminished (Supplementary Fig. S5B and S5C)

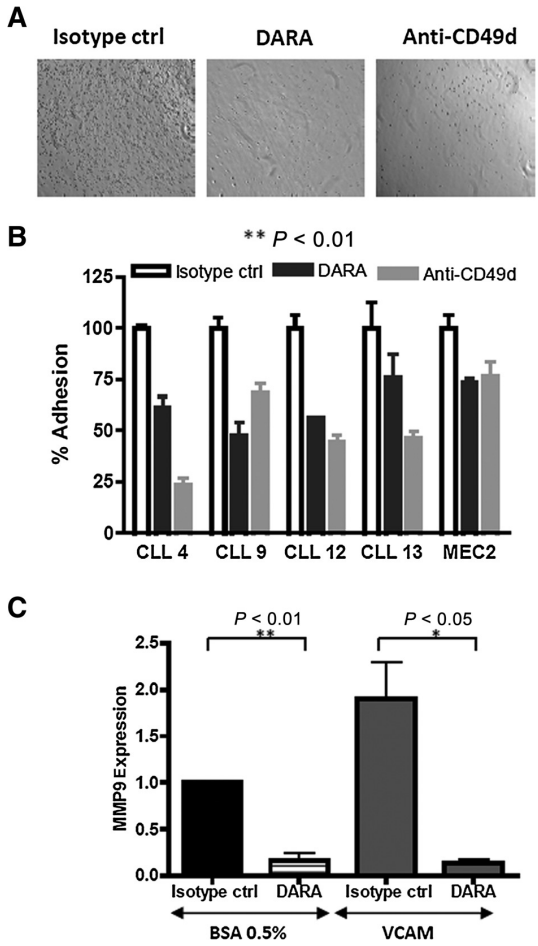

Figure 4.

Daratumumab hampers CLL adhesion to VCAM. Calcein-labeled primary CLL and MEC2 cells were preincubated with the corresponding antibodies (30 μg/mL) and left to adhere for 30 minutes to plates precoated with VCAM-1 or BSA (nonspecific adhesion). Anti-CD49d was used as positive control for inhibition of adhesion. Nonadhered cells were removed by extensive washing. **A,** Representative phase-contrast microscopy field images (100×) from adhesion to VCAM-1 of CLL9. **B,** Adhered cells were then lysed and supernatants analyzed in a fluorimeter. Percentage of VCAM-1 adhesion is expressed normalized to isotype control and after subtraction of BSA nonspecific adhesion ($P < 0.01$, one-way ANOVA comparing the 3 groups together). **C,** RT-PCR for *MMP9* was performed on the adhered cells to VCAM, using *mGUS* as endogenous control. $n = 3$, CLL4, CLL12, and CLL13. Expression levels for each sample are normalized to the corresponding isotype control and adhesion to BSA. **, $P < 0.01$; *, $P < 0.05$; unpaired t test.

Discussion

Targeted immunotherapy with mAbs has become the standard of care for successful treatment of many forms of cancer. In CLL, anti-CD20 antibodies (rituximab, ofatumumab, and obinutuzumab) have demonstrated therapeutic benefit, alone and in combination with chemotherapy (35, 36). Identification of new targets with a broader expression spectrum and potential for distinctive mechanisms could yield novel antibody therapeutics for a wider range of hematologic malignancies.

In the last years, CD38 has gained momentum as a novel therapeutic target for patients with hematologic malignancies, namely multiple myeloma (37, 38), CLL, and NHL. CD38 is an ectoenzyme belonging to the family of nucleotide-metabolizing enzymes, involved in the scavenging of extracellular nucleotides. CD38 catalyzes the synthesis of cyclic ADP-ribose and ADP-ribose from NAD, leading to an increase in cytoplasmic Ca^{2+} concentration. CD38 is used as a disease marker for leukemias and myeloma and it is considered a negative prognostic marker for CLL (4, 5). Moreover, recent evidence indicates that CD38 forms a complex molecular network delivering growth and survival signals in CLL cells. CD38 cooperates with chemokines and their receptors to influence cell migratory responses (11). These characteristics make CD38 an attractive target for CLL therapy. The use of an antibody such as daratumumab that specifically blocks CD38 might provide a new approach for interfering with deleterious growth circuits and for increasing the susceptibility of leukemic cells to conventional chemotherapy.

In this study, we have analyzed the potential therapeutic activity of daratumumab in CLL. Daratumumab showed limited CDC activity in both primary CLL cells and CLL cell lines. Complement activation is strongly regulated by CRPs *in vivo* to prevent its uncontrolled amplification, including CD46, CD55, and CD59 that have been shown to mediate resistance to CDC induced by rituximab and less by ofatumumab (39). In fact, these proteins are overexpressed in a number of tumor types, and their upregulation has been postulated to contribute to mAb resistance *in vivo* (40). Our data show that CLL cell lines and primary cells display very high expression of the CRP, which may explain the limited sensitivity to daratumumab-mediated CDC *in vitro*.

Our results are the first to provide strong evidence that daratumumab induces lysis of CLL cells by FcγR-mediated ADCC and ADCP through NK cells and macrophages, respectively. This cytotoxic effect is remarkable in CLL, where the mean ADCC induction was 35% at 0.1 μg/mL, being in the same range to those published for primary multiple myeloma (19, 32). In addition, ADCC does not correlate strictly with CD38 sABC in CLL cell lines or primary cells, indicating that at least in CLL, the number of CD38 molecules on the cell surface may not be the only factor in driving Fc-mediated cytotoxicity of daratumumab. This observation suggests that other molecules within the immune synapse must control the extent of ADCC/ADCP by daratumumab. Activating receptors expressed on NK cells include FcγRIIIA, activating forms of killer cell Ig-like receptors (KIRs; KIR2DS and KIR3DS), NKG2D, and the natural cytotoxicity receptors (NCR) called NKp30, NKp44, and NKp46 that are critical for optimal ADCC activity (41). NKG2D and NCRs are the most relevant receptors that stimulate responses to tumor target cells. In addition, inhibitory receptors counteract activating receptors as a means to tolerate mature NK cells. Thus, the overall makeup of these activating and inhibitory molecules on each individual CLL tumor cell may dictate the extent of ADCC by daratumumab (42). Along these lines, a recent study in multiple myeloma has shown that blocking inhibitory KIRs with IPH2102, a human IgG4 monoclonal antibody that blocks the interaction of the 3 main inhibitory KIRs with their ligands, improves ADCC induced by daratumumab against multiple myeloma cells (43).

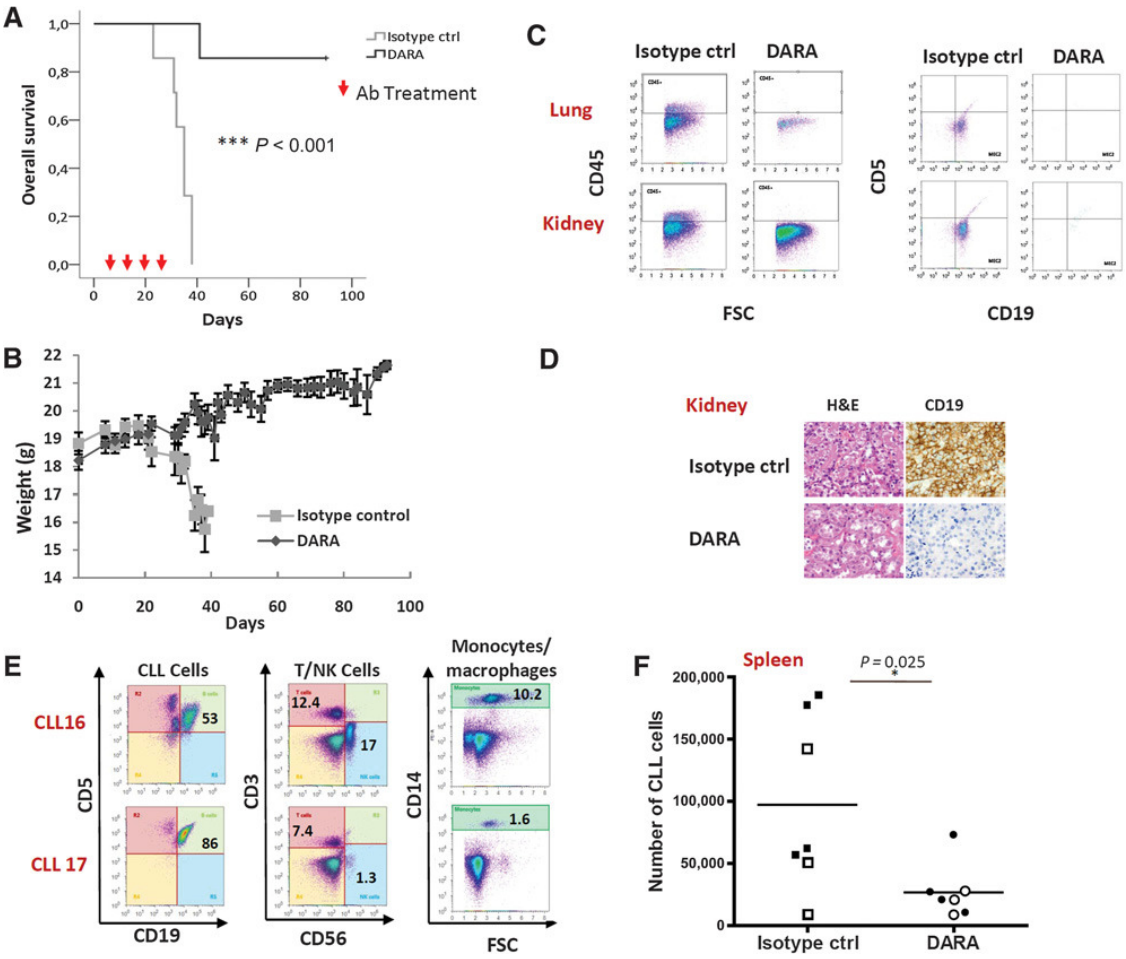

Figure 5.

In vivo efficacy of daratumumab (DARA). **A,** Kaplan–Meier survival curves for MEC2 systemic mouse xenografts. Mice received daratumumab ($n = 7$) or isotype control ($n = 6$) weekly for 4 weeks, starting 1 week after cell inoculation. Then, mice were monitored twice a week for any sign of disease and sacrificed when body weight decreased 15% to 20%. All control mice had to be sacrificed between days 23 and 38. One daratumumab-treated mouse became ill and was sacrificed on day 41. **B,** Body weight changes in the isotype and daratumumab-treated mice. **C,** Cells from lungs and kidneys were isolated and labeled with huCD45/CD19/CD5. The presence of MEC2 cells was evaluated by $CD19^+CD5^-$ (right) cells in the $CD45^+$population (left). Representative flow cytometric density plots for one mouse of each cohort sacrificed at day 30 are shown. **D,** IHC staining of H&E and CD19 of kidneys from isotype and daratumumab-treated mice (magnification, 400×). **E,** Density plots showing the percentage of CLL cells ($CD19^+CD5^+$), T cells ($CD3^+CD56^-$), NK cells ($CD3^-$ $CD56^+$), and monocytes ($CD14^+$). **F,** Total number of huCD45$^+$CD19$^+$CD5$^+$ recovered from the spleen of isotype control and daratumumab-treated groups (CLL16: open symbols; CLL17: closed symbols). Statistical differences between groups were assessed by unpaired *t* test.

Macrophages are tissue-resident immune cells that play a critical role not only in maintaining homeostasis and fighting infection but also in the progression of many pathologies including cancer (44). Human macrophages express both activating and inhibitory FcγR and are involved as most prominent effector cell populations in mAb-mediated tumor elimination *in vivo*. We have demonstrated that daratumumab induces phagocytosis of CLL by macrophages both *in vitro* and *in vivo*. In the *in vitro* model, CLL cells and macrophages were cocultured in the presence or absence of daratumumab. In the *in vivo* model, CLL cells were injected into the peritoneum of SCID *beige* mice, which are devoid of NK cells, but possess active macrophages in their peritoneal cavity.

Taken together, these results provide evidence that anti-CD38 therapy with daratumumab may be relevant for $CD38^+$ CLL cases.

We next explored whether these *in vitro* mechanisms translate to *in vivo* tumor growth inhibition. To accomplish this task, we developed 2 approaches. First, we successfully developed an MEC2 tumor model that showed engraftment efficacy of 100% and systemic disease involving mostly lungs, kidney, ovaries, parathyroid glands, enlarged lymph nodes, and bone marrow in a portion of the mice. This model is clinically relevant for CLL with

leukemic infiltrates in isolated organs similar to that reported in patients with CLL (45), presenting aggressive disease or transformation to Ritcher syndrome. In this model, treatment with relevant pharmacologic doses of daratumumab efficiently prevented tumor progression and significantly prolonged survival. Mice treated with daratumumab showed long-term survival even though daratumumab dosing was stopped after 4 weeks and the antibody concentrations in the serum were minimal, suggesting that these mice were free of disease. Second, we developed a CLL-PDX model using NSG mice, to avoid CLL clearance by mouse NKs or phagocytes, and inoculating selected CLL enriched in NKs and monocytes to provide the system with FcγR-bearing effector cells. In this model, daratumumab proved to reduce tumor burden in the mouse spleen, which constitutes the main infiltrated organ in this model (46). These results are the first to provide evidence that anti-CD38 therapy with daratumumab may be relevant for $CD38^+$ CLL.

As described in Introduction, daratumumab activity may extend beyond its effect on the tumor cells, as it shows immunomodulatory effects on CD38-expressing immunosuppressive regulatory T and B cells and myeloid-derived suppressor cells (23). We have also demonstrated additional activities of daratumumab besides ADCC and ADCP in CLL. Daratumumab has the potential to counteract microenvironment-derived signaling in protective cancer niches, such as lymph node and bone marrow. We have demonstrated that daratumumab interferes with *in vitro* cell migration and *in vivo* homing of CLL cells to spleen in NSG mice. Transendothelial migration and organ invasion of malignant cells require proteolytic degradation of the vascular basement membrane and extracellular matrix of lymphoid tissues and MMPs play a key role in these processes. MMP9 is the predominant MMP expressed in CLL and is physiologically regulated by CD49d/CD29 and CXCL12, playing a key role in cell invasion and transendothelial migration (47). Moreover, MMP9 correlates with advanced-stage disease and poor patient survival (48). We have demonstrated that daratumumab significantly reduces CD49d/CD29-mediated adhesion of CLL cells to VCAM-1 and, more importantly, downregulates both constitutive and adhesion-induced MMP9 expression. On the basis of the prominent role of MMP9 on CLL cell invasion, our results indicate that daratumumab treatment may impede CLL tissue infiltration that leads to progressive disease. Thus, in the era of BCR kinase inhibitors, daratumumab immunotherapy opens a new horizon offering unique effects on tumor dissemination against $CD38^+$ CLL cases. In conclusion, our results support daratumumab as a novel therapeutic approach for $CD38^+$ CLL by not only inducing the classical FcγR-mediated cytotoxicity but also harnessing microenvironment-derived survival signaling and blocking CLL dissemination to secondary lymphoid organs.

Disclosure of Potential Conflicts of Interest

A. Wiestner reports receiving commercial research grants from and is a consultant/advisory board member for Pharmacyclics. P.W.H.I. Parren is an employee of Leiden University Medical Center, and holds ownership interest (including patents) in Genmab. No potential conflicts of interest were disclosed by the other authors.

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Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): V. Rodriguez, H. Roca-Ho, P. Menéndez, P. Doshi, P. Pérez-Galán

Study supervision: J.L. van Bueren, P. Pérez-Galán

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